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Persistent Cell Proliferation of Terminal End Buds Precedes Radiation-induced Rat Mammary Carcinogenesis

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Abstract. *Background: The rat model of radiation-induced carcinogenesis is widely used to estimate breast cancer risk, but it is unknown whether mammary terminal end buds (TEBs), the hypothesized target of chemical carcinogens, are also targets of radiation-induced carcinogenesis. Materials and Methods: Female Sprague-Dawley rats were irradiated with X-rays (2 Gy). The morphology, bromodeoxyuridine (BrdU)-labelling index and pyknotic index of mammary ductal termini were investigated from 6 hours to 8 weeks post-irradiation. Results: The irradiated rats showed a significantly smaller decrease in TEB numbers than the controls 4-8 weeks after irradiation. The BrdU-labelling and pyknotic indices initially decreased and increased, respectively, recovered to control levels by 3 days post-irradiation, and remained significantly higher until 8 weeks and 4 weeks post-irradiation, respectively. One case of ductal hyperplasia was observed at 8 weeks. Conclusion: Irradiation resulted in a persistent proliferation of TEBs, which may be associated with neoplastic transformation via a temporal differentiation arrest.*

Ionizing radiation is a potent human carcinogen. A variety of epidemiological studies have shown that breast cancer is among the most frequent cancers that develop after radiation exposure and it is well established that ionizing radiation is a human breast carcinogen (1, 2). The rat has been used as a model to study the risk and mechanisms of breast carcinogenesis (3-5) because rat mammary cancers are comparable to human breast cancers in many respects, including hormone dependency and pathogenesis (6-8).

The initiation of mammary carcinogenesis by genotoxic chemicals has been intensively studied. The mammary glands grow rapidly during and after puberty, when the mammary ducts elongate and bifurcate to fill the subcutaneous mammary fat pads. This is a consequence of a controlled balance of cell proliferation and death within the terminal end bud (TEB), the club-shaped structure at the growing ductal terminus (9). These buds regress as the gland reaches full development and differentiate into terminal ducts and alveolar buds (10). Many lines of evidence suggest that TEBs are targets of chemical carcinogens. The susceptibility of the rat mammary gland to 7,12-dimethylbenz[*a*]anthracene (DMBA)- and 1-methyl-1-nitrosourea (MNU)-induced carcinogenesis is highest during the maturation period when numbers of TEBs are present (10, 11). In rats treated with MNU, the TEBs show pathological changes such as delayed regression and a high proliferation index (12, 13). Five to 12 weeks after MNU injection, most of the hyperplastic and premalignant lesions develop from TEBs (14). The susceptibility of TEB cells to carcinogenesis may be associated with their high proliferative activity and low degree of differentiation (5).

Despite our considerable knowledge of the role of TEBs in chemical carcinogenesis, little is known about the target structure of radiation carcinogenesis in the mammary gland. One report described hyperplastic alveolar nodules (HANs) that were induced by X-irradiation (15). Later, however, it was shown that HANs are not precursor lesions of rat mammary carcinoma (8). To test whether TEBs are a target of radiation carcinogenesis, the morphology and cell kinetics of TEBs after radiation exposure were examined.

Materials and Methods

Animals. Five-week-old female Sprague-Dawley rats (Jcl:SD; Clea Japan Inc., Tokyo, Japan) were housed in autoclaved cages with wood chips, in a room with controlled temperature (23±1˚C) and humidity (50±5%), under a regular 12-hour light/12-hour dark cycle. The rats were fed a standard laboratory diet (CE-2; Clea Japan) and were given water *ad libitum*. All the animals were treated according to the Guidelines on Animal Experiments of the National Institute of Radiological Sciences, Japan.

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Experimental schedule. At 7 weeks of age, half of the animals (n=21) were irradiated with 2 Gy of X-rays generated from an HF-320 X-ray machine (Pantak Inc., East Haven, CT, USA; 200 kVp, 20 mA, 0.725 Gy/min) with 0.5-mm copper and 0.5-mm aluminum filters. The other half (n=21) were left untreated as a control group. Irradiated and untreated rats (n=3 each) were sacrificed at 6 hours, 1 day, 3 days, 1 week, 2 weeks, 4 weeks and 8 weeks after irradiation. 5-Bromo-2′-deoxyuridine (BrdU, 50 mg/kg; Calbiochem, San Diego, CA, USA) was injected intraperitoneally (*i.p.*) 2 hours before sacrifice.

