REVIEW

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Progesterone metabolites in breast cancer

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Abstract

In the 70 years since progesterone (P) was identified in corpus luteum extracts, its metabolism has been examined extensively in many tissues and cell lines from numerous species. In addition to the reproductive tissues and adrenals, every other tissue that has been investigated appears to have one or more P-metabolizing enzyme, each of which is specific for a particular site on the P molecule. In the past, the actions of the P metabolizing enzymes generally have been equated to a means of reducing the P concentration in the tissue microenvironment, and the products have been dismissed as inactive waste metabolites. In human breast tissues and cell lines, the following P-metabolizing enzymes have been identified: 5α-reductase, 3α-hydroxysteroid oxidoreductase (3α-HSO), 3β-HSO, 20α-HSO, and 6α-hydroxylase. Rather than providing diverse pathways for inactivating and controlling the concentration of P in breast tissue microenvironments, it is proposed that the enzymes act directly on P to produce two types of autocrines/paracrines with opposing regulatory roles in breast cancer. Evidence is reviewed which shows that P is directly converted to the 4-pregnenes, 3α-hydroxy-4-pregnen-20-one (3α-dihydroprogesterone; 3αHP) and 20α-dihydro-progesterone (20αHP), by the actions of 3α-HSO and 20α-HSO respectively and to the 5α-pregnane, 5α-pregnane-3,20-dione(5α-dihydroprogesterone; 5αP), by the irreversible action of 5α-reductase. *In vitro* studies on a number of breast cell lines indicate that 3αHP promotes normalcy by downregulating cell proliferation and detachment, whereas 5αP promotes mitogenesis and metastasis by stimulating cell proliferation and detachment. The hormones bind to novel, separate, and specific plasma membrane-based receptors and influence opposing actions on mitosis, apoptosis, and cytoskeletal and adhesion plaque molecules via cell signaling pathways. In normal tissue, the ratio of 4-pregnenes:5α-pregnanes is high because of high P 3α- and 20α-HSO activities/expression and low P 5α-reductase activity/expression. In breast tumor tissue and tumorigenic cell lines, the ratio is reversed in favor of the 5α-pregnanes because of altered P-metabolizing enzyme activities/expression. The evidence suggests that the promotion of breast cancer is related to changes in *in situ* concentrations of cancer-inhibiting and -promoting P metabolites. Current estrogen-based theories and therapies apply to only a fraction of all breast cancers; the majority (about two-thirds) of breast cancer cases are estrogen-insensitive and have lacked endocrine explanations. As the P metabolites, 5αP and 3αHP, have been shown to act with equal efficacy on all breast cell lines tested, regardless of their tumorigenicity, estrogen sensitivity, and estrogen receptor/progesterone receptor status, it is proposed that they offer a new hormonal basis for all forms of breast cancer. New diagnostic and therapeutic possibilities for breast cancer progression, control, regression, and prevention are suggested.

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Introduction

The name 'progesterone' was first adopted in 1935 (Allen 1970), shortly after it was isolated from corpus luteum extracts (Allen & Corner 1929), purified (Allen 1930, Slotta *et al.* 1934), and structurally identified (Butenandt 1934). In the 70 years since its discovery, nearly 100 000 papers have been published dealing with progesterone P; (4-pregnene-3,20-dione) on many levels. As the name implies, its main actions have been linked primarily to human female reproductive aspects involving the uterine changes associated with the menstrual cycle and gestation. However, P is now known to influence (directly or indirectly) many other tissues and facets of regulatory physiology and endocrinology, including those of the mammary glands. Soon after its discovery, the metabolism of

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P began to be investigated, primarily with the aim of determining its route of inactivation. It has become apparent that many tissues have P-metabolizing enzymes, which can modify different parts of the molecule. Although the resulting metabolites have been shown, in some tissues, to be active molecules in and of themselves, for the most part there has been a reluctance to accept them as anything other than waste products, with their formation as a means of decreasing the local P concentrations.

PR has long been linked to the proliferative changes in the normal breast, but its role in breast cancer is unclear. Recent studies have provided evidence that P metabolites formed in breast tissue have regulatory functions with respect to breast cancer that may previously have been attributed to P. We first suggested (Wiebe *et al.* 2000) that the P metabolites produced within breast tissues might be independently active hormones functioning as cancer-promoting or -inhibiting regulatory agents. By this hypothesis, the maintenance of normalcy or progression to neoplasia would depend on the ratios of pro- to anti-cancer P metabolites in the local breast tissue microenvironment.

The aim of this review is to summarize observations which indicate that most (if not all) tissues/cells may have some capacity to convert P and that mammary tissue in particular has enzymes which catalyze the direct conversion of P to two classes of active metabolites. Evidence is reviewed that these P metabolites function as independent pro- or anti-cancer autocrine/paracrine hormones that regulate cell proliferation, adhesion, apoptosis and cytoskeletal, and other cell status molecules via novel receptors located in the cell membrane and intrinsically linked to cell signaling pathways. Current endocrine therapies are based on suppressing estrogen levels or inhibiting its actions. Unfortunately, only a fraction of all breast cancer patients respond to this estrogen-based therapy and the response is only temporary (McGuire 1987). As the breast tissue P metabolites act on breast cell lines regardless of their tumorigenicity, estrogen sensitivity and estrogen receptor (ER) and progesterone receptor (PR) status, they are suggested to provide a new endocrine-based explanation for progression to the various forms of breast cancer as well as for the maintenance of normalcy in breast tissues. Based on the findings, it is proposed that in breast tissue P serves as a precursor for active steroid hormones whose relative concentrations determine the levels of mitogenic, apoptotic, and metastatic activities locally within the tissue.

Progesterone is metabolized by many tissues

Soon after its identification, a large number of studies followed to determine the metabolism of P. In the early decades, many workers in the field identified and measured urinary metabolites of P with the aim of ascertaining how the body inactivated this progestagen. By 1954, almost 100 naturally occurring steroids had been isolated from tissue and urinary sources (Dorfman 1954). The urinary P derivatives were assumed to result from metabolism in the liver and included 5β-pregnanes such as pregnanediol (5β-pregnane-3α,20α-diol) and pregnanolone (5β-pregnan-3α-ol-20-one) as well as the 5α-pregnanes, 5α-pregnane-3,20-dione (5αP), 5α-pregnan-3α-ol-20-one, 5α-pregnan-3β-ol-20-one, and 5α-pregnan3-3α(β), 20α-diols (Atherden 1959). The rapid metabolism of intravenously administered [^{14}C]progesterone by eviscerated rats (Berliner & Wiest 1956, Wiest 1956) in which tissues such as liver, spleen, gut, and adrenals had been removed, showed that P conversion was also occurring extrahepatically. It then soon became apparent that P serves as the precursor for the major steroid hormones (androgens, estrogens, corticosteroids) produced by the gonadal and adrenal cortical tissues.

A large number of metabolism studies on a variety of reproductive tissue from various species and physiological states showed that P is not only converted to the well-known steroid hormones such as estradiol and testosterone, but also to various 21-carbon derivatives for which there were no well-defined functions (Fig. 1). Studies on uterine tissues from rats (Marrone & Karavolas 1981, 1982, Redmond & Pepe 1986), guinea pigs (Glasier *et al.* 1994, Hobkirk *et al.* 1997), and humans (Bryson & Sweat 1967, 1969, Pollow *et al.* 1975, Milewich *et al.* 1977, Arici *et al.* 1999), as well as placentae from humans (Little *et al.* 1959) and goats (Sheldrick *et al.* 1981), showed the presence of numerous P-converting enzymes. Similarly, incubations with ovarian tissues (especially granulosa cells) from rat (Zmigrod *et al.* 1972, Lacy *et al.* 1976, Nimrod 1977, de la Llosa-Hermier *et al.* 1983, Moon *et al.* 1986, 1987, Wiebe *et al.* 1994a), human (Sweat *et al.* 1960), and chicken (Marrone 1986, Wiebe *et al.* 1990), as well as incubations with testicular cells or homogenates from trout (Andersson & Rafter 1990), frog (Canosa *et al.* 1998), mouse (Kuwata *et al.* 1976), rat (Slaunwhite & Samuels 1956, Wiebe 1978, Wiebe & Tilbe 1979, Wiebe *et al.* 1980, Tilbe & Wiebe 1981), rabbit (Matsumoto *et al.* 1976), and human (Savard *et al.* 1956, Stegner & Lisboa 1984), have shown

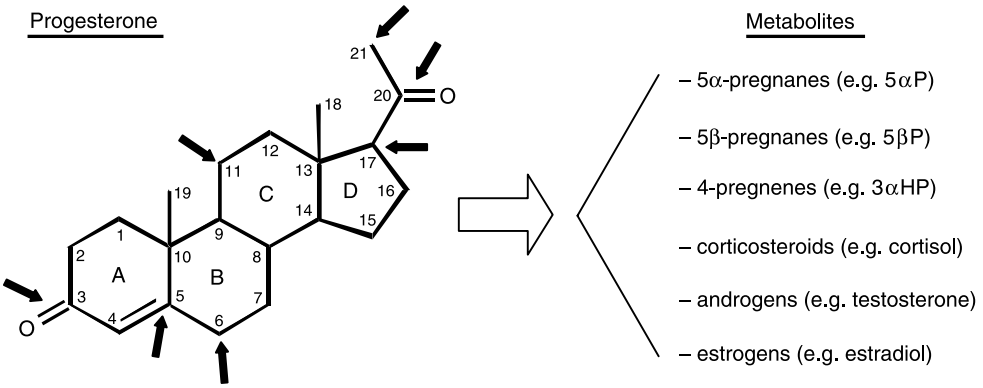

Figure 1 The structure of progesterone and the major classes of steroids resulting from its metabolism. P, the 21-carbon precursor of all the major steroid hormones produced in the gonads and adrenals, is also directly altered by enzymes within many, if not all, other tissues. The enzymes are specific for particular sites on the molecule (examples indicated by the arrows) and their actions lead directly to the 5α-pregnane, 5β-pregnane, and 4-pregnene metabolites of P and indirectly to the corticosteroids, androgens, and estrogens. In this review, evidence is presented that in human breast tissue 5α-pregnanes and 4-pregnenes are hormones whose actions may determine normalcy or progression to breast cancer.

the presence in these tissues of a number of enzymes capable of converting P to a variety of products.

