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Review

Estrogen and Progesterone Treatment Mimicking Pregnancy for Protection from Breast Cancer

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Abstract. *Early age at full-term pregnancy lowers the risk of breast cancer in women; lactation seems to be of marginal importance and aborted pregnancy is not associated with reduced risk. Although early full-term pregnancy provides protection against breast cancer, first full-term pregnancy in older women appears to increase the risk. The protective effect of pregnancy has also been observed in rats and mice; in these animals, lactation has an additive effect and interrupted pregnancy provides partial but significant protection. Pregnancy at a young age (≤3 months) is highly effective, but pregnancy in older animals (≥4 months) is less effective. Parity-induced protection against mammary cancer in rodents can be reproduced by short-term treatment (approximately equivalent to gestational period of rodent or shorter) with the pregnancy hormones, estrogen and progesterone. Administration of pregnancy hormones to nulliparous women may be a useful strategy for protection against breast cancer. However, estrogen and progesterone are thought to play major roles in promotion of the proliferation of breast epithelial cells. Thus, the duration of such treatment and the age at which it is administered are essential factors that require further study. Experimental data suggest that short-term treatment of older rats (aged 6 months) with estrogen and progesterone accelerates mammary carcinogenesis and that long-term (>20 weeks) treatment abolishes the cancer-suppressing effect or even accelerates mammary carcinogenesis. Thus, the available evidence suggests that age and duration of estrogen and progesterone treatment are particularly important factors for protection from breast cancer.*

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The etiology of human breast cancer is largely unknown. Genetic susceptibility, hormonal effects and environmental factors appear to be major determinants. However, known genetic risk factors are present in only 10% to 15% of breast cancer cases (1). Many aspects of hormonal effects confer increased risk of human breast cancer. The incidence of human breast cancer is 100-fold greater for females than for males and female reproductive history is a consistent risk factor for human breast cancer (2, 3). Studies indicate that early menarche and late menopause, both of which increase the duration of ovarian steroid exposure, positively correlate with increased risk; bilateral oophorectomy at an early age is associated with reduced risk (4). Although low physiological levels of ovarian steroids are necessary for breast cancer development and progression, young age at full-term pregnancy, which results in high physiological levels of ovarian steroid exposure during the gestational period, substantially lowers the risk of breast cancer (5-8). For women who undergo a full-term pregnancy before the age of 20 years, the risk of developing breast cancer is one-half as great as that of nulliparous women; lactation seems to be of marginal importance (9), while aborted pregnancy is not associated with reduced risk (5). Although early full-term pregnancy provides long-lasting protection against breast cancer, pregnancy after the age of 35 years appears to increase the risk (5-8, 10). Thus, parity-induced protection against breast cancer is principally dependent on the timing of the first full-term pregnancy rather than on its occurrence *per se*. Incidence of breast cancer varies worldwide and changes in breast cancer incidence among migrants provide important evidence for environmental factors in the genesis of breast cancer (11). Diet is thought to be an important environmental factor influencing breast cancer risk. Dietary components strongly associated with breast cancer risk include fat and phytochemicals, which alter the growth of the breast epithelium *via* various mechanisms (12). Humans consume a variety of foodstuff which may affect endogenous hormone levels and may modify breast cancer risk.

Parity (hormone)-induced protection against breast cancer is a universal phenomenon, common in women of all ethnicities, and is the only normal physiological condition that consistently protects against breast cancer without known side-effects. Protection against breast cancer is an important area of clinical and experimental investigation. The protective effect of pregnancy has also been seen in rats and mice. In chemical carcinogen-induced mammary carcinoma models in rodents, young animals (≤3 months of age) that undergo a full-term pregnancy before, during or shortly after carcinogen exposure, with or without lactation, have significantly reduced overall mammary cancer incidence and multiplicity, and have prolonged latency (13). In carcinogen-induced conventional rat and mouse mammary carcinoma models (13), and in genetically engineered mouse models (14), parity-induced protection against mammary cancer has been reproduced by short-term treatments (approximately equivalent to the gestational period of rodents: 21 days) with the pregnancy hormones estrogen and progesterone. An increasing percentage of women do not desire to bear children, increasing the attractiveness of mimicking the pregnancy environment by treating nulliparous women with estrogen and progesterone as a strategy for reducing the risk of human breast cancer. Repeated experiments have confirmed that pregnancy or short-term treatment with estrogen and progesterone at a young age can have a protective effect. However, because estrogen and progesterone are thought to play major roles in promoting the proliferation of mammary epithelial cells (15), safe use of such hormone treatment requires a thorough understanding of the effects of the age at treatment and the duration of the treatment. Moreover, despite the effectiveness of pregnancy and pregnancy hormone-induced refractoriness to mammary cancer, the cellular and molecular mechanisms of these effects are not fully understood. In the present review, we discuss the existing representative experimental data regarding pregnancy- and pregnancy hormone-induced mammary cancer protection, paying particular attention to the effects of age and the duration of pregnancy hormone exposure, and the underlying mechanisms.