Whole-mount and sectioned specimens. Inguinal and abdominal mammary glands were extended on glass slides and fixed in 10% neutral buffered formalin overnight. The fixed glands were defatted in 70% alcohol and acetone, stained with carmine alum solution, dehydrated through an alcohol series and *d*-limonene, and stored in mineral oil (Sigma, St. Louis, MO, USA). Photomicrographs were taken with a DC-70 digital CCD camera (Olympus Corporation, Tokyo, Japan). Areas outlined by the front tips of the mammary ductal network (one abdominal-inguinal mammary chain for each rat), as well as the diameters of ductal termini (n=40 for each rat) within 10-mm^2 fields of abdominal mammary glands, were measured on photomicrographs. Based on previous measurements (10), a TEB was defined as a ductal terminus that was ≥80 μm in diameter. Regions containing ductal termini were excised, embedded in paraffin, sectioned at 3-4 μm and used for standard hematoxylin-eosin (HE) and BrdU staining.

Cell kinetics. Incorporation of BrdU was detected immunohistochemically (Bromodeoxyuridine Immunohistochemistry System, Oncogene Research Products, San Diego, CA, USA). Stained epithelial cells, as well as cells with pyknotic nuclei, were counted in sections of each ductal terminus, and these numbers were divided by the total number of epithelial cells in the same field (1,700 cells/terminus, 10 termini/experimental group on average). The ApopTag *In Situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA, USA) was used, according to the manufacturer's instructions, for DNA nick end-labelling.

Results

TEBs persisted in irradiated rats. To investigate early changes in irradiated TEBs before the induction of mammary carcinomas, the rats were irradiated with a carcinogenic dose of X-rays (2 Gy) and were sacrificed at 6 hours to 8 weeks after irradiation. This dose of radiation is known to result in an ~50% incidence of mammary carcinoma within a year (16). The body weights of the irradiated rats were not different from those of the untreated controls (Figure 1A). Similarly, the extent of mammary gland growth, as indicated by the area of the mammary ducts, was not influenced by radiation exposure (Figure 1B). In control rats, the TEBs regressed normally with age and differentiated into terminal ducts and alveolar buds (Figure 2A-G). Interestingly, at later time-points, the size of the regressing terminal structures in irradiated rats was consistently larger than that of the control rats (Figure 2D-G, K-N). A morphometric analysis revealed the statistical significance of this difference (Figure 1C). Consistent with these data, the number of TEBs/mm^2 was not altered by irradiation, up to 2 weeks post-irradiation, but was significantly higher in the irradiated rats at 4 and 8 weeks post-irradiation than it was in the controls (Figure 1D). Thus, the TEBs persisted after irradiation as a consequence of their impaired regression.

The persistence of TEBs was associated with sustained cell proliferation. To examine if the persistence of the TEBs was related to altered cell kinetics, the BrdU-labelling index was determined to ascertain cell proliferation in the mammary duct terminal structures (Figure 3A). Initially, the TEBs showed a highly proliferative state with a BrdU-labelling index of 15-20% before irradiation. The index dropped significantly to 1-3% at 6 hours and 1 day after irradiation, indicating immediate cell cycle arrest. This was followed by a rapid recovery in 3 days to ~20%. Thereafter, the BrdU-labelling index maintained a proliferative level (15-20%) for several weeks in the irradiated rats, but in the control rats it gradually decreased to ~5%, as untreated TEBs regressed with age. We unsuccessfully attempted to label apoptotic cells by DNA nick end-labelling of whole-mount specimen samples, probably due to the fragmentation of DNA molecules during the course of specimen preparation and storage. Therefore, the cell death index was investigated by counting cells with pyknotic nuclei (Figure 3B), though this method is usually insensitive compared to the nick end-labelling method (17). Prior to irradiation, the percentage of cells with pyknotic nuclei was 2-3%; it then increased to ~6% at 6 hours post-irradiation, although this increase was not statistically significant. Then, the percentage of pyknotic cells returned to the control level of 2–3% by day 1. The control group showed a slight decrease to ~1% by 4 weeks, whereas the irradiated group remained at a significantly higher level (~2%). At 8 weeks post-irradiation, the pyknotic index was ~1% in both groups.

