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Progestin Regulation of Cellular Proliferation *

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I. Introduction

THE control of cellular proliferation is known to involve a delicate balance between the effects of different regulatory molecules including hormones and growth factors which provide, in the immediate environment of the cell, signals to stimulate or inhibit cellular replication (1). The past decade has seen significant progress in the identification and characterization of novel regulatory molecules, generally referred to as growth factors, and definition of their molecular mechanisms of action (2, 3). With the recent emphasis on peptide growth factors it is perhaps less well appreciated that some of the largest physiological changes (both positive and negative) in rates of growth and cell proliferation are mediated by steroid hormones. Recent studies on breast cancer cells (4) and immature rat uterus (5, 6), illustrating estrogenic control of autocrine and paracrine growth factor production, support the view that some of these steroid-induced responses are intimately linked to growth factor-mediated pathways although debate continues as to whether the growth factor response is the cause, or the consequence, of the estrogenic effect on cellular proliferation (7, 8). Generally speaking, the molecular mechanisms by which steroid hormones control proliferation are not well defined, with the most developed knowledge being in the area of estrogen action where a number of recent reviews are available (4, 9–11).

The regulation of growth and development of most female sex organs involves a balance between the actions of the two major female sex steroid hormones, estradiol and progesterone. While estrogen, acting in concert with other hormones and growth factors, appears to be the major drive to the proliferation of these tissues, progesterone is concerned principally with two major functions in normal mammalian physiology. First, progesterone is involved in preparing the uterus for implantation of the fertilized ovum and making nutrients available for its subsequent development. Second, progesterone causes the glandular elements of the mammary gland to grow

and develop into secretory epithelium with the ultimate effect of acting in concert with other hormones, particularly PRL, to facilitate milk production. In the most simplistic terms, progesterone might be seen as the "differentiating" female sex steroid which inhibits the "proliferative" effects of estrogen and directs the tissue toward its normal differentiated function. Biological regulation, of course, is never so simple, and progesterone is known to have a number of other normal physiological functions including the regulation of ovulation at both neural and ovarian loci, and major behavioral effects including the control of sexual receptivity. Furthermore, progesterone is not always "antiproliferative" and in some tissues induces proliferative responses of its own. In the case of the induction of stromal proliferation in the uterus, this represents a corollary of its primary function in facilitating implantation; in the case of its stimulation of lobuloalveolar proliferation in the mammary gland, such an action is a requirement for the development of lactation.

This review focuses specifically on the effects of progesterone on cell proliferation, an area of biology that has not been well studied from a mechanistic viewpoint and which has not been the subject of any recent in-depth review, although there are a number of recent reviews on progesterone receptor (PR) and progestin action (12–14). Emphasis is placed on the two most studied progestin target tissues, the uterus and the mammary gland, and on the human given the widespread pharmacological use of synthetic progestins. Such uses include treatment of endometrial and breast cancer, as combination oral contraceptive agents, and as adjuncts to estrogen in hormonal replacement therapy of postmenopausal women. While the benefits of progestins as first or second line therapy in the treatment of breast and endometrial carcinoma have been widely acknowledged, the long-term use of progestins as contraceptives or for the prophylaxis of the menopause has come under closer scrutiny. Although it is clear that the majority of women suffer no increased risk of breast or endometrial carcinoma as a consequence of combined oral contraceptive use, there is emerging concern that specific subgroups may have an increased risk of developing breast cancer [(15), reviewed in Ref. 16]. Progestin supplementation of estrogens in postmenopausal hormone replacement regimens has been shown repeatedly to mimic the premenopausal cyclical exposure to these hormones, and progestin use is advocated in order to protect against the unopposed effects of estrogen (17) on the development of endometrial carcinoma (reviewed in Ref. 18). However, the observation that breast epithelial proliferation is maximal in the luteal phase of the menstrual cycle (reviewed in *Section II. B.3*) raises the possibility that progestins may cause inappropriate proliferation in the breast. It has recently been suggested that progestin supplementation of estrogen replacement therapy could lead to an increase in breast cancer risk (19), and preliminary data in support of this hypothesis are emerging (20). In the light of these data, the effects of progestins on cellular proliferation in the breast require critical reexamination with a view to determining the effects of long-term prophylactic progestin use on subsequent risk of breast cancer development.

As mentioned above, much of the action of progesterone involves modulating the growth-stimulatory effects of estrogens. This could be achieved in a number of ways including progesterone-mediated changes in estrogen secretion, via effects on the hypothalamo-pituitary axis or directly at the site of synthesis, changes in the level of target tissue sensitivity by progestin-induced increases in the activity of estrogen-metabolizing enzymes, or decreases in estrogen receptor (ER) concentrations, effects on estrogen-induced paracrine or autocrine growth factor secretion and action, and direct antagonism of estrogen action at the postreceptor level. In addition, progestins might be expected to exert effects on cellular proliferation of target tissues independent of estrogen action. In other words, progestins may have direct growth-inhibitory actions apart from their ability to inhibit estrogen-mediated cell proliferation, and evidence is accumulating for such effects. In this review the evidence for these potential mechanisms of progestin action is critically evaluated with an aim to developing rational hypotheses to explain current knowledge on the action of progestins on cellular proliferation.

II. Effects of Progestins on Cellular Proliferation *in Vivo*

The effects of progestins on cell proliferation *in vivo* have been investigated in a number of progestin target tissues, with the majority of studies having concentrated on the uterus and mammary gland. Two principal approaches have been followed, involving either 1) the administration of progestins to immature, ovariectomized, or intact animals and assessing effects on cellular proliferation and differentiation; or 2) correlative studies relating changes in cell proliferation to changes in serum progesterone levels. In addition, there is an extensive literature on the effects of progestins on the growth and development of hormone-responsive tumors of the mammary gland, but in general such studies have not addressed basic mechanisms through which progestins control cell proliferation.

