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Histopathogenesis of 7,12-dimethylbenz[*a*]anthracene-induced rat mammary tumors

(experimental breast cancer/chemical carcinogenesis/hormone dependence/spontaneous tumor regression/preneoplastic lesions)

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ABSTRACT **The histopathogenesis and growth behavior of mammary tumors and dysplasias induced by a single intragastric dose of 7,12-dimethylbenz[*a*]anthracene in 50-day-old virgin female Lewis rats were examined both *in situ* and after transplantation into gland-free mammary fat pads of syngeneic hosts. Terminal mammary ductules are indicated as a site of origin of both ovarian hormone-dependent mammary tumors and spontaneously regressing mammary tumors, and terminal ductule hyperplasia appears to be an early stage in mammary tumor formation. The precancerous nature of hyperplastic alveolar nodules induced by dimethylbenzanthracene in rats has been further examined, and our studies indicate that these nodules are not significantly preneoplastic.**

Several studies indicate that the site of origin of human mammary carcinoma may be in lobules and terminal ductules of the mammary gland (1–6). It is known that in 30–40% of individuals mammary cancer is hormone responsive, whereas in the remainder it is not (7–9). At present it is not known if the histopathogenesis of these two forms of mammary cancer differ. For many reasons such studies are difficult to undertake in human beings, but the problem can be approached by using a suitable animal model.

When 7,12-dimethylbenz[*a*]anthracene (DMBA) is administered to the rat, numerous mammary gland dysplasias and mammary carcinomas occur (10–13). It has been reported that approximately 80% of the tumors arising are ovarian hormone-dependent (14, 15). Rat mammary carcinomas are claimed to arise in small mammary ducts (16–18) or from hyperplastic alveolar nodules (19, 20). The work presented here was undertaken to describe the histopathogenesis of rat DMBA-induced ovary-dependent tumors and ovary-independent mammary tumors in order to identify their site(s) of origin and to reexamine the preneoplastic nature of DMBA-induced mammary gland dysplasias.

MATERIALS AND METHODS

Inbred virgin female Lewis strain rats (Simonsen Laboratory, Gilroy, CA) at 50 days of age were fed DMBA (Sigma) intragastrically at 0.015 g/100 g of body weight, dissolved in 1 ml of sesame oil (groups A and C). Control rats received only an equivalent amount of sesame oil (groups B and D).

Histopathogenesis Study. Eight to 15 carcinogen-treated rats (group A) were killed 15, 30, 45, 60, 75, 90, 120, 150, 200, or 365 days after DMBA treatment. Eight to 10 control rats (group B) were killed 30, 60, 90, 150, 200, or 365 days after oil treatment. The thoracic, abdominal, and inguinal pairs of mammary glands were processed for whole mount examination according to the method of Beuving *et al.* (13). The occurrence and characteristics of mammary gland dysplasias and tumors were determined with the aid of a dissecting microscope. Tissues selected for histological examination were processed routinely (13).

Tumor Characterization. Parallel groups of rats, DMBA-treated and control (groups C and D), were palpated weekly for 12 months. Palpable tumors were recorded and classified as follows. Progressively growing tumors (PGTs) were those that, after reaching a palpable size, continued to grow. When these tumors reached 1.5–2.0 cm in average diameter, the rats were bilaterally ovariectomized. Those tumors that regressed to one-half or less of their average preoperative diameter were classed as ovary-dependent tumors (ODTs). Tumors that did not decrease in size or continued to grow after ovariectomy were classed as ovary-independent tumors (OITs). Spontaneously regressing tumors (SRTs) were those tumors that reached a palpable size then regressed to a nonpalpable state in the absence of any experimental intervention.

With one exception all tumors, including SRTs, proved to be carcinomas after histological examination of biopsy material; the exception was a fibroadenoma that arose 12 months after DMBA treatment.

