Mammary developmental fate and breast cancer risk

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Abstract

The ovarian hormones, estrogen and progesterone, play a pivotal role in normal and neoplastic development of the mammary gland. These hormones have a paradoxical role as long duration of estrogen and progesterone are associated with increased breast cancer risk, while short duration of pregnancy level doses are associated with a reduced breast cancer risk. The protective effects of estrogen and progesterone, as well as pregnancy, have been extensively studied in animal models. Recent studies have demonstrated that these hormones induce alterations in gene expression in the mammary epithelial cells which persist for a long time after the hormones are withdrawn from the host. It is postulated that hormones induce a switch in mammary developmental fate which decreases the risk of breast cancer over the lifetime of the host. Some of the possible cellular pathways persistently altered by short term hormone exposure are a decrease in growth factors and an increase in apoptosis. The expression of these genes, in turn, may be affected by alterations in genes regulating chromatin remodeling. The relative contributions of host-mediated factors and mammary cell intrinsic factors remain to be determined. The current studies have moved this research area from the biological to the molecular realm and offer the potential for directing prevention efforts at specific molecular targets.

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Introduction

Breast cancer is a major cancer of women in the United States and Western Europe with approximately 211 000 new cases predicted in the United States in 2005 (American Cancer Society 2005). Breast cancer accounted for approximately 32% of new cancers diagnosed in US women in 2004 (American Cancer Society 2005). The rate of increase of breast cancer incidence slowed in the 1990s; at the same time, the incidence of *in situ* breast lesions, mainly ductal carcinoma *in situ* (DCIS), increased so that approximately 59 000 new cases of *in situ* lesions occurred in 2004 (American Cancer Society 2005). The mortality rate was relatively constant through the last quarter of the 20th century before showing a significant decrease starting in the early 1990s. Early diagnosis and continuing new therapeutic approaches have managed to prevent the epidemic from causing a concomitant increase in death. Nevertheless, the death of 40 000 women due to invasive breast cancer remains a

sobering fact and indicates the need to understand this disease in greater depth and to develop new interventions, both preventive and therapeutic.

Current understanding of the central role of hormones in the genesis of breast cancer is based on over 100 years of studying breast cancer. Beatson in 1896 demonstrated that removing the source of steroid reproductive hormones by bilateral oophorectomy was an effective therapeutic treatment. The presence of estrogen receptor (ER) is the most widely used predictive factor of breast cancer response to treatment and was the basis for developing selective estrogen receptor modulators (SERMs; i.e. tamoxifen and raloxifene) and aromatase inhibitors (Jordan *et al.* 1991, Osborne 1998, Brodie *et al.* 1999, Goss 2004) as adjuvant therapy against breast cancer recurrence. Additionally, recent trials have documented that hormone replacement therapy that includes both estrogens and progestins imparts a greater breast cancer risk than estrogens alone (Santen 2003).

Epidemiological considerations

Breast cancer incidence rates are influenced by age, genetics, reproductive history, radiation, socio-economic status, place of residence, and ethnicity (McPherson *et al.* 2000). Reproductive history is the strongest and most consistent risk factor outside of genetic background and age (McPherson *et al.* 2000, Harris *et al.* 1992, Kelsey & Gammon 1991, Althuis *et al.* 2004, Ursin *et al.* 2004). Early menarche, late menopause, parity, and late age of first pregnancy are each independent risk factors. It has been estimated that breast cancer risk is reduced 10–20% for each year of delay in the onset of menarche (Henderson *et al.* 1991, Harris *et al.* 1992). It is of interest that the high calorie Western-style diet pre-disposes children to an earlier puberty (Harris *et al.* 1992). Additionally, the phenomena of a Western-style diet, decreased age of menarche, and increased breast cancer incidence are now occurring in Japan and other countries in Asia. The early age of menarche translates into earlier hormone exposure (estrogen and progesterone) and breast epithelial cell growth (Henderson *et al.* 1991). Epidemiological studies have indicated that early age of menarche (i.e. 12.8 years vs ≥ 17 years old) and height (90th vs 10th percentile) confer a 1.5-fold and 1.3-fold increase in risk, respectively (Harris *et al.* 1992). A recent study shows that height and body mass index (BMI) at age 14 are associated with an increased risk in adult life (Ahlgren *et al.* 2004). These latter two measurements are considered to be surrogate markers for growth stimulatory activities in the prepubertal and adolescent years.

Ironically, although early menarche and total years of hormone exposure are risk factors for increased incidence, early age of first pregnancy (≥ 20 years of age) is a strong protective factor. The protective factor is especially observed in postmenopausal women, the period of peak incidence. Parity-induced protection against breast cancer is principally dependent on the timing of the first full-term pregnancy but also is affected by total number of pregnancies (Albrektsen *et al.* 2005). The association is independent of the number of live births. Aborted pregnancies are not associated with decreased risk for breast cancer (Melbye *et al.* 1997, Beral *et al.* 2004). The protective effect of early first pregnancy has been repeatedly demonstrated in numerous epidemiological studies and provides a physiologically operative model to achieve practical and affordable prevention of breast cancer in humans (Henderson *et al.* 1991). The logic and rationale for understanding the

molecular basis for hormone-mediated prevention of breast cancer are based on the consistent observations in human epidemiological studies and the strong confirmatory experiments in rodent breast cancer models.