A ductal hyperplasia focus developed at 8 weeks post-irradiation. Persistent TEBs found in irradiated rats were morphologically normal and showed no signs of precancerous change (Figure 4A). One of three irradiated rats that were examined 8 weeks post-irradiation did, however, develop a focus of ductal hyperplasia, whereas none of the three untreated rats that were examined in parallel developed such lesions. As observed in the whole-mount preparation (Figure 4B), the focus consisted of hyperplastic ducts with very frequent branching and TEB-like termini. HE-stained sections of the lesion showed irregularly-shaped, multilayered ducts with intraductal papillary structures (Figure 4C, D).

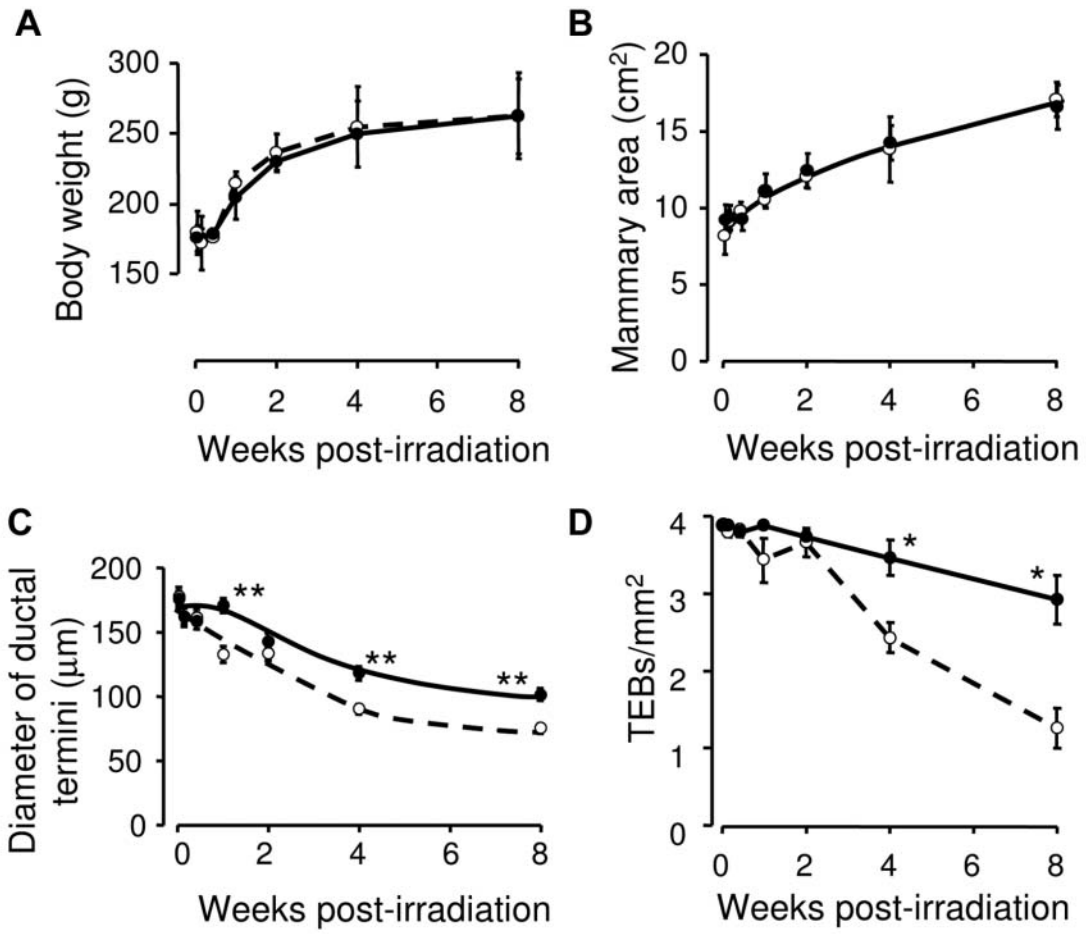

Figure 1. *Body weight, mammary gland growth, diameter of mammary ductal termini and the number of TEBs in control (open circles) and irradiated (filled circles) rats (n=3 each). A. Body weight at the time of sacrifice was not different between control and irradiated rats. B. Area delineated by the end tips of the mammary ducts, which is indicative of the extent of mammary gland growth, was not altered by irradiation. C. The diameter of ductal termini (n=120) decreased with age to a larger extent in control rats than in irradiated rats. D. The number of TEBs/mm^2 in whole-mount preparations was less in the control group than in the irradiated group at 4 and 8 weeks post-irradiation. The mean±SE is shown. *p<0.05, **p<0.001 irradiated vs. control by t-test.*

Discussion