A. Uterus

1. Mouse. The uterus of the ovariectomized mouse has been the most widely utilized experimental system for

studies on estrogen (reviewed in Ref. 21) and progesterone control of cellular replication *in vivo*. In this system estradiol alone causes a major mitogenic response in both luminal and glandular epithelial cells but not in the connective tissue stroma (22–24). Progesterone administration significantly alters the proliferative response to estradiol. Pretreatment with progesterone for 3 days completely inhibits epithelial cell proliferation while sensitizing the stromal cells to respond to estradiol with increased mitosis (22, 25–29). This progestin-induced switch in proliferation from epithelium to stroma is an essential prerequisite for implantation and decidualization in the mouse and rat (26). It is of major interest that in the mouse model, while progesterone inhibits estrogen-induced mitosis in the epithelium, it acts synergistically with estrogen to stimulate stromal proliferation. Progesterone alone is capable of inducing a small, but significant, number of mitoses in both the epithelium and stroma when administered alone (22, 25–29). The effects of progesterone, alone or in combination with estrogen, on cellular proliferation in the mouse uterus are summarized in Table 1.

These early studies also provided some insight into the cell kinetic basis of progestin antagonism of estrogen-induced epithelial cell proliferation by measuring the mitogenic response to estradiol when a single injection of progesterone is given at various times before or after the estrogen. While prior administration of progesterone (up to 10 h before estradiol) or simultaneous administration of the two hormones completely blocks the estrogenic response, no inhibition is seen when progesterone is given as little as 2.5 h after estradiol. Since estrogen induces synchronous progression of mouse uterine epithelial cells through the cell cycle it seems likely that progesterone inhibits mitosis by some action early in the G_1 phase and is without effect on progression through late G_1, S, and G_2 phases (26, 28, 29). This effect on cell cycle progression is accompanied by progesterone inhibition of estrogen-induced increases in acidic nuclear protein synthesis (30) but does not involve gross changes in protein synthesis, ribosomal RNA synthesis, polysome profiles, and accumulation of total protein and RNA (31) or changes in the nuclear or cytoplasmic binding of estradiol (32). Indeed, at early time points progesterone potentiates the effect of estradiol on macromolecular synthesis, indicating that progesterone inhibition of DNA synthesis is mediated independently of its regulatory effect on RNA and protein synthesis (31). The inhibition of epithelial proliferation is associated with morphological changes characteristic of epithelial differentiation (26).

TABLE 1. Cell proliferation in the mouse uterus

Cell type	Treatment					
	None	E	P	E > P	P > E	E + P
Ovariectomized[a]						
Epithelium						
Luminal	ND	++++	+	++++	$-^b$ $++^c$	++
Glandular	ND	++++	+	++++	$-^b$ $-^c$	++
Stroma	ND	−	+	ND	$+++^b$ $-^c$	+++
Neonatal[d]						
Epithelium	++++	++++	−	ND	ND	ND
Stroma	+	−	$+++^e$,$-^f$	ND	ND	ND

[a] Adapted with permission from Refs. 21–29.

[d] Adapted from Refs. 35 and 36.

E, estradiol; P, progesterone. (^{e}) 12–18 h after treatment, (^{f}) 24–48 h after treatment; E > P, estradiol followed by progesterone; P > E, progesterone followed by a single (^{b}) or multiple (^{c}) estradiol injections; E + P, combined estradiol and progesterone; ND, not done.

The subsequent effects of multiple administrations of estradiol on the progestin-pretreated uterus emphasize the differential cellular responses to these steroids within the one organ. Administration of estrogen to progesterone-pretreated animals results in little change in epithelial morphology and DNA synthesis after a single injection although protein synthesis increased to a greater extent than that observed after estrogen administration to progesterone-untreated animals. Interestingly, a second injection of estrogen 24 h later results in DNA synthesis and mitosis, and this occurs predominantly in the antimesometrial region of the luminal epithelium where implantation normally occurs. Thus, the antiproliferative and differentiation-inducing effects of progesterone can be reversed by estrogen in the luminal epithelium. This appears not to be the case in the glandular epithelium where, irrespective of the dose or the number of injections of estrogen, mitoses are rarely observed in progesterone-treated mice. In the progesterone-treated stroma, a single injection of estradiol results in synchronous entry of 30–40% of stromal cells into S phase while a second injection of estradiol produces no further effect. It thus appeared that progesterone stimulates resting stromal cells to enter the cell cycle where estrogen accelerates their passage through a single round of replication by shortening the G_1 phase. This single round of replication is thought to be a prerequisite for the differentiation of stromal cells into decidual cells and their withdrawal from the cell cycle (27). If true, the latter effect could explain the lack of mitogenic response to subsequent administration of estradiol. These results illustrate the complexity of progestin action *in vivo* with differential effects on different cell types within the same organ. The complexity of the uterine response to progestins is further complicated by the fact that the connective tissue stroma is a mixture of cell types including fibroblasts, blood vessels, macrophages, and lymphocytes, all of which may have the capacity to respond directly or indirectly to progestins.

These seminal observations of Martin and colleagues in the mouse uterus identified the critical issues that needed to be considered in attempting to define the molecular basis of progestin effects on cell proliferation. In particular, the complexity of the response was illustrated by observations on the cell type specificity of progestin responsiveness and the dependence of the proliferative response, not only upon the relative concentrations of estradiol and progesterone, but also upon the temporal relationship between their administration. In addition, documentation of the inhibitory effects of progestins on epithelial cell proliferation provided experimental evidence to support the pharmacological use of progestins in the treatment of endometrial carcinoma. Perhaps more importantly, from a mechanistic viewpoint, the data on cell cycle kinetics illustrate the inhibition of cell cycle progression in early G_1 phase in epithelial cells (28, 29) and activation of resting or G_0 stromal cells (27), and identified the fundamental paradox of progestin control of replication, *i.e.* these compounds appear to have both stimulatory and inhibitory effects on target cell proliferation. Thus, any unifying model of progestin action must accommodate this apparent paradox, a goal that has yet to be attained.