Protective effect of pregnancy

There are many different ways to prevent carcinogenesis. The concept developed by Wattenberg (1997) emphasizes the distinction between blocking agents and suppressing agents. The former prevent carcinogenic chemicals from interacting with target molecules, whereas the latter prevent the development and/or expression of the transformed cells. With respect to the mammary gland, agents such as retinoic acid (Welsch 1987, Wu *et al.* 2002), selenium (Ip & Medina 1987, Medina *et al.* 2001b), and D-limonene (Crowell *et al.* 1992) act as both blocking and suppressing agents, depending on the time intervals of application. It is more difficult to classify endogenous natural factors such as estrogen and progesterone. These agents do not fit the categories of suppressing or blocking agents since their effects can persist long after the withdrawal of the agents (Grubbs *et al.* 1985, Rajkumar *et al.* 2004). This consideration serves to emphasize the unique nature of hormonal prevention of methyl-nitrosourea (MNU)-induced carcinogenesis of the mammary gland. As illustrated in Table 1, prevention of mammary carcinogenesis can be attained by a single pregnancy or by hormone regimens comprising the two main hormones of pregnancy, estrogen and progesterone, or by human chorionic gonadotropin. Of the numerous experiments addressing this question, only the more pertinent experiments are summarized in Table 1.

There are two different experimental models that demonstrate parity/hormone-induced protection (Fig. 1). In the first model, one half of the animals undergo hormonal stimulation, the mammary gland is allowed to involute completely, and then the carcinogen is administered to the hormone-treated and AMV animals. This model is termed the 'pre-treatment model' and most of the experiments use this experimental protocol. In the second model, the animals are treated with a carcinogen, and then one half are exposed to hormone treatment for a specified time period. This model is referred to as the 'post-treatment' model. Unless otherwise stated, most comments refer to the pre-treatment model. The distinction is important as the underlying mechanisms for protection are likely to be different between the two models.

Table 1 Prevention of Mammary Tumorigenesis by Parity and Hormones

Hormone treatment	Carcinogen (days) ^a	Tumorigenesis (control/treated)		
		MC incidence ^b	Inhibition (%)	Ref.
Pregnancy	DMBA (21 d) ^c	71/0	100	(Moon 1969)
Pregnancy/lactation	DMBA (50 d)	71/11	84	(Moon 1969)
Pregnancy	DMBA (21 d)	48/6	88	(Russo & Russo 1980)
Pregnancy	DMBA (15 d)	70/14	80	(Sinha <i>et al.</i> 1988)
E + P (20 µg/4 mg)	MNU (21 d) ^d	61/13	79	(Grubbs <i>et al.</i> 1985)
E + P (5 µg/2 mg)	MNU (21 d)	61/19	69	(Grubbs <i>et al.</i> 1988)
hCG (10 lu)	DMBA (21 d)	44/18	52	(Russo IH <i>et al.</i> 1990)
Pregnancy/lactation	DMBA (100 d) ^e	38/16	58	(Russo IH <i>et al.</i> 1991)
Pregnancy	DMBA (130 d) ^e	38/18	53	(Russo IH <i>et al.</i> 1991)
Pregnancy	MNU	80/17	79	(Thordarson <i>et al.</i> 1995)
E + P (30 mg/30 mg)	MNU	55/0	100	(Guzman <i>et al.</i> 1999)
E + P (20 µg/20 mg)	MNU (28 d)	56/10	82	(Sivaraman <i>et al.</i> 1998)
Pregnancy/lactation ^f	MNU (168 d)	33/20	40	(Yang <i>et al.</i> 1999)
Pregnancy/lactation ^g	MNU (75 d)	54/8	85	(Yang <i>et al.</i> 1999)
Pregnancy ^h	MNU (75 d)	54/35	35	(Yang <i>et al.</i> 1999)
Pregnancy ⁱ	DMBA (15 d)	70/25	64	(Medina & Smith 1999)

^aInterval in days between end of E/P treatment, parturition (or weaning), and treatment with chemical carcinogen.

^bMC = mammary carcinoma; MC incidence in control rats/MC incidence in treated rats.

^cDMBA = 7,12-dimethyl benzantracene

^dMNU = methylnitrosourea

^eRats were 180–190 days of age when administered carcinogen.

^fExperienced one delivery of pups at 105 days of age (posttreatment model).

^gExperienced one delivery of pups at 113 days of age (pretreatment model).

^hExperienced one delivery of pups at 109 days of age without lactation (pretreatment model).