Animal Models for Mammary Carcinogenesis Evaluation Based on Parity or Pregnancy Hormone

Single injection of chemical carcinogens, such as 7,12-dimethylbenz(α)anthracene (DMBA) and *N*-methyl-*N*-nitrosourea (MNU), can effectively induce mammary carcinoma in susceptible strains of rats (16), while multiple injections of DMBA are necessary for mammary carcinoma induction in certain strains of mice (17, 18). Age at the time of carcinogen administration is a crucial factor in the susceptibility of the mammary gland to carcinogens (19, 20). Thus, hormone-unexposed age-matched virgins (AMVs) that receive the carcinogen at the same age must be included as controls. In addition to carcinogen-induced rodent models, genetically engineered mice that spontaneously develop mammary carcinomas have been created (21, 22) and have been used as models for evaluating pregnancy hormone-induced protection (14).

Suppression of Mammary Carcinomas in Rodents by Pregnancy or Treatment with Pregnancy Hormones before Exposure to Carcinogen

Hormonal stimulation, by pregnancy or pregnancy hormones (estrogen alone, or estrogen plus progesterone), in carcinogen-induced rodent models can be divided into pre- and post-treatment methods (23). In pre-treatment methods, the animals first undergo hormone treatment then, after their mammary glands have involuted, the carcinogen is administered. In post-treatment methods, the animals are first treated with carcinogen and are then exposed to hormone treatment.

Effect of pregnancy. The most impressive results obtained using pre-treatment methods are shown in Table I. These experiments differ in the species and strain of animals used, the type and timing of carcinogen exposure, the dose and duration of hormone treatment, or the duration of the experiment. Thus, it is not easy to compare the incidence of mammary carcinomas between the different experiments. In Table I, the mammary carcinoma incidence of hormone-exposed animals was statistically compared with that of AMVs using Fisher's exact test. The % inhibition was calculated as: % mammary carcinoma in [(AMV – hormone treated rats)/AMV] x100. Due to differences in group sizes, some experimental results showing considerable % inhibition are not statistically significant. According to Yang *et al.* (24), the mammary carcinoma incidence of Lewis rats that underwent pregnancy and lactation and then received MNU 9 weeks after delivery (age, 24 weeks) was significantly lower than that of the AMVs (22% *vs.* 73%; 70% inhibition). Compared with the AMVs, the average number of carcinomas per MNU-treated rat was significantly lower (0.22 *vs.* 0.86), and the latency was significantly longer (35.3 *vs.* 30.7 weeks). Compared with respective AMVs, Moon (25) observed 59% inhibition in parous Sprague-Dawley rats that underwent pregnancy and lactation followed by DMBA treatment, and observed 28% inhibition in rats that underwent pregnancy without DMBA treatment. Lactation seems to exert an additive effect on pregnancy-induced reduction of mammary carcinoma risk. In the experiment by Moon (25), carcinogen was administered long after pregnancy (the time interval between the delivery and the carcinogen exposure was 17 weeks) and the cancer

suppressing effect was still preserved, although it was not significant. In different experiments using Sprague-Dawley or Wistar/Furth rats (26, 27), when carcinogen (DMBA or MNU) was administered shortly after pregnancy (3-4 weeks after the delivery), pregnancy alone had a significant protective effect. When the time interval between delivery and carcinogen insult varied using the same experimental system (28), the protective effect was weakened by increasing the time between delivery and carcinogen insult (86% inhibition for a 3-week interval *vs.* 47% inhibition for a 9-week interval). A full-term pregnancy significantly inhibits subsequent development of carcinogen-induced mammary carcinomas when the time interval between delivery and carcinogen insult is short. A pregnancy interrupted at 5, 10 or 15 days resulted in 39%, 37% and 43% inhibition, respectively, compared with 83% inhibition after full-term pregnancy (33). Although aborted pregnancy in humans shows no protective effect, a small but significant inhibition of DMBA-induced mammary carcinogenesis has been observed in Sprague-Dawley rats (each, $p<0.01$). The protective effects of pregnancy may be strengthened in the last stage of pregnancy, when estrogen and progesterone levels are each at their highest. Thus, in pre-treatment models, pregnancy protects against mammary carcinogenesis in rats, lactation has an additive effect and interrupted pregnancy is also protective.

