REVIEW

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Pregnancy and the risk of breast cancer

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Abstract

It is well established that childless women and women having children later in life are at an increased risk of developing breast cancer. In particular, women having a first child before 20 years of age have a 50% reduction in lifetime breast cancer risk when compared with women who do not have children. This protective effect is specific for estrogen receptor positive breast cancer. Nevertheless, it remains unclear how parity decreases breast cancer risk. Possible mechanisms of action include changes to the hormonal profile of parous women, a more differentiated and so less susceptible mammary gland or changes within specific epithelial cell subpopulations. In this review, we discuss the epidemiological evidence for the protective effects of parity on breast cancer. We also explore the mechanisms by which parity protects, with a particular emphasis on the role of stem cells and the interactions between stem cells and estrogen.

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Introduction

The only factor known to consistently decrease lifetime breast cancer risk regardless of ethnicity is early childbirth (MacMahon *et al*. 1970, Henderson *et al*. 1974, Kelsey *et al*. 1993). Women who have undergone a first full-term pregnancy/birth (FFTB) before 20 years of age have a 50% reduced lifetime risk of developing breast cancer when compared with nulliparous women (MacMahon *et al*. 1970), whereas first full-term births over 35 years of age lead to an increased risk of developing breast cancer (Trichopoulos *et al*. 1983). However, the protective effect of pregnancy is not immediate. When compared with nulliparous women, uniparous women have an elevated risk of breast cancer soon after delivery, which only declines some years later. This increased risk is most pronounced in women who are aged 30 years or older at the time of their first delivery (Janerich & Hoff 1982, Lambe *et al*. 1994, Lambe *et al*. 1998, Schedin 2006). On average, the transient increase lasts ~10 years (Albrektsen *et al*. 2005) but is also dependent on age, being postponed an additional 10 years in women with FFTB after 30 years of age (Rosner *et al*. 1994). These parity-specific effects on breast cancer risk are limited to hormone-responsive breast cancer as highlighted in a recent meta-analysis. Ma *et al*. (2006) showed that, across eight separate clinical studies, parity-beneficial effects were confined to estrogen receptor positive/progesterone receptor positive (ER+/PR+) breast cancer not ER negative/PR negative (ER−/PR−) breast cancer.

Retrospective epidemiological studies have defined the contributions of parity, age at FFTB, and breastfeeding in breast cancer protection (MacMahon *et al*. 1970, Ursin *et al*. 2004); yet despite having this knowledge for more than three decades, the exact mechanisms involved remain unknown. Defining the role of parity-induced protection could lead to development of adjuvant therapies that specifically target the cellular processes underlying hormone-responsive breast cancer. In this review, we discuss the various mechanisms by which protection may be mediated including an altered hormonal milieu, increased differentiative phenotype of the gland, a protective change specifically in mammary stem cells or a change in estrogen responsiveness. We begin with a brief overview of mammary gland growth and development. This will cover both human and rodent mammary gland development. It is important to consider both systems, as although epidemiological and histopathological studies have examined the protective effects of pregnancy and breast cancer in humans, mechanistic studies and manipulations are only feasible in rodent models. Although there are some differences between development of the human breast and rodent mammary

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gland there are also strong similarities, which enable results to be compared between the two systems.

Mammary development in the human and rodent

The mammary epithelium has two main postnatal developmental stages in both humans and rodents. During puberty, the ductal elongation phase establishes a network of ducts which spread out from the nipple, driven by specialized growth structures at the tips of the elongating ducts, the terminal end buds (TEBs). These have been mainly described in rodents (Williams & Daniel 1983) although in humans similar structures have been observed (Anbazhagan *et al*. 1998). The TEBs consist of two morphologically distinct cell types, an inner layer of body cells and an outer layer of cap cells, which give rise to the luminal and basal cell layers respectively, of the subtending duct (Sapino *et al*. 1993, Srinivasan *et al*. 2003). It is also clear that somewhere within the TEBs there is a stem cell activity, most likely located within the cap cells, such that they also give rise to the body cells as well as the basal cell layer (Kenney *et al*. 2001). From the ductal network develops the future milk-producing structures of the gland. These are termed alveolar buds in the virgin rodent mammary gland and terminal ductal lobuloalveolar units (TDLUs) in the human breast (Cardiff & Wellings 1999, Smalley & Ashworth 2003). TDLUs consist of clusters of secretory alveoli – like bunches of grapes – whereas in the rodent the ABs are more evenly dispersed along the ducts. The extent of formation of ABs in the virgin rodent gland varies from strain to strain. In humans, TDLUs are always found in the virgin breast although they become more elaborate in response to pregnancy. Pregnancy is the second postnatal developmental stage seen in the mammary epithelium and its main feature is expansion and differentiation of the ABs/TDLUs under hormonal influence (Fendrick *et al*. 1998, Russo & Russo 1998). The mature ABs are termed lobular alveoli (LA). Notably, TDLUs are also the site of origin of most human breast cancers (Wellings 1980*a*,*b*, Russo *et al*. 1982). Following weaning of the young, the LA/TDLU structures regress in a process called involution. The involuted mammary gland retains some of the vestiges of the preceding pregnancy and in both mice and humans is clearly more differentiated when compared with its virgin counterpart (Russo *et al*. 1982, Cardiff & Wellings 1999). The proliferation–lactation–involution cycle, which the mammary tissue passes through with each pregnancy is described in Fig. 1.

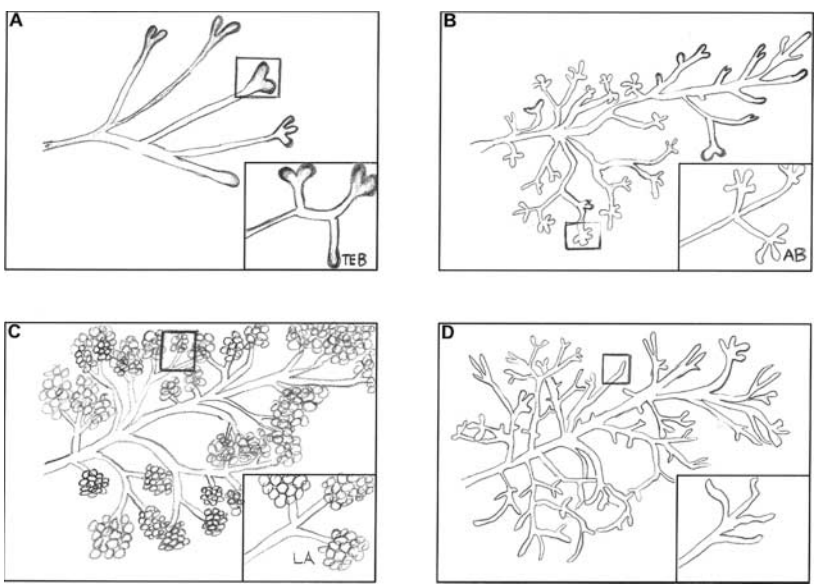

Figure 1 Development of the rodent mammary gland. (A) In the early weeks of postnatal life, the mammary gland consists of a single lactiferous duct that branches into 3–5 secondary ducts. The terminal end buds (TEB) form the growing tips of the ducts and contain the mammary stem cells. (B) The mature virgin gland consists of a branching ductal system within which the majority of the alveolar buds (AB) have now developed. These later form the milk-producing structures of the gland. Their differentiation during development begins from the nipple region and extends distally. The TEBs have regressed, leaving smaller terminal structures at the ends of ducts called terminal ducts (TD). (C) At pregnancy there is extensive epithelial cell proliferation and lobuloalveolar (LA) structures form from alveolar buds. (D) Following pregnancy the gland is remodeled, largely through apoptosis of the epithelial structures and subsequent phagocytosis. The gland resembles the virgin; however, the number of alveolar structures and degree of side branching tends to be higher, indicating greater differentiation.

The adult ductal and alveolar mammary epithelium in both humans and rodents consists of two basic cell layers – an inner (adjacent to the lumen) luminal epithelial layer and an outer (adjacent to the basement membrane and breast stroma) basal myoepithelial layer. Luminal cells line the inside of the ducts and form the differentiated milk-secreting cells in the LA/TDLUs. The basal layer is mainly composed of myoepithelial cells, which contract in response to oxytocin released during lactation to force milk down the ducts to the nipple. The basal cell layer also contains the stem cell compartment which maintains the epithelium (Shackleton *et al*. 2006, Sleeman *et al*. 2006, Stingl *et al*. 2006, Shipitsin *et al*. 2007).

Besides the developmental and biological similarities between the human breast and rodent mammary gland, there are also similarities between mouse and human breast cancer. These have been extensively reviewed previously (Wellings *et al*. 1975, Wellings 1980*a*,*b*, Cardiff 1996) and will not be discussed in detail here. However, for the purposes of this review, it is sufficient to note that 7,12-dimethylbenz[*a*]anthracene (DMBA) or *N*-methyl-*N*-nitrosourea (NMU)-induced tumors of rodents are hormone responsive and, as with humans, parity has a protective effect against tumorigenesis (Russo *et al*. 1990). Moreover, in rodents as in humans, the protective mechanism is greatest in younger individuals (MacMahon *et al*. 1970, 1982, Russo *et al*. 1982, Russo & Russo 1987*a*, Yuan *et al*. 1988, Kelsey *et al*. 1993). Rodent models provide, therefore, a good system to model the protective effect of pregnancy on breast cancer and to investigate its underlying causes.

