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# Human breast cell proliferation and its relationship to steroid receptor expression

*R. B. Clarke*

Breast Biology Group, Clinical Research Department, Christie Hospital, Manchester, UK

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## ABSTRACT

The steroidal regulation of proliferation and differentiation in the rodent mammary gland is well described, but how ovarian hormones regulate these processes in the human remains poorly understood. To investigate this, we developed the athymic nude mouse model in which intact normal human breast tissue is grafted subcutaneously and treated with estrogen and/or progesterone at human physiological serum levels. We demonstrated, first, that estrogen and not progesterone is the major epithelial cell mitogen in the adult non-pregnant, non-lactating breast, second, that estrogen induces progesterone receptor (PR) expression and, third, that PR expression is maximally induced at low estrogen concentrations while a higher amount of estrogen was required to induce proliferation. These data raised the question of whether one cell type possessed differential responses to high and low estrogen concentrations or whether PR expression and proliferation occurred in two cell populations. Using double-label immunofluorescence, we demonstrated that steroid receptor expression and cell proliferation (Ki67 antigen) occurred in separate cell populations in normal human breast epithelium, and that cells expressing the estrogen receptor-α (ERα) invariably contained the PR. We also found that this dissociation between steroid receptor expression and cell proliferation in normal epithelium was disrupted at an early stage in breast tumor formation. Recent findings presented herein support the proposal that some ERα/PR-positive epithelial cells are quiescent breast stem cells that act as 'steroid hormone sensors'. Such hormone sensor cells are likely to secrete positive or negative paracrine/juxtacrine factors dependent on the prevailing estrogen or progesterone concentration to influence the proliferative activity of adjacent ERα/PR-negative epithelial cells.

## INTRODUCTION

Ovarian steroids are essential for the development, proliferation and differentiation of the normal human breast[1]. There is much epidemiological evidence that ovarian hormones alter the risk of breast cancer. For example, an early menarche and a late menopause increase breast cancer risk, while an early menopause protects against breast cancer. This indicates that the risk of breast cancer relates to cumulative exposure to the secretion of ovarian hormones[2–4]. The greater risk from an increased reproductive life span may relate to the total number of times that the breast epithelium undergoes cyclical proliferation in response to ovarian hormones, which increases the chances of cancer initiation and promotion[5,6].

Correspondence: Dr R. B. Clarke, Breast Biology Group, Clinical Research Department, Christie Hospital, Manchester, M20 4BX, UK

REVIEW

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It is also clear from epidemiological studies that both pregnancy and breast-feeding are protective in terms of breast cancer risk[7]. This protective effect is thought to be related to the full lactational differentiation that the breast epithelium undergoes in response to hormones during pregnancy[8]. However, we still do not completely understand how hormones regulate normal human breast development, proliferation and differentiation, and how their effects on normal human breast epithelial cells relate to breast cancer risk. The purpose of this review is to describe normal human breast biology in relation to ovarian steroids, their receptors and proliferation.

## BREAST DEVELOPMENT

The breast is an unusual organ in that much of its development occurs during puberty or in the adult during pregnancy and lactation. The adult female breast consists of a branching, tree-like network of ducts lined by a double layer of epithelial cells that is surrounded by delimiting fibroblasts and embedded in an extracellular matrix[9]. The rudiments of the gland are developed during embryogenesis when newly formed breast epithelial cells become indented at the epithelial–stromal border, sprout and separate into 10–15 branches of the epithelial ducts that open separately onto the epidermal surface at the nipple. At puberty, the network of ducts leading from the nipple grows and divides into bundles of primary and secondary ducts lined with epithelial cells and ending with end bud structures[10]. It is from the end buds and ductal side-branches that the terminal duct lobulo-alveolar units (TDLU), or lobules, form. These are the principal, functional milk-producing units of the breast. These lobules exist initially as alveolar buds that mature following menarche into a variable number of blind-ending, secretory sacs known as acini, alveoli or ductules, which open into the intraobular terminal duct. The TDLU is the site from which many epithelial hyperplasias and carcinomas of the breast are thought to arise since this is where they are most often observed histopathologically[11]. In the mouse, estrogen induces growth of the ductal system during puberty, while progesterone stimulates growth of the lobules during pregnancy[12–15]. In contrast, human breast lobules form during and following puberty, and it is therefore not immediately clear which ovarian steroid regulates development of the human TDLU[1].

