N-methyl-*N*-nitrosourea-induced mammary carcinogenesis is promoted by short-term treatment with estrogen and progesterone mimicking pregnancy in aged female Lewis rats

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Abstract. We examined the effects of short-term estrogen and progesterone treatment mimicking pregnancy in aged female Lewis rats on the development of *N*-methyl-*N*-nitrosourea (MNU)-induced mammary carcinoma. Rats were administered a single intraperitoneal injection of 20 mg/kg MNU at 7 weeks of age and half of those rats were administered a sub-cutaneously implanted 21-day release pellet containing 0.5 mg 17ß-estradiol and 32.5 mg progesterone (E/P) at 24 weeks of age. The rats were then monitored for the occurrence of mammary tumors. Rats were sacrificed when the largest mammary tumor became ≥1 cm in diameter, or when the rat reached 48 weeks of age. Development of MNU-induced mammary carcinomas was accelerated after short-term E/P treatment, compared with E/P-untreated rats: the incidence of ≥1-cm mammary carcinomas tended to increase (60 vs. 44%); the latency tended to shorten (28.7 vs. 34.6 weeks); and cancer multiplicity (number of all-sized carcinomas per rat) significantly increased (1.8 vs. 0.8). In E/P-treated rats, comedo necrosis was frequently seen and the incidence of estrogen receptor and/or progesterone receptor-negative mammary carcinomas was significantly increased. Early age at full-term pregnancy or short-term hormone treatment mimicking pregnancy may suppress the risk of breast cancer, but the age of hormone exposure is a crucial factor, because hormone exposure mimicking pregnancy in aged individuals may exert effects opposite of those exerted in younger individuals.

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Introduction

Young age at full-term pregnancy is inversely related to the risk of breast cancer in women (1-4). The protective effect of early full-term pregnancy against breast cancer is universal for humans. Early full-term pregnancy is the only normal physiological condition that consistently prevents breast cancer in all ethnic groups without known side effects. Before the age of 35 years, any human full-term pregnancy is protective against breast cancer (1-3). This protective effect of pregnancy has also been seen in rats and mice (5,6). A full-term pregnancy or pregnancy and lactation before or soon after carcinogen exposure significantly reduces overall mammary cancer incidence and multiplicity in female rats (5,7). The protective effect of pregnancy against mammary cancer can be mimicked in rats by short-term treatment (approximately 21 days; the gestation period of rats is 21 days) with estrogen and progesterone. Short-term treatment of young adult rats (≤3 months old) with estrogen plus progesterone is highly effective in suppressing mammary carcinogenesis (8-15).

Mimicking the pregnancy environment by treating nulliparous women with estrogen and progesterone may be a useful method of suppressing the risk of breast cancer. However, after the age of 35 years, any full-term pregnancy appears to increase the risk of breast cancer, compared to age-matched nulliparous women (1-3,16). Parity-induced protection against breast cancer appears to be related to the age at the first full-term pregnancy. Moreover, pregnancy has a dual effect: early full-term pregnancy reduces the overall risk of breast cancer in later years, but it transiently increases the risk immediately after childbirth (3,17). In rats, while the overall mammary cancer incidence is reduced, the appearance of chemically induced mammary carcinomas is transiently increased during pregnancy (7), which is consistent with related findings for humans. There is no conclusive evidence as to how the risk of mammary cancer in aged rats is affected by pregnancy late in life or hormonal exposure mimicking pregnancy late in life. The effect of timing of estrogen and progesterone exposure on the occurrence of mammary carcinoma in aged rats is an issue that could have important implications for humans. The aim of the present

study was to assess the effects of short-term estrogen and progesterone exposure mimicking pregnancy late in life on the development of *N*-methyl-*N*-nitrosourea (MNU)-induced mammary carcinomas in aged female Lewis rats. We compared the incidence, multiplicity and latency of MNU-induced mammary carcinomas between hormone-treated and untreated rats. We also evaluated the characteristics of mammary carcinomas that developed in aged hormone-treated rats.