Hormonal control of mammary development

The factors that regulate embryonic mammary gland development have been reviewed recently (Parmar & Cunha 2004, Hens & Wysolmerski 2005). In brief, the Wnt signaling pathway, fibroblast growth factor signaling pathway, the Msx1/2 homeobox transcription factors, and parathyroid hormone-related protein play major roles in embryonic development and initial ductal growth (van Genderen *et al*. 1994, Satokata *et al*. 2000, Foley *et al*. 2001, Mailleux *et al*. 2002). However, the initial branching morphogenesis of the embryonic mammary gland is hormone independent, as mice that are deficient in either ER (α or β), the prolactin (PRL) receptor, the growth hormone (GH) receptor, or the PR have no obvious embryonic mammary phenotype (Hennighausen & Robinson 2001, Hovey *et al*. 2002).

Postpubertal ductal branching begins under the control of estrogens acting in concert with GH and insulin-like growth factor-I (IGF-I). Pubertal mammary development is impaired in mice lacking GH receptor (Gallego *et al*. 2001), IGF-I (Kleinberg *et al*. 2000), ERα (Curtis Hewitt *et al*. 2000) or aromatase (responsible for estrogen biosynthesis; Fisher *et al*. 1998). Similarly, in the absence of ovarian function in humans, there is complete absence of breast development. This can be restored by estrogen treatment. In contrast, pubertal mammary development was normal in mice lacking ERβ, PR, or the PRL receptor (Curtis Hewitt *et al*. 2000). A role for progesterone, however, was revealed in mice deficient for the two PR isoforms, PR-A and PR-B (Lydon *et al*. 1995), which failed to undergo side branching and alveolar development during pregnancy. Studies assessing PR protein localization and tissue recombination experiments indicate that epithelial rather than stromal PR stimulates lobuloalveolar development, although stromal PR-B may play a role in ductal branching (Humphreys *et al*. 1997, Brisken *et al*. 1998). The epidermal growth factor receptor (Egfr) is also involved in branching as shown by the ability of exogenous Egfr ligands to rescue ductal development in both ovariectomized (Coleman *et al*. 1988) and ERα-deficient mice (Kenney *et al*. 2003). Moreover, exogenous estradiol elicits Egfr activation in ovariectomized mice (Sebastian *et al*. 1998), suggesting that Egfr promotes mammary branching downstream of ERα. One ligand in particular, amphiregulin, appears to be particularly important as it is upregulated at puberty and is absolutely required for mammary development (Luetteke *et al*. 1999, Ciarloni *et al*. 2007).

Parity and the protection against ER+ PR+ breast cancers

Approximately 70% of human breast cancers express the ER and are hormone dependent (Masood 1992). This has been exploited in the development of antiestrogens such as tamoxifen and aromatase inhibitors, commonly used as adjuvant therapies for breast cancer. Epidemiological studies which have used both ER and PR to define hormone receptor status have provided evidence that parity specifically protects against ER+ PR+ breast cancers (Potter *et al*. 1995, Yoo *et al*. 1997, Huang *et al*. 2000, Britton *et al*. 2002, Cotterchio *et al*. 2003). For example, the Iowa Women's Health Study found no association with the risk of ER+/PR− breast cancers, but did show a decreased risk of ER+/PR+ breast cancers with a higher degree

of parity. Interestingly, parity has been associated with an increased risk of developing ER−/PR− breast cancer (Potter *et al*. 1995) although an assessment of the population-based case control Women's CARE Study (Marchbanks *et al*. 2002) showed that, while parity (as defined by pregnancies with a duration of more than 6 months) was associated with a decreased risk of ER+/PR+ breast cancers, it had no effect on ER−/PR− tumors. The Women's CARE Study also found that among parous women, older age at FFTB was associated with an increased risk of ER+/PR+ breast cancer (Ursin *et al*. 2005).

A recent meta-analysis of these two studies and seven others investigated parity and age at FFTB among ER+/PR+ and ER−/PR− breast cancers (Ma *et al*. 2006). This included two cohort studies, five population-based case control studies, and two hospital-based case control studies (Potter *et al*. 1995, Yoo *et al*. 1997, Huang *et al*. 2000, Britton *et al*. 2002, Cotterchio *et al*. 2003, McCredie *et al*. 2003, Colditz *et al*. 2004, Rusiecki *et al*. 2005, Ursin *et al*. 2005) For these studies, 65% of the cases had used available ER and PR data. This data were used to confirm that the protective effect of parity was confined to ER+/PR+ breast cancers. Each birth reduced the risk of breast cancer by 11%. The protective effect was maintained within the ER+/PR+ group even when the analyses were stratified by age. Furthermore, women in the oldest age at FFTB group were on average at 27% greater risk of developing ER+/PR+ breast cancers when compared with the youngest age group. There was no such effect on ER−/PR− tumor incidence.

In contrast, two studies have suggested that parity does not impart protection against the development of ER+/PR+ breast cancer. Britton *et al*. (2002), using data from the Women's Interview Study of Health (Brinton *et al*. 1995), showed that nulliparity was not associated with an increase in ER+/PR+ breast cancers, but rather an increased risk for all tumor types except ER−/PR+ breast cancers. Similarly, McCredie *et al*. (2003) found no significant difference in the incidence of ER+/PR+ or ER−/PR− breast cancers according to parity. However, both of these studies were performed in women who were premenopausal when diagnosed (age limits 20–44 and <40 respectively) and ER+/PR+ tumors tend to predominate in postmenopausal women.

Overall, the evidence strongly suggests that parity protects specifically against the development of sporadic ER+/PR+ breast cancers. Does this protection extend to familial breast cancer? It was originally thought that parity increased the risk of breast cancer development in BRCA mutation carriers (Jernstrom *et al*. 1999) or had no effect (Hartge *et al*. 2002, Tryggvadottir *et al*. 2003). However, recent studies (Cullinane *et al*. 2005, McLaughlin *et al*. 2007) using larger sample sizes and reporting separately on BRCA1 and two carriers, rather than on familial versus sporadic breast cancers, showed that parity did protect against breast cancer development in BRCA1 carriers (odds ratio=0.5). However, parity did not protect BRCA2 carriers, and in fact imparted a nonsignificant increased risk, which rose ∼15% for each additional pregnancy. In this group of women, three or more full-term births significantly increased their risk of breast cancer (odds ratio=2–3). Interestingly, this was largely attributed to the 70% increase in the 2 year period immediately following pregnancy and was specific to the development of early breast cancers (age <40 years). Considering the data on sporadic breast cancers, these results from the familial setting seem to be counterintuitive, as BRCA1 breast cancers tend to be ER− and BRCA2 tumors tend to be ER+ (Loman *et al*. 1998, Foulkes *et al*. 2004, Musolino *et al*. 2007). However, it is known that oophorectomy protects against breast cancer development in BRCA1 carriers (Rebbeck *et al*. 1999, Kauff *et al*. 2002, Eisen *et al*. 2005) so it is likely that there is an indirect (or ER-independent) effect of hormones on the development of these tumors, an effect which can be modulated by parity. The BRCA2 data are harder to explain. The cumulative increasing risk of pregnancy-associated breast cancer in parous BRCA2 carriers could be related to the possibility that mutations arising during the remodeling process following pregnancy accumulate in the context of impaired DNA repair in BRCA2-null cells. BRCA1/2 mutation carriers, already at a 40 and 20% respective increased risk of developing breast cancer (Ford *et al*. 1994, Risch *et al*. 2001), may benefit from more intensive surveillance following childbirth.

The requirement for a full-term pregnancy

If parity protects against breast cancer development, does the pregnancy need to end in full-term delivery, or is the initial differentiation of epithelial cells during pregnancy sufficient? The relationship between miscarriage, pregnancy termination, and breast cancer risk has been the subject of extensive research beginning in the late 1950s. Until the mid-1990s, the evidence was inconsistent. Findings from some studies suggested that there was no increase in risk of breast cancer among women who had undergone a termination, while findings from other studies suggested there was an increased risk (Pike *et al*. 1981, Daling *et al*. 1994, 1996). For most of

these studies, only small numbers of women were included and data were collected retrospectively, after the diagnosis of breast cancer. Women with breast cancer are more likely to report terminations when compared with their control counterparts (Lindefors-Harris *et al.* 1991, Jones & Forrest 1992), so the results from these case control studies should be interpreted with caution. More recent studies have examined large numbers of women, and collected data prospectively. Medical history information was gathered from medical records rather than from self-reports. These studies consistently showed no association between induced terminations and an elevated breast cancer risk (Michels & Willett 1996, Melbye *et al.* 1997, Tang *et al.* 2000, Goldacre *et al.* 2001, Erlandsson *et al.* 2003, Michels *et al.* 2007). However, the studies also demonstrated that an interrupted pregnancy was not sufficient for a protective effect.