Effects of estrogen and progesterone. The protective effects of estrogen and progesterone were initially observed by Huggins *et al.* (34). High levels of estrogen and progesterone administered for 30 days beginning 15 days after DMBA administration inhibited mammary carcinogenesis in Sprague-Dawley rats, compared with AMVs; reproductive function recovered soon after cessation of the treatment. The development of mammary carcinoma can be suppressed by estrogen and progesterone when they are administered before carcinogen exposure. After Huggins' report, different doses and administration methods for estrogen and progesterone were tested in different laboratories. In some experiments, estrogen and progesterone were packed in silastic tubes (18, 31, 35) or dissolved in beeswax (27, 32) and subcutaneously implanted, or dissolved in ethanol and then diluted with sesame oil (30, 36) and subcutaneously injected. In most of the experiments, circulating estrogen and progesterone levels were not recorded. However, in all experiments, evaluation of the mammary glands with whole-mount sections at the end of the hormone treatments showed alveolar differentiation comparable to that seen during pregnancy. Grubbs *et al.* (29, 30) administered estrogen and progesterone to Sprague-Dawley rats for 35 days, and achieved 79% to 82% inhibition of the incidence of MNU-induced mammary carcinoma, compared with AMV. When the duration of estrogen and progesterone administration was shortened to 21 days (27), 14 days (31), and 10 days (32), significant inhibition was still seen. However, estrogen alone was ineffective (30, 32). Moreover, in parous BD2fF1 mice after 7 days of lactation (17), and BALB/c mice treated with estrogen and progesterone for 13 days (18), mammary carcinoma incidence after carcinogen exposure was significantly reduced (64% and 73%, respectively), compared with respective AMVs. Thus, in pre-treatment models, both estrogen and progesterone are required for subsequent protection against mammary carcinogenesis and approximately 2 weeks of treatment can produce significant protection.

Suppression of Mammary Carcinoma in Rodents by Pregnancy or Treatment with Pregnancy Hormones after Exposure to Carcinogen

In humans, it is not known when mammary carcinogenesis is initiated by a carcinogenic insult, but it may occur early in life. Breast tissue of young women is more sensitive to radiation-induced carcinogenesis than that of older women (37); preneoplastic cells may be present before the first pregnancy. In post-treatment methods, carcinogen-treated animals are subsequently impregnated or treated with pregnancy hormones. Representative data for such experiments are shown in Table II.

Effects of pregnancy. In an experiment performed by Yang *et al.* (24), MNU exposure at 10 weeks of age followed by pregnancy and lactation resulted in significantly lower mammary carcinoma incidence than that of MNU-treated AMVs (25% *vs.* 94%; 73% inhibition). The number of carcinomas per rat was significantly lower (0.25 *vs.* 1.50), and the mean latency was significantly longer (34.3 *vs.* 22.1 weeks), compared with AMVs. Among pregnant rats without lactation, although it was not significant, the mammary carcinoma incidence was lower (76% *vs.* 94%; 19% inhibition), the number of carcinomas per rat was lower (1.50 *vs.* 1.12) and the latency was longer (22.1 *vs.* 26.7 weeks), compared with AMVs. In Grubbs' experiments (38, 39), both DMBA- and MNU-induced mammary carcinogenesis was suppressed by parity and although lactation had additive effects, pregnancy alone significantly suppressed mammary carcinoma incidence. In a comparison between the effects of early pregnancy with lactation (1 week interval from MNU to the onset of pregnancy; at 2 months of age) and those of late pregnancy with lactation (11 weeks interval from MNU to the onset of pregnancy; at 4 months of age), early pregnancy significantly inhibited the development of mammary carcinomas (70% inhibition), but late pregnancy did not significantly suppress carcinogenesis (29% inhibition) (39). As in humans, the earlier the age at first full-term pregnancy the lower the risk of mammary carcinoma in rats; the protective effect decreases when the first pregnancy occurs later in life.

Table I. *Inhibition of mammary carcinogenesis in rodents by parity and hormone treatment before carcinogen administration.*

Species	Strain	Treatment[1]	Interval[2] (weeks)	Carcinogen[3]	%MC in treated/AMV	%Inhibition[4]	*P*-values[5]	Reference
Rat	Lewis	Preg/Lac	9	MNU (24)	22/73	70	<0.01	24
Rat	Sprague-Dawley	Preg/Lac	17	DMBA (27)	16/39	59	NS	25
		Preg	17	DMBA (27)	28/32	28	NS	
Rat	Sprague-Dawley	Preg	3	DMBA (13)	57/100	43	<0.01	26
Rat	Wistar/Furth	Preg	4	MNU (13)	0/45	100	<0.01	27
Rat	Sprague-Dawley	Preg	3	DMBA (13)	6/44	86	<0.01	28
		Preg	9	DMBA (19)	10/19	47	NS	
Rat	Sprague-Dawley	Est/Prog (35)	3	MNU (13)	13/72	82	<0.01	29
Rat	Sprague-Dawley	Est/Prog (35)	3	MNU (13)	13/61	79	<0.01	30
		Est (35)	3	MNU (13)	48/61	21	NS	
Rat	Wistar/Furth	Est/Prog (21)	4	MNU (13)	10/57	82	<0.01	27
Rat	Lewis	Est/Prog (14)	5	MNU (14)	0/55	100	<0.05	31
Rat	Wistar/Furth	Est/Prog (10)	4	MNU (13)	26/59	56	<0.01	32
		Est (21)	4	MNU (13)	60/59	0	NS	
Mouse	BD2fF1	Preg/Lac	5	DMBA (15)	25/70	64	<0.01	17
Mouse	BALB/c	Est/Prog (13)	2	DMBA (8)	17/63	73	<0.01	18

[1]Treatment, with duration of treatment (days) in parentheses. Pregnancy or hormone treatment ended at ≤3 months of age; [2]weeks from day of delivery or end of the hormone treatment to carcinogen administration; [3](weeks), age at the start of treatment; [4]mammary carcinoma incidence in [(AMV-treated) /AMV rats] x100; [5]difference in mammary carcinoma incidence was compared between treated and AMV rats using Fisher's exact test. Abbreviations: MC, mammary carcinoma; AMV, age-matched virgin; Preg, pregnancy; Lac, lactation; MNU, *N*-methyl-*N*-nitrosourea; DMBA, 7,12-dimethylbenz(α)anthracene; NS, not significant; Est, estrogen; Prog, progesterone.