Materials and methods

Animals. Lewis rats were purchased from Charles River Japan (Atsugi, Japan). The animals were housed in plastic cages with wood-chip bedding, 3 to 4 rats/cage, at a room temperature- $(22 \pm 2^{\circ}C)$ and humidity- $(60 \pm 10\%)$ controlled animal room under a 12-h light/dark cycle. They were fed a commercial pellet diet (CMF; Oriental Yeast, Chiba, Japan) and water freely throughout the experiment. All procedures performed on experimental animals were approved by the Animal Experimentation Committee of Kansai Medical University.

Experimental procedure. At 7 weeks of age, 58 rats were injected intraperitoneally with 20 mg/kg MNU. The MNU was purchased from Chem Service (West Chester, PA, USA), stocked at -20°C in the dark and dissolved in physiological saline containing 0.05% acetic acid immediately before being injected. At 24 weeks of age, the rats were checked for palpable mammary tumors, all tumor-bearing rats (n=3) were eliminated from the study and the following procedures were performed. Of the remaining 55 rats, we arbitrarily selected 5 rats for sacrifice to examine their mammary glands. Half of the remaining 50 rats (E/P group; n=25), selected randomly, were implanted subcutaneously in the back with a slow-releasing pellet containing 0.5 mg 17ß-estradiol and 32.5 mg progesterone (Innovative Research of America, Sarasota, FL, USA). This pellet provides a steady release of the hormones for a 21-day period. The remaining 25 rats comprised the control group.

Mammary tumor detection and sacrifice. The rats were checked regularly once a week for mammary tumors by palpation. Rats were sacrificed when their largest mammary tumor reached a diameter of ≥1 cm. The rats that did not develop mammary tumors with a diameter of ≥1 cm were sacrificed 41 weeks after the MNU injection (at 48 weeks of age).

Histological examination. Mammary tumors, non-tumoral mammary glandular tissue and macroscopically abnormal organs and tissues were removed at autopsy, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned (thickness, 4 μm) and stained with hematoxylin and eosin (HE) for histological examination. Mammary tumors were classified according to criteria described elsewhere (18).

Immunohistochemistry. All ≥1-cm mammary carcinomas underwent immunohistochemical assays. Assays for expression of keratin 14 (K14; basal cell keratin), estrogen receptor (ER) α and the progesterone receptor (PgR) were performed using the labeled streptavidin-biotin (LSAB) method with an LSAB
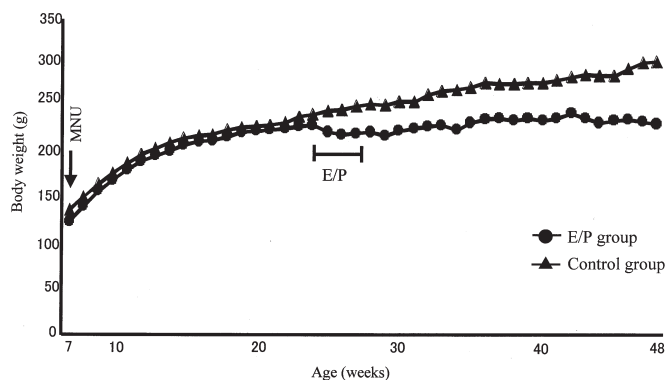