Two rodent models have been used to explore in more detail the requirement for a full-term pregnancy for parity-specific protection against breast cancer (Sinha *et al.* 1988, Russo *et al.* 1992). In the beginning of these studies, pregnancy interrupted prior to full term (21–22 days) and was able to partially protect against carcinogen-induced mammary tumor development. Sinha and colleagues showed that virgin mice treated with the chemical carcinogen DMBA developed tumors with an incidence of 70–88%. Age-matched animals that had completed a full-term pregnancy showed only 14% incidence. When pregnancy was interrupted at day 5, 10, or 15, breast cancer incidence was 48, 50, and 45% respectively. Thus, an interrupted pregnancy gave partial protection when compared with parous controls. In the second smaller study, pregnancy interrupted at day 12 failed to confer protection against breast cancer. Tumor development occurred in 70% of mice when compared with 79% in age-matched virgins (AMV). The differences between these studies may simply be due to the size of the study cohorts used, and hence differences in statistical power between them. Furthermore, the interval between the end of hormone stimulation and carcinogen treatment was different in the two studies (21 and 15 days respectively), which may suggest that the interval between involution onset and carcinogen exposure is important.

It is intriguing that as pregnancy progressed in the better-powered rodent model an increasing protective effect was seen, whereas in humans an interrupted pregnancy was not sufficient for protection. This suggests that there are late pregnancy events in the human that occur in a progressive fashion throughout the rodent pregnancy, which may be a key in understanding the underlying mechanism of protection. Additional studies to define the time course of pregnancy-induced protection in rodents are required as well as highly detailed studies of the comparative biology of the human and rodent during pregnancy to address this issue.

Mechanisms of parity-specific protection

Currently, there are four main schools of thought concerning how pregnancy-dependent breast cancer protection arises, although these theories do have common aspects (Fig. 2). First, protection may occur through parity-specific changes in levels of circulating hormones such as estradiol, PRL, and GH. Each of these has been associated with breast cancer risk (Emerman *et al.* 1985, Henderson & Feigelson 2000). Second, the extensive LA/TDLU development that occurs during pregnancy may result in epithelial cell differentiation that is maintained in those epithelial cells that remain after involution – 'maturing' of the gland in response to first pregnancy. The parous mammary gland may, therefore, contain epithelial cells with a more differentiated, and less proliferative character which are less susceptible to transformation. Third, there may be a specific effect of parity on mammary stem cells. Adult tissue-specific stem cells serve to replenish lost/damaged cells and in general maintain tissue integrity (Smalley & Ashworth 2003). Although our understanding of the identity and regulation of mammary stem cells and their relationship to the breast cancer stem cells is still limited, it is feasible that pregnancy may lead to a decrease in mammary stem cell numbers, and so a decrease in the pool of potentially transformation susceptible epithelial precursors. Finally, given that parity protects specifically against ER+ tumors, and the association with estrogen exposure and breast cancer risk, it is possible that parity protection may also be mediated via changes in the estrogen responsiveness of the mammary gland. These may take the form of changes

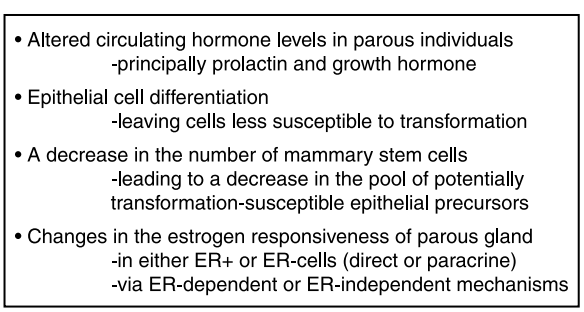

Figure 2 An 'at a glance' summary of the proposed mechanisms of parity-induced protection against breast cancer.

in the response of hormone-sensing cells to estrogen or of changes in paracrine interactions between hormone-sensing and stem cells. These paracrine interactions may themselves be direct or indirect and are mediated via cells in the stem cell niche. We will now discuss each of these four potential mechanisms in detail, with particular emphasis on the role of stem cells and estrogen responsiveness, as the paracrine nature of the regulation of mammary stem cells is an important emerging theme.

Altered hormonal profile

Results of mammary fat pad transplantation studies have indicated that the hormonal environment mediates protection. When isolated epithelial cells from mammary glands of virgin mice exposed to DMBA were transplanted into parous mice, the mammary glands showed reduced tumor development when compared with virgin mice transplanted with the same cells (Abrams *et al*. 1998). To define the hormones responsible, human epidemiological studies and rodent models have been investigated. Pregnancy exposes the body to a unique hormone profile including prolonged elevation of progesterone, the lactogenic hormone PRL, and placental lactogen, increasing titers of estrogens and altered glucocorticoid secretion and sensitivity (Numan 1994). Whether this leads to permanent changes in the hormonal profiles of parous women when compared with their nulliparous counterparts is not clear. The limited data available from women and the problems of assessing different hormones at different stages of the reproductive cycle makes it difficult to define a specific protective hormone profile. Parous women are reported to have reduced serum levels of PRL (Kwa *et al*. 1981, Musey *et al*. 1987, Eliassen *et al*. 2007), but permanent changes in estradiol (E_2) levels are not as reproducible (Bernstein *et al*. 1985, Musey *et al*. 1987, Ingram *et al*. 1990, Dorgan *et al*. 1995). E_2 levels are decreased in some studies (Bernstein *et al*. 1985, Dorgan *et al*. 1995) and unchanged levels in others (Musey *et al*. 1987, Ingram *et al*. 1990), is likely a function of the time and day of sampling in relation to the menstrual cycle and the age of the women assessed.

Despite the clear role of estrogen in inducing the parity-induced protection in rodents (Guzman *et al*. 1999, Rajkumar *et al*. 2001), as with humans, there is no consistent data in rodents to suggest that the permanent changes in either estrogen or progesterone mediate protection. The evidence for changes in PRL levels is also unclear (Thordarson *et al*. 1995), although PRL treatment in mice has been shown to greatly increase mammary tumors and PRL-suppressing drugs reduce tumorigenesis (Welsch & Nagasawa 1977). Parous rodents do have decreased GH levels (Bridges & Hammer 1992, Bridges *et al*. 1993, Thordarson *et al*. 1995). It has been shown that suppression of GH secretion causes regression of chemically induced mammary cancers (Rose *et al*. 1983) and that nulliparous GH-deficient rats are as refractory to mammary tumorigenesis as parous rats (Guzman *et al*. 1999, Swanson & Unterman 2002).

Differentiation and gene expression changes in the parous gland

The terminal differentiation hypothesis of breast cancer prevention predicts that the loss (through differentiation) of a population of susceptible cells and a general increase in the differentiation status of the gland following pregnancy results in protection from tumorigenic changes (Russo & Russo 1987*a*, 1997). Russo *et al.* have proposed that the differentiation state of the human breast may be defined by the degree of complexity of the secretory lobules. They categorize the lobules as types 1, 2, and 3 in the order of increasing complexity (defined as the number of clusters of ductules per lobule). They have suggested that type 1 and 2 lobules predominate in the nulliparous breast and the type 3 lobules (with up to 80 ductules per lobule) develop at pregnancy and are the most abundant in the breasts of parous women (Russo & Russo 1987*b*, de Waard & Trichopoulos 1988, Russo *et al*. 1992, Kelsey *et al*. 1993). It was also suggested that breasts from parous women with breast cancer were less differentiated, with levels of lobules type 1 and 2 similar to those of nulliparous women (Russo *et al*. 1990). However, given the difficulties of obtaining breast tissue in large enough numbers both before pregnancy and after post-weaning involution in the same women, these observations are interesting but not yet definitive.

Several gene expression array studies have been performed on nulliparous and nonpregnant parous mammary glands to identify functional changes within the gland. These have confirmed that the parous gland is more differentiated and less proliferative than its virgin counterpart and begin to suggest key molecular signatures. Ginger *et al*. (2001) used suppression subtractive hybridization to identify genes that are persistently upregulated in the glands of estrogen- and progesterone-treated Wistar-Furth rats when compared with AMV. They observed differences in several

distinct gene categories including markers of mammary differentiation, metabolism and homeostasis of the gland (metabolic enzymes and transport molecules), cell–cell contact and the extracellular matrix as well as regulatory factors (such as signaling molecules and transcription factors). D'Cruz *et al.* (2002) performed gene expression microarray analysis on whole mammary glands isolated from virgin and parous mice. Their study showed that parity-induced persistent downregulation of multiple genes encoding epithelial growth factors and led to an upregulation of the genes encoding the growth inhibitory molecule transforming growth factor β3 (*Tgfβ3*) as well as many of its downstream targets. In addition, they observed an increase in the differentiation state of the mammary gland, as demonstrated by increases in genes encoding milk proteins such as whey acidic protein, caseins, and adipocyte differentiation-related protein. Changes in the types of hematopoietic cell resident within the gland were also evident. There were significant increases in genes encoding B-cell-associated immunoglobulins, macrophage-specific genes, and T-cell-activating protein, which interacts with macrophages to induce inflammatory responses (Ashkar *et al.* 2000). Using several different rat strains this same group identified a core 70 gene parity-induced expression signature, conserved across strains (Blakely *et al.* 2006). This included increased expression of genes involved in mammary differentiation such as the milk proteins and, as observed previously, a change in the immune profile suggestive of an increase in plasma cells, macrophages, and T-cells. A decrease in several growth factor-related genes and a decrease in the GH/IGF axis was also noted.