Breast development achieves full maturity and function during pregnancy and lactation. The full development of the TDLUs is accelerated during pregnancy, as the breast lobules expand in terms of the number of epithelial cells and alveoli that they contain in preparation for lactation. At the termination of lactation, the lobules involute to resemble those present in the non-pregnant gland, although they may retain a larger number of individual alveoli per lobule than before[9,10].

## EPITHELIAL CELL PHENOTYPES

The terminal ducts and tubular alveoli within the lobules are lined by an inner layer of luminal epithelial cells surrounded by an outer layer of basal or myoepithelial cells and the basement membrane that separates them from the intralobular stroma. In the non-pregnant gland, the myo- and luminal epithelial cells are distinguished not only by their relative positions, but by the proteins that they express. The myoepithelium expresses a distinct subset of epithelial cytokeratins (CK 5 and 14), the common acute lymphoblastic leukemia antigen (CALLA) and smooth muscle actin[16–18]. In contrast, the luminal cell type can be distinguished by expression of a subset of epithelial cytokeratins (CK 8, 18 and 19), nuclear receptors for the ovarian steroid hormones estrogen and progesterone and low (but detectable) levels of milk proteins[16,17,19,20]. The luminal cells also account for more than 90% of the epithelial cell proliferation that is observed in the non-pregnant gland[21,22]. Significantly, more than 90% of breast tumors express cytokeratins distinctive of the luminal phenotype, and greater than 75% express steroid receptors, indicating that the luminal cell type is the major target for breast tumorigenesis[23].

## OVARIAN STEROID HORMONES AND REGULATION OF EPITHELIAL PROLIFERATION

In the adult, non-pregnant, non-lactating breast, epithelial proliferation is maximal approximately 1 week after ovulation, during the luteal phase of the menstrual cycle. This is when both estrogen and progesterone are being secreted by the corpus luteum[24–31]. The data on breast contrast with those from the endometrium, where estrogen drives proliferation during the follicular phase, and have led to the conclusion that, in the breast, progesterone is the major breast mitogen, possibly after estrogen priming. To study this experimentally, we developed a model in which small pieces of intact normal human breast tissue were implanted subcutaneously into adult female athymic nude mice. Intact pieces of tissue were used in

order to preserve the architecture of the tissue so that the epithelium and stroma remained in contact. Two weeks after tissue implantation, silastic pellets containing steroid hormone were inserted subcutaneously at the base of the tail. These pellets were tailored to give serum concentrations equivalent to those seen in the follicular or luteal phases of the menstrual cycle[32].

The human breast xenografts were removed 1, 2 and 3 weeks after the start of treatment and the percentage of proliferating epithelial cells was determined, either by tritiated thymidine incorporation during S-phase followed by autoradiography, or by immunohistochemical staining using the Ki67 antibody which recognizes a proliferation-associated nuclear antigen. In tissue removed from untreated control mice or those treated with luteal-phase levels of progesterone, breast epithelial proliferation rates were very low (Figure 1a). Follicular-phase estrogen levels induced low levels of proliferation, while luteal-phase levels of estrogen significantly increased proliferation. The addition of progesterone to luteal-phase estrogen levels had no additional effect in this model. Estrogen alone therefore appeared to explain the effect of the menstrual cycle on breast proliferation, since no effects of progesterone were observed. We therefore investigated whether there were more subtle effects of progesterone on proliferation, by combining different concentrations of the two hormones. No effect of luteal progesterone levels on low, follicular estrogen levels was observed (Figure 1b).

The conclusion from this study was that a low dose of estrogen equivalent to follicular-phase levels induced some proliferation, but higher-dose luteal-phase levels were necessary to maximally induce cell division and there were no obvious effects of progesterone[32–34].