Figure 1. Body weight gain in female Lewis rats injected intraperitoneally with *N*-methyl-*N*-nitrosourea (MNU) at 7 weeks of age, half of which were subcutaneously implanted with a 21-day-release estrogen and progesterone (E/P) pellet at 24 weeks of age (E/P group) and the other half of which were left hormone-untreated (control group).
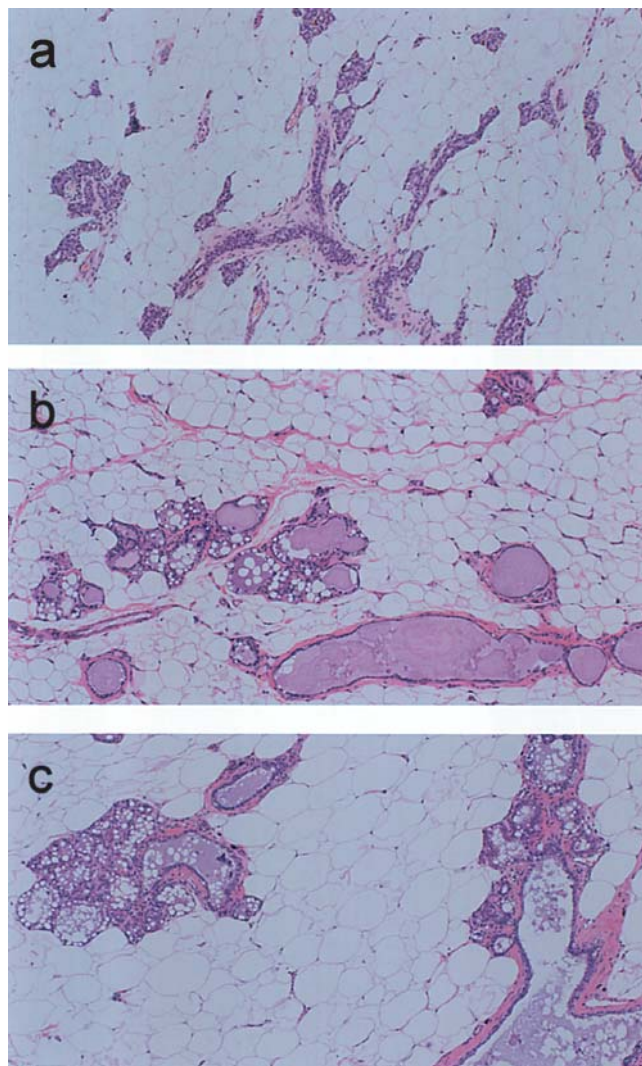

Figure 2. Mammary glands of female Lewis rats injected intraperitoneally with *N*-methyl-*N*-nitrosourea at 7 weeks of age. (a) Small lobules and thin ducts are visible (age, 24 weeks). (b) Estrogen and progesterone (E/P)-treated rat shows developed alveolar cells exhibiting secretory vacuoles and dilatated duct filled with secretion (age, 48 weeks). (c) Estrogen and progesterone (E/P)-untreated control rat shows grade of differentiation similar to that of age-matched E/P-treated rat (age, 48 weeks).
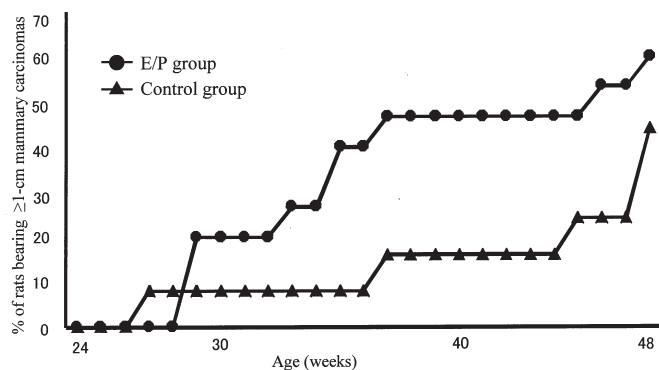
Figure 3. Effects of estrogen and progesterone (E/P) treatment on the cumulative incidence of *N*-methyl-*N*-nitrosourea (MNU)-induced mammary carcinomas with a diameter ≥1 cm in female Lewis rats.
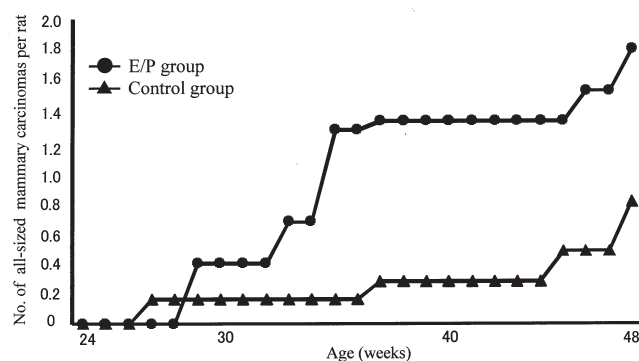

Figure 4. Effects of estrogen and progesterone (E/P) treatment on the cumulative number of *N*-methyl-*N*-nitrosourea (MNU)-induced mammary carcinomas of all sizes per rat.

Table I. Effects of short-term estrogen and progesterone treatment of aged female Lewis rats on occurrence of MNU-induced mammary carcinomas.