Numerous studies provided evidence that many of the same P-metabolizing enzymes also exist in tissues that are not directly associated with reproduction. These include various regions of the central and peripheral nervous systems (brain, cortex, spinal cord, olfactory bulb, optic lobe, medulla oblongata, cortex corpus callosum, pineal, hypothalamus, pituitary, telencephalon, and neuronal and glial cells) from quail (Ukena *et al.* 2001, Matsunaga *et al.* 2004), chicken (Balthazart *et al.* 1988, Pignataro *et al.* 1998), rat (Hanukoglu *et al.* 1977, Marrone & Karavolas 1981, 1982, Bertics *et al.* 1987, Korneyev *et al.* 1993, Martini *et al.* 1993, Stuerenburg *et al.* 1997, Wiebe *et al.* 1997, Pomata *et al.* 2000, Rhodes & Frye 2001), mouse (Korneyev *et al.* 1993), monkey (Korneyev *et al.* 1993), and human (Melcangi *et al.*1993, 1994, Patte-Mensa *et al.* 2005). P is also metabolized by diverse tissues/cells such as fibroblasts (Perlman *et al.* 1960, Zhang *et al.* 1999), heart cells (Desgres *et al.* 1980), blood cells (Seamark *et al.* 1970), skin (Frost *et al.* 1969, Mauvais-Jarvis *et al.* 1969), salivary glands (Ferguson & Bannon 1983, Laine & Ojanotko-Harri 1990), saliva (Laine & Ojanotko 1999), and amniotic fluid (Beling & Cederqvist 1978). Invariably, the products of P metabolism in this diverse array of tissues consist of 21-carbon compounds.

Thus, many metabolism studies from a large number of tissues and various species had indicated that, in addition to the gonads and adrenals, perhaps most, if not all, tissues have some capacity to convert P to other products. The studies had demonstrated the presence in tissues and cells of a number of enzymes capable of acting on various sites in the P molecule, leading to the formation of various classes of 21-carbon steroids, in addition to the known hormones, as illustrated in Fig. 1. These P-metabolizing enzymes included 5α-reductase, 5β-reductase, 3α-hydroxysteroid oxidoreductase (3α-HSO), 3β-HSO, 20α-HSO, 20β-HSO, 6α(β)-, 11β-, 17-, and 21-hydroxylase, and $C_{17–20}$-lyase. In spite of this large number of enzymes capable of local transformation of P, the 21-carbon P metabolites were for the most part considered to be waste products and the P-metabolizing enzymes as a means of controlling the local (in tissue) concentrations of P.

In terms of neoplasia, the presence of P-metabolizing enzymes had been demonstrated in rat testicular interstitial cell tumors (Chatani *et al.* 1990), androblastoma (Sertoli-Leydig cell tumor) (Stegner & Lisboa 1984), dimethylbenz(a)anthracene (DMBA)-induced rat mammary tumors (Mori *et al.* 1978, Mori & Tamaoki 1980, Eechaute *et al.* 1983), human endometrial carcinoma (Collins & Jewkes 1974, Pollow *et al.* 1975), human breast tissues (Lloyd 1979, Miller 1990), modified breast cancer cell lines (T47Dco) (Fennessey *et al.* 1986, Horwitz *et al.* 1986), and virally transformed adrenocortical cells (Wiebe *et al.* 1987). Although selective differences in P-metabolizing enzyme activities between normal and tumor tissues were noted in some of these studies, they were not linked to any potential effects of the metabolites themselves on cancer induction or promotion prior to our studies (Wiebe *et al.* 2000).

Progesterone metabolism in breast tissues and breast cell lines

P was known to be involved in normal breast development as well as in the proliferative changes that occur during the menstrual cycle, pregnancy, and lactation (Going *et al.* 1988, Potten *et al.* 1988). However, its direct role in mammary cancer was not clear (McGuire & Horwitz 1977, King 1993) and a number of studies provided conflicting results. Some reports indicated stimulation (Anderson *et al.* 1989), while others observed regression of, or no effect on, human tumors (Horwitz *et al.* 1985, Santen *et al.* 1990) resulting from treatment with P or synthetic progestins. Similarly, in other species such as rodents (Jabara 1967, Welsh 1982, Luo *et al.* 1997) and dogs (Segaloff 1975, Mol *et al.* 1996), progestins were shown to either stimulate or inhibit tumor growth. *In vitro* studies of the effects of progestins on human breast cancer cell lines likewise showed either stimulation or inhibition of cell proliferation and cell cycle progression (Braunsberg *et al.* 1987, Clark & Sutherland 1990, Cappelletti *et al.* 1995, King 1993, Pike *et al.* 1993, Musgrove & Sutherland 1994, Clarke *et al.* 1994, Groshong *et al.* 1997).

The conflicting results regarding the role of P in breast cancer, in addition to the lack of evidence that tumor progression could be substantially related to changes in *in situ* P levels, led us to speculate about the potential importance of further metabolism of steroids occurring locally within the tumor and its adjacent host tissue. This led us to hypothesize that P may be converted within breast tissue into several types of metabolites, some of which stimulate while others inhibit cell proliferation and tumorigenesis. By this hypothesis, P would serve as a precursor (or pro-hormone) and the metabolites as the active hormones in regulating breast cancer. The state or progression of mammary tumors could then depend on the ratio of cancer-promoting to cancer-inhibiting steroid compounds. If such P metabolites could be shown to exist, they might provide an alternate or additional endocrine explanation for the estrogen-sensitive and -insensitive breast carcinomas as well as for normalcy of breast tissues.

Breast tissues and breast cell lines convert progesterone to 5α-pregnanes and 4-pregnenes

To test the hypothesis, studies were conducted to determine the capacity of tumor and surrounding normal (nontumorous) breast tissues to metabolize [^{14}C]P. The paired tissue specimens came from premenopausal, menopausal and postmenopausal women with various subtypes and grades of infiltrating duct carcinomas and included tissues that were estrogen-receptor (ER) and progesterone-receptor (P) negative and/or positive (Wiebe *et al.* 2000). All the breast biopsies examined converted [^{14}C]P into at least ten different metabolites that could be grouped into two structurally different classes of steroids (illustrated in Fig. 2): those with a delta-4 double bond in ring A (the 4-pregnenes) and those that are 5α-reduced (the 5α-pregnanes). Reduction of P to 5α-pregnanes is catalyzed by 5α-reductase and the direct 5α-reduced metabolite of P is 5α-pregnane-3,20-dione (5αP). The 5α-reductase reaction is irreversible, but 5αP can in turn be altered to 3- and 20-hydroxy pregnanes by the reversible actions of 3α-HSO, 3β-HSO, and 20α-HSO (Fig. 2).

The two 4-pregnenes resulting from direct P conversion are 4-pregnen-3α-ol-20-one (3αHP) and 4-pregnen-20α-ol-3-one (20αHP), catalyzed by the actions of 3α-HSO and 20α-HSO respectively (Fig. 2).

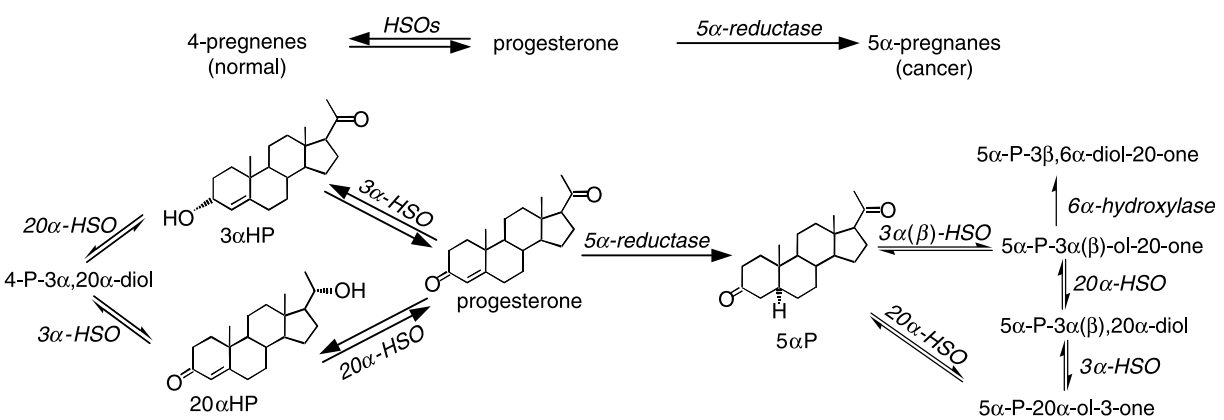

Figure 2 Progesterone conversion to 4-pregnene and 5α-pregnane metabolites by human breast tissues and cell lines. Note that 5α-reductase reaction is not reversible (see text for details; modified from Wiebe *et al.* 2005).

