

Ovarian cysts and granulosa cell tumors develop after sublethal total body irradiation in mice

Shiyun Xiao^{*1}, Yao Xiao², Xiaohong Xu³, Wen Zhang¹, Mandi M. Murph⁴, and Nancy R.

Manley¹

1. Department of Genetics, Paul D. Coverdell Center, 500 DW Brooks Drive, University of Georgia, Athens, Georgia 30602
2. Department of research, Child mind institution, 101 East 56th Street, New York, NY 10022
3. Department of the clinical laboratory, Zhejiang Cancer Hospital, Hangzhou, Zhejiang 310022, China
4. Department of pharmaceutical and Biomedical Sciences, Pharmacy South Building, 240 W Green Street, University of Georgia, Athens, Georgia 30602

*Correspondence should be addressed to: Email: shiyun@uga.edu (SX)

19 **Abstract**

20 **Background**

21 Localized and total body irradiation are used to treat certain cancers and also used prior to
22 transplantation of stem cells or organs. However, the use of radiation also induces collateral
23 damage to the cells of healthy tissue. Although the acute damage of radiation to oocytes is well
24 known, the long-term effects induced by radiation to stromal cells and their relationship with age
25 are still unclear.

26 **Methods**

27 A total of 206 two-month-old female mice were whole-body exposed to gamma rays at doses of
28 0, 0.5, 1, 2, or 4 Gy, respectively. The mice were sacrificed at 3.5, 9, 12, or 18 months of age and
29 pathological changes including cysts and tumors were assessed in the ovary and other organs.

30 **Results**

31 The overall incidence of visible pathological changes of mice receiving irradiation was 33.7% in
32 the ovary, but much lower in the liver, spleen, lung, thymus, and skin. Among these, the ovarian
33 cyst formation rate was 24.7%, and tumor lesions were 10.2%, respectively, compared to 5%
34 cyst formation and no tumor lesions among control, unirradiated mice. Statistical analysis
35 showed that cyst formation was age, but not dose-dependent, whereas the formation of tumor
36 lesions was dependent on both age and radiation dose. Pathology analysis indicated that most
37 ovarian cysts originated from follicles and both tumor lesions analyzed originated from
38 granulosa cells.

39 **Conclusion**

40 Ovaries are highly susceptible to the effects of radiation. Long-term damage is increased after
41 total body irradiation in mice, manifested by higher incidences of cyst formation and tumor

42 lesions. The ovarian stromal-derived granulosa cells might play an essential role in these changes.

43

44 **Introduction**

45 Ionizing irradiation is formed by charged energy particles, which can directly or indirectly cause
46 damage to the DNA when it passes through the body [1]. Following radiation exposure, DNA
47 repair mechanisms are initiated in the cell. In general, a lower-absorbed dose of radiation causes
48 repairable DNA damage, whereas a higher-absorbed dose causes significant DNA damage and
49 tends to kill the irradiated cells [2, 3]. Proper DNA repair restores the cells and tissues to normal;
50 mis-repaired or un-repaired DNA causes cell death, which can lead to damage
51 or failure of the tissue or organ. In some cases, the cells with the mis-repaired or un-repaired
52 DNA can survive after irradiation, and can be a substrate for tumor/cancer development [2, 4].

53

54 Different organs, tissues, and cells, and even the same type of cell in a different phase, appear to
55 have different radiation sensitivity [5-8]. The ovary is the female gonad and the primary
56 reproductive organ, which is composed of an outer cortex and an inner medulla, and the port of
57 entry into the ovary is known as the ovarian hilus [9]. The outer cortex contains developing
58 follicles at various stages and is the most radiation-sensitive part [10]. The cortical primordial
59 follicle pool is nearly destroyed in young adult female mice two weeks after a single total body
60 exposure to the dose of 0.1 Gy [11]. The complete destruction of the primordial follicle pool was
61 reported in neonatal mice five days after exposure to doses as low as 0.45 Gy [12]. In contrast,
62 the partial primary, secondary follicles, and large follicles still survived, showing more resistance
63 to the radiation than the primordial follicles [7, 10, 12, 13]. The inner medulla contains the major
64 blood and lymphatic vessels connected to the hilus and cortex stromal compartment, known as
65 the ovarian niche, which supports the development of oocyte follicles. These are largely
66 considered to be radiation resistant. However, the radiation damage to the follicles may destroy

67 not only the cortical construction but also the medulla stromal cells. It is these changes that
68 usually cause ovarian failure or dysfunction and therefore lead to premature reproductive
69 senescence or permanent menopause after irradiation [14-16].

70

71 Ovarian cysts are a frequent problem among reproductive-aged females undergoing ovulation
72 cycles and aging rodents with hormone variations [17, 18]. During each cycle, mature follicles
73 forms lumps on the ovary and rupture at ovulation, releasing an oocyte. Rodents have an estrous
74 cycle that lasts 4 to 5 days with mice ovulating an average of 11 oocytes. Fluid-filled cysts stem
75 from mature follicles that do not rupture to release the oocyte after the period of follicular growth
76 and development [19].

77

78 Granulosa cells play a critical role in this dynamic process. In order for successful ovulation and
79 follicular development to occur, granulosa cells surrounding oocytes must replicate to form
80 multiple layers and continually alter their morphology. In fact, a major characteristics of mature
81 follicle formation includes a fluid-filled vesicle that materializes as a result of proper granulosa
82 cells' organization [19]. If the mature follicle does not rupture appropriately, a fluid-filled cyst
83 remains, however, cysts are considered physiological changes and often resolve naturally over 1-
84 3 months [20, 21]. Interestingly, granulosa cells secrete growth-promoting factors along with
85 their receptors. As such, they are the main source of estradiol, insulin-like growth factor and
86 others that stimulate follicular growth and survival [22].