Group	No. of rats examined	No. of rats with ≥l-cm carcinomas (%)	No. of all-sized carcinomas	All-sized carcinomas per rat	Latency (weeks)
E/P group	15	9 (60)	27	1.8±0.5[a]	28.7±1.8
Control group	25	11 (44)	20	0.8±0.2	34.6±1.7

[a]$P<0.05$ compared with control group.

staining kit (Dako, Carpinteria, CA, USA) according to the manufacturer's instructions. The primary antibodies used for K14, ERα and PgR were LL004 (Novocastra, Newcastle upon Tyne, UK), 6F11 (Novocastra) and 10A9 (Biodesign, Saco, ME, USA), respectively. For visualization of positive staining, the antigen retrieval technique was performed using a citrate buffer (pH 6.0) and a microwave oven. Tumors in which >80% of cells were positive for ER or PgR were considered positive for the respective receptor (5).

Statistical analysis. All values were expressed as mean ± standard error (SE). The final body weight, multiplicity and latency were analyzed using the 2-tailed, independent Student's t-test for unpaired samples after assuring homogeneity of variance. The incidence of mammary carcinoma and the incidence of ER- and/or PgR-positive carcinoma were analyzed using the Chi-square test. A probability value of p<0.05 was considered to indicate statistical significance.

Results

Body weight gain. During the study, 10 rats in the E/P group died and were excluded from the analysis. Body weight gain was less in the E/P group than in the control group (Fig. 1) and the final body weight of the E/P group was significantly less than that of the control group (234±3 vs. 303±8 g; p<0.01).

Structure of normal mammary glands. In the 5 rats sacrificed at 24 weeks of age (time of E/P pellet implantation; 17 weeks

after MNU), no microscopic or macroscopic tumors were detected and the mammary glands were composed of thin ducts with small lobules (Fig. 2a). In the rats sacrificed at 48 weeks of age (end of the experiment; 41 weeks after MNU), mammary glands of both the E/P (Fig. 2b) and control groups (Fig. 2c) showed developed alveolar cells with secretory vacuoles and dilatated ducts filled with secretory product.

Development of mammary carcinomas. All mammary tumors were confirmed histologically to be papillary, cribriform or comedo carcinomas. The E/P group developed ≥1-cm carcinomas more rapidly than the control group (Fig. 3) and had a 60% incidence of ≥1-cm mammary carcinomas, with a mean latency of 28.7 weeks (Table I). In contrast, the control group had a 44% incidence of ≥1-cm mammary carcinomas, with a mean latency of 34.6 weeks. E/P treatment increased the incidence and shortened the latency of ≥1-cm mammary carcinomas. To evaluate cancer multiplicity, we compared the number of all-sized carcinomas per rat between the groups. The E/P group developed all-sized carcinomas more rapidly (Fig. 4) and had a significantly greater number of all-sized carcinomas per rat (1.8 vs. 0.8) (Table I). Thus, short-term E/P treatment of aged rats accelerated their development of MNU-induced mammary carcinomas and significantly increased their multiplicity.