The 4-pregnenes can be further reversibly converted to 4-pregnene-3α(3β),20α-diol. The same metabolic pathways were subsequently demonstrated in four different breast cell lines (Wiebe & Lewis 2003) and had been previously identified in a number of tissues, including gonads, pituitary, and hypothalamus (Wiebe 1997). In addition, in the human breast cell lines, the final major product was 5α-pregnane-3β,6α-diol-20-one, indicating the presence of 6α-hydroxylase, an enzyme that was also present in tissues at minor activity levels. Thus, the P-metabolizing enzyme activities identified in human breast tissues and cell lines were: 5α-reductase, 3α-HSO, 3β-HSO, 20α-HSO, and 6α-hydroxylase (Fig. 2).

Changes in progesterone metabolite ratios and metabolizing enzyme activities

Although both normal (nontumorous) and tumorous breast tissues converted P to the two classes of metabolites, there were significant quantitative differences. In normal breast tissue, conversion to 4-pregnenes greatly exceeded the conversion to 5α-pregnanes, whereas in tumorous tissue, conversion to 5α-pregnanes greatly exceeded that to 4-pregnenes (Fig. 3a). The differences in amounts of 5α-pregnanes and 4-pregnenes were mainly due to changes in the amounts of 5αP and 3αHP (Fig. 3b) and the ratio of 5αP:3αHP was nearly 30-fold higher in tumorous than in normal breast tissues. The results indicated that P 5α-reductase activity is significantly higher, whereas P 3α-HSO and 20α-HSO activities are significantly lower in tumor than in normal tissues (Wiebe *et al.* 2000). Earlier studies with cell-free homogenates of breast tissues (Lloyd 1979, Miller 1990) and chemically induced rat mammary tumors (Mori *et al.* 1978) had also shown higher 5α-reductase and lower 20α-HSO activities in tumors than in normal glands.

Confirmation of a shift in actual amounts of P metabolites in the breast microenvironment has been provided, in part, by measurements of 5αP and 3αHP levels in breast tissue and nipple aspirate fluids (J P Wiebe, E Sauter & G Zhang unpublished results). The amounts of 5αP and 3αHP in a paired tissue sample, determined by gas chromatography–mass spectrometry, showed that levels (ng/mg protein) were 15.5 and 4.3 for 5αP and 5.5 and 12.7 for 3αHP respectively in the tumor and adjacent nontumor portion, confirming a higher 5αP:3αHP ratio in the tumor portion of the breast. An indication of the molar concentrations of P and the metabolites, 5αP and 3αHP, in breast microenvironment was obtained by RIA measurements of breast nipple aspirate fluids from four tumorous breasts (Table 1). Of note is that the concentrations in the aspirate fluid are high, being in the micromolar range. Although the values for 5αP varied considerably (perhaps due to the lack of specificity of the 5αP antibody), on average the levels of 5αP were higher than the levels of 3αHP; the concentrations of 5αP were 5.23 ± 2.51 μM and those of 3αHP were 1.03 ± 0.08 μM. The differences in levels suggest active metabolism of the

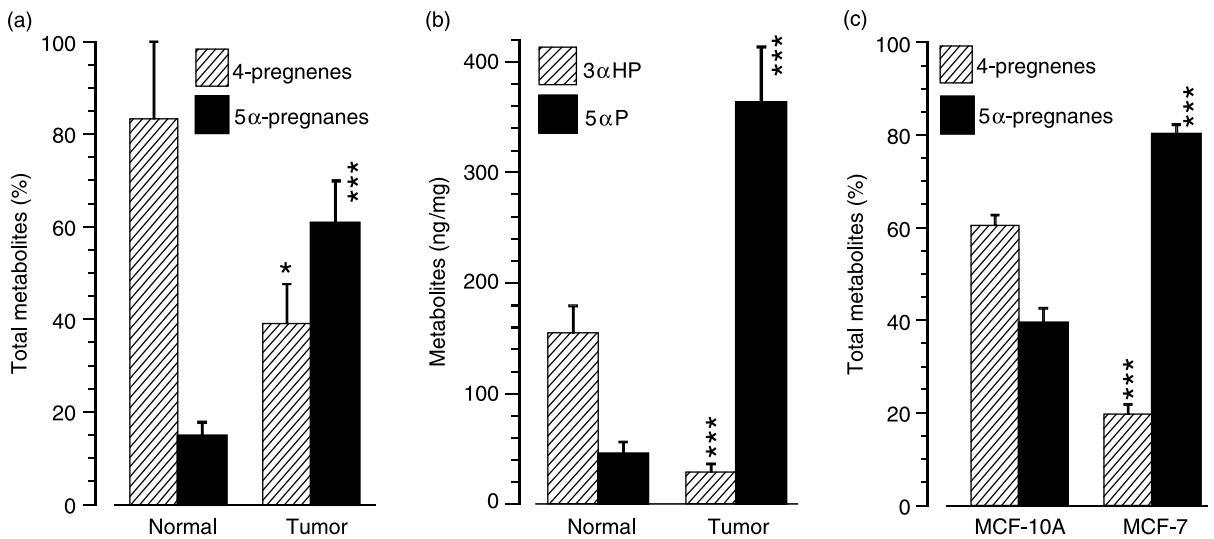

Figure 3 Differences in P metabolism between paired nontumorous (normal) and tumorous (tumor) human breast tissues ((a) and (b)) and between nontumorigenic (MCF-10A) and tumorigenic (MCF-7) human breast cell lines (c). In (a) and (c), the conversion to 4-pregnenes and 5α-pregnanes is presented as percentage of total P metabolism. In (b) the amounts of the specific metabolites, 3αHP and 5αP, formed from P by tissues is given as nanogram/milligram protein. *, significantly different from normal at $P<0.05$; ***, significantly different from normal ((a) and (b)) or from MCF-10A (c) at $P<0.001$. (Modified from Wiebe *et al.* 2000, Wiebe & Lewis 2003.)

Table 1 Levels of 5αP, 3αHP, and progesterone in breast nipple aspirate fluid samples from tumorous breasts

	5αP		3αHP		Progesterone	
Specimen[a]	(ng/μl)	(μM)	(ng/μl)	(μM)	(ng/μl)	(μM)
1	1.10	(3.48)	0.38	(1.2)	3.10	(9.87)
2	1.22	(3.86)	0.36	(1.14)	1.72	(5.48)
3	0.33	(1.04)	0.30	(0.95)	5.56	(17.7)
4	3.96	(12.53)	0.26	(0.82)	1.70	(5.41)
Mean±S.E.M.	1.65±0.79	(5.23±2.51)	0.33±0.03	(1.03±0.08)	3.02±0.91	(9.62±2.89)

[a]Breast nipple aspirate fluid samples (provided by Dr E. Sauter, University of Missouri, Coumbia, MO, USA), were extracted and steroids separated chromatographically and measured by RIAs by methods similar to those described (Wiebe *et al.* 1991).

locally available P (also present at micromolar concentrations) and the ability of the cells to alter the microenvironment in terms of the P metabolites.

To determine if breast cell lines exhibit differences in direction of P metabolism related to tumorigenicity, estrogen response and/or ER/P status, four breast cell lines with varying characteristics were used (Wiebe & Lewis 2003). Three of the cell lines (MCF-7, MDA-MB-231, T47D) are known to be tumorigenic in immunodeficient mice (Anderson *et al.* 1984, Soto *et al.* 1986); among these, MCF-7 and T47D cells are ER/P-positive (Horwitz *et al.* 1975) and estrogen-dependent for tumorigenicity, whereas MDA-MB-231 cells are ER/P-negative and develop tumors spontaneously without estrogen. The fourth cell line, MCF-10A, is ER/P-negative and considered to be nontumorigenic (Soule *et al.* 1990). The results showed that production of 5α-pregnanes was higher and that of 4-pregnenes was lower in tumorigenic (e.g. MCF-7) than in nontumorigenic (e.g. MCF-10A) cells (Fig. 3c), while differences in ER/P status did not appear to play a role (Wiebe & Lewis 2003). The 5α-pregnane-to-4-pregnene ratios were 7- to 20-fold higher in the tumorigenic than in the nontumorigenic cell lines, providing essentially the same pattern of results as for the tissues.

Overall, the metabolism studies showed that the altered direction in P metabolism, and hence in metabolite ratios, was due to significantly elevated 5α-reductase and depressed 3α- and 20α-HSO activities in breast tumor tissues and tumorigenic cells. It appeared, therefore, that changes in P-metabolizing enzyme activities might be related to the shift toward mammary cell tumorigenicity and neoplasia. The changes in enzyme activity might reasonably be expected to be due to changes in expression of the enzyme genes.