87

88 Exposure to radiation is a known cause of cancer, whereas reducing the overall number of
89 lifetime ovulations, which occurs with pregnancy or the use of oral contraception, decreases the

90 risk of ovarian cancer [23, 24]. Most previous studies on ovarian damage induced by radiation
91 have focused on the acute effects of follicle deletion and its related mechanisms, few have
92 focused on niche changes and long-term radiation effects on the ovary. Thus, our study provided
93 an opportunity to investigate the ovary for relationships between radiation damage, cyst
94 formation and tumor development.

95

96 In this study, two-month-old female mice were exposed to gamma rays (Co-60) at sub-lethal
97 doses ranging from 0.5 to 4 Gy, and radiation effects were analyzed at 3.5, 9, 12, and 18 months
98 of age by comparison to 0 Gy control animals. We previously reported late effects of radiation
99 on the immune system [25]; in the course of that study, we also observed pathological changes
100 like cysts and tumors increasing significantly in the mouse ovary, but not in other organs. Our
101 results herein demonstrate that the ovary is a highly radiation-sensitive organ in mice, and
102 damage sustained after a single total body irradiation (TBI) increases ovarian cysts and tumor
103 lesions. Visible ovarian cysts (the size $> 1 \text{ mm}^3$) were observed starting at 9 months of age (7
104 months after the irradiation). Cyst formation was age-dependent but not dose-dependent; tumor
105 formation showed both age-dependence and a dose threshold. Our studies are the first to provide
106 evidence that single sub-lethal TBI can cause long-term ovarian damage like cysts and tumors in
107 mice. The mouse model presented herein may be a useful tool for mechanistic studies on the
108 development of ovarian cysts and tumors, particularly with their shorter life and estrous cycle.

109 **Materials and Methods**

110 **Mice**

111 The mice used in this study were part of a larger study, which was supported by the grant RFP
112 NIAID-DAIT-NIHAI2008023. According to the original design, a total of 1000 mice were
113 irradiated including males and females. More specifically, 210 C57BL6/J female mice were
114 purchased from The Jackson Laboratory (Bar Harbor, ME) and 200 mice were divided into four
115 age groups. To irradiate the mice, 50 mice at 2 months of age were brought into the radiation
116 room each time, 10 mice in each of 5 groups were exposed to doses of either 0.5, 1, 2, or 4 Gy,
117 with the remaining 10 mice (the 0 Gy group) not exposed to radiation as a control. Six additional
118 mice were exposed to 0.5 or 1 Gy under these same conditions. Therefore, we irradiated a total
119 of 166 mice and had 40 unexposed mice as 0 Gy control. The mice in each dose were
120 subsequently sacrificed at 3.5, 9, 12, or 18 months of age and tissues harvested for assessment.
121 All mice were maintained in a specific pathogen-free facility at the University of Georgia before
122 and after irradiation. The experiment was approved by the Institutional Animal Care and Use
123 Committee of the University of Georgia.

124

125 **Irradiator survey and calibration**

126 The radiation exposure device used in this study is a Type 60 Co gamma (Nordion Canada),
127 Model Gammacell 200. To calibrate radiation exposure doses, OSL nanoDot dosimeters
128 (Landauer Inc., Glenwood, IL) were implanted in a mouse cadaver, which were then exposed at
129 the indicated doses of 0.5, 1, 2, and 4 Gy. After irradiation, the dosimeters were sent back to
130 Landauer Inc. for measurement. Based on the results of these measurements, we generated a
131 linear formula to calculate the exposure times for each dose ($Y(\text{dose})=2.16t+10.98$; S1 Dataset).

132 The standard error of the dose for each irradiation is within 5% [26]. Details of the calibration
133 protocols and evaluation of linear response were previously published [26].

134

135 **Measurement of cyst and tumors**

136 Mice in each dose group were sacrificed by CO₂ inhalation followed by cervical dislocation at
137 3.5, 9, 12, or 18 months of age and tissues harvested for assessment. The peritoneal and thoracic
138 cavities of the mice were opened, and the ovary, spleen, liver, gut, thymus, lung, and other
139 organs were evaluated for visually apparent anomalies. To measure ovarian cysts and tumors, the
140 ovaries were completely exposed along the uterus on both sides. Two individuals independently
141 assessed and measured cyst formation and tumor lesions. The visible cysts and tumors with a
142 size > 1 mm³ and any visible pathological changes in the ovaries and other organs were recorded
143 (S2 Dataset). In this study, the analysis of 3.5-month-old mice at 1.5 months after irradiation was
144 defined as "short-term" (to contrast with "acute"). We defined "long-term" effects as occurring at
145 or after the 9 months age at analysis, more than 6 months after exposure.

146

147 **H&E staining**

148 For histological analysis, ovarian samples included unirradiated control (0 Gy), irradiated
149 without visible cysts or tumors, irradiated with visible cysts or tumors were assessed. Tissues
150 were fixed with 4% paraformaldehyde (PFA) overnight, dehydrated in gradient ethanol solutions,
151 and embedded in paraffin. Ten μ m sections were cut and stained with hematoxylin and eosin
152 (H&E). Imaging was done on a Keyence BZ-X700 microscope (Osaka, Japan).

153

154 **Statistical analysis**

155 One-sided Fisher's exact tests were used to compare the overall incidence rates between the
156 irradiated and control group. Within the irradiated group, two-sided Fisher's exact tests were
157 used to assess the dependence of incidence rates across the age and dosage groups respectively.
158 Furthermore, simple logistic regression was used to assess the magnitude and directionality of
159 significant associations with age. These analysis were performed in R using functions from the
160 'stats' package version 3.6.1. (S3 Dataset). The rates of pathological changes in the liver, spleen,
161 lung, thymus, and skin (Table 1) was analyzed using Contingency table analysis, and Fisher's
162 exact test using PrismTM software (GraphPad Software, San Diego, CA).