ⁱAll experiments used rats except this experiment, which used two different mouse strains.

Effect of pregnancy, pregnancy and lactation

Table 1 clearly demonstrates that pregnancy is an efficient physiological process to reduce mammary carcinogenesis. Most of the pregnancy experiments have been performed in outbred Sprague-Dawley rats, inbred Lewis and Wistar-Furth rats, and inbred mice. In most of the studies there is greater than 75% inhibition of the incidence of carcinomas. Pregnancy alone and pregnancy and lactation, occurring both prior to carcinogen administration and following carcinogen exposure, significantly reduce the incidence of mammary carcinomas and the number of palpable carcinomas per rat, and significantly prolong the latency. Epidemiological literature on the history of breast-feeding in relation to breast cancer risk in humans also shows that prolonged breast-feeding is additionally protective against breast cancer among premenopausal women (Althuis *et al.* 2004, Perez-Escamilla & Guerrero 2004). In rodents, the protective effect of pregnancy was observed even when the carcinogen was administered 100–130 days after the first parturition, indicating a long-lasting alteration in the state of sensitivity of the mammary gland. Thus,

the rodent model represents an extensively validated model for the human paradigm.

In most experiments pregnancy was as effective as pregnancy and lactation (86% vs 83% inhibition respectively), so that the complete functional differentiation of the mammary gland would appear not to be necessary for protection from the carcinogenic insult. Pregnancy can be mimicked by estrogen and progesterone administration. It is known that estrogen can increase prolactin levels, so the role of prolactin has to be considered in any discussion of mechanisms. However, two studies using the NMU-rat model have shown that continuous treatment with perphenazine, a prolactin-elevating drug, does not lead to a protective state. The studies were performed in both the post-treatment model (Guzman *et al.* 1999) and the pre-treatment model (D Medina, unpublished observations). The length of pregnancy necessary to confer protection has yielded contradictory results. On the one hand, the experiments of Sinha *et al.* (1988) suggest that interrupted pregnancies of 5, 10, and 15 days resulted in 48% tumor incidence versus 79% tumor incidence for age-matched controls and 14% tumor incidence for full-term pregnancy. In a different

Figure 1 The two experimental treatment models for studying the effect of hormones on chemical carcinogen-induced mammary tumorigenesis in the rodent. The carcinogen, methyl nitrosourea (MNU) is more commonly used in current rat studies, whereas 7,12-dimethylbenz(α)anthracene is used in mouse studies. Pregnancy or specific hormone treatment like estrogens (E) or progestins (P) occur prior to carcinogen (pre-treatment model) or after carcinogen administration (post-treatment model). In either model, a sufficient period of time is allowed between hormone and carcinogen exposure.

study (Russo *et al.* 1992), pregnancy interruption at day 12 did not confer any difference in protection compared with full-term pregnancy (78% vs 71% respectively). There were only two basic differences in the experiments. Much larger group sizes were used (25–27 vs 7–9 rats respectively) in the Sinha experiment, and the interval between end of hormone stimulation and carcinogen treatment was 15 versus 21 days respectively. One can argue that either the larger group size or the longer time interval was the important variable. The larger group size (>25 animals per group) of the first study yields more powerful statistical data than the nine animals in one pregnancy-interrupted group. Additionally, the fact that only 5 days of hormone stimulation of pregnancy was sufficient to reduce the risk by 39% is startling. This result suggests that the initial act of hormone stimulation of mammary gland development induces sufficient changes in cell type and/or cellular regulatory pathways to create a significantly resistant cellular phenotype. After 5 days of stimulation, DNA synthesis is just beginning, morphological differentiation is minimal (Grubbs *et al.* 1988, Medina *et al.* 2001a), and subsequent epithelial cell involution is rapid. Therefore, it is unlikely that the resistance to carcinogen-induced transformation could be attributed to the presence of functionally differentiated lobuloalveolar cells. Furthermore, the perphenazine experiments

referred to in the preceding paragraph indicate that lobuloalveolar differentiation *per se*, is not a critical or determining factor in resistance to carcinogen-induced tumorigenesis. It is more likely that hormones upregulate a specific regulatory pathway(s) which persist in the cells and progeny thereof.

Table 1 also shows that hormonal treatment to mimic pregnancy using either estrogen and progesterone or human chorionic gonadotropin is also effective in reducing mammary tumor incidence in rodents. Human chorionic gonadotropin causes a rise in endogenous sex steroids. Like pregnancy, hormone mimicry of pregnancy has been investigated in both the pre-treatment and post-treatment models of prevention. It is clear that both estrogen (E) and progesterone (P) are required to induce the maximum resistant phenotype; however, the use of single hormone treatments has yielded tantalizing results (Grubbs *et al.* 1985). Estrogen treatment (20 μ g E/day $\times$ 35 days) did not significantly alter tumor incidence or the average number of tumors per rat. Progesterone treatment (4 mg/day $\times$ 35 days) delayed both the mean number of tumors per rat (0.55 vs 1.45, $P < 0.05$) and the mean latency of tumor appearance (120 vs 108 days). However, the incidence of cancers was not significantly lower compared with animals administered hormone vehicle alone. A recent study by Medina *et al.* (2001a) shows that rats treated with either estrogen (20 μ g) or progesterone (20 mg) alone for 21 days in a pre-treatment model was insufficient to confer protection. This result supports the earlier conclusion of Grubbs *et al.* (1988) that both hormones are necessary for the protective effect in a pre-treatment model.