While these gene expression studies are consistent with processes occurring within the mammary gland at this time, they are complicated by the fact that whole mammary glands, containing heterogeneous cell populations, were assessed. This means that only average changes in gene expression can be assessed. Small parity-associated changes in gene expression may be masked by larger changes in cell proportions (such as changes in the relative numbers of luminal epithelial cells when compared with basal/myoepithelial cells or stromal cells). Another problem with the previous studies is that both were performed in animals after 21–28 days of involution. As we have discussed above in humans, there is actually an initial increase in breast cancer risk associated with parity (Schedin 2006), which followed some 6–10 years later by a decrease in breast cancer risk and it is not clear whether the time point at which the animal studies were carried out models the early increased or the later decreased human risk period. In many cases, such studies may actually reflect a period during which the gland is undergoing involution and remodeling, rather than changes in the 'resting' uniparous gland, when compared with virgin tissue. Involution of the mammary gland is a complex process of controlled apoptosis and tissue remodeling. Significant immune responses occur during involution, including a primary neutrophil activation and secondary macrophage activation, a local acute-phase response and a late B-lymphocyte response (Stein *et al.* 2004). Stein and colleagues revealed a subset of genes which were induced during involution and remained elevated at involution day 20 when compared with nulliparous controls, with most other genes returning to pre-pregnancy levels. These genes were all immunoglobulin-related genes and collectively indicated a sustained B-cell response. The presence of such a strong immune profile in the previous gene expression studies on parity (Ginger *et al.* 2001, D'Cruz *et al.* 2002, Blakely *et al.* 2006) suggests that these experiments may have been performed when the gland is still involuting and do not truly reflect parity-specific changes that protect against breast cancer.

Only one study has been performed assessing the gene expression profiles of breast tissue from parous when compared with nulliparous women. Russo *et al.* assessed gene expression changes in reduction mammoplasties in postmenopausal women (Balogh *et al.* 2006). They observed that epithelial cells from parous women had increased innate immune response proteins, namely T-cell receptor protein, IL22R, and MHC class 1 HLA. DNA repair proteins and chromatin remodeling proteins such as Sox2, P300, and suppressor of Ty3 were also upregulated. While limited in size (parous, $n=5$; nulliparous, $n=2$) and by the fact that such studies cannot be carried out in the same individuals both prior to and after pregnancy, their study is strengthened by their cell-specific analysis (epithelial and stromal cells) and timing of tissue collection (in postmenopausal women, where the protective effect is apparent; Schedin 2006).

Cumulatively these studies suggest that the parous mammary gland is more differentiated than the virgin. However, differentiation of the mammary gland *per se* may not mediate the protective effect of pregnancy. The compound perphenazine causes acute release of PRL from the anterior pituitary (Ben-David 1968) and results in proliferation and differentiation of the mammary cells to a near lactational state. Rats treated with estradiol plus progesterone displayed a 96% reduction in mammary cancers when compared with controls whereas those rats that were treated with

perphenazine showed a similar incidence to age-matched controls, although with a slight decrease in tumor number per animal (Guzman *et al*. 1999). Therefore, complete differentiation of the mammary gland could not protect against tumor formation, but estradiol and progesterone treatment could. This has been confirmed by recent studies in which exogenous estradiol plus progesterone had a protective effect without inducing full lobuloalveolar differentiation (Sivaraman *et al*. 1998). Similarly 5 days of pregnancy in a rodent model was able to provide a partial reduction in breast cancer risk (see above; Sinha *et al*. 1988) despite the minimal DNA synthesis and morphological differentiation of the gland at this early stage (Grubbs *et al*. 1988, Medina *et al*. 2001). Likewise, a study assessing the ability of estrogen to mimic parity showed that lower doses of estrogen which did not confer complete differentiation were just as effective as higher doses at preventing carcinogenesis (Rajkumar *et al*. 2001).

Changes to the mammary stem cell population

Several lines of evidence suggest that mammary stem cells are targets for tumorigenesis. First, stem cells are thought to be long lived and are, therefore, able to accumulate the multiple mutations required for tumor formation. To counter this, they are likely to have developed specific protective mechanisms, such as a preference for undergoing apoptosis rather than DNA repair in response to DNA damage (Roos *et al*. 2007) and selectively retaining their template DNA during cell division (Smalley & Ashworth 2003, Booth & Smith 2006, Cairns 2006, Shinin *et al*. 2006). This last mechanism is predicted to keep replication-related mutations to a minimum and would explain why breast cancer incidences are not higher than they are as well as the ability of developmental insults (radiation exposure and hormonal treatments) to affect disease states later in life. Selective template DNA strand segregation is based on nonrandom, age dependent template segregation, which has been demonstrated in several systems however (Cuzin & Jacob 1965, Lark & Bird 1965, Lark *et al*. 1966), is not uniformly accepted (Lansdorp 2007, Rando 2007). Second, stem cells are thought to have a high proliferative potential and therefore an increased ability to drive tumor growth; however, in most cases, the actual proliferative compartment is likely to be the transit amplifying population derived from the stem cell, rather than the multipotent progenitors themselves. Third, mammary stem cells possess self-renewal capacity, which is also a hallmark of tumor cells. Finally, epidemiological studies suggest that the breast is at particular risk from acquiring deleterious genetic changes before or during puberty, which is thought to be a period of stem cell expansion. Stem cells have the ability to undergo both asymmetric and symmetric cell division. In asymmetric stem cell division, one of the two progeny is identical to the initial stem cell (resulting in self-renewal of the parental stem cell), while the other becomes a committed progenitor cell and ultimately generates all the differentiated cell types formed in the tissue. However, during puberty, stem cell numbers must be expanded to provide the tissue with its required adult complement of stem cells. This occurs through symmetric division resulting in the production of two identical daughter stem cells (Kimble 1981, Morrison *et al*. 1995). During this period of symmetric cell divisions, any DNA damage that occurs (or has previously occurred) to a stem cell will become fixed in the expanded stem cell compartment and retained for long periods of time, in contrast to mutations occurring in short-lived transit amplifying or terminally differentiated cells. Therefore, mutagenic insults or protective factors specifically operating before or during puberty are likely to have profound consequences for breast cancer later in life. The epidemiological evidence supports this. Young women (<20 years of age) exposed to radiation during the Hiroshima and Nagasaki atomic bombs were the age group most likely to develop breast cancer in later years with a 13-fold excess relative risk for early onset (<35 years) breast cancer and twofold for later onset breast cancer (>35 years; Tokunaga *et al*. 1994, Land *et al*. 2003). If the age at exposure is further subdivided into 0–4, 5–9, 10–14, and 15–19 years then the excess relative risk tends to be higher at 0–4 and 10–14; however, there were slight increases at the intervening ages, so no clear variation is seen below 20 years (Land *et al*. 2003). Similarly, breast cancer is the most common second primary neoplasm among survivors of Hodgkin's disease in childhood and adolescence who have been treated with chest irradiation (Aisenberg *et al*. 1997, Horwich & Swerdlow 2004) with an estimated risk of 15–33% of developing the disease by 25 years of follow-up. Protective factors (like a diet high in soy protein; Wu *et al*. 2002) are also likely to have a higher impact during adolescence, when the stem cell population is expanding. A population-based, case–control study of breast cancer among Chinese, Japanese, and Filipino women in Los Angeles found that after adjustment for age, specific Asian ethnicity, education, migration history, and menstrual and reproductive factors, women who reported high soy intake during adolescence showed a significantly lower

risk of breast cancer. This effect was greater than the effect for women with a high soy intake only in adulthood (Wu *et al*. 2002). Collectively, these studies suggest that at stages of mammary development associated with specification of mammary stem cells (prepubertal) and stem cell expansion and differentiation (pubertal/adolescence), the gland is more sensitive to known cancer causing agents than later in life.

However, although there is evidence that stem cells are targets for tumorigenesis, a direct effect of pregnancy, either conferring resistance to transformation in mammary stem cells, or reducing stem cell numbers, and thereby reducing the target cell population for transformation, remains to be demonstrated. Only recently have cell surface markers such as CD24 and either CD49f or CD29 become available to allow the isolation of mammary epithelial cell subpopulations highly enriched for *in vivo* stem cell activity in the mouse (Shackleton *et al*. 2006, Sleeman *et al*. 2006, 2007, Stingl *et al*. 2006). Similar strategies have been employed for identifying stem cells in the normal human breast (Shipitsin *et al*. 2007) and will now enable the comparison of stem cell-enriched populations in the breast of nulliparous and parous women. The fact that many genes with cell cycle functions consistent with a role in stem cell division are regulated by estrogen (Table 1) would suggest that estrogenic regulation of stem cells during pregnancy is plausible. Mammary stem cells are ERα-negative (Asselin-Labat *et al*. 2006, Sleeman *et al*. 2007), so if this was true then it would need to be mediated by ERα-independent pathways (possibly via growth factor-related crosstalk or GPR, G-protein coupled receptors) or via paracrine signaling intermediates.