163

164

165 **Results**

166 **Overall pathological changes in the ovary and other organs after TBI.**

167 To evaluate the effects of radiation with aging, two-month-old female mice were total body
168 exposed to a single radiation dose of 0, 0.5, 1, 2, or 4 Gy, and analyzed at 3.5, 9, 12, and 18
169 months of age. We previously reported that a single TBI could cause long-term deficits in
170 thymus function by reducing lymphoid progenitors in bone marrow [25]. Interestingly, we also
171 found visible pathological changes in the ovary in these mice (Fig. 1). Most ovarian cysts were
172 unilateral, a single round shape, within the bursa, and filled with transparent fluid, with some
173 displaying vascularization (Fig. 1A); a few appeared as large oval shapes formed by multiple
174 cysts (Fig. 1B). Some cysts or masses were bilateral or appeared deep brown or black due to
175 being filled with blood (Fig. 1 C). The ovarian tumors appeared as a large mass of tissue or black
176 masses covered by the bursa (Fig. 1 D). We also observed that some mice had enlarged spleens
177 or tumor lesions in the liver, lung, thymus, and/or skin after TBI. The overall incidence of visible
178 pathological changes after irradiation per ovary was 19.9%; The rates of pathological changes
179 were much lower in the liver, spleen, lung, thymus, and skin (Table 1). Contingency table
180 analysis using Fisher's exact test showed that pathological changes were significantly different
181 between the ovary and other organs ($P<0.001$). Our results indicate that the ovary is
182 significantly more sensitive to radiation compared to the other organs examined.

183

184 **Fig 1. Gross anatomy of ovarian cysts and tumors from BL6 mice after TBI. (A).** Single
185 round ovarian cysts were filled with transparent or hemorrhagic serous fluid. **(B).** Oval ovarian
186 cysts filled with transparent fluid and formed by multiple cysts. **(C).** Ovarian cysts or lumps were

187 filled with hemorrhagic serous fluid. **(D)**. Ovarian tumors were covered with a membrane.

188 Normal ovary (blue arrow); Cysts or tumors (yellow arrow). Size of bars: 10 mm.

189

190 **Table 1. Visible pathological changes after TBI.**

191

Organ	Non-irra.	Irradiated groups (all doses)					
		Ovary ^a	Ovary	Liver	Spleen	Thymus	Lung
No. of total organs examined ^b	80	332	166	166	166	166	166
No. of organs with cyst	2	49	0	0	0	0	0
No. of organs with tumor	0	17	4	3	2	1	1
% Path-change ^c	2.5%	19.9%	2.4%	1.8%	1.2%	0.6%	0.6%

192

193 Non-irra: Non irradiated group; Path-change: Pathological change

194 ^a Non-irradiated control mice had no visible changes in any other organs.

195 ^b As there are two ovaries/mouse, the numbers are double

196 ^c The overall frequencies of visible pathological changes observed per organ

197

198 **TBI increased cyst formation in the ovary.**

199 Next, we measured cyst formation and observed that it is a prominent pathological change in the
200 ovary after TBI. There were no visible cysts observed in either the control or irradiated groups at
201 3.5 months of age (1.5 months after irradiation), and cysts were observed to be present at 9
202 months of age (7 months after irradiation) in the irradiated group. The overall rate of cyst
203 formation in irradiated mice is 24.7% compared to 5% in non-irradiated control mice (Table 2,

204 P=0.003 Fisher's exact test one-sided), indicating that TBI exposed mice were more likely to
205 develop ovarian cysts. Among irradiated mice, we found that the rate of cyst formation was age
206 dependent (Table 2, P<0.001 Fisher's exact test two-sided). Furthermore, logistic regression
207 showed that age was positively associated with the rate of cyst formation (Odds Ratio=1.15,
208 P<0.001). We also found the rate of cyst formation to be independent of dose (Table 2, P=0.166
209 Fisher's exact test two-sided).

210

211 **Table 2. Ovarian cyst formation observed per animal by age and dose after TBI.**

Age	Dose	Non-irra.	Irradiated groups (Gy)				
			0	0.5	1	2	4
3.5	0 ^a /10 ^b	0/10	0/10	0/10	0/10	0/10	0% (0/40)
9	0/10	4/10	2/10	2/10	2/10	2/10	25% (10/40)
12	1/10	2/10	9/12	1/10	4/10	4/10	38.1% (16/42)
18 (M)	1/10	1/12	3/12	4/10	7/10	7/10	34.1% (15/44)
% mice with cysts by dose		5%	16.7%	31.8%	17.5%	32.5%	24.7% (41/166) ^c

212

213 Non-irra: Non irradiated group

214 ^a Number of mice with cysts

215 ^b Number of total mice

216 ^c Proportion of irradiated mice with cyst

217

218 **TBI induced tumor lesion in the ovary.**

219 Tumor lesions are frequently observed as a long-term consequence after exposure to radiation.

220 Visible tumor lesions (Size > 1 mm³) in the ovary were observed to be present at 9 months of age

221 in the irradiated group. The overall rate of tumor lesions in irradiated mice is 10.2% compared to
222 0% in control mice (Table 3, P=0.024 Fisher's exact test one-sided), indicating that TBI exposed
223 mice were significantly more likely to develop tumor lesions in the ovary. Among irradiated
224 mice, we found that the rate of tumor lesions was age dependent (Table 3, P<0.001 Fisher's
225 exact test two-sided). Furthermore, logistic regression showed that age was positively associated
226 with the rate of tumor lesions (Odds Ratio=1.37, P<0.001). Tumors were observed at the doses
227 of 0.5, 1, and 2 Gy respectively, while no tumor lesions were observed in the mice exposed to the
228 4 Gy dose, suggesting that tumor incidence had a threshold-like effect that was significantly
229 correlated to dose (Table 3, P <0.001 Fisher's exact test two-sided).