Our experimental studies in the athymic nude mouse model indicate that estrogen is a prime inducer of breast epithelial cell proliferation and may regulate the cyclical variation in breast cell proliferation during the menstrual cycle. Estrogen has also been reported to induce ductal elongation during puberty in the mouse and expression of the receptors for progesterone, a prerequisite for its activity[12,13]. Progesterone is certainly involved in the ductal side-branching and alveolar development that occurs during pregnancy in the mouse mammary gland[14,15]. However, in the human, alveolar development occurs during puberty and therefore no strict parallel can be drawn[9,10]. Some modulation of human breast cell growth is suggested by the existence of estrogen-dependent cancer cell lines in which progesterone can induce

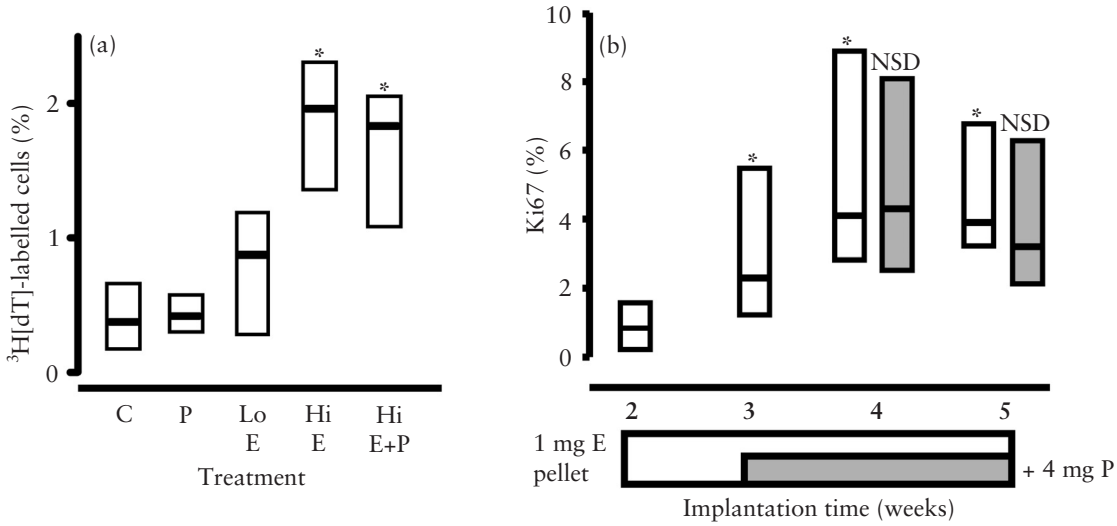


**Figure 1** The graphs demonstrate the effects of human physiological serum levels of estrogen (E) and progesterone (P) on the epithelial proliferation of normal breast tissue xenografted into the athymic nude mouse. This was measured (a) by incorporation of $^3$H[dT] and autoradiography or (b) by Ki67 immunohistochemistry. The medians (bars) and interquartile ranges (columns) of the percentage-labelled cells are shown for treatments ($n = 10$ per treatment group). (a) C, control (untreated); P, 7 days progesterone (4 mg pellet) treatment; E, 7 days estradiol (Lo, 0.5 mg pellet, Hi, 2 mg pellet) treatment; *, significantly different from C ($p < 0.01$). (b) Treatment from 2 weeks with 1 mg E pellet and/or treatment with a 4 mg P pellet. *, significantly different from 2 weeks untreated control ($p < 0.01$); NSD, not significantly different from 4 and 5 weeks E alone

a single cell division before acting as an inhibitor of cell proliferation[35]. The contribution of progesterone to the signal for proliferation therefore remains controversial[1].

The data presented above on the effects of progesterone in human breast epithelium implanted into the athymic nude mouse model are not in agreement with several recent publications on the risk of breast cancer in relation to hormone replacement therapy (HRT). These indicate that it is the combination of estrogen and progesterone in long-term HRT that correlates most strongly with an increased risk of breast cancer[36–39]. Two groups have investigated this further by looking at the proliferation rates of normal breast epithelium removed from postmenopausal women on estrogen only, or estrogen + progesterone HRT and comparing them to untreated postmenopausal women of a similar age[40,41]. A clear effect of the length of HRT administration was observed. Less than 5 years use of HRT of any type had no effect on proliferation. In contrast, more than 5 years on combined estrogen + progesterone HRT significantly increased breast epithelial proliferation rates compared to the untreated women[41]. The increased proliferation seen in normal breast epithelium taken from postmenopausal women being treated with combination estrogen + progesterone HRT correlates to increased breast cancer risk. Since the risk of breast cancer may be linked to the proliferation of normal epithelium, these data suggest that long-term progesterone treatment has an effect in the breast that we have not observed in our model of short-term treatment. We conclude from this that long-term and short-term progesterone have different effects in postmenopausal breast, especially when given as a long-term treatment.