Table II. *Inhibition of mammary carcinogenesis in rodents by parity and hormone treatment after carcinogen administration.*

Species	Strain	Carcinogen[1]	Interval[2] (Weeks)	Treatment[3]	%MC in treated/AMV	%Inhibition[4]	*P*-values[5]	Reference
Rat	Lewis	MNU	2	Preg/Lac	25/94	73	<0.01	24
			2	Preg	76/94	19	NS	
Rat	Sprague-Dawley	DMBA	1	Preg/Lac	41/93	56	<0.01	38
Rat	Sprague-Dawley	MNU	1	Preg/Lac	27/90	70	<0.01	39
			1	Preg	45/90	50	<0.01	
			11	Preg/Lac[6]	64/90	29	NS	
Rat	Sprague-Dawley	MNU	1	Est/Prog (40)	14/90	84	<0.01	36
			1	Est (40)	43/90	52	<0.01	
Rat	Lewis	MNU	2	Est/Prog (21)	11/100	89	<0.01	31
			2	Est (21)	38/100	62	<0.05	
Rat	Lewis	MNU	2	Est/Prog (14)	20/87	77	<0.01	35
			2	Est/Prog (7)	21/87	76	<0.01	
			2	Est (7)	38/87	56	<0.05	

[1]Administered at 7-10 weeks of age; [2]time from carcinogen administration to the beginning of pregnancy or hormone treatment; [3]pregnancy or hormone treatment ended at ≤3 months of age; (days), duration of treatment; [4]mammary carcinoma incidence in [(AMV-treated)/AMV rats] x100; [5]difference in mammary carcinoma incidence was compared between treated and AMV rats using Fisher's exact test; [6]pregnancy occurred at ≥4 months of age. Abbreviations: MC, mammary carcinoma; AMV, age-matched virgin; MNU; *N*-methyl-*N*-nitrosourea; DMBA, 7,12-dimethylbenz(α)anthracene; Preg, pregnancy; Lac, lactation; NS, not significant; Est, estrogen; Prog, progesterone.

Effects of estrogen and progesterone. In post-treatment models using rats, estrogen and progesterone treatment lasting 40 days (36), 21 days (31), 14 days or even 7 days significantly suppresses mammary carcinoma (35). In contrast to results of pre-treatment methods, estrogen alone is also effective in post-treatment models, but a combination of estrogen and progesterone is more effective (31, 35, 36). Like rats of the Sprague-Dawley, Lewis, and Wistar/Furth strains, rats of the Fisher 344 and Copenhagen strains exhibit a significant degree of protection against mammary carcinoma in response to treatment with estrogen and progesterone (40). Thus, pregnancy- or pregnancy hormone-

induced protection appears to be a universal phenomenon in rats, and pregnancy with lactation appears to be more effective than pregnancy alone. In post-treatment methods, the combination of estrogen and progesterone appears to be more effective than estrogen alone.

Although post- and pre-treatment methods significantly suppress mammary carcinoma incidence, pregnancy and pregnancy hormones promote the proliferation of mammary epithelial cells, suggesting that carcinogen exposure concomitant with pregnancy or pregnancy hormones would accelerate the development of mammary carcinomas. However, despite the increased mammary epithelial cell proliferation during pregnancy, mammary carcinogenesis was inhibited when DMBA was injected into rats during pregnancy (41). Moreover, when MNU was administered during the first, second and third week of treatment with estrogen and progesterone, researchers observed 81%, 87% and 100% inhibition of mammary carcinoma, respectively, compared with AMVs (42). Thus, carcinogen exposure during pregnancy or during treatment with estrogen and progesterone does not accelerate mammary carcinogenesis in rats, but rather suppresses it.

Pregnancy Hormone-induced Protection in Genetically Engineered Mouse Models

In human breast cancer, the oncogene *neu* is frequently overexpressed and the major tumor suppressor gene *p53* is frequently inactivated. Two genetically engineered mouse models have been used to test the effects of hormones (14). The genetically engineered FVB mouse carries an activated *neu* gene. In another model, *p53*-null mammary epithelium was transplanted into cleared mammary fat pads of wild-type-*p53* isogenic BALB/c mice. In mice carrying an activated *neu* gene, early short-term exposure to estrogen (aged 7-10 weeks) with or without progesterone significantly suppressed mammary carcinogenesis (63-67% inhibition), compared with AMVs. In *p53*-null mammary epithelial transplant mice, both early (aged 5-7 weeks) and late (aged 23-25 weeks) short-term treatment with estrogen and progesterone significantly inhibited mammary carcinogenesis in *p53*-null mammary gland (70% and 60% inhibition, respectively), compared to AMVs. In this model, the age of the animals did not influence the cancer-suppressing effects of hormone treatment, unlike the case with humans.