Although the uterus of the ovariectomized mouse provides a well defined and attractive model with which to study the effects of progestins on cell proliferation, there have been few recent studies employing this experimental system. Cheng *et al.* (31) utilized the model to differentiate the effect of estrogens and progesterone on the accumulation of ribosomal RNA and protein, from those on DNA synthesis. The data showed, in contrast to some theories on cell cycle progression, that these parameters were regulated separately in mouse uterine epithelium, and led to the suggestion that progesterone inhibition of epithelial cell proliferation was mediated either by inhibiting the synthesis of messenger RNA (mRNA) for a protein intimately involved in the inititation of DNA synthesis, or by interfering with the interaction of such a protein with chromatin. A previously identified 32 kilodalton acidic nuclear protein (30), with properties similar to the proliferation-associated protein cyclin (33), was cited as a potential regulator of DNA synthesis in this model (31). A more recent publication has demonstrated that the antiprogestin/antiglucocorticoid, RU 486, completely inhibits the progestin-induced switch of estrogen-mediated cell proliferation from the epithelium to the stroma while at the same time inhibiting progesterone-induced differentiation of the epithelium (34). Such data provide strong evidence that the effects of progestins on cell proliferation in the adult ovariectomized mouse uterus are PR mediated.

In contrast to the situation in the adult ovariectomized mouse, the rate of luminal epithelial cell proliferation in the uterus of the neonatal mouse is high and appears to be estrogen-independent in that it is not reduced in the ovariectomized-adrenalectomized animal (35). These properties render the neonatal mouse uterus a convenient model for studying the effects of progestins on cell proliferation in an apparently estrogen-free environment. Such an approach has been adopted by Bigsby and Cunha (36) who studied the effects of progesterone and glucocorticoids in neonatal mouse uterus both *in situ* and when grafted under the kidney capsule of ovariectomized adult mice. Rates of cell proliferation, assessed by [^{3}H] thymidine labeling and mitotic indices, after progesterone treatment were significantly reduced in uterine epithelium, unaffected in vaginal epithelium, and stimulated in the stroma. The latter effect was transient with the labeling index in the stroma returning to control levels at 24 h. Interestingly, the dose dependence of the inhibitory and stimulatory effects on the epithelium and stroma were markedly different as was the steroid specificity of the response. One hundred fold higher doses of progesterone were needed to inhibit epithelial proliferation than were required for stimulation of stromal proliferation. Since the former effect could be reproduced with dexamethasone and to a lesser extent with cortisol, it appears that inhibition of epithelial cell proliferation in the neonatal mouse was mediated via the glucocorticoid receptor (GR) while progesterone stimulation of stromal proliferation was via PR. Progesterone antagonism of luminal epithelial proliferation was also apparent when neonatal uteri were grafted into adult ovariectomized animals (36). The same authors confirmed potent progesterone antagonism of estrogen-independent growth in an ovariectomized rat model when epithelial cell proliferation was stimulated by intraluminal administration of cholera toxin (37). Both these sets of data provide strong evidence for progestin effects on proliferation that are independent of progestin effects on estrogen-mediated proliferation.

2. Rabbit. Although studies on the effects of progesterone on uterine cell proliferation are most detailed in the mouse model, there are published studies using several other species. It is beyond the scope of this review to summarize studies that document the effects of progestins on DNA synthesis in the whole organ; rather, discussion will be confined to data on the cell proliferation kinetics of individual cell types within the uterus. In this regard the rat uterus appears to respond in a similar manner to the mouse (38–41); in marked contrast to these species, however, progesterone stimulates endometrial proliferation in the rabbit, illustrating significant species differences in the proliferative response to progestins. The rabbit is unusual in that it is in almost constant estrus, and does not ovulate unless mating takes

place. It is likely, therefore, that progesterone secretion from the ovary consequent to mating and ovulation plays an important role in the proliferation of the uterus in preparation for implantation.

In intact rabbits, progesterone stimulates the labeling index of both luminal and glandular epithelium; the latter is also stimulated by estradiol, but to a lesser extent. Simultaneous administration of both estradiol and progesterone abolishes the proliferative response (Table 2). Foci of active epithelial cell proliferation are always found in the luminal epithelium after progesterone treatment and are postulated to be related to gland formation (42). In a subsequent publication (43) the same authors demonstrated that rabbit uterine epithelium consists of quiescent and cycling cells with differential proliferative responses to estrogen and progesterone. The progesterone-induced increase in proliferation in both luminal and glandular epithelium was attributed to shortening of the cell cycle, although an effect on the recruitment of quiescent cells into the cell cycle is not ruled out. In ovariectomized rabbits estrogen alone causes slight epithelial proliferation with no effect on the stroma and myometrium. Progesterone alone has no effect on the epithelium unless the animals are primed with estrogen. Under this treatment regimen, *i.e.* estrogen priming followed by progesterone administration, maximal DNA synthesis and mitosis occur in the epithelium with smaller responses in the stroma and circular muscle (44). The effect was not maintained since after a third daily injection of progesterone, cell proliferation declined in accordance with the low level of cell division in mid to late pregnancy in the rabbit.

The proliferative responses to estrogen and progesterone in the rabbit uterus need to be considered in the light of the differential distribution of ER and PR in this tissue. The mean intensity of ER immunostaining is highest in the luminal epithelium, which paradoxically failed to proliferate in response to estrogen, and then in decreasing order: glandular epithelium > myometrium > stroma (45). Furthermore, PR concentrations are low or barely detectable in luminal and glandular epithelium (45, 46), where the greatest proliferative effect of progesterone is noted. The highest PR levels are found in myometrium and stroma, and the possibility that progesterone is acting on these cells to induce the synthesis of a paracrine factor responsible for epithelial cell proliferation cannot be excluded.

TABLE 2. Cell proliferation in the rabbit uterus

Cell type	Treatment		
	E	P	E + P
Intact[a]			
Epithelium			
Luminal	–	++	–
Glandular	++	++++	–
Ovariectomized[b]			
Epithelium	++	–	++++[c]
Myometrium	–	ND	+
Stroma	–	ND	+

Abbreviations are as for Table 1.