230 **Table 3. Ovarian tumor lesions observed per animal by age and dose after TBI.**

Age \ Dose	Non-irra.	Irradiated groups (Gy)					% mice with tumors by age
		0	0.5	1	2	4 (Gy)	
3.5	0 ^a /10 ^b	0/10	0/10	0/10	0/10	0/10	0% (0/40)
9	0/10	0/10	1/10	0/10	0/10	0/10	2.5% (1/40)
12	0/10	0/10	2/12	1/10	0/10	0/10	7.1% (3/42)
18 (M)	0/10	4/12	5/12	4/10	0/10	0/10	29.5% (13/44)
% mice with tumors by dose	0%	9.5%	18.2%	12.5%	0%	0%	10.2% (17/166) ^c

231

232 Non-irra: Non irradiated group

233 ^a Number of mice with tumors;

234 ^b Number of total mice

235 ^c Proportion of irradiated mice with tumor

236

237 **The overall visible pathological changes in the ovary after TBI.**

238 To evaluate the radiation-induced effects in the ovary, we combined both cyst formation and
239 tumor lesions to assess pathological changes after TBI in different ages and doses (Table 4). The
240 overall rate of pathological change among irradiated mice is 33.7% compared to 5% in non-
241 irradiated control mice (Table 4, $P<0.001$ Fisher's exact test one-sided), indicating that TBI
242 exposed mice were significantly more likely to develop any pathological change. Among
243 irradiated mice, we found that the incidence of pathological change is age dependent (Table 4,
244 $P<0.001$ Fisher's exact test two-sided) and the direction of association is positive (Odds
245 Ratio=1.27, $P<0.001$). We also found that the incidence of pathological change is independent of
246 dose (Table 2, $P=0.177$ Fisher's exact test two-sided).

247

248 **Table 4. Overall visible pathological changes in the ovary observed per animal by age and**
249 **dose after TBI.**

250

Age	Dose	Irradiated groups (Gy)					% mice with path. by age
		0	0.5	1	2	4 (Gy)	
3.5	0 ^a /10 ^b	0/10	0/10	0/10	0/10	0/10	0% (0/40)
9	0/10	4/10	3/10	2/10	2/10	2/10	27.5% (11/40)
12	1/10	2/10	10/12 ^c	2/10	4/10	4/10	42.9% (18/42)
18 (M)	1/10	5/12	8/12	8/10	7/10	7/10	61.4% (28/44)
% mice with path. by dose	5%	26.2%	47.7%	30%	30%	33.7% (57/166) ^d	

251

252 Non-irra: Non irradiated group

253 ^a Number of mice with visible pathological changes.

254 ^b Number of total mice

255 ^c One mouse in this group had both cyst and tumor in the left and right ovaries, respectively.
256 Therefore, the number of mice with pathological change was 1 less than the total number of cysts
257 and tumors at this time point.

258 ^d Proportion of irradiated mice with any visible pathological changes

259
260 **Both ovarian cyst formations and tumor lesions after TBI originated from granulosa cells.**
261 To measure the morphology, structural changes and identify the cells of origin in the cysts and
262 tumors, a subset of ovarian samples with cysts and tumors were collected. The unirradiated ovary
263 and irradiated ovary without visible cysts and tumors were set as controls. These samples were
264 fixed and the paraffin-embedded sections were processed for H&E staining. Under the
265 microscope, the unirradiated, morphological structure of the ovary included clear cortex and
266 medulla areas, whereas there were no follicles in the cortex, which were instead replaced by
267 corpora lutea (CL) (Fig 2 A and B). All irradiated ovaries including non-visible cystic (Fig 2 C
268 and D) and visible cystic ovaries (Fig 2 E-H) had lost the entire structure of cortex, medulla and
269 corpora lutea, but displayed increased cysts in the ovaries. The granulosa cells either surrounded
270 the cyst or were disorderly distributed in the residual ovarian tissues. Most cysts showed
271 follicular origin profiles lined by one or several layers of cuboidal or flattened granulosa cells
272 with a thin wall. Some granulosa cells were luteinized with eosinophilic cytoplasm (Fig 2 C-H
273 blue arrow). Some cysts were located within the ovary, with an epithelial cell cyst profile of a
274 thin wall lined by a layer of flattened epithelium that resembled ovarian surface epithelial cells
275 (Fig 2 C and D, red arrow).

276 Two massive ovarian tumors were sectioned and stained. Both tumors were composed of
277 cuboidal granulosa cells indicating they were granulosa cell tumors. In the first tumor, some

278 granulosa cells formed structures of the Call-Exner bodies that resemble immature primitive
279 follicles; some granulosa cells lined the cyst and presented in irregular aggregates in the wall
280 (Fig 2 I and J, blue circles). In the second tumor, the granulosa cells were luteinized with
281 moderated amounts of eosinophilic cytoplasm and showed the pale, oval, and angular nuclei in a
282 disorderly arrangement (Fig 2 K and L, blue arrow). Thus, irradiation destroyed the entire
283 morphological structure. In addition, the granulosa cell is a critical cell type involved in the
284 formation of ovarian cysts and tumor lesions over the long-term after TBI.