Outgrowth Behavior and Characterization of Transplanted Mammary Gland Dysplasias and Mammary Carcinomas. Tissues for transplantation were obtained from groups A and C. Mammary gland dysplasias in group A were identified *in situ* by morphological criteria after intraductal injection of saline-washed erythrocytes (13). Tissues were transplanted into the inguinal gland-free mammary fat pad, according to Beuving's modification of the mammary fat pad transplantation method (13). Recipients of tissue transplants were 3 or 8 weeks of age; previously no difference in outgrowth behavior was observed between these two age groups (13). One mammary gland dysplasia was transplanted into the right inguinal gland-free pad of each recipient and a randomly selected piece of mammary duct from the same donor was transplanted into the contralateral gland-free fat pad. Rats transplanted with tumors received one 2-mm fragment of tumor from group C in each inguinal fat pad. Tumors were transplanted into intact hosts as well as into hosts that were ovariectomized 2 days prior to transplantation. Dysplasias were transplanted 30, 45, 60, 90, or 150 days after carcinogen treatment. Transplants were palpated weekly, for as long as 12 months, and palpable tumors were classified according to the scheme described above. Hormone dependence of tumor fragments was further established by growth behavior after daily subcutaneous injections of 5 μg of 17β-estradiol dissolved in sesame oil.

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Abbreviations: DMBA, 7,12-dimethylbenz[*a*]anthracene; HAN, hyperplastic alveolar nodule; TDH, terminal ductule hyperplasia; PGT, progressively growing mammary tumor; SRT, spontaneously regressing mammary tumor; ODT, ovary-dependent mammary tumor; OIT, ovary-independent mammary tumor; RTS, regressed tumor site.