Characteristics of ≥1-cm mammary carcinomas. In the E/P group, the morphology of ≥1-cm carcinomas was mainly comedo (13/19) or papillary (6/19). In the control group, the ≥1-cm carcinomas were mainly cribriform (12/16), with a few
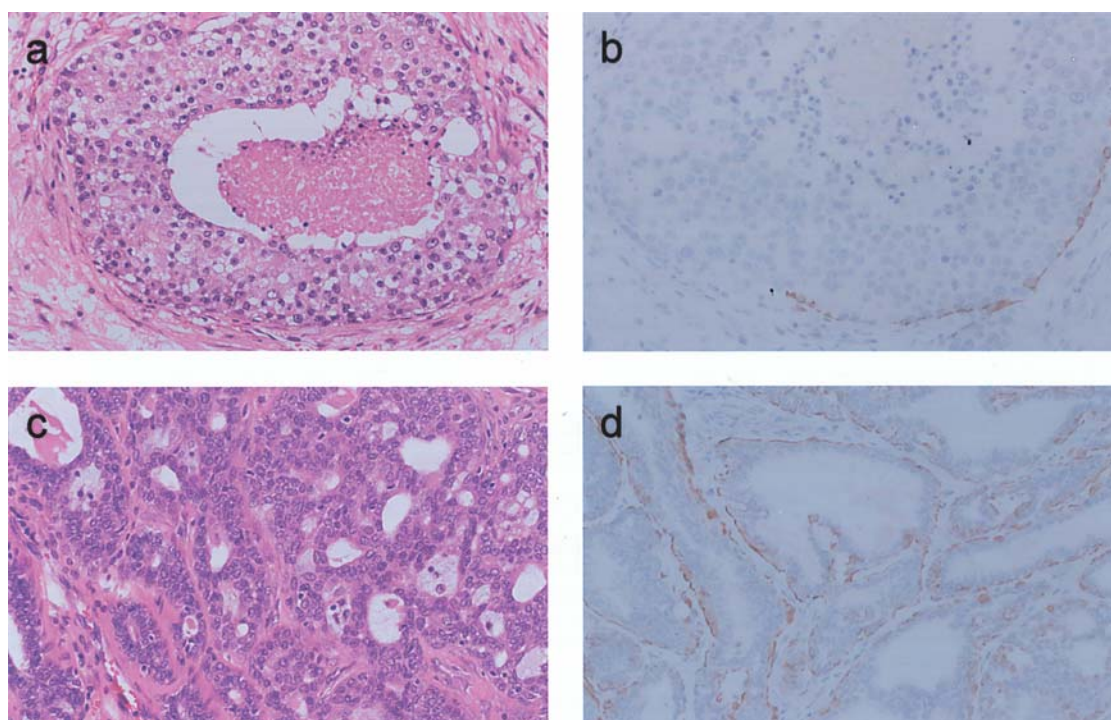
Figure 5. *N*-methyl-*N*-nitrosourea (MNU)-induced mammary carcinomas. (a) Comedo carcinoma shows necrotic cell debris (HE). (b) Basal cells focally surround cancer cell nests of comedo carcinoma (Keratin 14). (c) Cribriform carcinoma shows secondary lumina (HE). (d) Basal cells regularly surround cancer cell nests of cribriform carcinoma (Keratin 14).

Table II. Effects of short-term estrogen and progesterone treatment of aged female Lewis rats on immunohistochemical expression of ER and PgR on MNU-induced ≥1-cm mammary carcinomas.

Group	No. of carcinomas examined	No. of ER-positive carcinomas (%)	No. of PgR-positive carcinomas (%)
E/P group	19	5 (26)[a]	2 (11)[a]
Control group	16	14 (88)	10 (63)

[a]$P<0.01$ compared with control group.

papillary (4/16). Comedo necrosis was frequently seen in the E/P group. Cancer cell nests of comedo carcinoma accompanied by necrosis (Fig. 5a) were focally surrounded by scattered basal (myoepithelial-like) cells (Fig. 5b), whereas papillary and cribriform carcinomas (Fig. 5c) were more regularly surrounded by basal cells (Fig. 5d). Immuno-histochemically, the E/P group developed significantly fewer ER- and PgR-positive carcinomas (26 and 11%, respectively) than the control group (88 and 63%, respectively) (Table II). The only other abnormalities that were observed were uterine leiomyosarcoma and bilateral ovarian granulosa cell tumor; the former was found in a 43-week-old E/P rat and the latter was found in a 48-week-old E/P rat.

Discussion

Ovariectomy inhibits the development of mammary carcinomas, indicating that physiological levels of ovarian steroids are required for mammary carcinogenesis (19). However, mammary carcinogenesis can also be effectively suppressed by high physiologic levels of ovarian steroids during pregnancy (5,7), or short-term treatment with pregnancy levels of estrogen and progesterone (8-15). Estrogen treatment alone can provide protection from mammary carcinogenesis, but it is more effective in combination with progesterone (12). The hormonal milieu of pregnancy is involved in the protection against mammary carcinogenesis (20). However, whereas combined estrogen/progesterone treatment for ≤20 weeks has a pronounced protective effect against mammary carcinogenesis (21), treatment for >20 weeks has a markedly weaker effect (15) and even in parous rats, treatment for 33 weeks has a negligible effect (22). Thus, the duration of estrogen and progesterone treatment appears to be critical, in that short-term treatment has a much greater effect than long-term treatment.