[a] Adapted with permission from Ref. 42.

[b] Adapted with permission from Ref. 44.

[c] Declined after third daily progesterone injection.

3. Monkey. The necessity for cyclical renewal of the endometrium sets the primates apart from the species discussed above. If fertilization does not occur within a menstrual cycle, the endometrium regresses and is eliminated. However, progenitor cells remain and regenerate the endometrium in preparation for the subsequent cycle, in a process that begins while the serum concentration of progesterone is still elevated. The process of endometrial renewal in the monkey uterus has been delineated in seminal studies from Padykula and co-workers and has been recently reviewed (47). The Rhesus endometrium can be divided laterally into 4 zones that reflect its histological and functional compartmentalization. Zones I and II contain the luminal and upper glandular epithelium and surrounding stroma, and together make up the zona functionalis of the endometrium, which is shed during menstruation. Zones III and IV comprise the germinal zona basalis, which gives rise to the zona functionalis in the subsequent cycle.

Intravascular injection of [^{3}H]thymidine to Rhesus monkeys in midcycle, followed 1 hr later by removal of the endometrium by hysterotomy, was used to determine the epithelial labeling indices within each of the zones (47, 48). During the estrogen surge, epithelial labeling indices are higher overall in the functionalis than in the basalis, and furthermore within zones I, II, and III the magnitude of the response remains the same throughout the estrogen surge (Table 3). The fall in serum estrogen levels and the concomitant rise in progesterone levels after midcycle result in inhibition of epithelial mitoses in zones I-III. In marked contrast to the proliferative activity in zones I-III, the cells of the deep zona basalis (zone IV) show little labeling during the estrogen surge, but the number of dividing cells increases steadily as the serum concentration of progesterone rises. This distinct pattern of proliferative activity in response to progesterone suggests that this agent may be responsible for the production of progenitor cells leading to zona functionalis regeneration in the subsequent cycle.

4. Human. The cyclical histological changes in the endometrium during the human menstrual cycle are well documented and have been correlated with changes in the circulating concentrations of estrogen and progesterone. Cyclical changes in proliferation were first described

TABLE 3. Cell proliferation in the primate uterus

Cell type	Treatment		
	E	E > P day 19–22	E > P day 22–28
Human[a]			
Epithelium			
Functionalis	++++	–	–
Stroma			
Functionalis	+++	–	+++
Monkey[b]			
Epithelium			
Functionalis	++++	+	ND
Basalis IV	+	+++	ND

[a] Adapted with permission from Refs. 49–54. E, Estrogen exposure (proliferative phase of the menstrual cycle or estrogenized postmenopausal endometrium). E > P, Secretory phase (menstrual cycle days) or progestin-treated estrogenized postmenopausal endometrium.

[b] Adapted with permission from Refs. 47 and 48. E and E > P describe artificial menstrual cycles induced in ovariectomized animals.

by Nordqvist (49), who showed in whole endometrium that DNA synthesis was maximal around the time of ovulation, then decreased to a low level until the end of the cycle when a second wave of proliferation was noted. These changes were described in more detail by Ferenczy and co-workers, who examined [^{3}H]thymidine labeling of the endometrium at six time points (three proliferative, three secretory) during the menstrual cycle (50). DNA synthesis in both the glandular epithelium and stromal elements increased as the serum estrogen concentration increased, to reach a peak around the time of ovulation (Table 3). The rise in serum progesterone in the postovulatory phase resulted in the disappearance of mitoses in both epithelial and stromal cells, and this suggested that progesterone was able to inhibit estrogen-induced cell proliferation in these cells. The observation that DNA synthesis is high in the proliferative phase and low or absent after ovulation was confirmed in later studies (51–54). The hypothesis that progesterone inhibited estrogen-mediated DNA synthesis was tested directly by progestin treatment *in vivo* of estrogenized postmenopausal endometrium. This resulted in the rapid inhibition of epithelial [^{3}H]thymidine incorporation, to levels seen in the secretory phase of the premenopausal cycle (51, 52). The inhibition of mitosis was accompanied by induction of glandular secretory activity and demonstrated the ability of progestins to promote differentiated function in postmenopausal women on estrogen therapy.

The effect of progestins on stromal cells is complex. Although the significant stromal proliferation observed during the proliferative phase is decreased soon after ovulation, the stroma shows a second wave of proliferation, presumably in response to progesterone: stromal mitoses reappear in the last few days of the cycle in concert with the predecidual reaction observed in these cells. This was clearly demonstrated in hysterectomy specimens by Ferenczy and co-workers (50), who showed that stromal DNA synthesis was low from days 19–22, but increased markedly from days 23–28 of the cycle in the zona functionalis but not the zona basalis of the endometrium. This suggested that the second wave of endometrial DNA synthesis originally observed by Nordqvist (49) was in fact due to stromal proliferation. It is noteworthy also that mitotic activity is higher in the secretory, progesterone-dominated, phase of the cycle in uterine leiomyomas, which are thought to be derived from the same progenitor cells as the stroma (55). Interestingly, support for the onset of progestin action in the stroma late in the cycle is provided by the presence of PR at that time (56), presumably allowing continued progestin responsiveness and resulting in stromal proliferation and decidual transformation.

A number of other studies have also examined the effect of progestins on [^{3}H]thymidine incorporation in stromal cells. These demonstrated that progestins appeared either to increase or decrease stromal labeling indices; neither change reached statistical significance, possibly reflecting the paucity of specimens analyzed (54, 57). It is also probable that if the stimulatory effects of progestins on stromal cells are observed only late in the cycle, pooling of results from endometrial specimens obtained both early and late in the secretory phase, during which opposing effects of progestins on stromal proliferation occur, would lead to an inability to distinguish between the inhibitory and stimulatory effects of progestins.

The effects of progestins on stromal proliferation need to be reexamined, however, in the light of recent evidence that the wave of proliferation noted in the secretory phase of the cycle is confined to the lymphoid population of the stroma, with little or no proliferation being observed in cells not expressing lymphoid markers (58).