Changes in expression of progesterone-metabolizing enzymes

The above metabolic studies and *in vitro* enzyme kinetics studies showed that the activity of 5α-reductase is higher, whereas that of the 3α(20α)-HSOs is lower in tumor tissue and tumorigenic breast cell lines than in normal breast tissue and cell lines. Several factors can account for changes in enzyme activity. *In vivo*, changes in enzyme activity can result from changes in levels of the enzyme due to changes in expression of the mRNA coding for the enzyme, or from changes in the milieu in which the enzyme operates (such as temperature and pH, and concentrations of cofactors, substrates, products, competitors, ions, phospholipids, and other molecules). In *in vitro* experiments, the milieu is carefully controlled to be identical between incubations, and therefore, observed differences can be more easily ascribed to differences in enzyme amounts.

To determine if the differences in P-metabolizing enzyme activities between normal and carcinoma tissues/cells could be attributed to changes in enzyme mRNA expression, reverse transcriptase (RT)-PCR studies were carried out on breast tissues and cells lines. RT-PCR analyses on tissues from 38 patients showed significantly higher levels of expression of 5α-reductase type 1 (*SRD5A1*) and 5α-reductase type 2 (*SRD5A2*) mRNA and significantly lower levels of expression of the 3α-HSO type 2 (*AKR1C3*), 3α-HSO type 3 (*AKR1C2*) and 20α-HSO (*AKR1C1*) mRNAs in the tumor tissues than in the normal tissues (Lewis *et al.* 2004) (Fig. 4a). These results were similar to those from enzyme mRNA expression studies on breast cell lines (Wiebe & Lewis 2003), which showed higher 5α-reductase and lower HSO gene expressions in tumorigenic than in nontumorigenic cell lines (Fig. 4b). Other reports also indicate lower HSO mRNA expression levels in tumor than in normal portions of breast (Ji *et al.* 2004) and prostate tissues (Ji *et al.* 2003).

Overall, the enzyme activity and expression studies strongly suggest that 5α-reductase stimulation and 3α- and 20α-HSO suppression are associated with the transition from normalcy to cancer of the breast. It is tempting to speculate that factors in the mammary

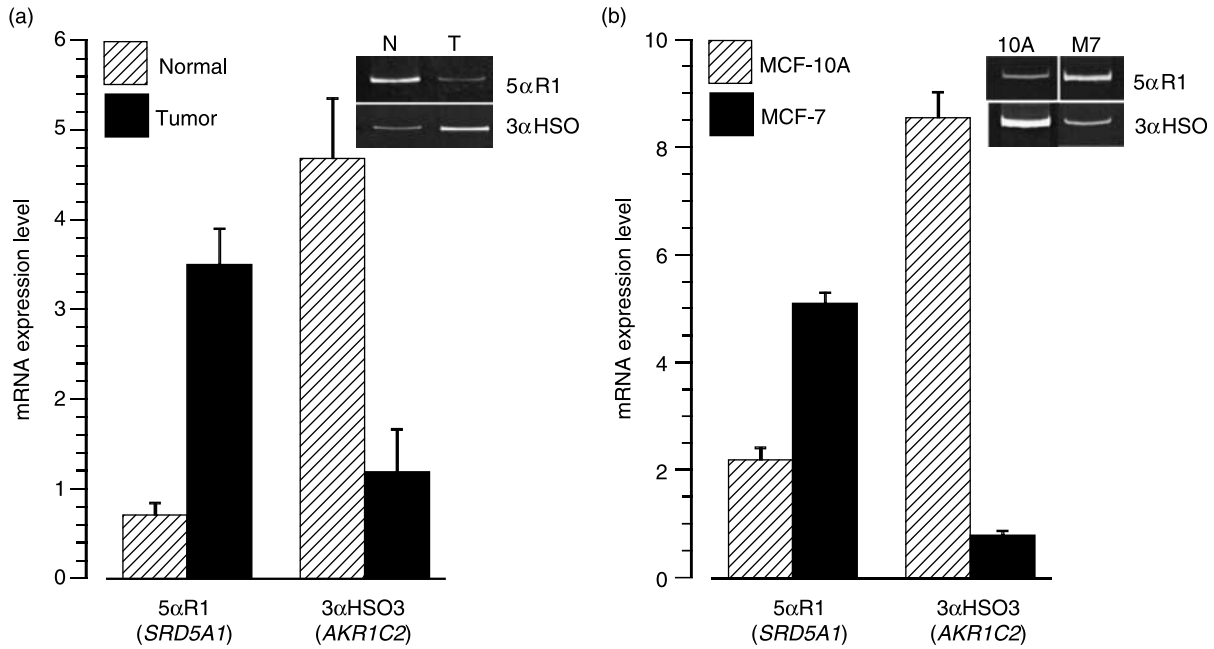

Figure 4 Examples of differences in progesterone-metabolizing enzyme expression levels (a) between paired normal and tumor breast tissues and (b) between nontumorigenic (MCF-10A) and tumorigenic (MCF-7) cell lines. For simplicity, only expression levels for 5α-reductase type 1 (5α-R1) and 3α-HSO type 3 (3α-HSO3) are shown; expression levels of the other enzyme genes (5α-R2, 3α-HSO2, and 20α-HSO) showed similar differences. Insets show representative RT-PCR results (gels) for (a) one tumor (T) and adjacent normal (N) breast tissue sample, and (b) MCF-10A (10A) and MCF-7 (M7) cells. Differences between normal and tumor and between MCF-10A and MCF-7 are significant at $P<0.001$. (See Wiebe & Lewis 2003, Lewis *et al.* 2004 for details.)

tissue milieu may be responsible for causing these changes in P-metabolizing enzyme gene expression and that these changes may be responsible for the transition. Steroid enzyme activities and gene expression have been shown in several tissues to be influenced by factors such as peptide hormones, cytokines, and steroids. For instance, prolactin acts as a paracrine/autocrine mutagenic agent in mammary cells (Clevenger & Plank 1997, Das &Vonderhaar 1997, Schroeder *et al.* 2002) and inhibits 20α-HSO expression in corpora lutea (Zhong & Vonderharr 1997). In mammary gland cells, cytokines have been shown to regulate activity and expression of 3β-HSO (Gingras *et al.* 1999) and 17β-HSO (Turgeon *et al.* 1998). The level of expression of 5α-reductase is up-regulated by estradiol and P in the uterus (Minjarez *et al.* 2001) and by 5α-dihydrotestosterone (DHT) in the prostate (Andersson *et al.* 1989, Ji *et al.* 2003). And the expression of 20α-HSO may be altered by P in corpora lutea (Sugino *et al.* 1997) and in endometrial cells (Nakajima *et al.* 2003). These examples suggest that the changes in P-metabolizing enzyme activity/expression that lead to higher ratios of 5α-pregnane:4-pregnene may be induced by an altered milieu within the breast. Identification of the factors that may be responsible for changes in P-metabolizing enzyme expression awaits future investigations.

The studies cited above provided evidence of selective changes in levels of enzyme activities/expression and in P metabolites formed in breast carcinoma, but there was as yet no evidence that P metabolites exhibited regulatory functions related to cancer. Some of the same P metabolites had been identified as active regulatory molecules in other tissues and with respect to other processes. For example, 5α-pregnanes such as 5αP (Selye 1942), 5α-pregnan-3α-ol-20-one (Majewska *et al.* 1986, Kavaliers & Wiebe 1987), and 3αHP (Wiebe & Kavaliers 1988) elicited marked anesthetic or analgesic effects via mechanisms involving calcium channels, the γ-aminobutyric acid(GABA)–benzodiazepine–chloride complex and endogenous opioid systems. 20αHP elevated serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels in rats (Gilles & Karavolas 1981), whereas 3αHP selectively suppressed basal and LH-releasing hormone-stimulated FSH secretion from primary cultures of anterior pituitary cells (Wood & Wiebe 1989) by nongenomic mechanisms at the level of the gonadotrope membrane, protein kinase C cell signaling pathway, and intracellular Ca^{2+} mobilization (Dhanvantari & Wiebe

1994, Wiebe *et al.* 1994*b*, Beck *et al.* 1997). The next step was to test the P metabolites for possible effects on mitogenic and metastatic parameters.

Cancer-related actions of the progesterone metabolites

Transformation of normal human cells into malignant cancers involves changes in, or deregulation of, a number of cell characteristics and processes (Hanahan & Weinberg 2000). Cardinal among these are: (a) proliferation rates, (b) cell-to-cell and cell-to-substrate adhesion, (c) cytoskeletal and adhesion molecules, (d) receptors that transduce growth regulating signals, and (e) mitogenic growth signaling pathways. A summary of the effects of the P metabolites on these parameters follows.