In contrast, the effects of E and P given singly were tested to determine the ability of these hormones to induce protection from mammary carcinogenesis in a post-treatment model (Rajkumar *et al.* 2001). It was shown that treatment with E2 alone, at a critical dose of 100 μ g/silastic tubing, provided a pregnancy level of circulating E2 and a protection from mammary carcinogenesis compared with the AMV. In a recent study (Rajkumar *et al.* 2004), these same authors demonstrated that a one week exposure to 100 μ g ethynyl estradiol plus 30 mg megestrol or norethindrone imparted a dramatic protective effect in the post-treatment model.

Mouse model for hormone-induced protection

Recently, we documented, in three different mouse strains, hormone-induced refractoriness of the involuted mouse mammary gland to chemical

carcinogenesis (e.g., 7,12-dimethylbenzanthracene, DMBA) under conditions that are similar to those reported in rats and presumed to be operative in humans (Medina & Smith 1999, Medina & Kittrell 2003). Using the BD2fF1 strain of mice, we showed that mammary tumor incidence was statistically significantly reduced from 70 to 25% upon pregnancy and involution. In the C3H/Sm mice, mammary tumor incidence was decreased from 57 to 21%, although the decrease did not reach statistical significance because of smaller group sizes. In BALB/c mice, mammary tumor incidence was decreased from 63% to 17%, ($P < 0.05$). The histopathology of the mammary tumors arising in parous versus AMV mice was similar and included both type B adenocarcinomas and squamous adenocarcinomas. An earlier study had concluded that parous BALB/c mice were resistant to mammary carcinogenesis induced by MNU (Swanson *et al.* 1995). These results demonstrate that the mouse is a suitable model to examine the mechanism of hormone-induced refractoriness to carcinogen-induced mammary tumorigenesis. This is an important piece of information because it now allows the use of gene KO and transgenic mouse models to test specific hypotheses regarding the function of genes involved in the protection, e.g. p53 KO, cyclin D1 KO mice, PR KO mice, SRC3 KO mice, etc.

Mechanisms of protection

Initiation vs promotion

A consideration of the majority of the literature indicates that experiments have not specifically addressed the question of whether hormone-induced refractoriness is targeted to the initiation stage or promotion stage of carcinogenesis. However, there are several recent results stated in the literature that address this question (Yang *et al.* 1999, Reddy *et al.* 2002, Nandi *et al.* 2005). The results demonstrate that the hormone-induced refractory gland, upon autopsy contains small, nonpalpable microtumors in the absence of palpable tumors. The frequency of the microtumors was less than or equal to that found in MNU-treated AMV virgins. These experiments, performed in the pre- and post-treatment models, definitely demonstrate that pregnancy primarily delays progression. Along these lines, the provocative experiments by Thordarson *et al.* (2001), that continuing the same doses of hormones for an extended period of time (20 rather than 3 weeks) results in abundant tumor development equivalent to the AMV, indicate that hormones can promote progression, if administered

continuously. A similar conclusion was reached in the parous mouse using pituitary isografts as the source of hormones (Swanson *et al.* 1995).

Induction of differentiation

Studies by Russo & Russo (1987, 1997) have led to the hypothesis that differentiation of the mammary gland, whether induced by pregnancy or hormones, results in the removal of the population of cancer-susceptible cells and hence confers protection against breast cancer. A gland that has encountered a full-term pregnancy has achieved a greater degree of terminal differentiation than that which is present in a similarly aged virgin gland. In this scenario, it has been postulated that the pregnancy-induced differentiation rids the gland of a specific population of susceptible cells (present in the terminal end buds (TEBs) and terminal ducts) which are considered targets of tumorigenesis (Russo & Russo 1987, 1997). The highly mitotic terminal ducts are differentiated to less mitotic alveolar cells and progeny thereof.

The 'terminal differentiation' hypothesis was an attractive but oversimplified hypothesis that cannot explain the beneficial effects of hormones for several reasons. First, the appropriate comparison is between the AMV, where TEB are few, and the hormone-treated gland. Second, the process of pregnancy does not lead to persistent morphologically recognizable differentiated cells, i.e. persistent lobules. In rats, there is no consistent alteration in morphological differentiation between the AMV gland and the parous involuted gland when surveyed over different strains (Sinha *et al.* 1988, Thordarson *et al.* 1995). Hence, differentiation *per se* cannot explain the mechanisms of hormone-induced protection. Third, placental lactogen, which promotes differentiation, has no protective effect against breast cancer. Fourth, as discussed earlier, perphenazine (PPZ) is a dopamine receptor inhibitor that causes acute release of prolactin from the anterior pituitary of rats. This release of prolactin causes proliferation and differentiation of the mammary gland to a near-lactational state. Despite inducing morphological and functional differentiation of the mammary gland, PPZ is ineffective in offering protection from MNU-induced carcinogenesis in rats in the pre- or post-treatment model (Guzman *et al.* 1999).