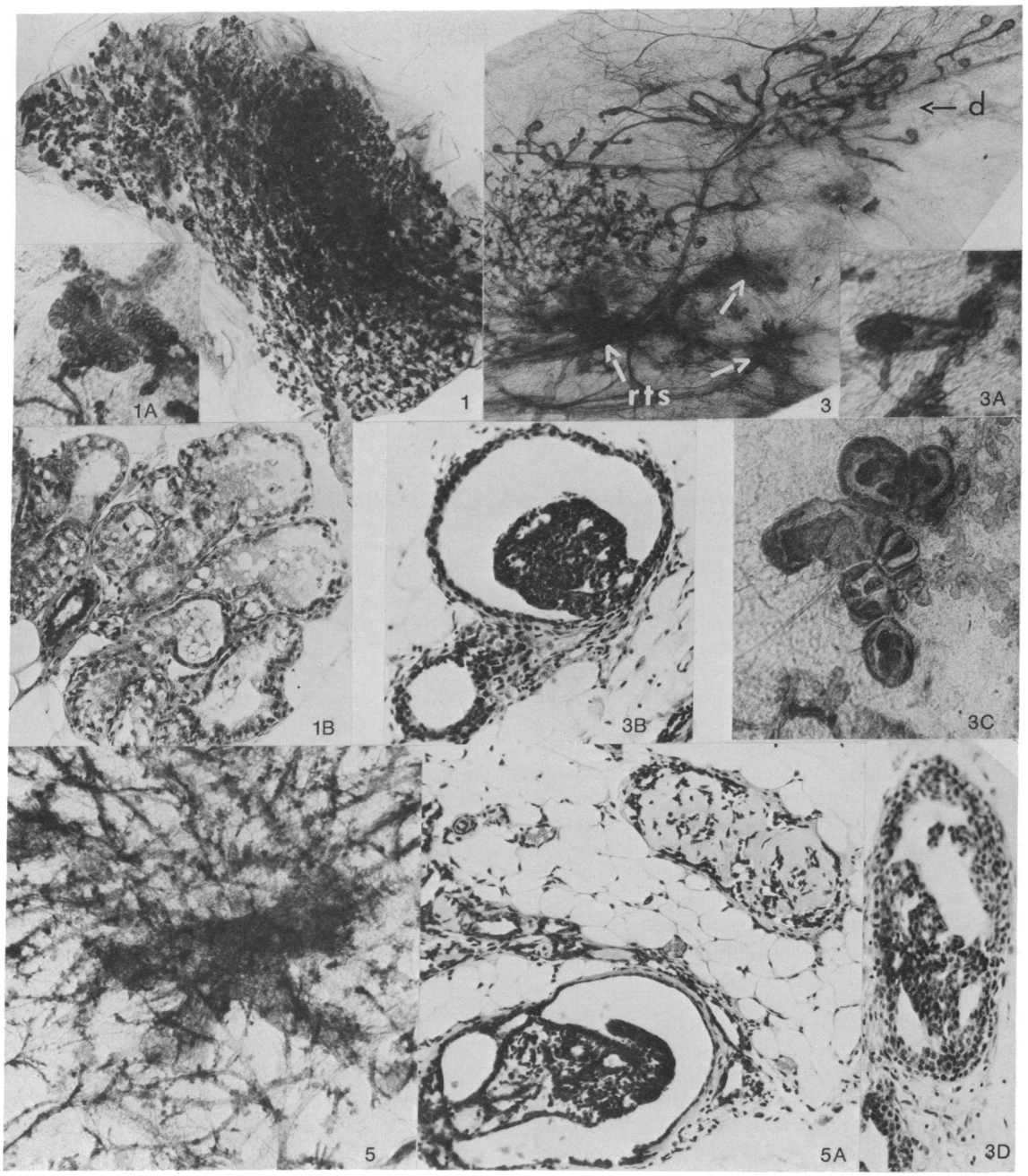

FIG. 1. Whole mount of mammary fat pad with lobuloalveolar outgrowth from transplanted HAN. (×2.9.) (*A*) Typical HAN *in situ* in whole mount. (×18.) (*B*) Section of HAN stained with hematoxylin and eosin; note secretory material within lumina. (×116.)

FIG. 3. Whole mount of mammary fat pad containing abnormal ductal outgrowth (d) and regressed tumor sites (rts) resulting from transplanted TDH. (×4.6.) Similar outgrowths are obtained from transplanted SRTs and ODTs. (*A*) TDH *in situ* in whole mount. (×37.) (*B*) Section of TDH in *A*. (×116.) (*C*) Larger TDH showing involvement of adjacent ductules. (×37.) (*D*) Section of TDH in 4*C*. (×116.)

FIG. 5. RTS *in situ* in whole amount. (×37.) (*A*) Section of a regressing tumor. Area at top right is fully regressed and is composed mainly of dense connective tissue. Lower left shows an adjacent small tumor focus undergoing regression. (×116.)

RESULTS

Mammary Gland Dysplasias. Three distinct classes of dysplasias were observed in the mammary glands of carcinogen-treated rats. The predominant dysplasia was the hyperplastic alveolar nodule (HAN). In whole mounts HANs resemble translucent dilated alveoli in the form of a single lobular unit (Fig. 1*A*) or of a cluster of lobular units interconnected by several ductules; in both cases they varied in size, degree of alveolar distension and symmetry and were sometimes cystic. Histologically HANs were composed of numerous alveoli, each surrounded by connective tissue and lined by a single layer of low cuboidal epithelial cells. The lumina were frequently filled with basophilic secretion (Fig. 1*B*). HANs increased from a mean number of 3 ± 1 per rat at 30 days to a mean of 115 ± 8 by 150 days (Fig. 2). In control rats, HANs were rarely present; they were not encountered before 120 days, and did not exceed three per rat (data not shown). Extensive examination of mammary gland whole mounts never revealed HANs *in situ*

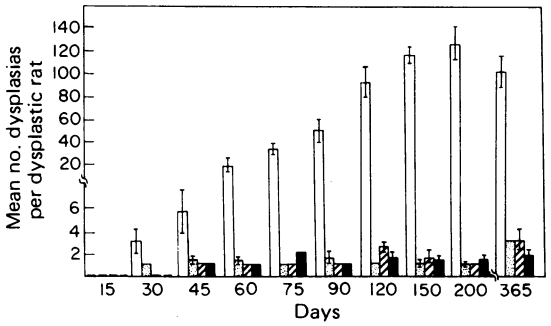

FIG. 2. Mean number (±SEM) of dysplasias and tumors occurring in mammary glands at various times after DMBA treatment. Empty bars, HAN; stippled bars, TDH; hatched bars, tumor; solid bars, regressed tumor.