Effects of Short-term Estrogen and Progesterone Treatment in Older Rats

Although early full-term pregnancy protects against the development of breast cancer in humans, pregnancy in older women accelerates development of breast cancer. The question arises whether pregnancy hormones suppress carcinogenesis to equal degrees in older and younger mammary glands (≤3 months of age) in carcinogen-induced rat models (as seen in the *p53*-null model), or exert opposite effects (as seen in humans).

Table III. *Circulating 17β-estradiol and progesterone levels in female Lewis rats implanted with a 21-day-release estrogen/progesterone pellet.*

E/P treatment[1]	Age (weeks)	Estradiol (pg/ml)	Progesterone (ng/ml)
Untreated virgin	8-14	30.3±12.7	8.3±1.4
2 weeks after E/P	8	415.9±86.9	50.3±19.0
8 weeks after E/P	14	20.4±4.5	5.6±2.0
Pregnant[2]		55-630	45-130

[1]Single estrogen and progesterone (E/P) pellet containing 0.5 mg of 17β-estradiol and 32.5 mg of progesterone (Innovative Research of America, Sarasota, FL, USA) was implanted at 6 weeks of age. Data are expressed as mean±SE; [2]collected data of different strains of rat which shows the range during pregnancy (based on Numan (43)).

When a single 21-day-release hormone pellet containing 0.5 mg of 17β-estradiol and 32.5 mg of progesterone (E/P pellet) was implanted into female Lewis rats, circulating 17β-estradiol and progesterone levels were significantly elevated, reaching pregnancy levels 2 weeks after implantation, and decreasing to control levels 8 weeks after implantation (Table III) (44). When Lewis rats were treated with MNU at 7 weeks of age, and this E/P pellet was implanted into them at 24 weeks of age, the MNU-induced mammary carcinogenesis was accelerated (45); the incidence of mammary carcinomas tended to increase (60% *vs.* 44%), the latency tended to shorten (28.7 *vs.* 36.4 weeks), and cancer multiplicity significantly increased (1.8 *vs.* 0.8), compared with AMVs. In the E/P-treated rats, comedo carcinoma was frequently observed, and the incidences of estrogen receptor (ER)- and progesterone receptor (PgR)-positive mammary carcinomas were significantly lower than those of the AMVs (26% *vs.* 88%, and 11% *vs.* 63%, respectively). In that study, carcinogen was administered long before the hormone treatment. Further studies are necessary to determine whether the interval between carcinogen insult and hormone treatment modifies the subsequent development of mammary carcinomas in rats. Administration of estrogen and progesterone treatment to older mice and rats has cancer-protective and cancer-promoting effects, respectively. Late first full-term pregnancy promotes human breast carcinogenesis, suggesting that older women should not be administered estrogen and progesterone treatment.

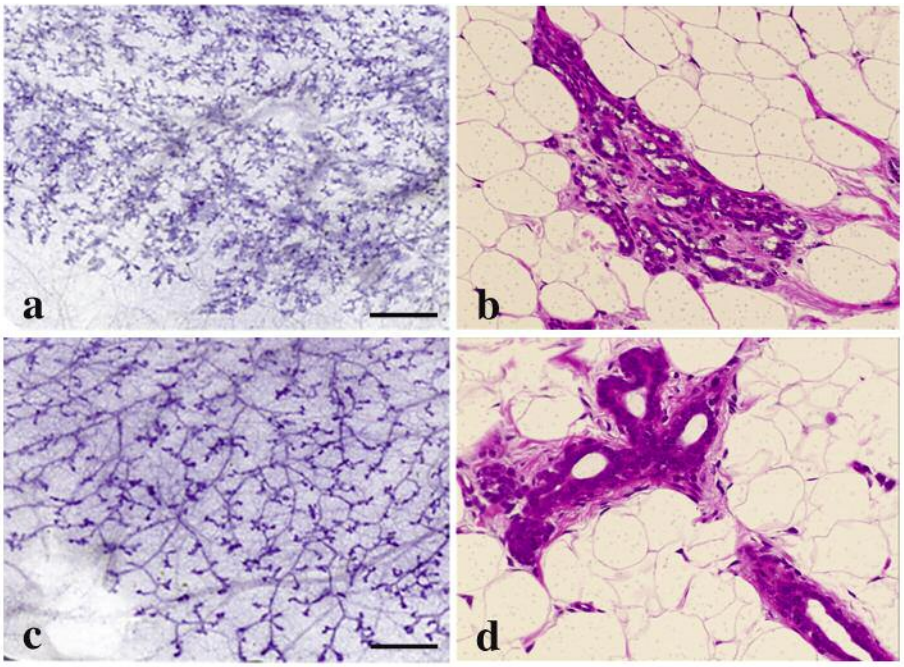

Figure 1. *Parous and age-matched virgin (AMV) mammary glands treated with N-methyl-N-nitrosourea (MNU). Parous rats nursed their pups for 3 weeks, followed by post-lactational involution for 4 weeks, followed by MNU injection, followed by sacrifice 3 weeks later. AMV rats received MNU and were sacrificed at the same age as the parous rats. Parous mammary gland showed increased ductal branching and alveolar development (a), and was composed of atrophic cells (b). AMV mammary gland exhibited a lower degree of branching (c) and a lower degree of alveolar formation (d). (a and c, Whole-mount section. Scale bars, 1.0 mm; b and d, Hematoxylin and eosin staining. Original magnification, x200).*