Human breast cancer risk tends to rise with increasing age of first delivery and elderly primipara (age ≥35 years) have a greater risk than nulliparous women (1-4,16). In humans, the time of carcinogenic insult (initiation of mammary carcinogenesis) is unknown, but may occur early in life. Pre-pubertal female breasts are susceptible to ionizing radiation

(23,24). MNU, an alkylating agent, can induce mammary carcinomas in sexually immature female rats (25), which are more susceptible to MNU than sexually mature female rats. However, 7,12-dimethylbenz(α)anthracene (DMBA), aromatic hydrocarbone, is much less carcinogenic to immature rat mammary glands than to mature rat mammary glands (26). In the present study, MNU was administered to mature female Lewis rats at 7 weeks of age, which is a risk window for MNU and DMBA in rats. Then, estrogen and progesterone were administered to half of the rats beginning when they were 24 weeks old, using a 21-day release pellet containing 0.5 mg 17ß-estradiol and 32.5 mg progesterone. The E/P pellet we used elevates serum 17ß-estradiol and progesterone, which increases levels comparable to those of pregnancy at 3 weeks after pellet implantation; the levels of the 2 hormones then drop until they are equal to those of the control group, at 8 weeks after pellet implantation (15). Thus, the present E/P regimen can effectively reproduce the hormonal milieu of pregnancy. The age of 24 to 27 weeks was selected because no major remodeling of mammary glands takes place in virgin rats during that period (27). Short-term treatment of the present aged rats with estrogen and progesterone mimicking pregnancy enhanced mammary carcinogenesis; thus, patterns seen in elderly human primipara can be reproduced in rats.

In rats, obesity increases susceptibility to development of mammary carcinoma (28). E/P-treated rats tend to have slower body weight gain than untreated control rats (15) and the present E/P group of rats had a significantly lower final body weight than the control rats. In the present study, body weight was inversely related to the development of mammary carcinoma; thus, body weight *per se* appears to have been a relatively weak factor in mammary carcinogenesis in the present E/P group. Studies indicate that differences in hormone-induced mammary glandular differentiation underlie differences in susceptibility to mammary carcinogenesis (29). In the present study, at 24 weeks of age (time at E/P pellet implantation), mammary gland morphology was composed of thin ducts with small lobules. Then, at 48 weeks of age (end of the experiment), in both the E/P and control groups, we observed alveolar cells exhibiting secretion and dilatated ducts filled with secretory materials; this morphology is similar to that reported elsewhere as normal physiological aging of the Lewis rat mammary gland (30). Thus, the present E/P treatment did not promote glandular differentiation, suggesting that mammary glandular architecture does not play a role in mammary carcinogenesis. However, further study of the relationship between the pituitary gland and the secretion observed in elderly Lewis rats may shed light on the relationship between mammary glandular architecture and mammary carcinogenesis (31).

The present E/P treatment influenced the histology of mammary carcinomas and comedo carcinoma (considered biologically malignant) was often seen in the present E/P group. Comedo-type necrosis is one of the most common features of ER-negative human breast carcinomas (32). Consistent with our previous findings (5), MNU-induced mammary carcinomas in the present control rats were mostly (>80%) ER-positive. However, it is noteworthy that mammary carcinomas in the E/P group were predominantly (74%) ER-negative. Parity is associated with a reduced risk of ER/PgR-positive human beast carcinomas, but not with a reduced risk of ER/PgR-negative carcinomas (33,34) and elderly primipara (35 years of age) have been found to have a greater incidence of ER/PgR-negative carcinoma than nulliparous women (35). Estrogen stimulates growth of both ER-negative and ER-positive breast carcinoma cells *in vitro* (36). However, ER-positive proliferating cells comprise the majority of proliferating cells in mature virgin rat mammary glands (i.e. the cells presumed to be the progenitors of ER-positive carcinomas) and E/P treatment blocks the proliferative response of ER-positive cells (37). Moreover, estrogen is tumorigenic and promotes the growth of ER-negative transformed human breast epithelial cells *in vivo* (38). These findings are consistent with the development of ER- and/or PgR-negative mammary carcinomas in the present E/P group.

Combined estrogen/progesterone treatment is as effective as ovariectomy at suppressing mammary cancer and does not cause the loss of ovarian function when given to young rats for a short duration. Thus, this treatment may be useful for human breast cancer control. However, as shown here, the timing as well as the duration of estrogen/progesterone treatment appears to be essential for effective breast cancer control, as the treatment may actually promote breast cancer when applied to aged individuals.

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