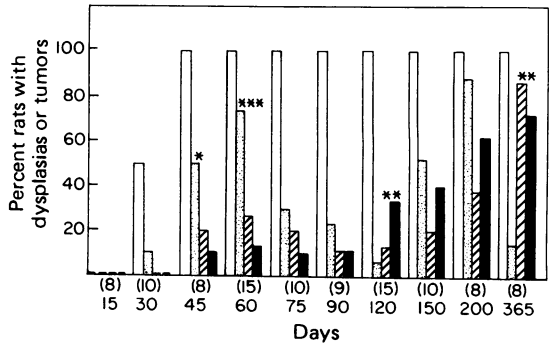

FIG. 4. Percent of rats with mammary gland dysplasias and tumors at various times after DMBA treatment at 50 days of age. Numbers of rats killed at each time point are given in parentheses. Empty bars, HAN; stippled bars, TDH; hatched bars, tumor; solid bars, regressed tumor. Probability that a percent is the same for TDHs, tumors, and regressed tumors: *, $P = 0.05$; **, $P = 0.025$–.01; ***, $P = 0.005$ (χ^2 test).

giving rise to small mammary carcinomas. Upon transplantation HANs grew to fill the fat pad and the outgrowths were lobuloalveolar, ductoalveolar, or cystic alveolar (Fig. 1). Histologically, therefore, these outgrowths resembled HANs. Infrequently, tumors occurred within HAN outgrowths. Six out of 113 outgrowths produced mammary carcinomas (5.3%) by 8 ± 0.6 months after transplantation. Of four tumors characterized, three were ODTs and one was an OIT. Randomly selected pieces of mammary ducts from the same DMBA-treated donors, transplanted to the contralateral fat pads, gave rise to tumors with a frequency that was not significantly different: four out of 125 (3.2%) at 10 ± 0.5 months after transplantation. These tumors were not palpable prior to termination but were found in whole mounts and consequently could not be characterized in terms of hormone dependence. No HANs were encountered in the ductal outgrowths.

A second class of dysplasia occurred in single terminal end-buds or within terminal ductules sprouting from end-buds. The ductules were enlarged and appeared to contain a plug of cells (Fig. 3*A*). Histological examination revealed hyperplasia of the epithelial cells lining these ductules. The proliferating cells grew inward, resulting in partial occlusion of the lumen (Fig. 3 *B* and *D*). These terminal ductule hyperplasias (TDHs) were surrounded by thin connective tissue, and at this stage there was no lymphocytic infiltration or stromal reaction. With time, further distension and epithelial hyperplasia occurred and neighboring ductules became included in the dysplasia (Fig. 3*C*). Within larger lesions there was a gradation from focal epithelial hyperplasia to carcinoma *in situ*. TDHs were noted first at 30 days and preceded the appearance of frank mammary carcinomas (Fig. 4). TDHs occurred with an average of one to two per rat (Fig. 2). TDHs may be microscopic foci of mammary carcinoma cells. After transplantation three out of six TDHs gave rise to small palpable mammary carcinomas which subsequently regressed. Outgrowths from TDHs were ductal; those with tumors showed areas of abnormal spacing and ductal hyperplasia (Fig. 3). The site of the regressed tumor was characterized by thickened connective tissue (Figs. 3 and 5); contracted ducts, ductules, and nests of epithelial cells were present, embedded within the connective tissue (Fig. 5*A*).

The third class of dysplasias were regressed tumor sites (RTSs). Morphologically these resembled areas of scar tissue (Fig. 5). RTSs ranged in appearance from well-regressed structures in which the predominant component was dense connective tissue to aggregations of small tumorous foci (Fig. 5*A*). These tumorous foci were frequently sites of lymphocytic infiltration. RTSs were first observed at 45 days (Fig. 4), averaging one to two per rat (Fig. 2).

Fig. 4 shows that the number of DMBA-treated rats bearing HANs reaches 100% by 45 days and remains constant. In contrast, the number of rats bearing TDHs and mammary tumors varies significantly at different times after DMBA treatment. TDHs precede the occurrence of mammary tumors; at 45 and 60 days more rats have TDHs than mammary tumors. By 75 and 90 days the number of rats bearing TDHs approaches the number that have mammary tumors; at 120 and 365 days the number of rats with mammary tumors exceeds the number with TDHs. Inasmuch as rats were killed at each time point, it was not possible to follow the fate of individual dysplasias and tumors; however, the overall general pattern suggests that terminal ductule hyperplasia is related to tumor development. PGT and SRT are histologically indistinguishable carcinomas, and such tumors appear to originate within terminal ductules. TDHs appear to be microscopic foci of tumor cells, which may continue to grow or which may regress spontaneously at some point. No TDHs, RTSs, or tumors were observed in controls.