Alternatively, the form of progesterone may be important. For example, it has been shown that 19-norprogestins that are used in some HRT formulations can induce proliferation in breast cell lines by directly activating the ERα[42–44]. Second, it has been reported that the endogenous enzyme 5α-reductase can convert progesterone to a 5α-metabolite that stimulates the growth of breast cancer cells in culture[45].

## STEROID RECEPTORS: LIGAND-ACTIVATED TRANSCRIPTION FACTORS THAT ACT AS CELLULAR SENSORS OF HORMONAL CUES

Estrogen and progesterone exert their effects by binding to receptors in the nucleus of the cell and altering the receptor structure such that they efficiently bind specific DNA sequences within gene promoter regions, termed steroid response elements. The ER and the PR are thus essentially ligand-activated transcription factors that regulate gene expression in the presence of the steroids[46]. The cells that express the classical ER and the PR are found within the luminal epithelial, but not the myoepithelial or stromal, cells of the human breast[19]. Recently, a second gene expressing the ER has been reported, and the classical ER has been renamed the ERα, the novel form being ERβ[47,48]. The ERα and the PR are known to be co-localized in a distinct subset of luminal epithelial cells, but their relationship to the cells that express the ERβ has yet to be fully described[49]. The PR is also expressed as two different protein isoforms, PRA and PRB, although both of these are transcribed from the same gene using different promoters. PRB is the longer version, containing an additional 164 amino acids at the *N*-terminal of the protein[50]. *In vitro* data support the view that PRB is the active PR, while PRA is either inactive or acts as an inhibitor of PRB[50]. However, in the normal physiology of breast epithelium, both PRA and PRB appear to be co-expressed in the same subset of cells and at similar levels[51].

We have demonstrated that approximately 10–20% of epithelial cells are ERα- and PR-positive and these are distributed regularly throughout the breast lobule, both in the intralobular duct and in the peripheral alveoli[52]. Approximately 2% of epithelial cells were proliferating and, rather surprisingly, these cells did not appear to contain receptors. We therefore used dual-label immunofluorescence to further determine the relationship between expression of steroid receptors and cell proliferation. This showed that proliferating cells (Ki67 antibody-positive) did not contain PR and constituted a separate population of cells. Since we have previously demonstrated that both proliferation and PR are induced by estrogen in normal breast tissue[34], we assumed that cell types would express the ERα. We therefore examined the co-expression of ERα and PR, again by immunofluorescence. When we quantified this, we showed that 96% of cells that express one receptor express both[52]. In contrast, when we examined the relationship between ERα and proliferating Ki67-positive cells, we saw no association. Supporting this