In the present study, the early changes in mammary TEBs of rats given a carcinogenic dose of X-rays were investigated. The change consisted of two phases: the first was observed from 6 hours to 1 day post-irradiation and was characterized by suppressed cell proliferation and increased cell death. The second phase was observed at 4 to 8 weeks post-irradiation and was marked by the persistence of TEBs within the mammary ductal network, with sustained cell proliferation. A ductal hyperplasia was observed in one rat at 8 weeks post-irradiation.

Early changes 1-3 days after carcinogenic exposure were reported by Sharkey and Bruce (18) in a previous study using the mouse mammary gland. Their report that nuclear aberrations in TEBs had increased after irradiation until 24 hours and then had decreased by 36 hours is consistent with our observations, but they did not examine changes thereafter. We showed that TEBs from 2 weeks post-irradiation forward exhibited an increased number, increased proliferation (BrdU-labelling index 15-20%) and more cell death (pyknotic index 2-3%). The development of a hyperplastic lesion was also observed. Delayed regression and a high proliferation index have been reported in TEBs of rats treated with MNU (12, 13). A hyperplastic lesion, similar to the one that we observed, was also reported for MNU-treated rats (14). It has been established that ductal hyperplasia represents a premalignant step in rat mammary carcinogenesis (8). Thus, radiation induces differentiation arrest and persistent proliferation of TEB cells, which may lead to preneoplastic changes.

A report that massive radiation-induced cell death of the intestinal epithelium is followed by a proliferative burst of stem cells that persists for more than a week (19) resembles our observation in mammary TEBs. Stimulation by intestinal