285
286

287 **Fig 2. H&E staining profiles of ovarian cysts and tumors. (A).** An ovary from an unirradiated
288 18 month-old mouse as 0 Gy control. **(B).** Higher magnification image of the indicated area in A,
289 showing cortex with corpora lutea (CL). **(C).** An ovary without a visible cyst from a mouse
290 exposed to 0.5 Gy, analyzed at 18 months. **(D).** Higher magnification image of the indicated area
291 in C, showing an ovarian inclusion cyst located in the ovarian tissue lined by a layer of flattened
292 epithelium resembling the ovarian surface (red arrow). **(E).** A huge follicular cyst located on the
293 ovarian surface with the residual ovary. **(F).** Higher magnification image of the indicated area in
294 E, showing the disorded structure, cyst and luteinized with eosinophilic cytoplasm granulosa
295 cells (blue arrow). **(G).** A huge follicular cyst located on the ovarian surface lined by several
296 layers of cuboidal granulosa cells (blue arrow) with a thin wall, surrounded by the ovarian
297 surface membrane (red arrow). **(H).** Higher magnification image of the indicated area in G,
298 showing several layers of cuboidal granulosa cells. **(I).** A massive ovarian tumor enriched by
299 granulosa cells. **(J).** Higher magnification image in the indicated area in I, with Call-Exner
300 bodies (blue circle or oval). **(K).** Another ovarian tumor, enriched by luteinized granulosa cells.
301 **(L).** Higher magnification image showing the indicated area of K; luteinized granulosa cells with

302 eosinophilic cytoplasm (blue arrow). All samples were collected from 18 month-old mice. A-B
303 from unirradiated mice. C-K from irradiated mice. Scale bars: A, C, E, G, I, K = 1mm; B, D, F,
304 H, J, L = 200 μ m

305
306

307 **Discussion:**

308 In this study, we provide novel evidence that ovarian stromal cells such as follicular granulosa
309 cells are highly sensitive to harm from sub-lethal doses of radiation, resulting in irreparable
310 cellular damage that can manifest much later in life. Ovarian cyst formation and tumor lesions
311 are two significant, long-term effects after TBI in mice.

312

313 Most ovarian cysts result when mature follicles fail to rupture and release the oocyte during
314 ovulation [17]. Left behind is an organized series of granulosa cells surrounding the oocyte and a
315 fluid-filled antrum. Other types of cysts, including corpus luteum and theca-lutein, result from an
316 exaggerated physiological response, like hormonal overstimulation of the ovary [27]. TBI or
317 pelvis irradiation can destroy all primordial follicles in mice, as well as later-stage follicles and
318 mature follicles. Because maintaining the ovarian environment requires functional interaction
319 between the follicle cells and stromal cells, damage from radiation destroys both ovarian
320 structure and activity during the estrous cycle, including ovulation [28, 29].

321

322 Our data suggests that radiation-induced follicle damage at sub-lethal doses entirely destroys the
323 cortex and medulla structures with a disorded granulosa arrangement and leads to follicular cyst
324 formation among aged ovaries after TBI. Our results show that most of these ovarian cysts have
325 follicular profiles and are lined by granulosa cells, indicating that most of these radiation-induced
326 ovarian cysts originated from follicles. Furthermore, cyst formation significantly increased with
327 age, suggesting a long-term impact after TBI includes enhances susceptibility to age-related cyst
328 formation. This is also consistent with estrous cycle changes observed in aging mice, whereby
329 the first phase is characterized by the presence of follicular cysts [30].

330

331 Incessant ovulation increases the risk of ovarian inclusion cysts, which are a possible source of
332 epithelial ovarian cancer in women [31]. In this study, we found that although the incidence of
333 ovarian tumors increased after TBI, most of them originated from granulosa cells rather than
334 epithelial cells. We hypothesize that the radiation-induced depletion of ovarian follicles and the
335 subsequent reduction in ovulation may cause this difference. Therefore, these results are
336 consistent with the decreased risk of epithelial ovarian cancer that coincides with ovulation
337 reduction [23, 24]. Ovarian lesions were dramatically more frequent compared to the other
338 organs we evaluated. This observation suggests that the ovary is disproportionately susceptible to
339 damage from radiation.

340

341 In contrast with cyst formation, which was age- but not dose-dependent, tumor formation was
342 both and age- and dose-dependent. The dose-dependent effect was mainly driven by in the 4 Gy
343 group, which showed no tumors, and thus was a threshold effect, rather than a linear or
344 exponential dose dependency as has often been reported for other tissues/organs [32, 33]. Since
345 higher radiation doses may directly cause cell death [2, 3], the 4 Gy dose may be sufficient to
346 directly kill sensitive ovarian follicle cells, including oocytes and granulosa cells [7].

347

348 While the ovarian tumors incidence was very low overall, we observed a much higher incidence
349 of cysts at 9 and 12 months of age. Numerous studies support that invaginated or trapped
350 inclusion cysts are a possible source of epithelial ovarian cancer and might increase the risk of
351 the disease [34, 35]. In this study, some of the H&E sections showed inclusion cysts, but we did
352 not see tumors of ovarian epithelial origin. Both tumors we analyzed originated from ovarian

353 granulosa cells; all profiles indicate that they were ovarian granulosa cell tumors [36-38].

354 Granulosa cell tumors are a rare type of slow-growing ovarian cancer, a part of the sex cord-

355 gonadal stromal tumor that accounts for 2% to 5% of all malignant ovarian cancers [39, 40].

356 Therefore, our data suggest that sublethal radiation damage is less likely to induce epithelial

357 ovarian cancer, but instead can induce granulosa cell tumors.

358

359 To summarize, in the present study, we provide new evidence that the ovary is disproportionately

360 sensitive to radiation, even at relatively low doses. Ovarian cyst formation and tumor lesions

361 both occur as long-term effects after TBI in mice. Granulosa cells are the primary target cells

362 that contribute to these changes. Because most cysts originate from follicles, this mouse model

363 may provide a way to analyze the underlying mechanisms of ovarian cyst formation caused by

364 radiation-induced oocytes/follicular cell damage, hormone imbalance, and ovulation cycle

365 disorders. Also, because we have no human clinical trial data to evaluate the genetic effects of

366 human exposure to ionizing radiation, extrapolating human genetic risk is based on mouse data.