Decreased proliferation activity

There is strong evidence to suggest that decreased susceptibility of the gland to carcinogenesis with increasing age is correlated with a decrease in the

highly proliferating cell populations of the mammary gland, i.e. the TEBs and terminal ducts. As a corollary to Russo's differentiation hypothesis, the differences in susceptibility to carcinogen-induced tumorigenesis between parous and age-matched virgin (AMV) glands could be explained by the differences in proliferation indices. This alternative explanation is unlikely. In 9–100-day old virgin rats, there are few TEBs and a very low cell proliferative index, yet the susceptibility is still very high (since 60–70% of the rats develop mammary adenocarcinomas within a short-time period after MNU). It has been reported that there are no differences in the overall cellular kinetics between parous rats and AMVs (Sinha *et al.* 1988, Sivaraman *et al.* 1998).

Altered hormonal milieu

Another hypothesis emphasizes that resistance to mammary tumorigenesis in parous rats may be caused by persistent changes in the parous rat mammary gland hormonal environment resulting in permanent biochemical alterations in the mammary epithelia (Musey *et al.* 1987, Thordarson *et al.* 1995). Circulating levels of prolactin appeared lower (although this was not statistically significant) in the parous rat compared with the AMV (Thordarson *et al.* 1995). Growth hormone (GH) concentration was significantly reduced in parous rats as compared with AMV rats (Thordarson *et al.* 1995). The parous animals also had decreased levels of ER and EGF-R (Thordarson *et al.* 1995). The reduction in the circulating levels of GH caused a reduced susceptibility of the parous rats to mammary carcinogenesis, possibly by decreasing the levels of ER, EGF-R and/or IGF-1 in the mammary gland. The role of the GH-IGF1 axis in hormone-induced protection is supported by two additional results (Thordarson *et al.* 2004a,b). First, NMU-induced mammary tumorigenesis is almost totally absent in GH-deficient spontaneous dwarf rats (SDR). SDR rats given GH or IGF-1 before and after NMU-treatment exhibited a normal (GH) or near normal (IGF-1) tumorigenic response. Estradiol (E2) and progesterone (P4) treatment did not restore tumorigenesis. Furthermore, E2+P4 blocked the response to GH. SDR rats have a normal circulating concentration of prolactin (PRL), indicating PRL was not a factor in the low tumorigenic incidence (Thordarson *et al.* 2004a). Second, IGF-1 treatment of parous rats for 60 days after NMU-treatment increased the mammary tumor incidence compared with untreated parous rats and to AMV rats (Thordarson *et al.* 2004b).

In some studies, the results show that pregnancy reduces the number of ER and/or PR positive cells in the normal mammary glands of the parous rat (Thordarson *et al.* 1995, Yang *et al.* 1999). An inadequate hormonal environment could contribute to low ER and PR levels, low levels of proliferation, and slow rate of progression. This hypothesis was developed in the post-treatment model and has not been tested in the pre-treatment model. In addition, parts of the hypothesis, i.e. the altered frequency of ER positive cells in parous rats, have not been reproduced in other rat strains (Sivaraman *et al.* 2001b). In addition, it is important to examine whether the stroma of parous animals supports proliferation of mammary epithelial cells in a quantitatively different manner than the stroma of AMVs. It has been demonstrated that the reproductive state does persistently alter mammary stroma extracellular matrix composition and function (Schedin *et al.* 2004).

Cell fate hypothesis

The cell fate hypothesis states that in the mammary gland of the immediate postpubescent female, hormones induce a molecular switch in stem cells that results in cells with persistent changes in the intracellular regulatory loops governing proliferation and response to DNA damage (Sivaraman *et al.* 1998). In this 'cell fate hypothesis' at a critical period in adolescence, the hormonal milieu of pregnancy affects the developmental fate of a subset of mammary epithelial cells. The consequence of hormone exposure is the induction of persistent differences in signal transduction pathways, growth factors, cell cycle regulators, transcription factors, and/or other unknown regulatory molecules between the epithelial cells in the hormone-treated and the mature virgin mammary gland. These molecular changes are manifested in at least three cellular responses that differentiate the two cell states: first, a block in carcinogen-induced proliferation (Sivaraman *et al.* 1998); second, a block in proliferation of estrogen-receptor positive cells (Sivaraman *et al.* 2001b); and third, the appearance of a new cell type, termed parity-induced mammary epithelial cells (PI-MEC) (Wagner *et al.* 2002).