Tumor Characterization and Occurrence of Palpable Tumors. The first palpable tumors also occurred 2 months after DMBA treatment. All these early tumors regressed spontaneously (SRT); they grew for an average of 2.5 ± 0.2 weeks and then decreased in size for 1.8 ± 0.2 weeks until they were no longer palpable. These tumors never exceeded an average diameter of 1.0 cm. Eight out of 20 rats developed SRTs (40%) with an average of 1.5 ± 0.3 per rat. Of a total of nine SRTs occurring, five remained permanently regressed and four regrew. Of the four regrowths, two regressed a second time and two grew progressively. One of these tumors was characterized as an ODT; the other rat died after ovariectomy. Histologically these tumors displayed heavy lymphocytic infiltration and intense stromal reaction.

Transplanted SRT fragments grew sporadically and underwent subsequent regression. Daily injections of estradiol for up to 8 weeks failed to reactivate tumor growth. Nine months after transplantation, 9/18 transplanted fragments grew progressively; two tumors were characterized as ODTs. Transplanted SRTs also gave rise to abnormal ductal outgrowths similar to those obtained from TDHs (Fig. 3).

The second class of palpable tumors grew progressively (PGTs). These tumors occurred first at 3 months and continued to occur over the 12-month experimental period. Thirteen out of 20 rats (65%) developed PGTs. Seventy percent of the PGTs ($N = 15$) were ODTs and 30% were OITs.

Total tumor incidence by 12 months was 70%. Of the 24 tumors arising 37% were SRTs, 42% were ODTs, and 21% were OITs. Thirty percent of the rats developed only PGTs, 5% de-

veloped only SRTs, and the remaining 35% developed both SRTs and subsequent PGTs. Mean number of tumors per rat was 2.1 ± 0.3.

Histological evidence indicates that PGTs frequently initially regress after transplantation. Of a total of eleven PGTs transplanted, seven resumed progressive growth 13–20 weeks after transplantation. After transplantation in intact rats, 36 fragments from two ODTs grew progressively as tumors all surrounded by some abnormal ductal outgrowth (Fig. 3). Fifteen out of 18 pieces of these ODTs failed to grow or give rise to outgrowth in ovariectomized recipients; three fragments of one ODT grew progressively. All transplanted ODTs growing in intact hosts regressed after ovariectomy, and growth was reactivated by daily injections of estradiol. These tumors regressed again when injections were discontinued. The regressed states of ODTs and SRTs, both *in situ* and after transplantation, were grossly and histologically similar (Figs. 3, 5, and 5A).

DISCUSSION

The data presented herein lead us to propose that the majority of rat mammary carcinomas arise within terminal ductules of the mammary gland and that there is a continuum from TDH to carcinoma *in situ*. The time course of TDH incidence and the close agreement between the number of such lesions and the number of mammary tumors indicate that TDHs may be microscopic foci of mammary tumor cells. Ductal origin of tumors is further substantiated by the similar ductal outgrowths obtained from ODTs, SRTs, and TDHs. Thus, our results support the proposed ductal origin of mammary carcinomas in DMBA-treated rats (16–18).

Studies of the transplantation behavior of *hormone-responsive* (pregnancy-dependent) virus-induced mammary tumors of the mouse also strongly indicate a ductal origin for those tumors (21). The mammary glands of mice treated with DMBA also develop ductal abnormalities and hyperplasias (22–25). However, at present it is not known if DMBA-induced mouse mammary tumors require hormones for continued growth. The present results are also in agreement with those studies of human mammary carcinoma that postulate a terminal ductule site of origin in the human disease, and also identify a similar pattern of epithelial hyperplasia as the earliest stage in possible tumor development (1–6). Therefore, it seems reasonable to conclude that terminal ductule hyperplasias are an early stage in the formation of hormone-dependent mammary carcinomas. No unique dysplasia or site of origin was identified for OITs. Although their histopathogenesis remains to be clarified, they do not appear to originate from HANs.