Progestins are also known to inhibit the growth of endometrial carcinoma tissue (see Refs. 59 and 60 and references therein), which provides the basis for their widespread use as therapeutic agents. The labeling index in endometrial carcinoma tissue was shown to be high and comparable to that of the normal endometrium in the proliferative phase of the cycle (54, 61). DNA synthesis in endometrial carcinoma tissue is lowered in the presence of progestins, and in this regard this tissue resembles the epithelial component of the endometrium from which it arises (62). These similarities in proliferative response between well-differentiated endometrial carcinoma in particular and normal endometrial epithelium extend to the likely mechanisms by which progestins inhibit endometrial carcinoma proliferation, and these are discussed in *Section V.C.*

B. Mammary gland

The hormonal control of mammary gland development and function has been the subject of extensive investigation over several decades. This has resulted in a voluminous literature covering a wide spectrum of biomedical disciplines including biochemistry, cell biology, endocrinology, pathology, and physiology. While the current review will be confined almost entirely to recent literature on the role of progesterone and its interaction with estrogen, in the control of cell proliferation within the mammary gland of selected species, the reader is referred to several detailed reviews on other aspects of the topic, particularly as they relate to the early literature (63–67). Although mammary epithelial development involves complex interactions between a number of different hormones, which may vary between species and at different stages of development, the principal role of progesterone appears to be in promoting lobuloalveolar development in the adult gland (66, 67). Progesterone does not appear to be necessary for ductal development, which occurs at adolescence mainly under the influence of estrogen in combination with pituitary factors, probably PRL and GH.

1. Mouse. As was previously noted with studies on the hormonal control of uterine proliferation and differentiation, the immature mouse and the ovariectomized adult mouse have provided the most extensively utilized experimental models for studies on the hormonal control of mammary gland development.

The mammary gland undergoes a series of well-coordinated phases of development which are known to have differing hormonal requirements. At birth the mammary epithelium of the female mouse consists of a cord of cells connected to the nipple at one end and terminating in a branched structure at the other. Development to this stage is thought to be determined predominantly by mesenchymal influences and may be independent of hormones. These immature ductal cells have, however, differentiated to the extent that they can synthesize casein under appropriate hormonal stimulation *in vitro* (66).

The next phase of mammary development, which occurs at sexual maturation (4–7 weeks of age in the mouse), involves the rapid growth of ducts to yield a characteristic tree-like morphology as a consequence of intense mitotic activity in the end buds of these ducts (68–70). The cap cells of the end bud are considered to be stem cells giving rise to myoepithelial and perhaps luminal epithelial cells (70). A minimal hormonal requirement for ductal development in most species appears to be estrogen and either PRL or GH (66). Little alveolar development occurs at this time.

The last major phase in mammary gland development in the mouse occurs at pregnancy with the development of lobuloalveolar structures which fill the interductal spaces. Lobuloalveolar growth in the ovariectomized, hypophysectomized, adrenalectomized adult mouse requires estrogen, either GH or PRL, and progesterone (66). Thus the major difference between stimulation of ductal and lobuloalveolar cell proliferation is the additional requirement for progesterone in the latter cell type.

Significant new information on the role of estrogens and progesterone in ductal and alveolar cell proliferation has been forthcoming from recent studies in mice (71–73). In a study designed to test whether the known effects of estrogen on ductal development (74) were mediated by a direct action on the mammary gland, young ovariectomized mice were treated with implants of estradiol directly into the gland (71). This resulted in end bud development in the vicinity of the implant but not elsewhere, indicating that the effect was mediated locally. Furthermore, the effect was blocked by the coimplantation of an antiestrogen, demonstrating that the effect was probably ER-mediated. Analysis of ER distribution using steroid autoradiography revealed ER in the luminal cells of the end bud, in ductal epithelium, and in stroma adjacent to the ducts but not in the rapidly proliferating cap cells. It was concluded that estrogen acted in conjunction with nonepithelial target cells to stimulate DNA synthesis in the end buds (71). The conclusion that estrogen may act via stromal intermediaries is compatible with *in vitro* studies showing that estrogen was mitogenic to cultured mammary epithelium only when the latter was cocultured with fibroblasts (75, 76).

Similar studies by Haslam (72), in which estradiol pellets were implanted sc (systemic) or within the mammary gland (local), demonstrated differential responses in epithelial DNA synthesis between immature and mature mice. The data indicated that estrogen acted locally to increase end bud growth in the immature gland, in agreement with the previous study (71), and systemically in the mature gland where the effect was to stimulate ductal side-branching. One potential explanation for these differences is that the epithelium in the immature mammary gland end bud is developmentally unique and may exhibit hormonal responses not shared by the adult gland. Furthermore, the systemic effect in the mature gland may be due to estrogen effects on PRL secretion and action, since it has long been known that estrogen is ineffective in restoring mammary growth in the ovariectomized-hypophysectomized animal (77), while PRL alone or in synergy with progesterone stimulates mammary epithelial cell growth *in vitro* (78).

Having established differential regulation of cell proliferation in the epithelium of immature and adult mouse mammary gland, Haslam (73) studied progesterone effects on mammary epithelial DNA synthesis under the

same experimental conditions. In the immature animal, progesterone alone had no effect on end bud epithelium, and estradiol alone resulted in a 3-fold increase in labeling index, a value that was slightly reduced in animals receiving both hormones. DNA synthesis in the ductal epithelium was affected differently; both estradiol and progesterone alone stimulated proliferation, but with different time courses, and at some times progesterone appeared to markedly inhibit the estrogen-induced response. In the ductal epithelium of the mature gland, progesterone and estradiol alone both stimulated proliferation, though progesterone was considerably more effective. Administration of the two steroids together resulted in a marked synergistic, although transient, effect in the mature gland, which was attributed in part to the ability of estrogen to increase PR and progestin responsiveness. These studies were interpreted as suggesting that in the mature mouse progesterone, rather than estrogen, has a major role in promoting DNA synthesis and epithelial cell proliferation (73). The effects of progesterone on cell proliferation of the mouse mammary gland are summarized in Table 4.