367 Our studies may provide a reference for the clinical protection of radiation therapy in females.

368 However, we should note that because the radiation sensitivity of follicle cells varies widely

369 depending on stages and species [8, 15], extrapolation of the ovarian data from mouse to humans

370 requires caution.

371

372

373 **Reference:**

- 374 1. Jackson SP, Bartek J. The DNA-damage response in human biology and disease.
375 Nature. 2009;461(7267):1071-8. doi: 10.1038/nature08467. PubMed PMID: 19847258;
376 PubMed Central PMCID: PMC2906700.
- 377 2. Hashimoto N, Endoh D, Kuwabara M, Satoh H, Sato F. Dose and dose-splitting effects
378 of X-rays on lung tumour induction in mice. International journal of radiation biology.
379 1990;58(2):351-60. doi: 10.1080/09553009014551681. PubMed PMID: 1974581.
- 380 3. Major IR, Mole RH. Myeloid leukaemia in x-ray irradiated CBA mice. Nature.
381 1978;272(5652):455-6. Epub 1978/03/30. doi: 10.1038/272455a0. PubMed PMID:
382 273147.
- 383 4. Rivina L, Davoren MJ, Schiestl RH. Mouse models for radiation-induced cancers.
384 Mutagenesis. 2016;31(5):491-509. doi: 10.1093/mutage/gew019. PubMed PMID:
385 27209205; PubMed Central PMCID: PMC6280996.
- 386 5. Delaney G, Jacob S, Barton M. Estimation of an optimal external beam radiotherapy
387 utilization rate for head and neck carcinoma. Cancer. 2005;103(11):2216-27. doi:
388 10.1002/cncr.21084. PubMed PMID: 15856428.
- 389 6. Hanoux V, Pairault C, Bakalska M, Habert R, Livera G. Caspase-2 involvement during
390 ionizing radiation-induced oocyte death in the mouse ovary. Cell Death Differ.
391 2007;14(4):671-81. doi: 10.1038/sj.cdd.4402052. PubMed PMID: 17082817.
- 392 7. Kerr JB, Hutt KJ, Michalak EM, Cook M, Vandenberg CJ, Liew SH, et al. DNA damage-
393 induced primordial follicle oocyte apoptosis and loss of fertility require TAp63-mediated
394 induction of Puma and Noxa. Mol Cell. 2012;48(3):343-52. doi:
395 10.1016/j.molcel.2012.08.017. PubMed PMID: 23000175; PubMed Central PMCID:
396 PMC3496022.
- 397 8. Adriaens I, Smitz J, Jacquet P. The current knowledge on radiosensitivity of ovarian
398 follicle development stages. Hum Reprod Update. 2009;15(3):359-77. doi:
399 10.1093/humupd/dmn063. PubMed PMID: 19151106.
- 400 9. Clement PB. Histology of the ovary. Am J Surg Pathol. 1987;11(4):277-303. doi:
401 10.1097/00000478-198704000-00006. PubMed PMID: 3565674.
- 402 10. Suh EK, Yang A, Kettenbach A, Bamberger C, Michaelis AH, Zhu Z, et al. p63 protects
403 the female germ line during meiotic arrest. Nature. 2006;444(7119):624-8. doi:
404 10.1038/nature05337. PubMed PMID: 17122775.
- 405 11. Morita Y, Perez GI, Paris F, Miranda SR, Ehleiter D, Haimovitz-Friedman A, et al.
406 Oocyte apoptosis is suppressed by disruption of the acid sphingomyelinase gene or by
407 sphingosine-1-phosphate therapy. Nat Med. 2000;6(10):1109-14. doi: 10.1038/80442.
408 PubMed PMID: 11017141.
- 409 12. Livera G, Petre-Lazar B, Guerquin MJ, Trautmann E, Coffigny H, Habert R. p63 null
410 mutation protects mouse oocytes from radio-induced apoptosis. Reproduction.
411 2008;135(1):3-12. doi: 10.1530/REP-07-0054. PubMed PMID: 18159078.
- 412 13. Kerr JB, Brogan L, Myers M, Hutt KJ, Mladenovska T, Ricardo S, et al. The primordial
413 follicle reserve is not renewed after chemical or gamma-irradiation mediated depletion.
414 Reproduction. 2012;143(4):469-76. doi: 10.1530/REP-11-0430. PubMed PMID: 22301887.
- 415 14. Hansen KR, Knowlton NS, Thyer AC, Charleston JS, Soules MR, Klein NA. A new
416 model of reproductive aging: the decline in ovarian non-growing follicle number from birth

417 to menopause. *Hum Reprod.* 2008;23(3):699-708. doi: 10.1093/humrep/dem408. PubMed
418 PMID: 18192670.

419 15. Wallace WH, Thomson AB, Kelsey TW. The radiosensitivity of the human oocyte.
420 *Hum Reprod.* 2003;18(1):117-21. doi: 10.1093/humrep/deg016. PubMed PMID: 12525451.

421 16. Meirrow D, Nugent D. The effects of radiotherapy and chemotherapy on female
422 reproduction. *Hum Reprod Update.* 2001;7(6):535-43. doi: 10.1093/humupd/7.6.535.
423 PubMed PMID: 11727861.

424 17. Kozak LJ, Owings MF, Hall MJ. National Hospital Discharge Survey: 2002 annual
425 summary with detailed diagnosis and procedure data. *Vital Health Stat* 13. 2005;(158):1-
426 199. PubMed PMID: 15853196.

427 18. Kon Y, Konno A, Hashimoto Y, Endoh D. Ovarian cysts in MRL/MpJ mice originate
428 from rete ovarii. *Anat Histol Embryol.* 2007;36(3):172-8. doi: 10.1111/j.1439-
429 0264.2006.00728.x. PubMed PMID: 17535347.

430 19. Vanputte C RJ, Russo A. *Seeley's Anatomy & Physiology*. 9th Edition ed2010.