Parity induces changes in estrogen responsiveness of the mammary gland

Nearly all aspects of mammary gland development are under hormonal control. Estrogens are thought to mediate various stages of mammary development and breast cancer risk has long been associated with estrogen exposure, although the change in risk depends on the age, dose, and duration of exposure (Table 2). If women were exposed *in utero* to elevated estrogen they showed increased breast cancer incidence later in life (Rothman *et al*. 1980, Thompson & Janerich 1990, Ekbom *et al*. 1992, Braun *et al*. 1995, Weiss *et al*. 1997), while the more restricted levels associated with preeclampsia may lead to decreased incidence (Braun *et al*. 1995). Women exposed to the environmental estrogen diethylstilbestrol (DES) also display increased incidence of breast cancer (Hatch *et al*. 1998, Palmer *et al*. 2002). The younger a woman's age at menarche the higher her breast cancer risk (Helmrich *et al*. 1983, Brinton *et al*. 1988, Kvale & Heuch 1988, Hsieh *et al*. 1990), which may be related to the increased levels of estrogen experienced directly following menarche (MacMahon *et al*. 1982, Apter *et al*. 1989) or to the earlier exposure to the regular ovulatory cycles of hormones. Similarly, the older a woman is at the time of menopause the higher her risk of breast cancer, the risk increasing 17% for each 5 year delay (Hsieh *et al*. 1990). Epidemiologic studies in dogs have shown that if oophorectomy is performed before the first estrus cycle then the relative risk for breast cancer is 0.005 (Schneider *et al*. 1969). The risk of developing breast cancer is therefore very low without exposure to ovarian hormones.

Besides duration of exposure, estrogen-dependent breast cancer risk may also be mediated by changes in

Table 1 Genes with possible roles in stem cell cycling which are regulated by estrogen

Gene	Role in stem cell kinetics	Effect of parity/estrogen	Refs
Sox2	Marker of neuroepithelial stem cells	Upregulated	Balogh *et al.* (2006)
Sox30	Marker of testicular germ cells	Upregulated	Balogh *et al.* (2006)
Odz	Implicated in hedgehog pathway (structural homolog of notch)	Decreased	Balogh *et al.* (2006)
BarH	Controls decisions of neuronal fate	Increased	Balogh *et al.* (2006)
JunB	Controls number of hematopoietic stem cells	Increased	Balogh *et al.* (2006)
TGFβ	Regulates cell cycle entry, regeneration and formation of niches, and telomerase	Increased	D'Cruz *et al.* (2002)
Notch 2	Controls cell fate decisions by influencing cell proliferation, differentiation, and apoptosis	Decreased by Genistein	Su *et al.* (2007)
Wnt5a	Involved in maintenance of stem cells via non-canonical Wnt pathway	Decreased by Genistein	Su *et al.* (2007)
Sfrp2	Negative regulator of Wnt signaling	Increased by Genistein	Su *et al.* (2007)
Cdc42	A Rho GTPase, which regulates the PAR complex (controls apical polarity, junction formation, and asymmetric division)	Increased by estrogen	Ginger *et al.* (2001)

Table 2 Effect of timing of estrogen treatment and breast cancer risk

Treatment	Effect on breast cancer	Refs
Humans		
Female twins exposed to elevated intrauterine estrogen	Increased incidence as adults	Ekbom *et al.* (1992), Braun *et al.* (1995), Weiss *et al.* (1997)
Women prenatally exposed to increased estrogens (older mothers have increased circulating estrogen at pregnancy)	Increased incidence as adults	Rothman *et al.* (1980), Thompson & Janerich (1990)
Women prenatally exposed to DES	Increased incidence as adults	Hatch *et al.* (1998), Palmer *et al.* (2002)
Women prenatally exposed to restricted placental estrogen (pre-eclampsia)	Decreased incidence as adults	Braun *et al.* (1995)
Pregnancy levels of estrogen early adulthood	Decreased incidence of ER+ cancers	Ursin *et al.* (2005), Ma *et al.* (2006)
Rodents		
Prenatal/neonatal treatment with DES (rats)	Increased incidence as adults	Rothschild *et al.* (1987)
E_2 to newborn female mice infected with mammary tumor virus	Increased incidence	Mori *et al.* (1976)
Neonatal treatment with DES (mice)	Increased sensitivity to hormones and carcinogens later in life	Bern *et al.* (1992)
E_2 injections d1–d30, or d2–d5	Inhibited tumor development in adults	Shellabarger & Soo (1973), Nagasawa *et al.* (1974), Yoshida & Fukunishi (1978)
DES exposure at mid-gestation (mice)	Decreased incidence as adults	Nagasawa *et al.* (1980)
Pregnancy or treatment with pregnancy levels of estrogen early in adulthood	Decreased incidence of ER+ cancers	Russo & Russo (1980), Sinha *et al.* (1988), Rajkumar *et al.* (2001)

DES, diethylstilbestrol; E_2, estradiol.

the estrogen responsiveness of the gland following parity. We will now discuss changes in the responsiveness of the mammary gland to estrogen as a mechanism mediating parity protection against breast cancer. We will start by introducing the different pathways of estrogen action and discuss the levels of ERs throughout mammary development.

The mechanism of estrogen – ER action

The mechanisms of estrogen action have been reviewed extensively (Kushner *et al.* 2000, Shang *et al.* 2000, McDonnell & Norris 2002, Bjornstrom & Sjoberg 2005, Moriarty *et al.* 2006). The majority of the effects of estrogens are mediated via two distinct, yet similar intracellular receptors, ERα and ERβ. In the classical mode of action, estrogen-ER binds to estrogen response elements (EREs) in target promoters and causes up- or downregulation of gene transcription. However, estrogen-ER complexes can alter transcription of genes using response elements other than EREs (AP-1 and SP-1), where DNA-bound transcription factors (Fos/Jun) tether the activated ER to DNA. Growth factors can also activate intracellular kinase pathways leading to phosphorylation and activation of ER. This phosphorylation occurs via one of the many cellular kinases at a specific position within the activation function region of ER (reviewed in Lu & Giguère 2001).

In addition to these genomic pathways, a number of other effects of estrogens are so rapid that they cannot depend on the activation of RNA and protein synthesis. These actions are known as non-genomic pathways. One potential example of this is estrogen activation of a membrane-associated binding site, GPR30, which is linked to intracellular signal transduction pathways. Estrogen stimulation of GPR30 results in transactivation of the EGFR, via G-protein activation (Filardo *et al.* 2000, Filardo 2002, Maggiolini *et al.* 2004) explaining observations, which suggested interactions between estrogen and epidermal growth factor (DiAugustine *et al.* 1988, Yarden *et al.* 1996). While membrane-mediated estrogen action is not universally accepted, the potential importance of this pathway has been recently highlighted by studies showing that it may promote endocrine-insensitive breast cancer cell growth (Hutcheson *et al.* 2003).

ERα and ERβ do not regulate gene expression alone but require the action of co-regulatory proteins (McKenna *et al.* 1999). Binding of agonists (such as estradiol and DES) to ER induces a conformational change in the receptor that permits coactivator recruitment (Heery *et al.* 1997, Feng *et al.* 1998), while anti-estrogens (such as tamoxifen and raloxifene) do not allow binding of coactivators (Shiau *et al.* 1998). Several reviews have recently described the expression, function, and clinical relevance of

co-regulators in breast cancer and tamoxifen resistance (Smith & O'Malley 2004, Hall & McDonnell 2005, Girault *et al*. 2006). Coactivators such as AIB1 have been shown to be amplified and overexpressed in breast cancer cell lines and breast cancer biopsies. They also appear important in tamoxifen resistance, which occurs in 30–50% of treated ERα-positive breast cancer patients (Group 1998, Girault *et al*. 2003, Osborne *et al*. 2003).

Finally, in addition to full-length ER-mediated effects there are more than 20 different variants of human ERα (Poola *et al*. 2000) occurring in normal and neoplastic breasts (Poola & Speirs 2001) and at least 10 human ERβ variants (Poola *et al*. 2002) have been reported. Splice variants for both ERs have also been identified in rodents (Chu & Fuller 1997, Lu *et al*. 1998, Kos *et al*. 2000). Studies are ongoing to determine the clinical significance of expression of ER variants (Ko *et al*. 2002, Secreto *et al*. 2007).