The present data lead to concurrence with the conclusion of Dao and his colleagues (17, 26) that HANs, which can occur in large numbers in DMBA-treated rat mammary glands and in small numbers in control rats, do not appear to be a locus of origin of mammary carcinoma. Sinha and Dao (17) have shown that ODTs can be induced by directly applying DMBA to the mammary gland; with this method no HANs develop. Therefore, they conclude that the formation of HANs is not essential for tumor development. Only infrequently do HANs give rise to tumors after transplantation; in the present series of experiments they do not differ significantly in this respect from randomly selected segments of carcinogen-exposed mammary ducts. On the other hand, HANs were found to be significantly preneoplastic in the rat in previous experiments by Beuving and coworkers (12, 13, 19, 20). In the Lewis rat strain, 8 out of 37 (25%) transplanted HANs gave rise to mammary tumors, and no tumors arose from 37 duct transplants (19, 20). In the Fischer rat strain, none of 16 HAN or 17 duct transplants gave rise to tumors by 12 months after transplantation (13). It was proposed that because Fischer rats are less susceptible than Sprague–Dawley rats to tumor induction by DMBA, HANs arising in Fischer rats might have a low tumorigenic potential (13). However, the tumor incidence reported here for Lewis rats is not different from that reported by Beuving for Fischer rats (13). It is possible that TDHs, inadvertently included within tissue taken with HANs for transplantation, increased the frequency of tumor development in the Beuving series of experiments. In any case, ductal elements are always included in HANs taken for transplantation and are present within HAN outgrowths; therefore, they cannot be eliminated as a possible site of tumor origin within outgrowths.

Hormone-dependent rat mammary carcinomas contain specific cytoplasmic estrogen-binding proteins (8, 27, 28). Dao *et al.* (26) have examined HANs for the presence of these estrogen receptors and find that they are absent or present in low concentration as compared with normal rat mammary gland or hormone-dependent rat mammary carcinomas. This further indicates that HANs are not likely to be precursors of rat mammary carcinomas, which are predominantly hormone-dependent. However, HANs may be more susceptible to neoplastic transformation upon subsequent exposure to carcinogenic agents (29).

The occurrence of spontaneously regressing mammary carcinomas is a frequent phenomenon in DMBA-treated rats and contributes significantly to the number of tumors arising. Although this phenomenon has been previously reported and is not unique to the Lewis strain rat, it has received little attention and has not been adequately studied (8, 15, 30, 31). The regressed site (state) of these tumors can be readily identified and does not appear to differ histologically from the regressed condition of ODTs. However, the present results indicate that mechanisms causing regression in these two cases may be different, because ODT growth can be readily reactivated after ovariectomy by treatment with 17β-estradiol, whereas SRT growth cannot be so reactivated.

Because of the potential importance for therapy and prophylaxis, the mechanism of this regression requires further investigation; examination of human mammary cancer for evidence of comparable spontaneous regression would be desirable.

In conclusion, DMBA-induced hormone-dependent mammary carcinomas appear to be of terminal ductule origin. TDHs are possible microscopic foci of tumor cells. If this is correct, mammary cells exposed to DMBA may be transformed directly to neoplastic cells, and no intermediate, preneoplastic step(s) would be required. Almost half of the tumors that develop undergo spontaneous regression by an undefined mechanism. Although HANs arise as a consequence of carcinogen treatment and are numerous in the mammary glands of carcinogen-treated rats, they did not prove to be significantly preneoplastic. Even though HANs represent a mammary gland dysplastic response to carcinogen exposure, they are apparently not related to tumor development.

Note Added in Proof. M. Miyamoto (personal communication) has found ductal dysplasias in the mammary glands of virgin, virus-positive GR mice. Pregnancy-dependent (*hormone-responsive*) and pregnancy-independent mammary tumors develop within some of the abnormal ductal outgrowths derived from transplanted ductal dysplasias.

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