Duration of Estrogen and Progesterone Exposure Required for Protection against Mammary Carcinoma in Rats

In DMBA-treated Sprague-Dawley rats, a 12-week exposure to pregnancy levels of estrogen completely abolished the development of mammary carcinomas (46). In growth hormone-deficient spontaneous dwarf rats (derived from the Sprague-Dawley strain), which are refractory to carcinogen-induced mammary carcinomas, a 20-week exposure to growth hormone restored mammary carcinoma incidence to that of normal Sprague-Dawley rats (47). In these dwarf rats, treatment with a combination of estrogen, progesterone and growth hormone abolished the growth hormone-induced increase in the incidence of MNU-induced mammary carcinomas, whereas small numbers of mammary carcinomas began to appear after 20 weeks of hormone treatment. In a study by Yuri *et al.* (44), when MNU was injected into 4-week-old Lewis rats and a 21-day-release E/P pellet containing 0.5 mg of 17β-estradiol and 32.5 mg of progesterone was implanted at 6 weeks of age and replaced every 3 to 4 weeks for 23 weeks, development of mammary carcinoma was suppressed at the beginning of the experiment but abruptly increased from 21 to 23 weeks after the start of E/P treatment, and the cancer-suppressing effect eventually disappeared.

Pituitary isografting can produce continuous elevation of estrogen, progesterone and prolactin to levels similar to those that occur in mid-pregnancy. When *p53*-null mammary epithelial transplant mice underwent pituitary isografting at 5 weeks of age, they began to develop mammary carcinoma in p53-null epithelium at about 28 weeks of age, and they exhibited accelerated overall mammary carcinogenesis, compared with AMVs (100% *vs.* 62%) (48). Moreover, parous Sprague-Dawley rats refractory to carcinogen-induced mammary carcinomas regained high susceptibility to MNU-induced carcinogenesis after 33-weeks exposure to estrogen and progesterone and develop an even higher incidence of mammary carcinomas than AMV rats (92% *vs.* 64%) (49). Long-term exposure to pregnancy levels of estrogen and progesterone canceled out the mammary cancer-inhibiting effect or even accelerated mammary carcinogenesis. Thus, the above findings indicate that exposure of rats to estrogen and progesterone mimicking the pregnancy milieu only protects against mammary carcinogenesis if the duration of the exposure is <20 weeks.

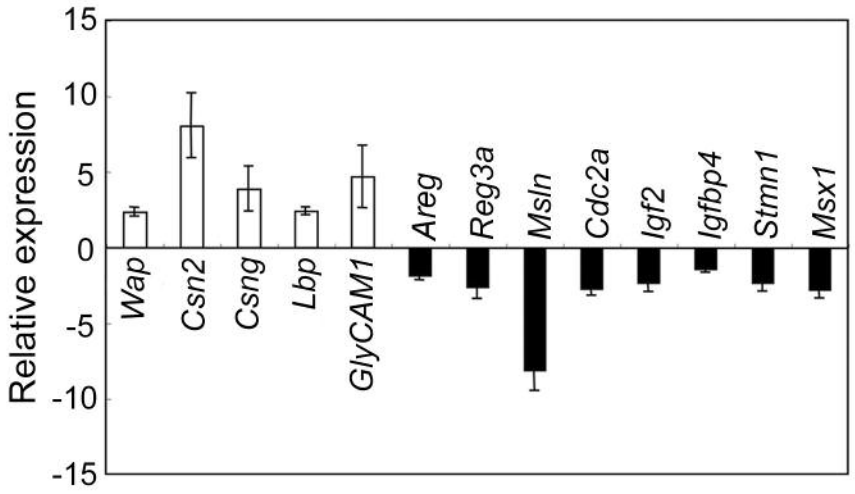

Figure 2. *Changes in differentiation- and growth-related gene expression in parous compared with age-matched virgin (AMV) mammary glands in response to N-methyl-N-nitrosourea (MNU). Parous rats nursed their pups for 3 weeks, underwent post-lactational involution for 4 weeks and then received N-methyl-N-nitrosourea (MNU). Age-matched virgin (AMV) rats received MNU at the same age as the parous rats. Both parous and AMV rats were sacrificed 3 weeks after MNU injection. Changes in gene expression levels of parous mammary glands were compared with those of AMV mammary glands using quantitative real-time PCR.*

Mechanisms of Parity- and Pregnancy-induced Protection

The cellular and molecular mechanisms that underlie parity-induced protection against breast cancer are largely unknown. Studies of those mechanisms have involved systemic alterations of the host environment and/or local changes in the mammary gland. Changes in host hormonal environment and structural or functional alteration of the mammary gland have been proposed to explain these mechanisms and several candidate genes have been isolated.