Effects of progesterone metabolites on cell proliferation, mitosis, and apoptosis

Uncontrolled cell proliferation is one of the hallmarks of cancer, and factors which affect cell proliferation rates are known to affect cancer rates (Cohen & Ellwein 1990, Pike *et al.* 1993, Hanahan & Weinberg 2000). Initial studies conducted on MCF-7 cells showed significant, but opposite, effects on cell proliferation; 3αHP inhibited whereas 5αP-stimulated proliferation dose-dependently between 10^{-9} and 10^{-6} M (Fig. 5a). In this concentration range, estradiol resulted in weak stimulation at 10^{-8} M and either no effects or slight inhibition at higher concentrations (Fig. 5a). Stimulation in cell numbers was also observed when cells were treated with other 5α-pregnanes, such as 5α-pregnan-3α-ol-20-one, 5α-pregnan-20α-ol-3-one, and 5α-pregnane-3α,20α-diol, whereas other 4-pregnenes such as 20α-HP and 4-pregnene-3α,20α-diol resulted in suppression of cell proliferation similar to that of 3αHP (Wiebe *et al.* 2000). Stimulation of cell proliferation with 5αP and inhibition with 3αHP were also observed in all other breast cell lines examined, whether ER/P-negative (MCF-10A, MDA-MB-231) or ER/P-positive (T47D, ZR-75-1) and whether requiring estrogen for tumorigenicity (MCF-7, T47D) or not (MDA-MB-231), or whether they are nontumorigenic (MCF-10A) (Wiebe *et al.* 2000, Pawlak *et al.* 2005, G Zhang & J P Wiebe, unpublished results).

Increases in cell numbers can result not only from increased rates of cell division, but also from decreases in rate of cell attrition via programmed cell death (apoptosis) (Thompson 1995). A balance of proliferation and apoptosis provides the homeostasis in normal tissues and alteration in this balance is postulated to set off a series of changes ultimately leading to malignancy. Studies on cell lines (Zhang *et al.* 2005, G Zhang & J P Wiebe, unpublished results) using several methods of evaluating apoptosis and proliferation/mitosis showed that 3αHP resulted in significant increases in apoptosis and decreases in mitosis, leading to significant decreases in total cell numbers. In

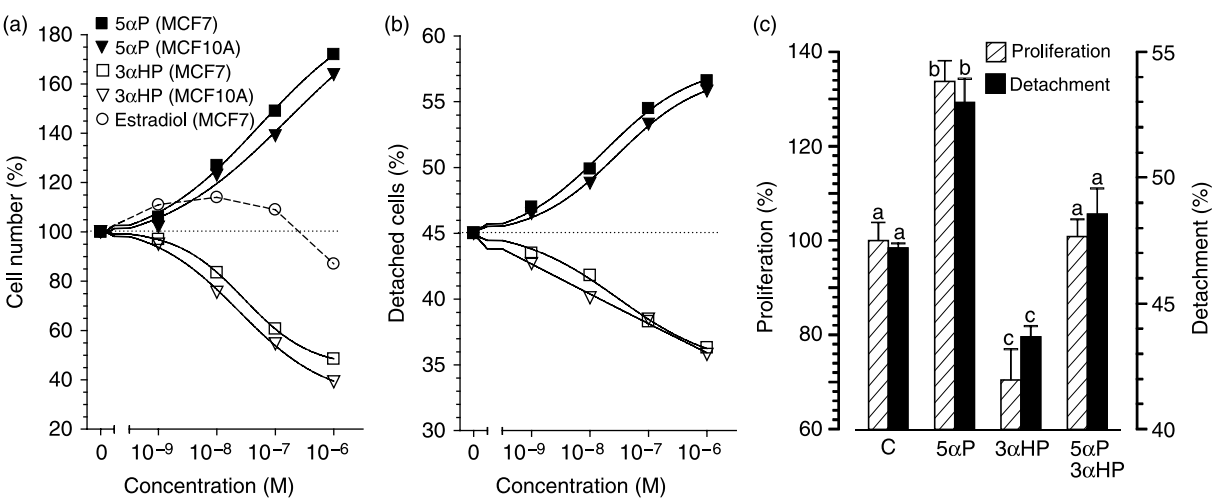

Figure 5 Effects of 5αP and 3αHP exposure (72 h) on proliferation (cell number) and adhesion (detachment) of MCF-7 and MCF-10A human breast cell lines. Values are presented as percent in relation to controls (0, C). In (a) and (b) cells were exposed to 0 or 10^{-9}–10^{-6} M 5αP or 3αHP and results show highly significant dose-dependent effects. In (a) MCF-7 cells were also exposed to estradiol for comparison purposes. The effect of combining 5αP and 3αHP (each at 10^{-7} M) on proliferation and detachment of MCF-7 cells are shown in (c); different letters (a, b, and c) on bars denote significant differences ($P<0.05$) within proliferation or detachment results. (Modified from Wiebe *et al.* 2000, Wiebe & Muzia 2001, Pawlak *et al.* 2005.)

contrast, treatment with 5αP resulted in decreases in apoptosis and increases in mitosis. Thus, with respect to overall cell proliferation effects, the results indicated that the actions of 3αHP and 5αP are diametrically opposed and involve both cell division and cell death. The results correlated with the metabolism studies in that the levels of the proliferation-inducing hormone (5αP) were higher and those of the proliferation-suppressing hormone (3αHP) were lower in tumorous tissue and the reverse was true for normal tissue.

Effects of progesterone metabolites on cell adhesion

Cellular adhesion is a critical aspect of cancer biology. In culture conditions, normal cells of mesenchymal or epithelial origin generally depend on anchoring to a solid substratum for cell division. This dependence on support by solid substrates for cell proliferation is lessened as cells become neoplastic and metastatic (Raz 1988). Some time during the development of most types of human cancer, pioneer cells are spawned that are capable of moving out of the primary tumor masses and of traveling to distant sites where they may succeed in founding new colonies. It is these distant settlements of tumor cells — metastases — that are the cause of about 90% of human cancer deaths (Sporn 1996). The capability of escaping the primary tumor mass and colonizing new terrain involves a number of cellular changes, not the least of which are cell–cell and cell–substrate adhesion characteristics. To allow the initial escape, adhesion must be decreased and attachment severed.

To determine whether P metabolites might play a role in the acquisition of metastatic potential, their effects on cell adhesion were examined (Wiebe *et al.* 2000, Wiebe & Muzia 2001) by quantitative cell–substrate attachment and detachment assays that had been developed earlier (Dinsdale *et al.* 1992) for baby hamster kidney cells. The first tests were on MCF-7 cells and the results showed that 5αP caused significant dose-dependent decreases in attachment to, and increases in detachment from, the substratum (Fig. 5b). The opposite effect was observed with 3αHP, which promoted cell attachment and decreased cell detachment (Fig. 5b). Similar effects have also been demonstrated recently in MCF-10A, T47D, and MDA-MB-231 cells (Wiebe *et al.* 2004, Pawlak *et al.* 2005). The opposing actions of 5αP and 3αHP on both cell anchorage and proliferation strengthen the hypothesis that the direction of P metabolism *in vivo* toward higher 5α-pregnane and lower 4-pregnene concentrations could promote breast neoplasia and lead to malignancy.

Proof of principle

Confirmation of the hypothesis that the move from normalcy to neoplasia in breast cells is influenced by the *in situ* increase in the 5α-pregnane:4-pregnene ratio requires studies in which 5α-reductase activity is blocked, as well as paradigms where various concentrations of a 5α-pregnane and a 4-pregnene are used in combination and in various temporal sequences. We have used the 4-azasteroid dutasteride, a known inhibitor of 5α-reductase types 1 and 2 (Bramson *et al.* 1997) that has been employed in trials to inhibit the 5α-reduction of testosterone to DHT in men with benign prostate hyperplasia (Brown & Nuttal 2003, Clark *et al.* 2004) and prostate cancer (Andriole *et al.* 2004, Iczkowski *et al.* 2005). First, we demonstrated that in MCF-7 cells dutasteride at 10^{-6} M inhibited P conversion to 5α-pregnanes by $>95\%$ and at the same time increased 4-pregnene production. Next, it was demonstrated that treatment of cells with P alone, without medium change for 72 h, resulted in significant conversion to 5α-pregnanes and concomitant increases in cell proliferation and detachment. These increases in proliferation and detachment were blocked in cells incubated with P plus dutasteride. In turn, the suppression by dutasteride was overridden by the addition of 5αP. The results are seen as providing proof of the principle that the effects on proliferation and adhesion were not due to P, but due to the 5α-reduced metabolites (Wiebe *et al.* 2006).

To confirm the hypothesis that the ratio of 5α-pregnanes:4-pregnenes is a determinant of the degree of cell proliferation and adhesion, detailed studies will need to be carried out using various concentrations of 3αHP and 5αP in combination and in various temporal sequences. Similar studies could also determine if the progression toward neoplasia can be impeded or even reversed by high 3αHP:5αP ratios, i.e. ratios of P metabolites that favor the 4-pregnenes. Data from studies in which cells were treated simultaneously with both 3αHP and 5αP show that the independent effects of the individual hormones on proliferation and adhesion are cancelled out when present in equal concentrations (Fig. 5c) (Pawlak *et al.* 2005) and support the view that the overall effects may depend on the relative concentration of each in the milieu.