431 20. group Sm. Ovarian cyst 2014. Available from:
https://www.summitmedicalgroup.com/library/adult_health/obg_ovarian_cysts/

432 21. Cole L. *ovaian cystic disorders* 2016. Available from:
<https://www.sciencedirect.com/topics/medicine-and-dentistry/follicle-cyst>.

433 22. Matsuda F, Inoue N, Manabe N, Ohkura S. Follicular growth and atresia in
434 mammalian ovaries: regulation by survival and death of granulosa cells. *J Reprod Dev.*
435 2012;58(1):44-50. Epub 2012/03/28. doi: 10.1262/jrd.2011-012. PubMed PMID:
436 22450284.

437 23. Tsilidis KK, Allen NE, Key TJ, Dossus L, Lukanova A, Bakken K, et al. Oral
438 contraceptive use and reproductive factors and risk of ovarian cancer in the European
439 Prospective Investigation into Cancer and Nutrition. *Br J Cancer.* 2011;105(9):1436-42. doi:
440 10.1038/bjc.2011.371. PubMed PMID: 21915124; PubMed Central PMCID: PMC3241548.

441 24. Fathalla MF. Incessant ovulation--a factor in ovarian neoplasia? *Lancet.*
442 1971;2(7716):163. doi: 10.1016/s0140-6736(71)92335-x. PubMed PMID: 4104488.

443 25. Xiao S, Shterev ID, Zhang W, Young L, Shieh JH, Moore M, et al. Sublethal Total Body
444 Irradiation Causes Long-Term Deficits in Thymus Function by Reducing Lymphoid
445 Progenitors. *J Immunol.* 2017;199(8):2701-12. doi: 10.4049/jimmunol.1600934. PubMed
446 PMID: 28931604; PubMed Central PMCID: PMC5659725.

447 26. Seed TM, Xiao S, Manley N, Nikolic-Zugich J, Pugh J, Van den Brink M, et al. An
448 interlaboratory comparison of dosimetry for a multi-institutional radiobiological research
449 project: Observations, problems, solutions and lessons learned. *Int J Radiat Biol.*
450 2016;92(2):59-70. Epub 2016/02/10. doi: 10.3109/09553002.2015.1106024. PubMed
451 PMID: 26857121; PubMed Central PMCID: PMCPMC4976771.

452 27. DiSaia PJ CW. *Clinical Gynecologic Oncology*. 9th ed: Elsevier; 2018.

453 28. Mishra B, Ripperdan R, Ortiz L, Luderer U. Very low doses of heavy oxygen ion
454 radiation induce premature ovarian failure. *Reproduction.* 2017;154(2):123-33. doi:
455 10.1530/REP-17-0101. PubMed PMID: 28528322; PubMed Central PMCID: PMC5598766.

456 29. Ortega HH, Marelli BE, Rey F, Amweg AN, Diaz PU, Stangaferro ML, et al. Molecular
457 aspects of bovine cystic ovarian disease pathogenesis. *Reproduction.* 2015;149(6):R251-64.
458 Epub 2015/03/15. doi: 10.1530/REP-14-0618. PubMed PMID: 25767139.

461 30. Cruz G, Fernandois D, Paredes AH. Ovarian function and reproductive senescence in
462 the rat: role of ovarian sympathetic innervation. *Reproduction*. 2017;153(2):R59-R68.
463 Epub 2016/11/02. doi: 10.1530/REP-16-0117. PubMed PMID: 27799628.

464 31. Tan OL, Hurst PR, Fleming JS. Location of inclusion cysts in mouse ovaries in relation
465 to age, pregnancy, and total ovulation number: implications for ovarian cancer? *J Pathol.*
466 2005;205(4):483-90. doi: 10.1002/path.1719. PubMed PMID: 15685692.

467 32. Brenner DJ, Doll R, Goodhead DT, Hall EJ, Land CE, Little JB, et al. Cancer risks
468 attributable to low doses of ionizing radiation: assessing what we really know. *Proc Natl*
469 *Acad Sci U S A*. 2003;100(24):13761-6. Epub 2003/11/12. doi: 10.1073/pnas.2235592100.
470 PubMed PMID: 14610281; PubMed Central PMCID: PMCPMC283495.

471 33. Scott BR. Low-dose radiation risk extrapolation fallacy associated with the linear-
472 no-threshold model. *Hum Exp Toxicol*. 2008;27(2):163-8. Epub 2008/05/16. doi:
473 10.1177/0960327107083410. PubMed PMID: 18480143.

474 34. Feeley KM, Wells M. Precursor lesions of ovarian epithelial malignancy.
475 *Histopathology*. 2001;38(2):87-95. doi: 10.1046/j.1365-2559.2001.01042.x. PubMed PMID:
476 11207821.

477 35. Radisavljevic SV. The pathogenesis of ovarian inclusion cysts and cystomas. *Obstet*
478 *Gynecol*. 1977;49(4):424-9. PubMed PMID: 854244.

479 36. Ahmed E, Young RH, Scully RE. Adult granulosa cell tumor of the ovary with foci of
480 hepatic cell differentiation: a report of four cases and comparison with two cases of
481 granulosa cell tumor with Leydig cells. *Am J Surg Pathol*. 1999;23(9):1089-93. Epub
482 1999/09/09. doi: 10.1097/00000478-199909000-00012. PubMed PMID: 10478669.

483 37. Irving JA, Young RH. Granulosa cell tumors of the ovary with a pseudopapillary
484 pattern: a study of 14 cases of an unusual morphologic variant emphasizing their
485 distinction from transitional cell neoplasms and other papillary ovarian tumors. *Am J Surg*
486 *Pathol*. 2008;32(4):581-6. Epub 2008/02/28. doi: 10.1097/PAS.0b013e31815c186f.
487 PubMed PMID: 18301054.