First, the number of apoptotic cells is low and comparable in the hormone-exposed and AMV glands at the time of carcinogen challenge, and remains low 8 days after MNU. The number of BrdU-labeled cells at the time of carcinogen challenge is low in both the AMV (1.8%) and hormone-exposed (0.8%) animals (Sivaraman *et al.* 1998). On the eighth day after MNU

treatment, cell proliferation in the AMV (5.7%) was significantly different from the parous involuted (1.2%) and E/P-treated animals (1.5%). This same relationship was seen in DMBA-treated BALB/c mice (Sivaraman *et al.* 2001a). Therefore, it appears that hormone treatment results in persistent alterations in intracellular pathways governing proliferation responses to carcinogens. The difference in proliferation frequency disappears by 20 days after carcinogen challenge. There are likely other cellular responses that are different between the two cell states, but these have not been elucidated yet.

Second, ER+ and PR+ proliferating cells are rare in the developing mammary gland of a young virgin rat, and the two markers, receptor expression and proliferation, are dissociated from each other (Russo *et al.* 1999). This result is in accord with the current hypothesis that steroid receptor dependent regulation of proliferation is paracrine in the normal developing mammary gland (Anderson *et al.* 1998). The percentage of dual-labeled cells significantly increased in the mammary gland of a mature virgin rat (96-day old rat), and the two markers were positively associated. However, prior exposure of the mammary gland to pregnancy levels of E/P results in the failure of the mammary gland to attain a positive association between steroid receptor expression and proliferation (Sivaraman *et al.* 2001b). These results have two important implications. The co-expression of steroid receptors and proliferation may be an early manifestation of an important molecular change in steroid receptor-dependent regulation of proliferation that increases susceptibility to tumor formation. As well, the ability to block association between the two markers might be one way in which pregnancy induces protection against breast cancer.

Third, the altered cell fate hypothesis is supported by recent studies demonstrating that pregnancy induces the appearance of a stable cell subpopulation that persists after involution (Wagner *et al.* 2002). The authors termed these cells parity-induced mammary epithelial cells (PI-MEC) and demonstrated that these

cells provide the cells generated in subsequent pregnancies. Transplantation experiments demonstrate that these cells possess progenitor capabilities as they produced both luminal and myoepithelial cells. These PI-MEC are pluripotent and self-renewing (Boulanger *et al.* 2005). Interestingly, G.B7, one of the genes persistently elevated by pregnancy, colocalizes in the same tissue compartment occupied by the parity-induced cells (Ginger *et al.* 2001).

Molecular studies

Gene Expression

Several different groups have published extensive gene expression profiles of parous versus AMV mammary epithelial cells in rodent models. The results are summarized in Table 2. Ginger *et al.* (2001) used suppression subtractive hybridization (SSH) and identified over 200 genes that were persistently upregulated in the hormone-treated involuted glands of Wistar-Furth female rats. One gene, RbAp46, functions in chromatin remodeling. The other gene of interest is designated G.B7 and is a noncoding RNA. The importance of the upregulation of these unique genes is that they suggest epigenetic mechanisms for the persistent changes found in the hormone-treated mammary epithelial cells and for the altered cell fate. A similar mechanism may operate with hCG-induced protection. In this model, the effect of hCG is correlated with increased expression of inhibin and downstream activation of c-myc and other genes (Jiang *et al.* 2002). The authors show that the transcription of these genes is accompanied by increased acetylation of histones in a non-tumorigenic human breast cell line MCF-10F when treated with either hCG or inhibin.

In an elegant and comprehensive study by D'Cruz *et al.* (2002), the authors used DNA microarray technology to identify differentially expressed genes common in the parous involuted glands in multiple strains of both rats and mice. The authors identified 38 differentially expressed genes common to both species

Table 2 Evidence for altered gene expression in hormone-treated mammary gland

Gene(s)	Method	Result	Reference
Growth factors, TGF β_3	DNA microarrays	$\uparrow\downarrow$ in parous gland	(D'Cruz <i>et al.</i> 2002)
G.B7, RbAp46 (chromatin remodeling)	Suppression subtractive hybridization	$\uparrow$ in parous gland	(Ginger <i>et al.</i> 2001)
Growth factors, pro-apoptotic	Agilent DNA microarray	$\uparrow\downarrow$ in E2 exposed gland	(Rajkumar <i>et al.</i> 2005)
Pro-apoptotic	Northern blots	$\uparrow$ in hCG treated gland	(Srivastava <i>et al.</i> 1997)
p53	Immunohistochemistry	$\uparrow$ in parous gland	(Sivaraman <i>et al.</i> 1998)

that defined the parous involuted gland. The genes included increased expression of TGF β 3 and several of its downstream targets. The result of this upregulation would be inhibitory for cell growth. Additionally, there was decreased expression of several growth factors that included IGF-1 and amphiregulin. The result of the decreased expression of these genes would be a lowered stimulus for proliferation. The net result is a low, steady state for growth. One can speculate that responses to specific stimuli for proliferation might also be diminished. In fact, the response to chemical carcinogen-induced proliferation is reduced in the parous involuted glands of both rats and mice (Sivaraman *et al.* 1998, 2001a). Also, one has to consider that some of these genes (e.g. IFG1) might be downregulated due to decreased pituitary growth hormone as demonstrated by Swanson *et al.* (1995), Thordarson *et al.* (2004b).