2. Experimental mammary tumors. Hormone-dependent mammary tumors can be induced in rodents by chemical carcinogens, radiation, and alterations in the endocrine environment induced by pregnancy, estrogen administration, and pituitary isografts (reviewed in Refs. 79 and 80). The growth of such tumors is regulated by a number of hormones, of which estrogen and PRL are considered to be the most important. The relative contributions of these two hormones may vary with the model; of the two most widely studied models in rats, dimethylbenzanthracene (DMBA)-induced tumors are predominantly PRL dependent (81) while those induced by nitrosomethylurea (NMU) are thought to be more estrogen dependent (82).

The role of progestins in the control of growth of rodent mammary tumors has been addressed, but there

TABLE 4. Cell proliferation in the mouse mammary gland

Cell type	Treatment								
	E			P			E + P		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Immature[a]									
Epithelium									
end bud	+++	+	–	–	–	–	++	–	–
ductal	+++	+++	+++	–	+++	–	+	++	–
Mature[a]									
Epithelium	–	+	–	+++	+	+	+++	+++ +++ +++	–

Abbreviations are defined in Table 1.

[a] Adapted with permission from Ref. 73.

are few data on the actual mechanisms by which progesterone controls tumor cell replication. Interestingly, progesterone has been reported to both stimulate and inhibit the growth of experimental tumors depending upon the dose and the experimental preparation. Progesterone stimulation of growth has been demonstrated in carcinogen-induced and transplantable rat tumors (83–89), spontaneous mouse mammary tumors of pregnancy (90, 91), and in murine tumors induced by pituitary isografts (92). Furthermore, in a number of mouse mammary tumors, including GR/A and TPDMT-4, a combination of estrone and progesterone either induces tumors or enhances growth while each agent is ineffective alone (80, 93). In the well studied DMBA model estrogen alone or in combination with progesterone sustained growth in ovariectomized and adrenalectomized rats while administration of progesterone alone did not (94). More recent data demonstrate that progesterone can partially reverse the effects of the antiestrogen, tamoxifen, on inhibition of tumor development and growth in the DMBA model (89).

In contrast, progesterone has also been reported to inhibit the development of carcinogen-induced mammary tumors. This effect was first described by Huggins *et al.* (85) but was complicated by the simultaneous administration of a pharmacological dose of estrogen. Further experimentation revealed that the effect of progesterone was dependent upon the time of administration relative to the carcinogen [(87, 95), reviewed in Ref. 96] and that the effect may be related to effects of progesterone on mammary gland differentiation. This conclusion has recently been elegantly demonstrated in studies in which pretreatment of rats with a combination of estradiol and progesterone, sufficient to induce a highly differentiated state in the mammary gland, markedly reduced the ability of NMU to induce tumors (97). Thus, the protective effect of estradiol and progesterone would appear to result from the reduction in the number of end buds, *i.e.* the sites of rapid cell division and the point of action of the carcinogenic insult.

A similar protective effect of progesterone was observed with estrogen-induced mammary tumors in ovariectomized W/Fu rats (98). Interestingly, estrogen-induced pituitary tumorigenesis as well as mammary tumorigenesis was inhibited by tamoxifen, but the former was not inhibited by progesterone, indicating that the protective effect of the progestin was tissue specific and unlikely to be due to inhibition of estrogen action at the receptor level. Again, the synergistic effect of estrogen and progesterone on mammary gland differentiation is a likely explanation for this result (98).

There is little information on the effects of progesterone alone on DNA synthesis in mammary carcinoma. A recent study (99) assessed the effect of progesterone on

the thymidine labeling index in the MXT transplantable murine tumor and demonstrated a mitogenic effect similar to that observed after estrogen treatment. This effect, however, was transient and had returned to control levels after 48 h of progesterone treatment. This may have been due to the rapid clearance of the progesterone or to the ability of the steroid to stimulate only a single round of replication. A further study from the same group evaluated the effect of estradiol and progesterone *in vitro* on the thymidine labeling index in a small series of canine mammary tumors of mixed histological type. Estradiol was stimulatory in 90–100% of cases while progesterone induced a significant increase in thymidine labeling in only 10–30% of tumors. Response was not correlated with ER and PR status (100). More detailed studies are urgently needed to ascertain the true effects of progesterone alone and in combination with other hormones, particularly estrogen and PRL, on cell proliferation in experimental mammary tumors.

3. Human breast. The human breast is known to respond to the fluctuations in serum hormone levels during the menstrual cycle with cyclical changes in breast volume and cellular morphology. Contributing to these effects are cyclical changes in the mitotic activity of the epithelium, which have been measured either by direct counting of mitoses or by [^{3}H]thymidine incorporation. DNA synthesis measured by the latter technique in two groups of patients was high in the early follicular phase; it then decreased in the late follicular phase only to markedly increase again in the latter part of the secretory phase, reaching a maximum in the last 6–8 days of the cycle (101–103). In the breast epithelium, the highest proliferative activity was noted in the intralobular terminal ducts (104). Studies in which mitoses were evaluated histologically showed that both mitosis and cell loss through apoptosis varied in a cyclical manner during the cycle, with mitoses clearly being maximal on days 23–26 (53, 105, 106), although there was variation in the ability to detect mitoses in the early follicular phase.

Increases in DNA synthesis in the secretory phase have been consistently observed in all studies, but the evidence that there is increased DNA synthesis in the follicular phase has been conflicting.