observation, the cells co-expressing ERα and PR were always found to contain $p27^{KIP1}$, a marker of non-proliferative cells[53]. However, proliferating cells were often adjacent or in close proximity to cells containing ERα/PR[52]. This dissociation between steroid receptor expression and proliferation in the mammary epithelium has since been demonstrated in both rats and mice[54–56] and suggests a model where ovarian steroids stimulate proliferation via paracrine signals secreted by steroid receptor-positive cells. Supporting evidence for this model has demonstrated that receptors are not being down-regulated during proliferation, but are expressed in separate cells. For example, mouse mammary epithelium in which the PR gene has been deleted, so-called PR knockout (KO) mice, normally fail to undergo alveolar development. However, this growth deficit can be overcome by mixing the PR KO cells together with wild-type cells[57]. This suggests that the wild-type cells communicate with the PR KO cells via a paracrine growth signal. Indeed, the same group of researchers identified that this signal was mediated, at least in part, by the Wnt4 gene product[58]. We have recently carried out microarray analyses to identify the growth signals induced by estrogen in human breast implanted into our athymic nude mouse model. Previously identified estrogen-induced genes, such as PR and pS2, were amongst the most highly upregulated, along with genes encoding the growth factor amphiregulin, intracellular pathway molecules (that mediate signalling from cell surface receptors), such as MAP kinase, JAK and STAT, transcription factors that induce growth such as Myc, Myb and ETS, and the cell cycle gene cyclin D1 (Figure 2). Amphiregulin, which binds the epidermal growth factor receptor, provides a good candidate for the molecule that relays the paracrine signal generated in the human breast in response to estrogen from the ERα-positive cell to an adjacent cell that proliferates. More recently, we reported that the separation between steroid receptor expression and cell proliferation observed in the normal mammary epithelium is altered at an early stage in human breast tumorigenesis such that estrogen directly drives cell proliferation in ERα-positive cancers[59]. This alteration in estrogen action may increase the sensitivity of breast tumor cells to estrogen and explain why estrogen-dependent breast tumors arise postmenopausally, at a time when the normal epithelium undergoes a process of involution as ovarian steroid levels decline.

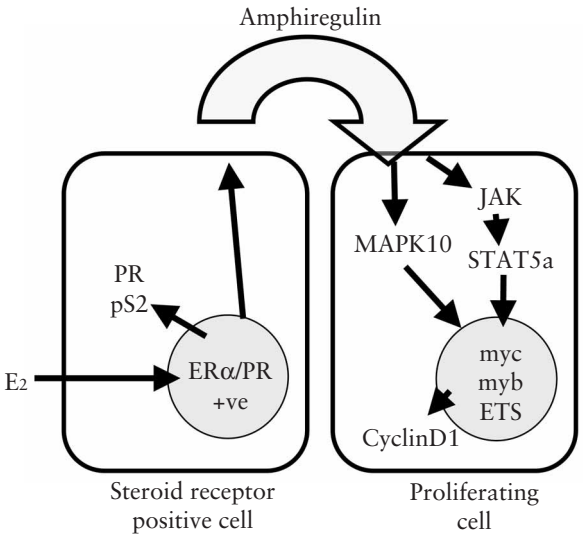


**Figure 2** Results of recent gene microarray experiments superimposed on a model of steroid hormone stimulation of normal breast cell growth by paracrine signaling (see main text for explanation). Estrogen (E) diffuses into breast cells and binds to estrogen receptor-α (ERα) in the 10–20% of cells that express ERα and progesterone receptor (PR). Ligand-activated ERα transactivates specific genes including PR, pS2 as well as growth factors such as amphiregulin, which is secreted by the cell and acts in a paracrine fashion on an adjacent cell to induce its proliferation. Proliferation may be induced via intracellular signaling pathways which lead to activation of nuclear transcription factors and consequently cell cycle genes. The signaling pathway molecules (MAPK10, JAK and STAT5a), transcription factors (Myc, Myb and ETS) and cell cycle gene (cyclinD1) were significantly induced in E-treated breast tissue compared to control tissue in gene expression studies performed in our laboratory

## STEROID RECEPTOR-POSITIVE CELLS INCLUDE A POPULATION WITH STEM CELL CHARACTERISTICS

Breast epithelial stem cells have been hypothesized to be the primary targets in the etiology of breast cancer. There is accumulating evidence for the existence of stem cells, both in normal mammary epithelium and in breast cancer[60–66]. Since breast cancers are predominantly steroid receptor-positive, we examined the biology of steroid receptor-positive cells and their relationship to stem cells in normal human breast epithelium. We employed several complementary

approaches to identify putative stem cell markers, to characterize an isolated stem cell population and to relate these to cells expressing steroid receptors. Using DNA radiolabelling in human tissue implanted into athymic nude mice, a population of label-retaining cells (LRCs) were shown to be enriched for cells expressing the putative stem cell markers $p21^{CIP1/WAF1}$ and Musashi-1 (Msi1). Human breast epithelial cells with Hoechst dye-effluxing 'side-population' properties characteristic of both mammary and hematopoietic stem cells in mice[67,68] were demonstrated to be undifferentiated cells by lack of expression of myoepithelial (CALLA) and luminal (MUC1) membrane markers, to express high levels of $p21^{CIP1/WAF1}$ and Msi1, and to be six times enriched for ERα and PR expression. Steroid receptor-positive cells also co-expressed stem cell markers, including cytokeratin (CK) 19, $p21^{CIP1/WAF1}$ and Msi1[62]. These data suggest a model where scattered ERα/PR-positive cells are stem cells that self-renew through asymmetric cell division and generate patches of transit amplifying and differentiated cells (Figure 3).