ER activity is, therefore, dependent not only on the receptor isoforms, their relative levels of expression, and the presence of splice variants, but also on ligand-dependent or independent activation and the complement of co-regulatory molecules present. This complex regulation is likely to be why different doses of estrogen can have distinct effects in different tissues. The levels of estrogen experienced at pregnancy are some 10–100 times higher than those normally experienced in reproductive life (Shaikh 1971, Watson *et al*. 1975, Moore *et al*. 1978, Adeyemo & Jeyakumar 1993, Guzman *et al*. 1999, Offner *et al*. 2000) and may elicit completely different effects than the lower levels normally present during development. The complexity of the estrogen dose–response is exemplified in a recent study assessing the response of the mammary gland and uterus of ovariectomized mice to increasing concentrations of estrogen. Using both gene/protein expression and tissue architecture as end points, Vandenberg *et al*. (2006) showed that the uterus responded increasingly strong to increasing doses of estrogen (a sigmoidal dose–response curve) for both gene expression and tissue architecture. However, while the mammary gland showed higher levels of gene expression (*Msx2, Wnt4*, and *PR*) in response to increasing concentrations of estrogen (a sigmoidal dose–response curve), tissue architecture followed a polynomial dose–response. Low to moderate doses of estrogen induced TEB formation and ductal elongation, while higher doses inhibited these processes. In studies which examined mammary carcinogenesis in response to estrogen treatment, continuous administration of supraphysiological doses of estrogen led to a high percentage of mammary adenocarcinomas (Young & Hallowes 1973), while low doses given over long periods induced fibroadenomas (Geschickter *et al*. 1934). This might suggest that the epithelial and stromal compartments are differentially responsive to estrogen, although it is important to note that these studies were not performed in parallel. It has also been shown that heightened sensitivity to the mitogenic effects of estrogen occurs in MCF7 breast cancer cells after a period of estrogen withdrawal (Masamura *et al*. 1995) and in the normal mammary epithelial cells of long-term ovariectomized mice (Raafat *et al*. 1999). These data suggest that the high levels of estrogen experienced by the breast at pregnancy may result in an altered response to estrogen, which could result in permanent changes that persist after pregnancy when high hormone levels are no longer present.

ER levels during mammary development

The mammary gland shows altered responsiveness to estrogen at different developmental stages (Haslam & Shyamala 1980, Haslam 1989). In the mouse, ERα is first expressed postnatally at 3 days of age, in 8% of epithelial cells and 4% of stromal cells. In the epithelium at this stage, it is observed only in basal ductal cells (Haslam & Nummy 1992). However, the receptors are nonresponsive at this stage as competence to proliferate in response to estrogen starts only at 3 weeks, and the ability of estrogen to increase PR expression starts at 7 weeks (Haslam & Shyamala 1980, Haslam 1989). It is important to note that PR is used as an indicator of intact estrogen action, and in all the studies mentioned hereafter the PR subtype has not been defined by the investigator. As discussed later, the specificity of the induction of PR subtypes can now be explored, as subtype-specific antibodies have been developed (Mote *et al*. 2001). By 7 days of age, ERα has increased twofold in epithelial cells and is now located in both basal and luminal cells. During the next 5 weeks of development, the ERα expression in epithelial cells remains relatively constant; however, the proportion of ERα-positive stromal cells increases to 16%. Variations in the reported percentage of ERα-positive cells exists in the literature (Haslam & Shyamala 1980, Haslam 1989, Haslam & Nummy 1992, Saji *et al*. 2000, Shyamala *et al*. 2002); however, a general expression pattern with age is evident (Table 3). Within the TEBs, the cap cells are ERα-negative and only the inner body cells stain positively (Zeps *et al*. 1998). The majority of ERα cells occurred in clusters, rather than being evenly dispersed among negative cells. At 7–10 weeks of age, the percentage of

Table 3 Estrogen receptor localization within the breast during reproductive life

	D3-7	Pre-puberty	Adult	Pregnancy	Lactation	Post-lactation
ERα[a]	Low	15–40%[b]	20%	Low	High	Low
ERβ[a]	Unknown	60%	Unknown	High	High	High ERβ1, ERβ2
ERαβ[c]		25%		Few cells	High	Little
PR status		Negative	Positive	High	Low	High
Downstream effects	No proliferation	No proliferation	Cyclic proliferation	Rapid growth	Gland is insensitive to E_2	No proliferation remodeling

Data compiled from results within studies by Haslam & Nummy (1992), Haslam & Shyamala (1980), Haslam (1989), Shyamala *et al.* (2002) and Saji *et al.* (2000).
[a]Results from single stained staining.
[b]Results differ depending on study.
[c]Results from double immunofluorescence.

ERα cells in such clusters is significantly increased, when compared with younger ages (Haslam & Nummy 1992). This indicates either division of preexisting ERα cells, directly stimulated by estrogen acting on the classical receptor pathway, or differentiation of ERα cells from ER− stem/progenitor cells, stimulated through paracrine interactions.

The largest developmental study of ER expression levels in women to date was carried out by Bartow (1998) using autopsy material. In the early neonatal period, ERα was detected and localized to the nuclei of luminal cells of the duct. ERα-positive cells were present in all ductal areas, but appeared to cluster at budding points. At this stage no PR was present; however, TGFα-positive epithelial cells were noted. Breast tissue at 2 and 4 months of age had no detectable ERα, no PR, and weak if any staining for TGFα. Up to 7 years of age, immunohistochemistry showed only faint staining for nuclear ERα. In prepubescent girls, ERα protein was absent but PR was observed. In pubescent girls, low numbers of epithelial cells expressing ERα and abundant PR staining were seen. In adult women, some ERα staining was observed in the follicular phase and some PR, but no ERα was observed in the luteal phase, despite PR still being expressed. ERα was absent throughout pregnancy. In the postmenopausal breast, when circulating estrogen levels are low, there was marked involution of the TDLU and levels of ERα mRNA were low, but ERα and PR protein was common in luminal epithelial cells (Bartow 1998). A few smaller studies also exist which provide both supporting and conflicting observations (Petersen *et al.* 1987, Jacquemier *et al.* 1990, Ricketts *et al.* 1991, Williams *et al.* 1991, Clarke *et al.* 1997, Keeling *et al.* 2000), but it is difficult to draw general conclusions across these studies on the ERα expression pattern in the normal human breast due to differences in methodologies (Shimada *et al.* 1985, Ricketts *et al.* 1991). However, it seems clear that 6–15% of normal human breast epithelial cells stain positively for ERα. ERα-positive cells are predominately nestled within ER− epithelial cells and consistent with findings in mice, the percentage of contiguous ERα-positive cells increases with age and cancerous progression (Shoker *et al.* 1999).

ERβ has not been as extensively analyzed in humans as the classical ERα; however, two studies assessing its expression in normal human breast sections do exist. Speirs *et al.* (2002) showed widespread expression throughout the breast using a monoclonal ERβ antibody on reduction mammoplasty samples, but Shaw *et al.* (2002) reported more varied results, with 1–75% of epithelial cells staining positively for ERβ. While it is generally accepted that ERα is the most important subtype for determining estrogen action in the breast, a more detailed developmental study of ERβ expression to confirm its expression pattern is necessary to help determine its role and functional interactions with ERα. This may be important as recent gene expression arrays and clinical data have shown that ERβ exhibits growth inhibitory effects in ERα-positive breast tumor cells. Moreover, expression profiles of tumors clustered as a function of ERβ expression. Those with high ERβ downstream gene expression profiles had significantly higher probability of disease free survival when compared with low ERβ profiles (Lin *et al.* 2007). We believe that while not as important as ERα for predicting clinical outcome, ERβ may provide additional clues to deciphering parity-induced protection via estrogen.

Most of the protein tissue localization studies in rodents have examined only ERα. However, one detailed comparative analysis of ERα and ERβ throughout mouse mammary development has been carried out (Saji *et al.* 2000). This study showed that prepubertally, when estrogen does not induce

proliferation in the epithelium (Haslam 1989), both ERα and ERβ are present within the mammary gland, with co-expressing cells accounting for 25% of epithelial cell nuclei. At pregnancy, when estrogen causes rapid growth and maturation of the mammary gland and PR levels are high, ERβ is present in the majority of epithelial cells and ERα is scarce. Only a few cells express both receptors. During lactation, when the breast is insensitive to estradiol and PR levels are low, both ERα and ERβ are high and a high level of coexpression exists. Following lactation, ERα levels are extremely low and there is little colocalization of the two receptors (Table 3).

Unfortunately, while knockout mice have clearly demonstrated the role of ERα in promoting mammary epithelial proliferation and mammary ductal growth (reviewed in (Couse & Korach 1999), the data for $ERβ^{-/-}$ animals have been less clear. Prepubertal $ERβ^{-/-}$ females appear to have a normal mammary histology (Krege *et al*. 1998) with unaffected ductal outgrowth of the mammary gland anlage. However, because corpora lutea are rare in these animals, in contrast to their wild type littermates, little progesterone is produced in the ovaries and $ERβ^{-/-}$ mammary glands fail to develop ductal side branches and alveoli after puberty. Progesterone administration restores side branching leaving mammary glands morphologically indistinguishable from those of their wild type littermates (Palmieri *et al*. 2002). Forster (Forster *et al*. 2002) examined the possibility that ERβ is required for terminal differentiation of the mammary gland. Histomorphological comparison of $ERβ^{-/-}$ lactating glands with wild type controls revealed that ERβ was essential for the complete differentiation of the gland during pregnancy and lactation. $ERβ^{-/-}$ mice had incomplete penetration of the fat pad by the epithelial tissue, an increase in lumen size, a reduction in the number of alveoli, a reduction in the content of secretory epithelium, and a reduction in the width of the basement membrane. These mice also showed a reduction in expression of collagen in the extracellular matrix and in E-cadherin, integrin α2, occludin, connexin-32, and smooth muscle actin, markers of differentiation in the different mammary epithelial populations. Proliferation levels (assessed by Ki67 staining) were also increased in the adult gland (Forster *et al*. 2002). Cumulatively, these changes suggest that the mammary gland of lactating $ERβ^{-/-}$ mice is less well differentiated than that of wild type mice. If ERβ does function to promote mammary epithelial differentiation then it could have a key role in mediating the protective effects of parity against breast cancer in mice.