Several explanations have been advanced for this variability including: differences in the sensitivities of the methods used to measure mitosis, technical limitations of the *in vitro* [^{3}H]thymidine incorporation method, limitations in the accurate dating of menstrual cycle day, and differences in the breast tissue adjacent to the normal sections selected for study. The first two issues are inherently problematical and will not be discussed further. However, difficulties in accurately dating breast samples with respect to stage of the menstrual cycle are generally acknowledged, due to individual fluctuations in cycle length and lack of well-defined histological markers analogous to those used for dating the endometrium. Furthermore, most of the studies reported to date have used normal breast tissue obtained from women undergoing biopsies, mammoplasties, or mastectomies for a number of indications, and the normal tissue examined was therefore potentially adjacent to abnormal regions, which may have influenced proliferative behavior in the remainder of the breast including the normal regions studied. These issues were addressed in one study, which used autopsy material without clinical or histological evidence of breast abnormality, and dated the breast samples by examination of endometrial samples from the same subject (53). This study clearly showed that epithelial mitoses were present between days 22–28 of the cycle and were undetectable at any other time (Fig. 1).

A recent study has confirmed that women with natural cycles, irrespective of parity, all show maximal epithelial proliferation in the late secretory phase of the cycle (107). Parous women using oral contraceptives had a blunted proliferation profile in comparison, with no marked "late secretory" peak. However, nulliparous women on oral contraceptives had persistently greater basal proliferative activity than women with natural cycles and displayed a dramatic increase in proliferation in the equivalent of the late secretory phase of the artificial cycle (107). The estrogen concentration in the oral contraceptive formulation was found to be important, whereas the progestin concentration was not, and the mean thymidine labeling index increased progressively with increasing estrogen content of the formulation in these nulliparous women. The authors suggested that the primary function of estrogen may be to increase the PR concen-

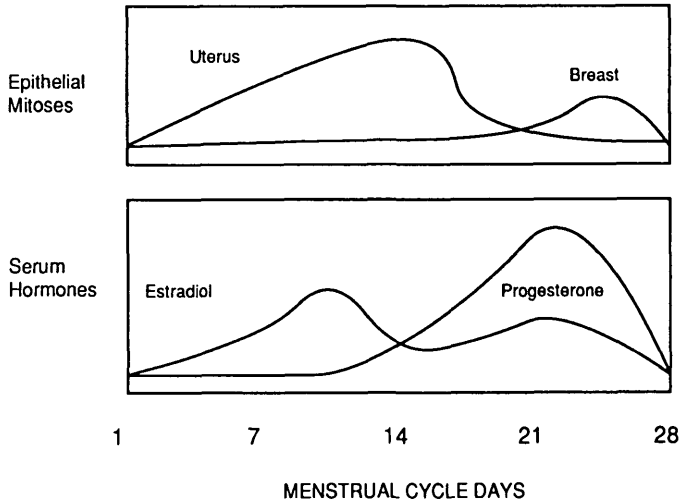

FIG. 1. Changes in uterine and breast epithelial mitoses and serum gonadal steroid hormones during the human menstrual cycle [Adapted with permission from T. A. Longacre and S. A. Barlow: *Am J Surg Pathol* 10:382–393, 1986 (53).]

tration in a dose-dependent manner, assuming that the lowest administered progestin dose had a maximal effect, in order to explain the lack of a dose-related progestin effect. This intriguing suggestion awaits further analysis. Therefore, the stimulus for this wave of epithelial DNA synthesis in the late secretory phase of the cycle is presently unknown. The effect coincides with, or immediately follows, a rise in the serum concentrations of both estrogen and progesterone, from which the inference can be drawn that these hormones, alone or in combination, directly or indirectly, may be responsible.

The potential role of estrogen and/or progesterone in mediating breast epithelial DNA synthesis has yet to be investigated directly in humans, but the issue has been examined using *in vitro* techniques such as organ culture, transplantation of normal human breast into nude mice, and primary cell culture (108–110). The consensus to emerge from these *in vitro* studies is that estrogen is capable of stimulating breast epithelial growth. Progesterone stimulated either good (108), modest (110, 111), or no growth (109) or was growth inhibitory (112). Where tested, progestins either were (112) or were not (110, 111), able to inhibit the estrogen-mediated effect. However, several factors, such as the cellular heterogeneity of the breast epithelium and the presence or absence of ER and/or PR in cells which demonstrate DNA synthesis, have to be addressed before these *in vitro* studies can be extrapolated to the human breast *in vivo,* and before any general conclusions can be drawn concerning the likely mitotic stimulus in the secretory phase of the cycle. The breast epithelium is composed of ductal, lobular, and alveolar elements, encased by myoepithelial cells, and it is likely that these cellular elements respond differently to growth stimuli, particularly in the various phases of the cycle. For instance, myoepithelial cells seldom divide, if at all, once mature (113) and, in addition, the exact cellular composition and therefore the likely responsiveness of breast explants after *in vitro* culture, or growth in nude mice, has not been described. It is furthermore unclear which, if any, of the breast epithelial elements contain receptors for estrogen or progesterone, and whether these receptors are maintained after transplantation or culture, although there is recent evidence that PR is present in nonmalignant epithelial cells adjacent to PR-positive carcinoma (114). Finally, it has yet to be demonstrated that steroid hormones act directly on the human breast epithelium, rather than through a paracrine mechanism involving the stromal elements.

4. Human mammary carcinoma. The synthetic progestins, megestrol acetate (MA) and medroxyprogesterone acetate (MPA), are effective agents in the treatment of metastatic breast cancer. Response rates similar to those observed with the antiestrogen tamoxifen (see Refs. 115–118 and references therein), and similarly predicted by the presence of ER and PR, are observed. Clinical data relating objective responses after progestin treatment to steroid hormone receptor status reveal that, in both ER+ and ER− tumors, the additional presence of PR assures a significantly greater response to therapy (117, 118). Despite the widespread clinical use of progestins and in contrast to their well-defined antiproliferative effects on normal and neoplastic endometrium, relatively little information is currently available concerning the molecular mechanisms of action of progestins as antitumor agents in breast cancer. Both direct effects on tumor cells (reviewed in *Section III*) and indirect effects on the hypothalamo-pituitary-adrenal axis (*Section V.C*) have been implicated.