Effects of progesterone metabolites on cytoskeletal and adhesion complexes

The transformations in morphology, replication, and adhesion during the transition from normal to cancerous cell have been shown to be accompanied by rearrangements of cytoskeletal and adhesion structures. The cytoskeletal organization differs between normal and cancerous cells (Ben-Ze'ev 1985, Holme 1990, Holth *et al.* 1998) and between high- and low-metastatic cells (Suzuki *et al.* 1998). For example, the level of organization of the actin cytoskeleton observed in normal cells (Bershadsky *et al.* 1995, Helige *et al.* 1997) is characterized by higher levels of polymerized actin, whereas transformation to the metastatic condition may be accompanied by disruption and/or visible disappearance of actin filaments (Suzuki *et al.* 1998). Similarly, vinculin, a protein that is associated with cell-to-cell and cell-to-substrate adhesion sites (Wilkins & Lin 1982, Luna & Hitt 1992, Humphries & Newham 1998), may show alterations. In normal cells, vinculin may be readily detected, while in highly malignant cell lines its organization may be significantly altered (Schliwa *et al.* 1984) or it may not be detected at all (Sadano *et al.* 1992), suggesting that depolymerization or suppression of vinculin expression may be closely related to progression of malignancy.

To determine the cellular sites of action of the proliferation- and detachment-promoting P metabolite, 5αP, its effects on MCF-7 cell morphology, F-actin expression, polymerization, and filament distribution, as well as vinculin expression and vinculin-containing adhesion plaque numbers, were examined by immunohistochemistry, morphometry, and western blotting (Wiebe & Muzia 2001). Figure 6a shows typical distribution of polymerized actin filaments and terminal vinculin molecules in a normal cell. Treatment of cells with 5αP resulted in dose-dependent decreases in vinculin-containing adhesion plaques and vinculin expression (Fig. 6b), as well as in polymerized actin stress fibers (Fig. 6c). Similar results were observed with MCF-10A, MDA-MB-231, and T47D breast cell lines (Wiebe *et al.* 2004), again confirming that the P metabolites appear to be able to target a variety of human breast cells. The results suggest that the observed decreases in adhesion and increases in cell proliferation following 5αP-treatment may be related to depolymerization of actin and decreased expression of vinculin.

Receptors for progesterone metabolites in human breast cells

Localization and characterization of progesterone metabolite receptors

The actions of hormonal steroids are considered to generally require complexing with specific-binding sites (receptors) on target cells. Therefore, an important step in elucidating the mechanisms of action of a regulatory hormone is the identification of such receptors. To identify potential binding sites for P metabolites in mammary cells, competition radioreceptor assays were

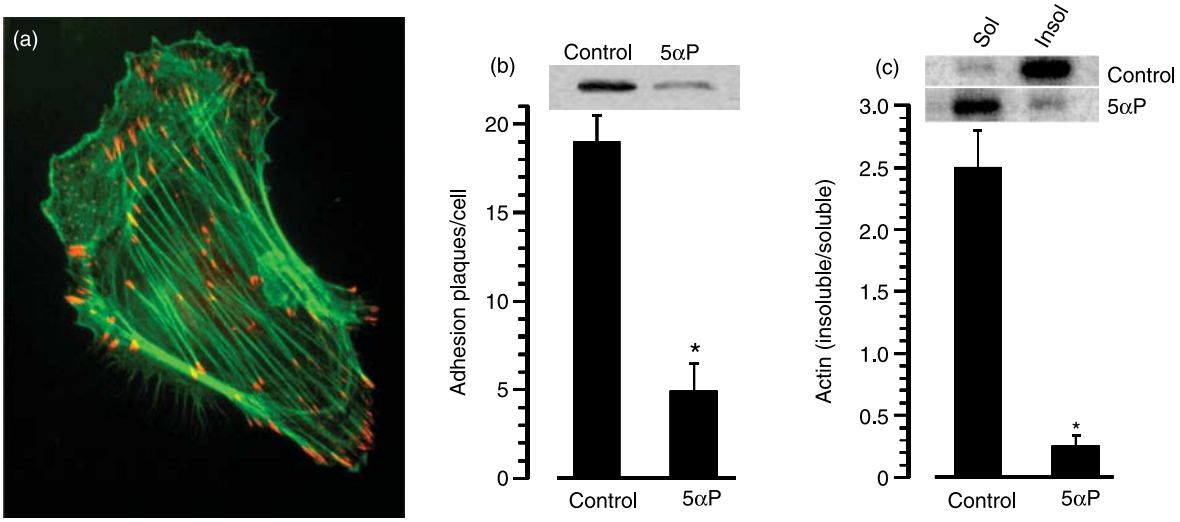

Figure 6 Examples of effects of 5αP on cytoskeletal and adhesion complex molecules in MCF-7 cells. (a) The arrangement of vinculin-containing adhesion plaques (red) and polymerized F-actin fibers (green) is shown in a control cell. The effects of 5αP (10^{-6} M) treatment result in marked reduction in vinculin expression (inset) and in number of vinculin-containing adhesion plaques (b), and depolymerization (insoluble to soluble change) of F-actin (c). (From Wiebe & Muzia, 2001 and unpublished results).

conducted on nuclear, cytosolic, and membrane fractions of MCF-7 and MCF-10A breast cell lines using [^{3}H]-labeled 5αP and 3αHP (Weiler & Wiebe 2000, Pawlak *et al.* 2005). The studies showed that binding of 5αP or 3αHP occurs in the plasma membrane fractions, but not in the nuclear or cytosolic compartments (Fig. 7a). Saturation and Scatchard analyses indicated separate high-specificity, high-affinity, low- capacity receptors for 5αP and 3αHP that are distinct from each other and from the well-studied nuclear/cytosolic P, estrogen, and androgen and corticosteroid receptors; binding of [^{3}H]5αP or [^{3}H]3αHP was not displaced by 200 to 500-fold concentrations of P, estradiol, androgens, corticosteroids, and other P metabolites. In turn, binding of [^{3}H]P or [^{3}H]estradiol to cytosolic or nuclear fractions was not displaced by excess 5αP or 3αHP. The binding studies showed that the criteria of high affinity, specificity, saturability, and association and dissociation kinetics required of receptor designation (Laduron 1984, Limbird 1996) were met. The studies thus provided the first demonstration of the existence of specific P metabolite receptors. Identifying the presence of distinct and separate receptors for 3αHP (3αHPR) and 5αP (5αPR) in human breast cells is important in light of the findings that the two P metabolites exert opposing actions with respect to cell proliferation and adhesion.

Regulation of progesterone metabolite receptor levels

Since the action mechanisms of hormonal steroids are generally initiated by the binding to specific receptors, the level of cellular response to steroids is limited not only by the local concentration of the hormone, but also by the receptor number (Vanderbilt *et al.* 1987, Webb *et al.* 1992). Due to the potential importance of 5αP in promoting breast cancer via the binding to its membrane-based receptors, the role of mitogenic (estradiol, 5αP) and anti-mitogenic (3αHP, 20αHP) endogenous steroids on 5αPR levels in a tumorigenic (MCF-7) and a nontumorigenic (MCF-10A) breast cell line were explored (Pawlak *et al.* 2005). Exposure of MCF-7 cells for 24 h to estradiol or 5αP resulted in significant dose-dependent increases in 5αPR levels (Fig. 7b), whereas 3αHP or 20αHP resulted in significant dose-dependent decreases in 5αPR levels (Fig. 7c). Treatment with two mitogenic (estradiol or 5αP) or two anti-mitogenic (3αHP or 20αHP) hormones resulted in additive effects on 5αPR numbers (Fig. 7b and c), whereas treatment with one mitogenic and one anti-mitogenic hormone abolished the mitogen-induced increases (Fig. 7d). In addition, preliminary experiments in which MCF-7 cells were exposed to 1.0 nM estradiol for 24 h showed a 60% decrease in 3αHPR numbers (Weiler & Wiebe 2000).

The nontumorigenic breast cell line, MCF-10A, was also shown to posses specific, high-affinity plasma membrane receptors for 5αP that are up-regulated by estradiol and 5αP and down-regulated by 3αHP (Pawlak *et al.* 2005). Estradiol binding was demonstrated in MCF-10A cell membrane fractions and may explain the estradiol action in these cells, which reportedly lack intracellular ER. In both MCF-7 and MCF-10A cells, the increases in 5αPR due to estradiol or 5αP and decreases due to 3αHP or 20αHP correlated with respective increases and decreases in cell proliferation as well as detachment (Pawlak *et al.* 2005), indicating the functional relevance of

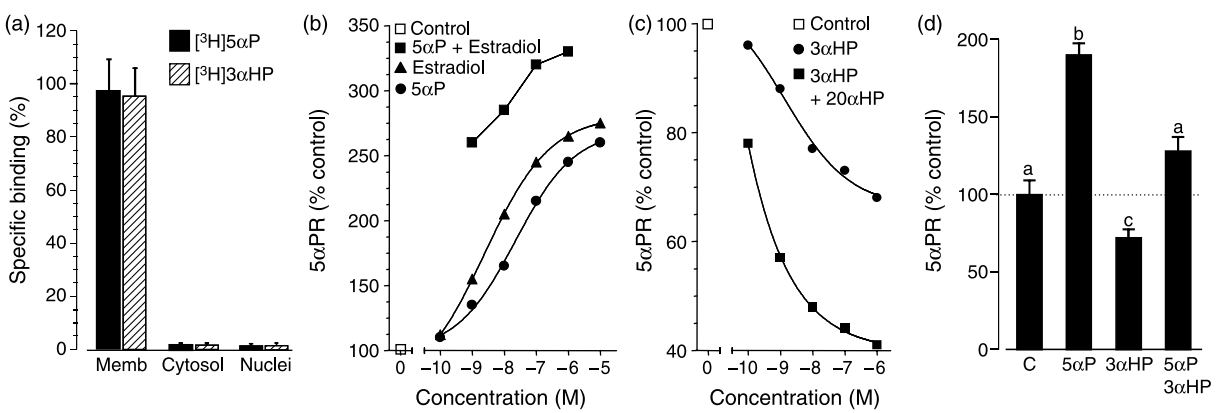

Figure 7 Binding sites (receptors) for progesterone metabolites in MCF-7 and MCF-10A human breast cell lines. Specific, saturable-binding sites (receptors) for 5αP and 3αHP are located only in the membrane fraction (a). 5αP receptors (5αPR) are significantly, dose-dependently and additively up-regulated by estradiol and 5αP (b) and down-regulated by 3αHP and 20αHP (c). The increases and decreases in 5αPR numbers due to 5αP and 3αHP respectively are abolished when cells are treated simultaneously with both hormones (d). (From Weiler & Wiebe, 2000 and Pawlak *et al.* 2005.)

alterations in 5αPR concentrations. Together, the receptor results suggest that the putative tumorigenic actions of 5αP may be significantly augmented by the estradiol-induced increases in 5αP binding and decreases in 3αHP binding.