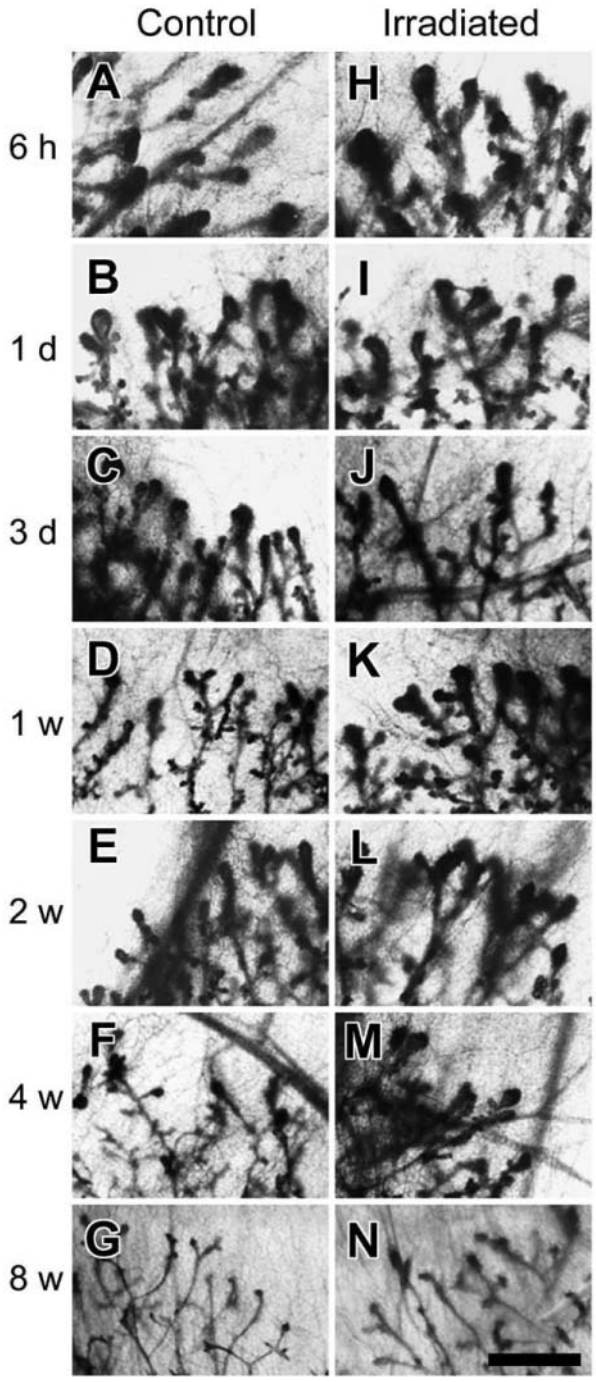

Figure 2. *Photomicrographs of mammary ductal termini stained with carmine alum in whole-mount preparations. Rats were irradiated at 7 weeks of age and were sacrificed at 6 hours (h), 1 day (d), 3 days, 1 week (w), 2 weeks, 4 weeks and 8 weeks after irradiation (H–N, respectively). (A-G) Control rats of the same age. Bar=1 mm.*

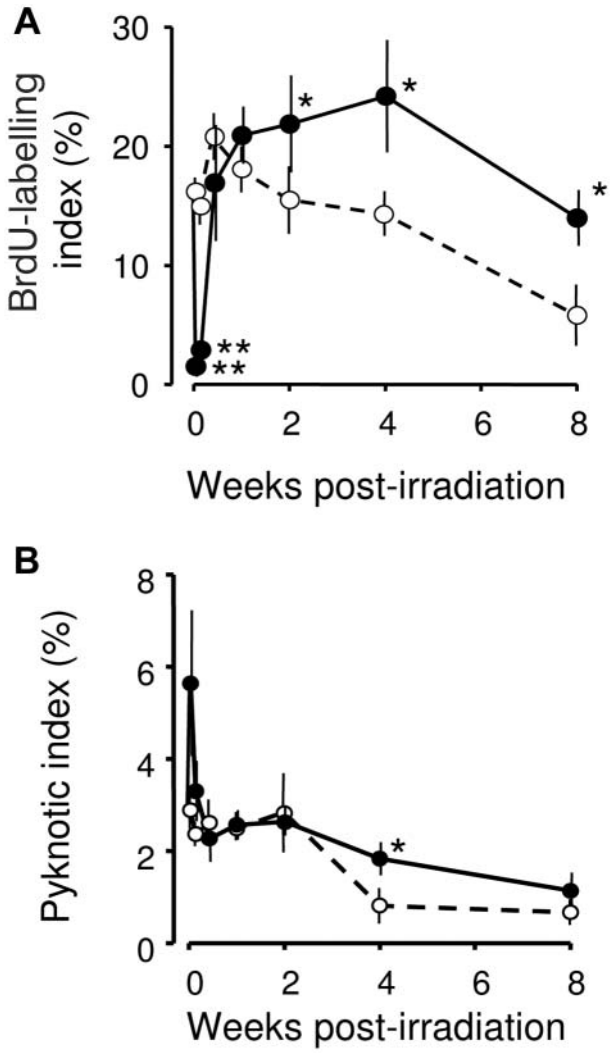

Figure 3. *Cell kinetics of mammary ductal termini. A. The index for BrdU staining was examined as an indicator of cell proliferation. B. Changes in the pyknotic cell count were examined. Open circles, control rats; filled circles, irradiated rats. The mean±SE (n=8-13 termini) is shown. *p<0.05, **p<0.001 irradiated vs. control by Mann-Whitney U-test.*

growth factors was involved in the process of regeneration of the intestine after irradiation (20). Similarly, the growth of mammary TEBs was regulated by a wide range of growth factors, and irradiation altered the expression of various growth factors (*e.g.*, transforming growth factor α) and activated their receptors (*e.g.*, epidermal growth factor receptors and estrogen receptors) in breast cancer cells (21, 22). The persistent proliferation of TEBs, therefore, might be a result of growth factor production after irradiation.