## SUMMARY

Over recent years, there has been considerable controversy about which of the ovarian steroids, estrogen or progesterone, regulates breast epithelial cell proliferation and, consequently, breast cancer risk. Our data and those of others lead us to the conclusion that estrogen is the major mitogen in the non-pregnant, premenopausal breast, whereas progesterone may have a more significant long-term role in the postmenopausal breast, where estrogen levels are much lower. Clearly, recent reports on estrogen + progesterone HRT indicate that progesterone contributes to breast tumorigenesis. Breast epithelium does not appear as sensitive an estrogen target organ as the endometrium, and our data suggest that this decreased steroid responsiveness may be due to an indirect effect on proliferation which requires paracrine factors to mediate their signal. Recent evidence for this and the preliminary characterization of stem cells in breast epithelium suggest that an increased understanding of normal breast biology may provide us with new opportunities for the prevention of breast cancer through

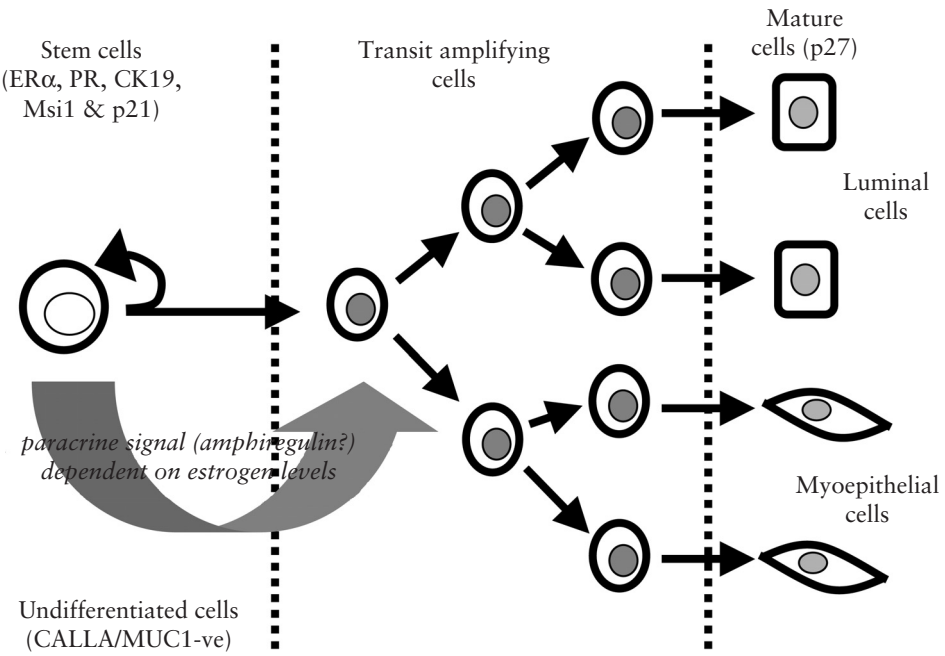


**Figure 3** A model of cellular hierarchies in human breast epithelium. Hoechst dye-effluxing SP cells include mainly undifferentiated breast cells (CALLA and MUC1 negative) and steroid receptor-positive cells which express CK19, $p21^{CIP1/WAF1}$ and Msi1, all markers of putative stem cells. The proliferation of the transit amplifying cells would be regulated by paracrine factors such as amphiregulin secreted from steroid receptor-positive cells depending on prevailing serum steroid levels (see Figure 2). After a small number of cell divisions, transit amplifying cells exit from the cell cycle, switch on expression of the CDKI $p27^{KIP1}$ and differentiate into myoepithelial or luminal cells

specific targeting of the cell population that is susceptible to cancer.

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