Progesterone as a downstream target

ERα is expressed in 75% of primary breast cancers and over 50% of these also express PR (McGuire 1978). When PR was identified as an ERα-regulated gene, it was hypothesized that it would indicate an intact ER (Milgrom *et al*. 1973, Leavitt *et al*. 1977) and so predict the tumors that were more responsive to endocrine therapies (Horwitz & McGuire 1975). This has been supported by retrospective studies showing that patients whose tumors contain both the ERα and the PR have the greatest probability of responding to tamoxifen therapy and have a better prognosis than those whose tumors do not contain steroid receptors (Osborne *et al*. 1980, Gross *et al*. 1984, Ravdin *et al*. 1992, Elledge *et al*. 2000). More recent clinical advances have also suggested that PR expression can be used to define the clinical relevance of aromatase inhibitors rather than tamoxifen as first line therapy.

As with the studies on the ER, the focus has been one of the subtypes of the PR. Two PRs exists (PRA and PRB), which are transcribed using alternate promoters of the same gene (Conneely *et al*. 2003). While structurally similar, the PRs have different functions. PRB is a strong transactivator whereas PRA is a transrepressor and can specifically inhibit both ERα and PRB (Meyer *et al*. 1992). Studies assessing PR protein expression within the mammary gland have shown that across mammals, PRA is abundantly expressed throughout development with PRB predominating during pregnancy. This is consistent with the expression of PRB in alveolar epithelial cells. Studies assessing their temporal and cell-specific expression patterns in the mammary gland are confusing due to the proportion of studies carried out before reagents capable of distinguishing the two isoforms became available (Mote *et al*. 2001) as eluded to earlier. Some confusion has also arisen in terms of PR regulation in mouse and human, due to the more dispersed alveolar architecture of the virgin mouse gland. However, once this is taken into account, it is evident that PR subtype protein expression levels are comparable in both species (Kariagina *et al*. 2007). Whether the PR isoform ratio has any bearing on response to endocrine therapy remains to be determined. However, it is clear that increased PRA is associated with poor prognosis, presumably via its repression of ER, while elevated levels of PRB are positively correlated with a more differentiated tumor phenotype and negatively correlated with high levels of HER2 expression/amplification (Bamberger *et al*. 2000, Mote *et al*. 2002).

Parity-specific changes in estrogen responsiveness

It is difficult to draw general conclusions about the effects of parity on the estrogen sensitivity of the mammary gland as existing studies have used different experimental regimes (varying ages of subjects and type and length of treatment) and different analysis end points (immunohistochemical analysis of ER/EGFR, gene expression, or hormonal response) or analysis times (following involution, during subsequent pregnancies, or during later life). However, cumulatively, the data suggest that the parous mammary gland may have altered levels of estrogen signal transducing machinery when compared with the nulliparous gland. Whether this results in an overall increased or decreased sensitivity to estrogen is still unclear (Table 4). ERα and Egfr expression levels were significantly reduced in mammary glands of parous rats when compared with age-matched nulliparous animals (Thordarson *et al*. 1995). Parous rats have fewer ERα-positive cells (Yang *et al*. 1999). However, to our knowledge, the levels of ERα in virgin versus parous breasts in humans have not been definitively assessed. Consistent with a decrease in ERα the levels of PRA-positive epithelial cells are decreased in parous rats suggestive of decreased ER-mediated estrogen action and PRB+ cells are increased consistent with increased differentiation (Aupperlee *et al*. 2005). Expression of ERα and ERβ splice variants 1 and 2 were shown to be greater in luminal cells of multiparous rodents on D9 of pregnancy in a comparison of nulliparous and uniparous groups. In addition, *ERβ1* and *2* mRNA expressions were upregulated in multiparous rats. In the interlobular stroma ERα and ERβ were increased in multiparous animals (Kass *et al*. 2004). GPR30, the cell surface receptor, which may crosstalk with Egfr in the nonclassical estrogen-responsive pathway, was increased in parous tissue when compared with nulliparous controls (Balogh *et al*. 2006). Furthermore, *JunB*, which is involved in AP1-mediated estrogen action, was increased in parous stroma, while the co-regulator *p300/CBP* was increased in parous epithelial tissues (Balogh *et al*. 2006). It is not clear from these studies that, however, whether changes in ER (or GPR30) expression are due to changes in numbers of ER/GPR30 expressing cells or to changes in levels of ER receptors within the same cell populations. Whether the effects on ER levels are differentially affected by single versus multiple pregnancies is also not clear.

In assessing the role of estrogen responsiveness in parity-induced protection, it is also important to note that the expression of *Tgfβ*, has been consistently shown to increase in parous mammary glands (D'Cruz *et al*. 2002, Blakely *et al*. 2006). Tgf β1 blocks the proliferation of ERα-positive mammary epithelial cells (Ewan *et al*. 2005). Studies in mice with differential Tgfβ1 levels have confirmed this relationship (Kulkarni *et al*. 1993, Pierce *et al*. 1993), with Tgfβ1 depletion (assessed in TGFβ1$^{+/-}$, which have 10–30% of wild type levels) promoting proliferation in ERα-positive cells, and MMTV-Tgfβ1 transgenic mice showing decreased colocalization of ERα and KI67 (Ewan *et al*. 2005). A parity-induced increase in TGFβ is consistent with the decreased level of ERα-positive epithelial cells observed in parous women (Russo *et al*. 1999) and the decrease in proliferating ERα-positive epithelial cells in parous rats (Sivaraman *et al*. 1998, Yang *et al*. 1999).

Parity appears to change the estrogen responsiveness of the breast at several different levels: ER expression, changes to the growth factor regulation of ER expressing cells, as well as changes in downstream estrogen transducing machinery such a GPRs and coactivators. Despite this, the nature of the changes and their biological basis remain poorly defined and this limits our understanding of the physiological consequences.

Stem cells and estrogen regulation

As discussed above, the epidemiological evidence suggests that the breast is at particular risk from environmental mutagens at or just prior to a time at which the stem cell population is likely to be most actively expanding. It is also established that estrogen is required for mammary epithelial development, that exposure to estrogen alters breast cancer risk depending on the time of exposure and that the response is likely to be determined by levels of expression of different ER isoforms and co-receptors. We now explore the relationship between stem cells, ER, estrogen, and parity. For a more detailed discussion of mammary stem cells, see previous reviews (Smalley & Ashworth 2003, Dontu *et al*. 2005, Visvader & Lindeman 2006, Wicha 2006).

Recently, the prospective isolation of adult virgin mouse mammary epithelial populations highly enriched for stem cell activity has been reported (Shackleton *et al*. 2006, Sleeman *et al*. 2006, 2007, Stingl *et al*. 2006). Single cells from these populations can be successfully transplanted into mammary fat pads, regenerating the glandular tissue and self-renewing (as shown by their ability to be serially transplanted; Shackleton *et al*. 2006, Stingl *et al*. 2006). The basal cell population, rather than the luminal cell population, appears enriched for stem cell activity in the adult virgin mouse. As ERα is known

Table 4 Parity-induced changes in estrogen responsiveness

Refs	Treatment	Technique	ER/EGF/cofactors	Analysis
Thordarson *et al.* (1995)	Mated at 7 week 15 d lactation 35 d involution	Radioreceptor assay	Decreased cytoplasmic ER in parous Decreased nuclear EGFR in parous	Proestrus 17 weeks
Kass *et al.* (2004)	Age of mating unknown 21 d lactation 14 d involution Assessed at 11–12 months	IHC RT-PCR	Luminal ERα increased in multiparous ERα and β increased in inter-alveolar stroma in multiparous Decrease in PR+ luminal cells in multiparous No differences in myoepithelial ER ERβ1 and ERβ2 mRNA increased in multiparous only	D9 of pregnancy
Yang *et al.* (1999)	Mated 15 weeks 15 d lactation NMU at 24 week NMU at 10 weeks Mated at 15 weeks	IHC Normal regions of tumor bearing mammary gland	Trend for ERα to be decreased in parous PR decreased Trend for ERα to be decreased in parous PR not changed	60 weeks 47 weeks
Bridges & Byrnes (2006)	Age at mating unknown 21 d lactation 14 d involution 14 d recovery then EB	Ability of EB to	Nulliparous more responsive at low doses of EB, multiparous more responsive to superphysiological doses 16% reduction plasma E_2 in primiparous	1–2 d after EB
Balogh *et al.* (2006)[a]	FFTB <24 years No data on lactation assessed at menopause	Gene expression microarray	*p300/CBP*, *GPR30* increased in parous epithelial cells, *JunB* increased and *PFTK1* decreased in parous stroma	55–60 years
D'Cruz *et al.* (2002)	Mated at 4 weeks 21 d lactation 28 d involution	Gene expression microarray	*Areg* and *IGF-I* decreased in parous Increase in *Tgf-3* and *cyclin D1*	14 weeks
Ginger *et al.* (2001)	42 d at E/P treatment (for 21 days) 28 d rest period	Subtractive suppressive hybridization	*S1-5/T16* (EGF-like protein) altered[b]	13 weeks