Some evidence suggests that ionizing radiation induces mammary carcinogenesis independently of TEBs. Susceptibility to radiation-induced mammary carcinogenesis is not reduced at 22-36 weeks of age or during lactation, when TEBs have already regressed (4, 23). These studies suggested that radiation-induced mammary cancers can also develop from structures other than TEBs, such as terminal

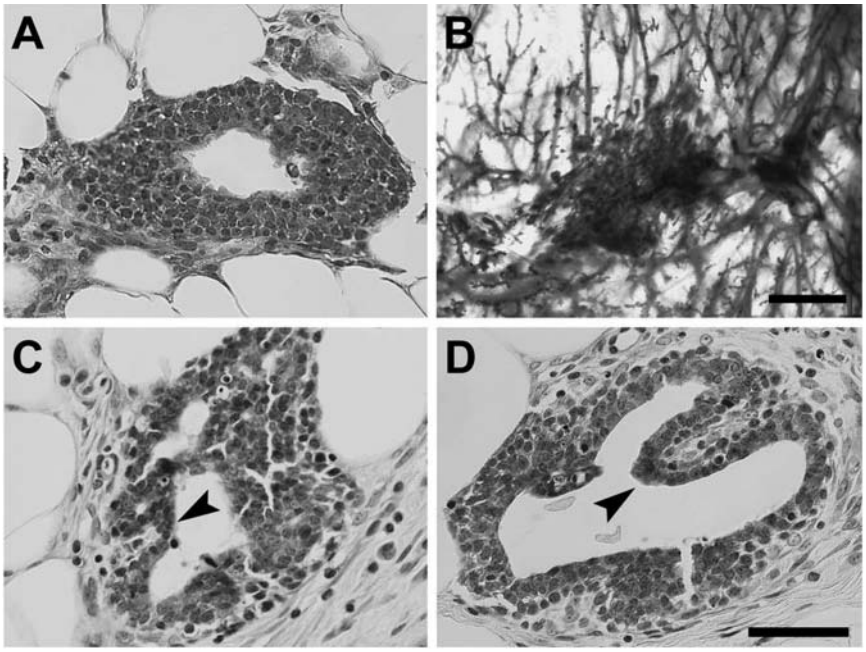

Figure 4. *Normal and preneoplastic mammary ducts observed in irradiated rats. A. A mammary terminal end bud from a rat at 4 weeks post-irradiation. B. A lesion of ductal hyperplasia observed in a whole-mount preparation from a rat at 8 weeks post-irradiation. Bar=2 mm. C and D. HE-stained sections of ducts from the lesion shown in B. Arrowheads indicate intraductal papillary structures. Bar in D=50 μm (for A, C and D).*

ducts or alveoli. Our results, however, support the idea that TEBs can be one target of radiation carcinogenesis, at least when young animals are irradiated. Taken together, our results suggest that the radiation-induced persistent proliferation of mammary TEBs that lasts for several weeks is associated with preneoplastic progression.

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References

1 Ronckers CM, Erdmann CA and Land CE: Radiation and breast cancer: a review of current evidence. Breast Cancer Res *7*: 21-32, 2004.

2 Land CE, Tokunaga M, Koyama K, Soda M, Preston DL, Nishimori I and Tokuoka S: Incidence of female breast cancer among atomic bomb survivors, Hiroshima and Nagasaki, 1950-1990. Radiat Res *160*: 707-717, 2003.

3 Shellabarger CJ, Cronkite EP, Bond VP and Lippincott SW: The occurrence of mammary tumors in the rat after sublethal whole-body irradiation. Radiat Res *6*: 501-512, 1957.

4 Bartstra RW, Bentvelzen PA, Zoetelief J, Mulder AH, Broerse JJ and van Bekkum DW: Induction of mammary tumors in rats by single-dose gamma irradiation at different ages. Radiat Res *150*: 442-450, 1998.

5 Pei RJ, Sato M, Yuri T, Danbara N, Nikaido Y and Tsubura A: Effect of prenatal and prepubertal genistein exposure on *N*-methyl-*N*-nitrosourea-induced mammary tumorigenesis in female Sprague-Dawley rats. In Vivo *17*: 349-357, 2003.