Role of progesterone metabolites in regulating ER levels

Estradiol can influence mitogenicity of ER-positive mammary cells and therefore the regulation of ER levels may be important for the progression of estrogen-dependent mammary neoplasias. Estradiol and P are known to play a role in modulating ER concentrations (Shyamala *et al.* 2002). To determine if P metabolites affect ER levels, MCF-7 cells were exposed for 24 h to 5αP, 3αHP, 20αHP, and estradiol, or combinations of these steroids, and ER concentrations were determined in cytosolic and nuclear fractions by specific-binding of [^{3}H]estradiol (Pawlak & Wiebe 2005). Estradiol and 5αP resulted in significant dose-dependent increases, whereas 3αHP and 20αHP each resulted in dose-dependent decreases in total ER as well as inhibition of estradiol- or 5αP-induced ER levels. In combination, estradiol + 5αP or 3αHP + 20αHP resulted in additive increases or decreases respectively in ER numbers.

The results are the first to show that the pro- and anti-cancer P metabolites have also marked selective (up or down) regulatory effects on ER levels in ER-positive MCF-7 breast cancer cells. The suggested implications for breast cancer are that the stimulatory and inhibitory effects of 5αP and 3αHP respectively on cell replication and cell detachment might be significantly modified by exposure to estradiol, 4-pregnenes, and 5α-pregnanes and, in turn, that the P metabolites may significantly affect ER response in estrogen-targeted cells.

Effect of the progesterone metabolite, 5αP, on cell signaling pathways

The location of the receptors for 5αP and 3αHP on the cell membrane suggests involvement of nongenomic mechanisms of action via cell signaling pathways. Modes of action via plasma membrane-based binding sites and cell signaling pathways have been suggested for estradiol (Watson *et al.* 1999, Keshamouni *et al.* 2002, Purves-Tyson & Keast 2004, Simoncini *et al.* 2004), corticosteroids (Wehling 1997, Croxtall *et al.* 2000), 3αHP (Dhanvantari & Wiebe 1994, Beck *et al.* 1997, Wiebe 1997), and neurosteroids such as the P metabolite, 5α-pregnan-3α-ol-20-one (allopregnanolone) (Majewska *et al.* 1986). Signaling pathways that control cell proliferation and adhesion involve the mitogen-activated protein kinase (MAPK) pathway and, in turn, deregulation of this Ras-Raf-MEK-MAPK cascade plays a central role in human cancer (Chang & Karin 2001, Pearson *et al.* 2001, Santen *et al.* 2002). Studies on serum-starved MCF-7 cells showed that treatment with 5αP for as briefly as 5 min resulted in significant, dose-dependent increases in activated (phosphorylated) MAPK (Erk1/2) (Wiebe *et al.* 2005, Cialucu & J P Wiebe, unpublished results). Treatment with the MEK inhibitor, PD98059, resulted in significant suppression of the 5αP-induced MAPK activation. Similarly, in concomitant cell proliferation ([^{3}H]thymidine uptake) and detachment assays, 5αP resulted in significant increases in cell proliferation and detachment, whereas PD98059 significantly suppressed the 5αP-induced increases. The data suggest that the action of 5αP on breast cancer cells involves modulation of the MAPK signaling pathway. Whether other cell signaling pathways are involved or 5αP and 3αHP act via different pathways in promoting or inhibiting neoplasia in breast cells remain to be explored.

Implications of changes in progesterone 5α-reductase activity for androgen action in breast cancer

Although the majority of primary human breast cancers express androgen receptors, no direct association with any androgen and breast cancer growth and progression has been convincingly established (Bradlow & Sepkovic 2004). How might the up-regulation in 5α-reductase in neoplastic breast tissue influence androgen metabolism in the breast and in turn affect the role of transformed androgens in breast cancer?

Suzuki *et al.* (2001) have suggested that increased conversion of testosterone to DHT resulting from increased 5α-reductase activity should inhibit cancer cell proliferation in human breast carcinoma. However, studies with ZR-75-1 (Poulin *et al.* 1988, Birrell *et al.* 1995, Kandouz *et al.* 1999), T47-D (Birrell *et al.* 1995, Ortmann *et al.* 2002), MDA-MB-231 (Di Monaco *et al.* 1995, Ortmann *et al.* 2002), MFM-223 (Hackenberg *et al.* 1991), and CAMA-1 cells (Lapointe & Labrie 2001) and with DMBA-induced rat mammary tumors (Boccuzzi *et al.* 1995) have shown that both testosterone and DHT inhibit cell growth more or less to the same extent. This is in marked contrast to the actions of P metabolites, where the 5α-pregnanes stimulate and the 4-pregnenes inhibit cell proliferation. Also, 5α-reductase type 2 (SRD5A2), which catalyzes

reduction of testosterone to DHT in androgen-dependent tissues such as the prostate, is present in very low levels in breast tissue (Ji *et al.* 2004, Lewis *et al.* 2004) and human breast cancer cell lines (Wiebe & Lewis 2003). In breast tissue, 5α-reductase type 1 (SRD5A1) is predominant and it may be that P is a better substrate than testosterone for this isoenzyme. Overall, current evidence does not appear to support the notion that increased 5α-reductase activity/expression might significantly alter androgen influences on breast tumor growth.

Implications of progesterone-metabolizing enzymes for synthetic progestin-based contraceptives and hormone-replacement therapy drugs

The synthetic progestins used for contraception and hormone replacement therapy (HRT) do not behave like P in terms of their metabolism and probably not with respect to their actions at the level of the breast tissue microenvironment. As different formulations may exhibit marked differences in chemical structure, metabolism, and pharmacodynamic actions, it is not possible to generalize about them. The effects of the drugs at the level of the breast tissue will be governed by the molecular form and bioavailability, but unfortunately these are areas that remain unexplored. At the outset, the level of metabolism may vary greatly, depending on whether the route of administration is oral, transdermal, subcutaneous, or intravaginally (Fotherby 1996). When taken orally, many drugs are readily metabolized in the gastrointestinal tract and/or liver and the degree and site of metabolism varies substantially between different compounds. Some contraceptive and HRT drugs (for example, desogestrel, norgestimate, mestranol, norethisterone acetate, and ethinylestradiol-3-methyl ether) are in fact pro-drugs and are converted into their active metabolites when taken orally (Fotherby 1996, Henzl 2001). On the other hand, compounds like Nestorone must be administered parenterally due to their rapid hepatic metabolism and apparent inactivation (Sitruk-Ware 2004). The different formulations also exhibit great variation in level of binding to serum proteins (Kuhl 1996, Hammond *et al.* 2003), potential action via estrogen, androgen, P, and corticosteroid receptor binding and consequent androgenic, estrogenic, and progestational potency, and actions on enzymes (Kuhl 1996). To ascertain the possible role of the contraceptive and HRT drugs in breast cancer regulation via the P metabolites, it will be necessary to measure their levels and composition in the breast microenvironment to determine their effects on P metabolism in breast tissue and/or cell lines and to establish whether the P-metabolizing enzymes can further alter the drugs to pro- or anti-cancer moieties.

Summary, significance, and future prospects

Mammary gland cells show cyclicity and respond to steroid hormones. Normal breast tissue goes through cycles of imbalance between proliferation and apoptosis during menstrual periodicity, pregnancy, and lactation, but regularly corrects these temporary imbalances. In cancer, changes have occurred such that overall increases in cell numbers continue and result in the development of tumors. The normal changes are believed to be due to the changes in concentration of the ovarian hormones, estradiol and P. Since estrogens have been shown to increase proliferation in some cells, and because about one-third of breast cancer patients show some responses to anti-estrogen therapies, estrogens have been considered the primary hormonal cause of breast cancer. In time, however, estrogen-sensitive neoplasms become unresponsive and the patients experience relapse. Overall, this means the existence of an overwhelming majority of breast cancers for which the current estrogens based explanations and therapies are inadequate. Since P appears to be involved in the normal cyclical changes, it too has been implicated in breast neoplasia, but its role has been unclear and no specific categories of breast cancers have been shown to respond unambiguously to P or to anti-progestins. The end result is that for the majority of breast cancers, current estradiol/P-based explanations are inadequate and therapies ultimately ineffective. Moreover, estradiol and P do not provide hormone-based explanations for those breast tissues that do not become cancerous.