488 38. Nogales FF, Concha A, Plata C, Ruiz-Avila I. Granulosa cell tumor of the ovary with
489 diffuse true hepatic differentiation simulating stromal luteinization. *Am J Surg Pathol*.
490 1993;17(1):85-90. Epub 1993/01/01. doi: 10.1097/00000478-199301000-00011.
491 PubMed PMID: 7680545.

492 39. Jamieson S, Butzow R, Andersson N, Alexiadis M, Unkila-Kallio L, Heikinheimo M, et
493 al. The FOXL2 C134W mutation is characteristic of adult granulosa cell tumors of the ovary.
494 *Mod Pathol*. 2010;23(11):1477-85. Epub 2010/08/10. doi: 10.1038/modpathol.2010.145.
495 PubMed PMID: 20693978.

496 40. Pisarska MD, Barlow G, Kuo FT. Minireview: roles of the forkhead transcription
497 factor FOXL2 in granulosa cell biology and pathology. *Endocrinology*. 2011;152(4):1199-
498 208. Epub 2011/01/21. doi: 10.1210/en.2010-1041. PubMed PMID: 21248146; PubMed
499 Central PMCID: PMCPMC3206711.

500

501 **Acknowledgments**

502 We thank Dr. Scott Noakes (Center for applied isotope studies, University of Georgia) for setting
503 up the irradiation of mice. And thank Dr. Thomas Seed (Tech micro services Co, Bethesda, MD)
504 for the irradiator calibration.

505

506 **Authorship and Contributions**

507 S.X. designed the study, performed all experiments, analyzed data, and wrote the manuscript.
508 Y.X. performed statistical analysis on the data. X.X. analyzed H&E staining images. W.Z.
509 helped to perform all experiments and took care of mice. M.M.M. participated in discussions
510 about the data and wrote the manuscript. N.R.M. designed the study and discussed the results
511 and wrote the manuscript. All authors read and edited the manuscript before submission.

512

513 **Conflict of Interest Disclosure**

514 The authors declare no competing financial interests.

515

516 **Supporting information**

517

518 **S1 Dataset. Calibration of radiation dose rate.**

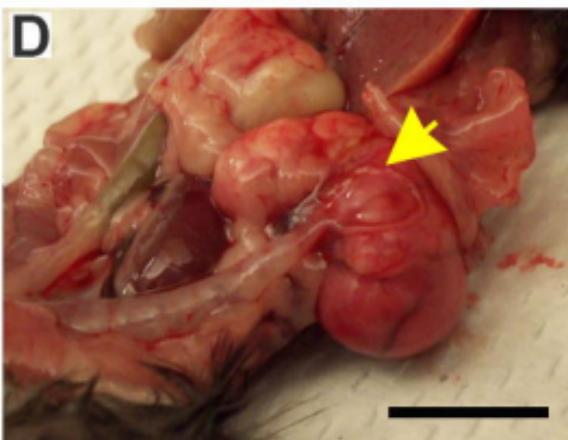
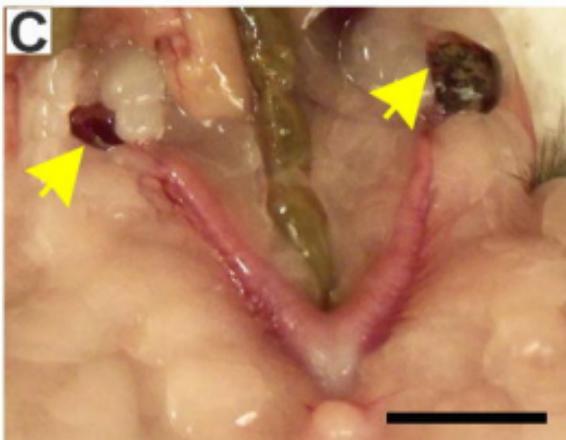
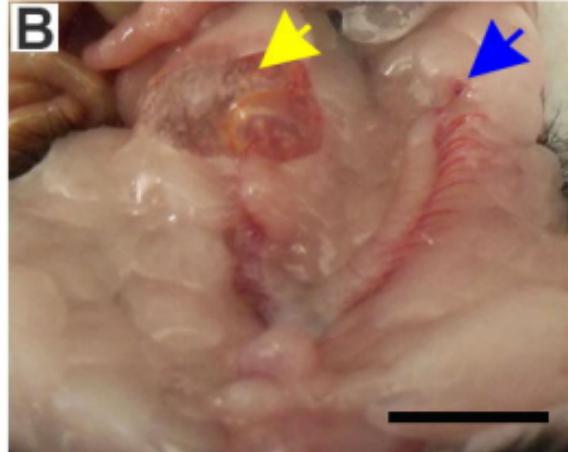
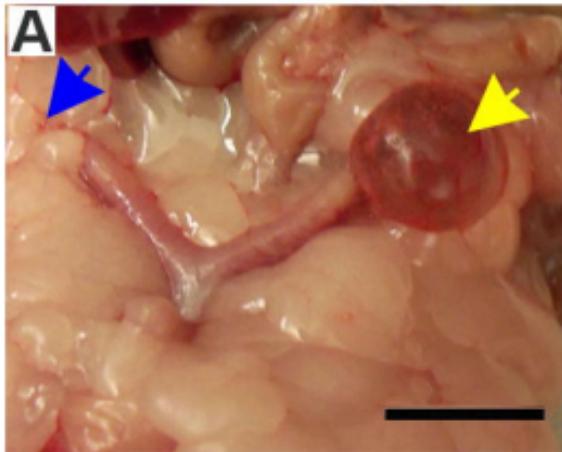
519 **S2 Dataset. Sample record of pathological changes in ovaries and other organs.**

520 **S3 Dataset. Report of statistical analysis result.**

521

522

523



4 X

20 X

4 X

20 X