In a third study using the post-treatment model in the rat, Rajkumar *et al.* (2005) using Agilent rat cDNA microarrays, identified a gene signature conferred by a protective dose of 200 μ g E2. Totally 20 genes were differentially expressed between the hormone treated and untreated groups. The genes included both pro-apoptotic (upregulated) and cell cycle regulatory (downregulated) genes.

Alterations in other genes have been shown by a variety of methods. In the post-treatment model where hCG induces a protective state, enhanced apoptosis is present during 40 days of treatment, but declines upon cessation of treatment. Northern blot studies indicate that mRNA of interleukin-1- β -converting enzyme, testosterone repressed prostate message 2, p53, c-myc and bcl-XS were upregulated and correlated nicely with the enhanced apoptotic activity in the gland (Srivastava *et al.* 1997). The expression of these genes reached a peak at 40 days of treatment and was still elevated 20 days after cessation of treatment. Some of these genes, such as bcl-XS, p53, c-myc and inibin were also found in a cDNA microarray analysis (Russo *et al.* 2005). As in the initial Northern blot study, these genes reached the highest peak at 21 days, but were decreasing by 42 days post hCG treatment. However, there was a small set of genes that were persistently differentially expressed at 42 days of hCG treatment that the authors suggest confer a genetic signature for hCG treatment (Russo *et al.* 2005).

A common element that appears in these various studies is the downregulation of growth regulatory genes in the protected state, particularly in the pre-treatment models studied by Ginger *et al.* (2001) and D'Cruz *et al.* (2002). In the post-treatment model, there is the additional element of pro-apoptotic genes

that are upregulated (Rajkumar *et al.* 2005, Russo *et al.* 2005). The molecular studies support the observations at the cellular level where it was concluded that downregulation of the proliferative response was prevalent in the pre-treatment model (Sivaraman *et al.* 1998), whereas upregulation of apoptosis was observed only in the post-treatment model (Srivastava *et al.* 1997, Sivaraman *et al.* 1998).

p53

A striking cellular alteration present in the parous involuted glands in the pre-treatment model is the block in carcinogen-induced proliferation (Sivaraman *et al.* 1998). Alterations in apoptosis were not detected. This block is detected in both Wistar-Lewis rats and BALB/c mice. Concomitantly with this block in proliferation is a significant increase in the level and nuclear localization of the tumor suppressor protein, p53, in the parous involuted gland. The increase occurs as early as 3 days after hormone treatment and is persistent until 28 days of involution and for 3 days after NMU treatment (Sivaraman *et al.* 1998, 2001a). Kuperwasser *et al.* (2000) have previously discovered that wildtype p53 is inactive in the virgin gland, but is functionally activated by hormone-exposure. The molecular mechanisms of this activation have not been elucidated yet, but the demonstration of hormonal activation of p53 supports a hypothesis for the role of p53 in the hormone-induced protective state. Additionally, it has been demonstrated that p53 functions in the DNA repair of polycyclic aromatic hydrocarbon adducts (Lloyd & Hanawalt 2000).

The importance of p53 for the hormone-induced protective state has been directly tested using the p53-null BALB/c mouse mammary epithelial cell model. The experiments demonstrated that the absence of p53 gene function abrogated the protective effect of hormones against carcinogen (DMBA)-induced mammary carcinogenesis (Medina & Kittrell 2003). To date, this study represents the first and only identification of a specific gene product that mediates the protective effect of hormones. However, the development of several new and elegant conditional transgenic mouse models for c-myc, wnt, Brca-1 and c-neu offer the promise of similar experiments (Deng 2002, Gunther *et al.* 2002, Moody *et al.* 2002, Gunther *et al.* 2003, Boxer *et al.* 2004, Chodosh 2005). Additionally, the identification of new candidate genes by microarray studies suggests that numerous genes can be tested for their roles in the hormone-induced protective state using transgenic mouse models.

Beyond the current paradigms

There are several important questions that need to be examined before we have a fuller understanding of the mechanisms and translatability of the hormone-induced protective state. First, a critical testing is needed of the obligatory role of the genes and regulatory pathways involved in protection. Second, a dissection is required of the relative roles of the molecular changes intrinsic to the target cells for neoplastic transformation, i.e. mammary epithelium versus alterations in the pituitary gland and in the mammary stroma that affect progression of initiated mammary cells. Third, is the paradigm that has been developed using chemical carcinogen treated rats and mice applicable to mammary tumor models that are independent of chemical carcinogen initiation? This latter question is of interest for two reasons. First, the only experiment in the published literature using irradiation as an initiating agent for mammary tumorigenesis in the rat failed to demonstrate a hormone-induced protective effect (Holtzman *et al.* 1982). Second, polycyclic aromatic hydrocarbons, like DMBA, and nitrosoureas have not been demonstrated to be carcinogenic agents for the human breast.