Host environment. Resistance to mammary carcinogenesis in parous rats may be caused by persistent changes in the host environment. When isolated virgin rat mammary epithelial cells, previously exposed to MNU, are transplanted into isogenic parous and AMV hosts, the cancer development is lower in the parous rats than in the AMVs; the host environment appears to be the determining factor (50). As described above, parous rats regain high susceptibility to MNU-induced mammary carcinogenesis after treatment with estrogen and progesterone for a long period (49); thus, protection against mammary carcinomas in parous rats is not permanent but plastic. Reduced circulating levels of growth hormone and prolactin in parous rats suggest that hormones act at the systemic level when reducing the susceptibility of parous rats to mammary carcinogenesis (51). This evidence suggests that changes in the host hormonal environment are responsible for the resistance of parous mammary glands to mammary carcinogenesis.

Mammary gland structure. Structural changes in the mammary gland caused by hormones are thought to underly the protective effect seen in parous rats; *i.e.* protection is due to the pregnancy-induced differentiation of the target structures for carcinogenesis, which are terminal end buds (TEBs) and terminal ducts (TDs) (52). Some reports indicate that the structure of the involuted parous mammary gland is a highly branched epithelial tree that is more complex than are AMV glands (24, 28, 40, 44, 51, 53, 54). However, other reports indicate that there is no significant structural difference between fully involuted hormone-exposed mammary glands and AMV glands (27, 32, 33). When MNU-treated rats were treated with estrogen and progesterone or perphenazine (a dopamine receptor inhibitor) for 3 weeks, mammary carcinoma was suppressed after estrogen and progesterone treatment but not after perphenazine treatment (31). Although perphenazine causes mammary gland differentiation to a near-lactational state, the finding that it does not protect against mammary cancer suggests that structural differentiation *per se* does not have protective effects.

Hormone receptor status. Parity decreases the number of ER- and PgR-positive cells in normal mammary glands; in animal studies, this decrease happens whether the pregnancy occurs before or after carcinogen administration (24, 51). Interaction between estrogen and progesterone and their respective receptors is an important factor in proliferation of mammary epithelial cells. The majority of mammary carcinomas in rats are hormone-dependent and

neither pregnancy nor administration of estrogen and progesterone affects the receptor status of subsequent mammary carcinomas (24, 42). These findings suggest that the cancer-suppressing effects of pregnancy and treatment with estrogen and progesterone are due to reduced numbers of ER- and/or PgR-positive cells, which are the presumed progenitors of hormone-dependent mammary carcinoma.

Proliferative potential. One of the cellular mechanisms underlying the protective effect of parity involves a diminution of the proliferative potential of the mammary epithelial cells (52). In a study using a *p53*-null model, the proliferative activity of estrogen and progesterone-treated mammary glandular cells was reduced shortly after cessation of hormone treatment, compared with AMVs (14). Proliferating cell nuclear antigen (PCNA) labeling in parous mammary glands is significantly lower than in AMV glands, even long after delivery (at the termination of the experiment) (24, 31). This change in proliferative potential includes suppression of carcinogen-induced cell proliferation. The bromodeoxyuridine (BrdU) or PCNA labeling index is lower in parous mammary glands than in AMV mammary glands and whereas proliferation in AMV glands is significantly increased after carcinogen challenge, parous glands do not exhibit a proliferative response after carcinogen challenge (27, 54, 55). Before MNU treatment, the level of PCNA-positive cells was lower in parous mammary glands (3.3%) than in AMV gland (5.6%); after MNU treatment, a significant increase in PCNA labeling occured in AMV glands (13.7%), whereas the labeling index remained low in parous mammary glands (3.6%) (54). Thus, the increase in proliferation triggered by carcinogen treatment appears to be retained by AMV mammary glands, while it is attenuated in parous glands. Parity protects against the occurrence of frank mammary carcinomas, but it does not significantly suppress the development of small non-palpable microcarcinomas (24). Most strikingly, in a *p53*-null epithelial model, hormone treatment suppressed the development of large palpable carcinomas, but did not suppress the development of non-palpable premalignant lesions (14). These findings strongly suggest that parity-induced protection against mammary carcinogenesis involves the post-initiation stage of carcinogenesis, rather than the initiation stage; the decrease in proliferation potential may prevent the initiated cells from growing into frank carcinoma.

Molecular analysis. The hormonal milieu of pregnancy may cause persistent molecular changes in the mammary gland. After pregnancy or short-term treatment with estrogen and progesterone, characteristic changes in the mammary gland include up-regulation of specific genes involved in epithelial differentiation, immune regulation and TGFβ signaling, and down-regulation of genes involved in epithelial proliferation (40, 53, 54, 56). Molecules involved in immune responses, such as molecules expressed in hematopoietic cells, may participate in the immune environment of the mammary gland, and may modify the carcinogenic response. TGFβ directly affects growth of mammary epithelial cells, and inhibits mammary carcinogenesis (57). Consistent with this is the finding that normal mammary glands of multiparous women are characterized by elevated expression of differentiation-related genes, compared to nulliparous mammary glands (58).