6 Gould MN: Rodent models for the study of etiology, prevention and treatment of breast cancer. Semin Cancer Biol *6*: 147-152, 1995.

7 Nandi S, Guzman RC and Yang J: Hormones and mammary carcinogenesis in mice, rats, and humans: a unifying hypothesis. Proc Natl Acad Sci USA *92*: 3650-3657, 1995.

8 Thompson HJ and Singh M: Rat models of premalignant breast disease. J Mammary Gland Biol Neoplasia *5*: 409-420, 2000.

9 Humphreys RC, Krajewska M, Krnacik S, Jaeger R, Weiher H, Krajewski S, Reed JC and Rosen JM: Apoptosis in the terminal endbud of the murine mammary gland: a mechanism of ductal morphogenesis. Development *122*: 4013-4022, 1996.

10 Russo J, Tay LK and Russo IH: Differentiation of the mammary gland and susceptibility to carcinogenesis. Breast Cancer Res Treat *2*: 5-73, 1982.

11 Ariazi JL, Haag JD, Lindstrom MJ and Gould MN: Mammary glands of sexually immature rats are more susceptible than those of mature rats to the carcinogenic, lethal, and mutagenic effects of *N*-nitroso-*N*-methylurea. Mol Carcinog *43*: 155-164, 2005.

12 Korkola JE and Archer MC: Resistance to mammary tumorigenesis in Copenhagen rats is associated with the loss of preneoplastic lesions. Carcinogenesis *20*: 221-227, 1999.
13 Osborne MP, Ruperto JF, Crowe JP, Rosen PP and Telang NT: Effect of tamoxifen on preneoplastic cell proliferation in *N*-nitroso-*N*-methylurea-induced mammary carcinogenesis. Cancer Res *52*: 1477-1480, 1992.
14 Shilkaitis A, Green A, Steele V, Lubet R, Kelloff G and Christov K: Neoplastic transformation of mammary epithelial cells in rats is associated with decreased apoptotic cell death. Carcinogenesis *21*: 227-233, 2000.
15 Faulkin LJ Jr, Shellabarger CJ, DeOme KB: Hyperplastic lesions of Sprague-Dawley rat mammary glands after X irradiation. J Natl Cancer Inst *39*: 449-458, 1967.
16 Imaoka T, Nishimura M, Teramoto A, Nishimura Y, Ootawara M, Osada H, Kakinuma S, Maekawa A and Shimada Y: Cooperative induction of rat mammary cancer by radiation and 1-methyl-1-nitrosourea *via* the oncogenic pathways involving c-Myc activation and H-ras mutation. Int J Cancer *115*: 187-193, 2005.
17 Gavrieli Y, Sherman Y and Ben-Sasson SA: Identification of programmed cell death *in situ via* specific labeling of nuclear DNA fragmentation. J Cell Biol *119*: 493-501, 1992.
18 Sharkey SM and Bruce WR: Quantitation of nuclear aberrations as a screen for agents damaging to mammary epithelium. Carcinogenesis *7*: 1991-1995, 1986.
19 Potten CS: A comprehensive study of the radiobiological response of the murine (BDF1) small intestine. Int J Radiat Biol *58*: 925-973, 1990.
20 Ruifrok AC, Weil MM, Mason KA and Thames HD: Induction of transforming growth factor alpha in irradiated mouse jejunum. Int J Radiat Oncol Biol Phys *42*: 1137-1146, 1998.
21 Schmidt-Ullrich RK, Valerie K, Chan W, Wazer DE and Lin PS: Expression of oestrogen receptor and transforming growth factor-alpha in MCF-7 cells after exposure to fractionated irradiation. Int J Radiat Biol *61*: 405-415, 1992.
22 Bowers G, Reardon D, Hewitt T, Dent P, Mikkelsen RB, Valerie K, Lammering G, Amir C and Schmidt-Ullrich RK: The relative role of ErbB1-4 receptor tyrosine kinases in radiation signal transduction responses of human carcinoma cells. Oncogene *20*: 1388-1397, 2001.
23 Inano H, Suzuki K, Onoda M and Yamanouchi H: Susceptibility of fetal, virgin, pregnant and lactating rats for the induction of mammary tumors by gamma rays. Radiat Res *145*: 708-713, 1996.

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