The P metabolites, produced within breast tissues, are put forward as potential candidates that could up- or down-regulate mitogenic and metastatic processes in various (perhaps all) mammary tissues, resulting in maintenance of normalcy or in progression to cancer. The suggestion is based on the following lines of evidence and summarized in Fig. 8: (1) Breast tissue, like many other tissues, has a number of enzymes capable of catalyzing the conversion of P to various metabolites, which can be grossly grouped into 5α-pregnanes and 4-pregnenes. (2) In breast tumor tissue and tumorigenic cell lines, 5α-reductase activity and mRNA expression are significantly higher, whereas 3α- and 20α-HSO activities and mRNA expression are significantly lower than in normal breast tissue and

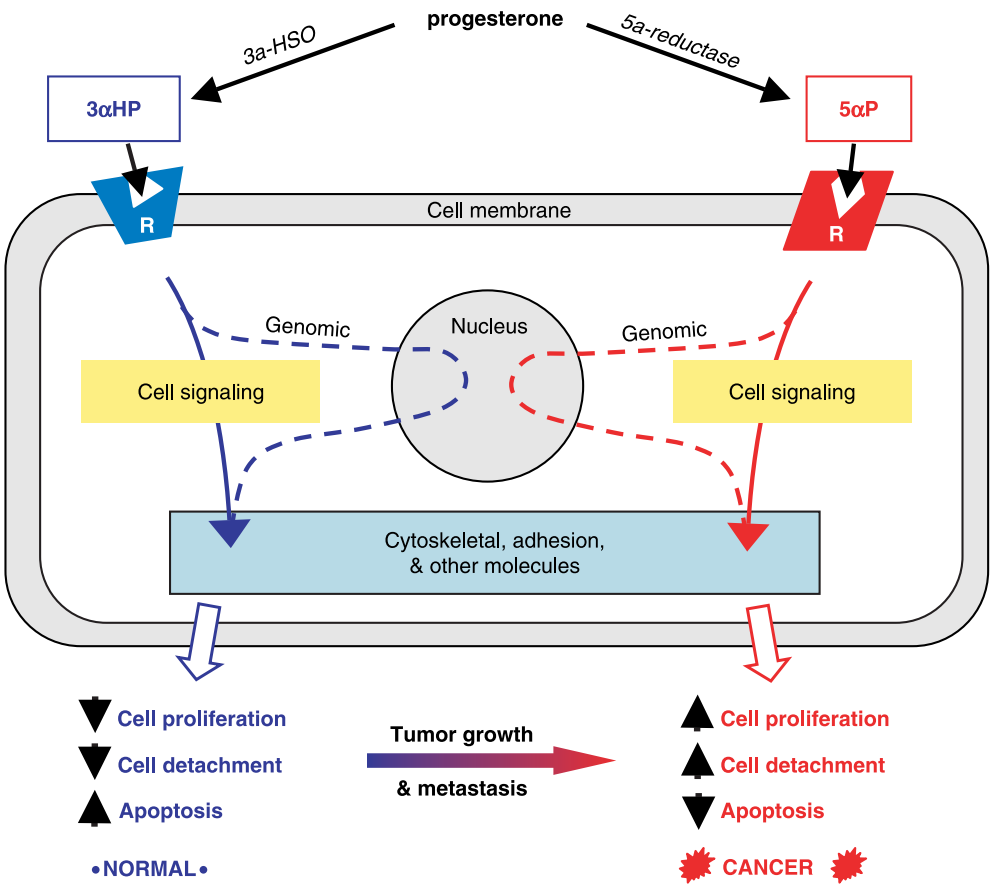

Figure 8 Summary of actions and action pathways of the progesterone metabolites 5αP and 3αHP in a stylized human breast cell and their proposed roles in maintaining normalcy or promoting cancer. Following binding to separate and specific receptors in the plasma membrane, 5αP and 3αHP act via cell signaling, and potentially indirectly via genomic, pathways to effect independent and opposite changes resulting in increases (↑) or decreases (↓) in cell proliferation, apoptosis and adhesion. Maintenance of normalcy depends on higher levels of 3αHP, and progression to neoplasia and metastasis are promoted by increased levels of 5αP.

nontumorigenic cells. (3) The result is that in para- and intra-cell environments of localized regions of the breast, the levels of 5α-pregnanes such as 5αP are increased, whereas those of the 4-pregnenes like 3αHP are decreased. (4) Studies using various breast cell lines have shown that 5αP and 3αHP have opposing actions in terms of cell proliferation and adhesion; 5αP stimulates cell proliferation (through increased mitosis and decreased apoptosis) and cell detachment, whereas 3αHP suppresses cell proliferation (through decreased mitosis and increased apoptosis) and detachment. (5) Separate mechanisms of action of 5αP and 3αHP are proposed, involving binding to separate, specific, and novel membrane receptors that are up- or down-regulated by estradiol and the P metabolites and that are linked to cell signaling pathways which transcribe different effects on cytoskeletal and adhesion molecules. (6) Based on the *in vitro* results, the paracrine/autocrine functions of 5αP are cancer-promoting and those of 3αHP are cancer-inhibiting. Changes from normal status to progression through increasing degrees of neoplasia are determined by changes in the relative concentrations of the pro- and anti-cancer hormones in the microenvironment. (7) As the P metabolites affect cell lines with various characteristic (ER/P-positive or -negative, tumorigenic or nontumorigenic, estrogen-sensitive or -insensitive), it is suggested that they may be general determinants of normalcy or cancer of the human mammary gland (Wiebe 2005). They may thus provide a new endocrine basis for the majority of human breast cancers that do not respond to ER-based therapy and also an alternate one for those that do.

The work on the potential role of P metabolites in promoting normalcy or cancer of the breast is in its infancy and a number of issues need to be addressed. First, all the observations summarized in this review about the effects/actions of the P metabolites were made *in vitro* on breast cell lines in culture.

To substantiate the hypothesis that the P metabolites play a role in mammary cancer, it is necessary to demonstrate their effects *in vivo*. Such experiments would test the P metabolites, 5αP and 3αHP, for their independent and combined effects on promotion or inhibition of growth of mammary tumors in mouse models resulting from human cell line inoculates and/or derived spontaneously or by chemical induction. Encouraging evidence that P metabolites can have similar effects *in vivo* and *in vitro* has come from a pilot experiment conducted by Drs R Schillaci and P Elizalde (NRC, Buenos Aires). They showed (personal communication) that C4HD murine cells inoculated into BALB/c mice developed into substantial palpable tumors if treated with 5αP (40 mg depot). Tumor growth rate was about the same (or slightly higher) with 5αP as with an equivalent dose of medroxyprogesterone acetate, a known tumor inducer in this model (Lanari *et al.* 1986). Secondly, in terms of potentially preventing, suppressing, or regressing breast tumors, more attention (both *in vivo* and *in vitro*) needs to be directed at the presumptive anti-cancer P metabolite, 3αHP, as well as 5α-reductase inhibitors. Thirdly, the structural characterization of the novel receptors located in the cell membranes would help in understanding the molecular mechanisms of action and in turn provide a basis in designing 5αPR binding antagonists and 3αHPR agonists. Fourthly, more information is needed on the mechanisms of action and the involved cell signaling pathways, particularly for 3αHP. Fifthly, with respect to their metastatic potential, effects of the P metabolites on angiogenesis and cell-to-cell as well as cell-to-matrix interaction molecules need to be explored. Sixthly, the identification of factors that alter expression of 5α-reductase and HSOs, resulting in changes in 5α-pregnane:4-pregnene ratios, may give insight into processes that initiate deregulation of P metabolite balance.

In addition to raising the status of the P metabolites from waste products to active hormones and to providing an alternative endocrine-based hypothesis for human breast cancer, the findings suggest new biomarkers, diagnostic tests, and therapeutic regimens that may be applicable to both estrogen-sensitive and -insensitive normal and cancerous human breast tissues (Wiebe *et al.* 2005). Biomarkers and diagnostic tests might be based on measurements of P metabolite concentrations in nipple aspirates, changes in 5α-reductase and HSO activities and gene expression, and/or 5αP receptor concentrations in biopsies. Therapeutic regimens might involve (a) actively decreasing 5αP and increasing 3αHP by blocking 5α-reductase and stimulating 3α-HSO activities and gene expression, (b) blocking the binding of 5αP to its receptor, and (c) down-regulating 5αP receptor and up-regulating 3αHP receptor levels.

In light of the findings regarding the P metabolites in relation to breast cancer, it appears pertinent to stress the importance of the intra- and para-cellular metabonomic microenvironments generated by the cells and potentially responsible either for maintaining normalcy or for transition/progression to neoplasia. It would seem propitious to consider therapies for breast cancer to be applied directly to the affected tissues via local depots or targeted infusions rather than the whole-body-every-tissue mode of current ingestion routes. Thus, it is hoped that the evidence presented in this review will stimulate further research into the potential roles of P metabolite hormones in breast cancer and generate new ideas for its control, regression, and prevention.

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