The first two issues are being addressed by current research but it will be some years yet before there is a clear understanding of these issues. The third issue has been recently examined in the p53 null mammary epithelial model. The absence of p53 protein function confers an increased risk for breast tumorigenesis (Jerry *et al.* 2000). This is accompanied by chromosomal instability (Goepfert *et al.* 2000, Pati *et al.* 2004) and as yet, undefined secondary alterations in gene function. The model allows a test for the issue discussed above. If BALB/c wildtype mice bearing p53-null normal mammary epithelial cells are exposed to E plus P for only 2 weeks during the duct elongation phase at 5 to 7 weeks of host age, (i.e. akin to immediate post-pubertal growth phase), then tumorigenesis is reduced over 90% over an ensuing 40 weeks (D Medina, unpublished observations). Interestingly, the inhibition of tumorigenesis is accompanied by a decreased steady state proliferation rate. These results need to be repeated in other models (e.g. c-myc, c-neu) in order to document conclusively the importance and relevance of the hormone-induced protective state as a prevention modality.

The cell fate hypothesis and the demonstration that both chemical carcinogen and oncogene models are subject to hormone-mediated protection raises a more fundamental question. Is there a unique developmental window that is particularly sensitive to

hormone-mediated prevention of mammary cancer? For instance, would hormones induce protection in the mature virgin gland, say at 20–26 weeks of age? It is known in the pre-treatment model that hormones given at 7 to 10 weeks of age induce a protective state against DMBA-induced tumorigenesis whether DMBA is given at 14 weeks or 26 weeks of age (Moon 1969). The question is whether hormones affect the developing gland (4–12 weeks of age) differently than the mature gland (≥ 20 weeks of age). There is considerable evidence in the literature that events occurring in the mammary gland during the development phase confer the greatest relative risk for tumorigenesis (Colditz & Frazier 1995). For instance, for human breast cancer, the greatest risk in individuals exposed to a single large dose of radiation (i.e. Hiroshima and Nagasaki atomic bomb survivors) occurred in females ≤ 20 years of age (Tokunaga *et al.* 1994, Land *et al.* 2003). The protective effects of a soy-based diet depend on soy intake during the adolescent years (Wu *et al.* 2002). Finally, the recent demonstration that the highest quintile of height and BMI at age 14 is associated with subsequent breast cancer risk suggests the importance of growth stimuli during the peri-pubertal years (Ahlgren *et al.* 2004). These considerations are critical for understanding the importance and relevance of the cell fate hypothesis discussed earlier. A corollary to the hypothesis is that the developing gland (immediately post-pubertal) represents a unique window for altering cell fate and subsequent response to oncogenic initiating stimuli. If validated, then the optimum period for long term prevention modality would be in these years. A short time period (months) of exposure to non-invasive, normal cycling hormones would induce a long lasting protective effect. This hypothesis can be tested thoroughly in current experimental models and within 2 years, the validity of the hypothesis can be proven or disproven.

Conclusion

The area of hormone-induced protection of mammary cancer has been studied for over 30 years. The current studies have moved the area from the biological to the molecular realm. The central hypothesis has been redefined from a vague 'promoting differentiation' to a molecular-based 'altered cell fate.' It is likely that systemic and even stromal alterations also affect the fate of altered mammary epithelial cells. In its current form, the hypothesis states that upon exposure to hormones, the mammary epithelial cells comprising the young virgin gland undergo a series of changes in gene

Figure 2 The 'Cell fate' hypothesis. In the mammary gland of the immediate postpubescent female, hormones stimulate the proliferation of a specific stem cell population (PI-MEC) which is accompanied by a molecular switch in these cells that results in persistent changes in intracellular regulatory loops governing proliferation and response to DNA damage. The hormonal milieu of pregnancy affects the developmental fate of a subset of mammary epithelial cells such that they become resistant to neoplastic transformation (either initiation and/or promotion). (Modified from Ginger & Rosen 2003.)

expression affecting specific regulatory pathways resulting in a cell population of the parous involuted gland that is distinct from that of the age matched virgin (Fig. 2). In effect, the mammary epithelial cell has attained its complete developmental fate; a unique situation compared with most organogenesis where development is completed in the fetal stage or early postnatally. The consequences for prevention of human breast cancer are potentially enormous because they provide the basis for a non-invasive strategy using normal, endogenous hormones. The challenge will be to translate the basic concepts developed in the animal models to the human population where the affected individual is of young age and non-cancer bearing. However, there are very high risk human populations of young age where these prevention modalities could be justified.

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