Uehara *et al.* (54) compared gene expression between parous and AMV mammary glands of Lewis rats after MNU treatment. Representative morphologies used for molecular analysis are chosen in Figure 1. The parous mammary glands treated with MNU showed increased branching and alveolar development (Figure 1a), compared with AMV glands (Figure 1c and d), and were composed of atrophic cells (Figure 1b). In a study in which microarray expression profiles were used to assess changes in global gene expression in response to hormone and carcinogen treatments, selected genes were further analyzed by quantitative real-time PCR. Parous and AMV mammary glands differ significantly in their expression of several genes in response to carcinogenic challenge. Figure 2 summarizes the expression of differentiation- and growth-related genes in parous compared with AMV mammary glands in response to MNU. Parous mammary glands treated with MNU exhibit up-regulation of differentiation-related genes such as *whey acidic protein (Wap), casein beta (Csn2), casein gamma (Csng), lipopolysaccaride binding protein (Lbp)* and *glycosilation-dependent cell adhesion molecule 1 (GlyCAM1),* and exhibit down-regulation of growth-related genes such as *amphiregulin (Areg), regenerating islet-derived 3 alpha (Reg3a), mesothelin (Msln), cell division cycle control 2 homolog A (Cdc2a), insulin-like growth factor 2 (Igf2), insulin-like growth factor binding protein 4 (Igfbp4), stathmin 1 (Stmn1), homeobox,* and *msh-like 1 (Msx1)*. Down-regulation of growth-related genes in parous mammary glands may be related to blockage of carcinogen-induced cell proliferation. A parity-induced decrease in expression of *Areg*, an epidermal growth factor receptor (EGFR) ligand, has been observed in many strains of rats and mice (40, 53). There have been no reported findings regarding *Reg3a* expression in the mammary gland. *Msln* promotes anchorage-independent growth and prevents anoikis, a form of apoptosis induced by detachment from the substratum, in human breast cancer cells (59). Cyclin-dependent kinases (CDKs) are critical regulators of cell cycle progression and overexpression of *Cdc2* (*CDK1*) has been observed in human breast cancer cell lines (60). *Igf2* is up-regulated in DMBA-induced mammary carcinomas (61) and transgenic mice that express *Igf2* develop multiple mammary carcinomas that have metastatic potential (62).

Igf2 is a potent mitogen in human breast cancer cells and Igf2 activity is influenced by its binding proteins (Igfbp); *Igfbp4* expression is related to growth of MNU-induced mammary carcinomas (63). Overexpression of *Stmn1* has been observed in DMBA-treated normal rat mammary gland (64) and has been observed in highly proliferative human breast carcinomas (65). *Msx1* plays a role in morphogenesis of the mammary gland and may be involved in carcinogenesis *via* cyclin D1 expression (66). Molecular studies suggest that parous mammary glands are more differentiated than AMV mammary glands and that parous mammary glands are refractory against carcinogenic stimuli due to their lower expression of genes involved in cell proliferation. In attempts to create a cell phenotype of refractoriness against carcinogenic challenge, the aforementioned genes are potentially important targets.

Conclusion

Pregnancy hormone treatment that produces circulating levels of estrogen and progesterone similar to those of pregnancy replicate some effects of pregnancy and can result in natural chemoprevention without adverse side-effects when given to young female mammals for a short duration. Thus, such treatment may be useful as a method of protecting against breast cancer. In studies using molecular analysis, parous mammary gland exhibit up-regulation of differentiation-related genes and down-regulation of growth-related genes. It appears that parity-induced protection against breast cancer involves blockage of carcinogen-induced cell proliferation, and that such blockage involves genes, such as *Areg, Reg3a, Msln, Cdc2a, Igf2, Igfbp4, Stmn1* and *Msx1*. The systemic hormone milieu and local hormone receptor status in the mammary gland may also be involved in parity-induced protection. In rodents, when treatment with estrogen and progesterone is used to mimic the pregnancy milieu, treatment for durations shorter than the period of gestation can provide significant protection. In pre-treatment methods, both estrogen and progesterone are required for protection, whereas in post-treatment methods, treatment with estrogen alone can be effective; addition of progesterone can enhance the effect of estrogen in post-treatment methods. Thus, although it is unclear when mammary carcinogenic initiation occurs in humans, it appears that the most suitable option for hormone-induced protection against mammary carcinogenesis is early administration of both estrogen and progesterone. In animal experiments, effective protection against mammary carcinogenesis has been obtained by administering estrogen and progesterone beginning at $\leq$3 months of age for a duration of $<$20 weeks. However, short-term administration of estrogen and progesterone to older rats accelerates the development of carcinogen-induced mammary carcinomas with low ER and PgR levels, frequently with comedo necrosis, which suggests a poor prognosis. Moreover, the protective effects of estrogen and progesterone treatment decrease with increasing duration of treatment and sufficiently long treatment can actually increase susceptibility to mammary carcinogenesis. Thus, age and treatment duration are particularly important factors for effective protection from breast cancer.

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