[a]This study is performed in humans.
[b]Direction of change not mentioned in report. EB, estradiol benzoate; EGFR, epidermal growth factor receptor; E/P, estrogen/progesterone; ER, estrogen receptor; E_2, estradiol; FFTB, first full-term pregnancy; GPR, G-protein coupled receptor;
IGF-I, insulin-like growth factor; IHC, immunohistochemistry; NMU, *N*-methyl-*N*-nitrosourea; PR, progesterone receptor; PRL, prolactin; PFTK1, PFTAIRE protein kinase 1; RT-PCR, reverse transcription-polymerase chain reaction.

to be found specifically within the luminal epithelial cells, this would suggest that the mammary stem cells are ERα-negative. This was directly demonstrated by Asselin-Labat *et al.* (2006), who assessed the ERα, PR, and Egfr levels in sorted single cells by qPCR and immunohistochemistry. They found that it was the luminal population of epithelial cells that was enriched for ER and PR, rather than the stem cell-enriched fraction. They did, however, find that that the stem cell-enriched population was Egfr-positive (Asselin-Labat *et al.* 2006). The presence of Egfr in the stem cell-enriched mouse mammary basal epithelial cells is consistent with profiling studies showing that it is overexpressed in breast cancer with a basal-like subtype (Ansquer *et al.* 2005, Hu *et al.* 2006, Livasy *et al.* 2006), which is the cancer subtype most likely to be directly derived from normal breast stem cells (Yehiely *et al.* 2006). Furthermore, prospective isolation and transplantation of mammary epithelial basal cells, ERα-negative luminal cells, and ERα-positive luminal cells confirmed that the ER+ luminal population has little or no transplantation capacity, whereas the basal population was enriched for stem cell activity (Sleeman *et al.* 2007). Therefore, estrogen is unlikely to directly stimulate stem cells and is likely to be operating via a paracrine mechanism. The paracrine relationship between ER+ cells and stem cells may also explain how estrogen can promote the growth of ER− tumors in a xenograft mouse model of pregnancy-associated breast cancer (Gupta *et al.* 2007) and why oophorectomy can be protective against ER− breast cancers (Nissen-Meyer 1964*a*,*b*, Group 1992*a*,*b*).

Paracrine stimulation of mammary stem cell function was directly demonstrated by transplantation of wild type mammary epithelial cells and marked mammary epithelial cells from ERα knockout mice. The marked cells were only able to contribute to epithelial outgrowths when the two populations were co-transplanted, demonstrating that the stem cells from the ERα knockout mice required a signal from the ERα wild type cells (Mallepell *et al.* 2006). This signal was identified as the EGF family ligand amphiregulin (Areg). It may be acting directly on the mammary stem cells themselves, as mammary epithelial cells with *in vivo* stem cell activity express Egfr (Asselin-Labat *et al.* 2006) or indirectly via Egfr in the stroma and a second set of paracrine messengers, which then signal from stroma to the stem cell compartment (Sebastian *et al.* 1998, Wiesen *et al.* 1999, Sternlicht *et al.* 2005). Areg has been known for some time to be important for early mammary gland development. Genetic disruption of Areg in mice caused dramatic defects in ductal outgrowth (Luetteke *et al.* 1999). Areg is expressed by the cap cells of the TEBs as well as myoepithelial and luminal cells of prepubescent mice and is also seen in the stroma adjacent to migrating TEBs in pubertal mice (Kenney *et al.* 1995). Areg has been shown to mirror the ability of estrogen to rescue ductal growth and TEB development in ovariectomized mice (Kenney *et al.* 1995) and is induced 50-fold upon estrogen stimulation in these mice. The requirement for Areg seems to be restricted to pubertal duct formation and growth as estrogen was unable to stimulate ductal growth and TEB formation in the absence of Areg (Kenney *et al.* 2003, Ciarloni *et al.* 2007) but side branching and alveolar formation could proceed normally. This suggests that at different stages of development, the actions of estrogen are mediated via alternate downstream pathways and paracrine signals. Interestingly, *Areg* has been consistently shown to be decreased in the parous mammary gland (D'Cruz *et al.* 2002, Blakely *et al.* 2006). Whether or not this is a potential causative mechanism in parity-dependent breast cancer protection, considering that Areg only appears important for the ductal outgrowth stage, or simply another marker of increased differentiation remains to be determined.

Another signal likely to be involved in the paracrine regulation of stem cell activity is Tgfβ. Stem cell behaviors such as cell cycle entry, regeneration, and formation of niches have been suggested to involve regulation by Tgfβ1 (Booth *et al.* 2000, Dao *et al.* 2002). Telomerase activity, postulated as a characteristic of stem cells, is also regulated by Tgfβ1 (Rama *et al.* 2001, Yang *et al.* 2001). Interestingly, constitutive activation of TGFβ1 in the mammary gland led to decreased serial transplantation capacity, hypothesized to be a result of premature stem cell senescence (Boulanger & Smith 2001) and when administered via slow release pellets caused end bud regression in mice (Silberstein & Daniel 1987). This ligand has also been shown to have concentration-dependent effects on ductal development in other systems (Montesano *et al.* 2007). TGFβ3 and its transcriptional targets were upregulated in parous glands (D'Cruz *et al.* 2002) and it is therefore possible that the parity directly results in a decrease in stem cell numbers as a result of TGFβ upregulation, although this needs to be directly tested.

Although adult mouse mammary stem cells are ER−, and the majority of ER+ cells do not colocalize with markers of cell proliferation (Clarke *et al.* 1997, Russo *et al.* 1998), there is direct evidence that some ER+ cells (∼2% of the ER+ fraction) in the mammary gland are dividing, and can take up a DNA label and pass it on to daughter cells (Smith 2005, Booth & Smith 2006), at least during the pubertal ductal expansion phase. This suggests that a subfraction of ER+ cells in the mouse mammary

gland form a progenitor population and are not terminally differentiated. ER+ progenitors may also occur in the human breast. Clarke and colleagues identified stem cells as a function of their ability to retain a radioactive DNA label (^{3}H-dT) and then colocalized putative mammary stem cell markers ($p21^{CIP1}$ and Msi1) as well as ERα. This demonstrated that a population of cells enriched for the putative stem cell markers, $p21^{CIP1}$ and Msi-1, were also steroid receptor-positive (Clarke *et al.* 2005). This led the authors to suggest that, within the human breast, scattered steroid receptor-positive cells are stem/progenitor cells that self-renew through asymmetric cell division and generate patches of transit amplifying and differentiated cells (Clarke *et al.* 2005). In contrast to these findings, immunohistochemical studies have shown that ERα and PR are found within the luminal epithelial, but not the basal myoepithelial or stromal, cells of the human breast (Petersen *et al.* 1987) and recent data analyzing separated epithelial populations from breast cancers and the normal breast support the notion of a basal stem cell population (Shipitsin *et al.* 2007). Therefore, as with the mouse studies, it may be that a sub-fraction of ER+ luminal cells in the human breast have progenitor activity without being true stem cells. Progenitor cells are likely to have limited self-renewal capacity and may also be targets for tumorigenic change. Luminal ER+ progenitors may, therefore, be direct targets for the protective changes in the response of the breast to estrogen as a result of pregnancy, quite apart from indirect effects on basal stem cells.

Concluding comments

Parity protects women against the development of hormonally responsive breast cancer, and the earlier the first full-term birth occurs, the greater the protection. Mouse models have shown that estrogen is the driving force behind this protection although several possible mechanisms are suggested to underlie this. The parous mammary gland appears more differentiated when compared with its virgin counterpart, and this is supported by altered gene expression profiles. Whether the increased differentiation of the gland *per se* induces protection is questioned by studies showing that differentiation of the gland by agents other than estrogen, do not confer protection against cancer development. Unique hormonal changes occur at pregnancy and may lead to permanent changes in the hormonal milieu of parous women. In particular, the altered levels of Prl and GH in parous individuals observed in some studies fits with the roles of these hormones in normal mammary growth and cancer development. However, studies at specific time points within the reproductive cycle, and at a time point where protection is evident, are required in order to ascertain whether a parous hormone profile actually exists. In keeping with the link between lifetime estrogen exposure and breast cancer risk, parity-induced protection may also be mediated via changes in the estrogen responsiveness of the gland. Emerging studies suggest that the levels of the estrogen transducing machinery in cells such as ERs, growth factors, growth factor receptors, as well as GPRs, are altered in parous glands. The significance of these changes is still unclear. Analysis in individuals who have resumed cycling, at various time points within the reproductive cycle, is required and would be aided if multiple levels of the estrogen pathway were assessed simultaneously. A role for stem cells in parity-induced protection against breast cancer is less clear, but would be consistent with the proposed role of mammary stem cells in cancer susceptibility and development. Without definitive mechanistic studies, however, a link between parity protection and stem cells is speculative, but plausible. Overall, estrogen seems key to understanding parity-induced protection, but whether the mechanism is through permanent gene expression changes, changes in the way estrogen is sensed or changes in the way stem cells respond to it, remains to be determined.

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