While the relationship between responsiveness and PR status might be interpreted as providing support for a direct inhibitory effect of progestins on tumor cell growth *in vivo,* other potential mechanisms need to be considered. Synthetic progestins, while having high affinity for the PR, have also been shown to compete for binding to the GR and androgen receptor. In one clinical study in which responses to MA treatment were correlated with different steroid hormone receptors in breast cancer patients, responsiveness was significantly associated with high levels of androgen receptor, was weakly related to GR, and unrelated to ER and PR (119). Furthermore, 7 of the 17 responders lacked detectable levels of PR in their tumors suggesting that PR may not always be implicated in the antitumor effects of MA. Interpretation of these data is complicated by the fact that the levels of different steroid hormone receptors among human breast cancer biopsies and cell lines appear to be correlated (119–121), and this may be due to the coordinated control of steroid hormone receptor gene expression (121). Under such circumstances it is difficult to place a mechanistic interpretation on relationships between receptor levels and hormone responsiveness.

There have been few studies addressing the effects of progestins on cell proliferation in human breast tumors (122, 123). The initial study of Dao *et al.* (122) revealed that in 7 of 10 patients treated with a physiological dose of estradiol plus progesterone, the *in vitro* thymidine labeling index rose significantly even though 4 of the 7 responsive tumors lacked ER; PR status was assessed in only 4 cases, and, although proliferation increased in 3 of these, all 4 lacked PR. When breast tumors were removed from patients and the effects of estrogen and progesterone on the growth fraction assessed *in vitro,* a spectrum of effects was observed (123). Estradiol decreased the growth fraction in 6 of 7 tumors and increased it in the remaining sample. A similar pattern was observed when estradiol and progesterone were admin-

istered together. Dose-response studies on a small number of biopsies demonstrated variable responses: 4 of 6 tumors treated with progesterone alone had markedly reduced growth fractions while the other 2 were stimulated; combined treatment with estradiol and progesterone increased the growth fraction in 4 of 7 cases at low doses, but treatment with 10- to 100-fold higher concentrations of both steroids invariably led to a decrease in proliferation. Again, responsiveness was unrelated to steroid hormone receptor status (123). Interpretation of these *in vitro* data was complicated by the presence of 10 μg/ml insulin, a potent mitogen for breast cancer cells, in the culture medium. On face value, these studies call into question the role of PR in mediating the responsiveness of human breast cancers to progestin therapy. However, the small number of patient samples in these studies and the significant limitations, both in the measurement of PR in the face of high circulating progestin levels and in the maintenance of stable PR levels in organ culture of malignant tissue (124), suggest that these *in vitro* studies should be interpreted with caution.

The transplantation of breast cancer biopsies or cell lines into nude mice provides an attractive model for studying the effects of hormones on breast cancer cell proliferation *in vivo*. Unfortunately, there is only one study describing the effects of progesterone in such a system (125). Administration of progesterone alone to nude mice carrying an MCF 7 breast carcinoma did not stimulate growth. However, progesterone and estrogen had a synergistic effect on tumor burden, which was attributed to a decrease in the latency period rather than an effect on tumor incidence or growth rate.

C. Chicken oviduct

The chick oviduct represents one of the best defined systems for studying the effects of steroid hormones on growth and differentiation (126–129). In the immature chicken, the magnum region consists of a single layer of columnar epithelial cells seated upon a dense stroma. After stimulation with estrogen this epithelial layer can differentiate into three distinct cell types: tubular gland cells which produce egg white proteins under stimulation by estrogen and/or progesterone; goblet cells which are progestin responsive and synthesize avidin when progestin stimulated; and ciliated cells (126–129). Although this system has been used almost exclusively for studies on egg white protein gene expression, a number of studies have addressed the issue of estrogen and progesterone effects on mitosis. The study that most comprehensively and clearly defined the effects of progesterone on cell division in this organ was undertaken by Socher and O'Malley (130).

A single injection of progesterone to previously untreated immature chicks resulted in a 2-fold increase in the mitotic index of the surface epithelium 12–18 h later. Mitoses fell below the low, but significant, level in untreated chickens at 24 h and further declined to undetectable levels at 48 h. A second injection of progesterone at 24 h after the initial injection did not result in further mitoses. These data suggest that progesterone alone is able to induce a single round of replication in a small population of immature epithelial cells but thereafter has no effect on cell division in the magnum. In contrast, estradiol increased the mitotic index to a 7- to 8-fold maximum at 18 h, and repeated 24 hourly injections led to further rounds of replication. It is unclear whether the progestin-responsive cells are also part of the estrogen-responsive pool. When estrogen and progesterone were administered together, the effect, during the first round of replication, *i.e.* during the first 24 h, was indistinguishable from that with estradiol alone. However, readministration of this combination at 24 h, in marked contrast to estradiol alone, failed to induce further mitoses, *i.e.* under these circumstances progesterone inhibited the estrogen-induced replication.

This study (130) also examined the persistence of the progesterone-induced suppression of mitosis by rechallenging progesterone-treated, or estrogen plus progesterone-treated, animals with estradiol at 24 h intervals. These experiments revealed a time-dependent return to estrogen responsiveness after progesterone-induced suppression of mitosis, indicating that the ability of oviduct epithelium to divide was not altered permanently by progesterone treatment. A similar result was previously seen in the adult ovariectomized mouse luminal epithelium (27).

When chickens are treated continuously with estrogen, the immature magnum differentiates into a series of tubular gland cells, which demonstrate responses to the mitogenic effects of estrogen and the antiproliferative effects of progesterone, identical to those seen in the surface epithelium. The continued presence of estrogen maintains a high mitotic index in both cell types, and this is significantly reduced by administration of progesterone. Finally, when oviducts are fully differentiated by 10 daily injections of estrogen then withdrawn from treatment for a similar period, and the hormone-depleted animal rechallenged with hormone, estrogen but not progesterone (in contrast to the situation in the immature gland), leads to an increase in mitosis. Simultaneous administration of the two steroids results in complete attenuation of the estrogenic effect by progesterone. Thus, while progesterone demonstrates some mitogenic activity in the immature magnum, once the organ has differentiated progesterone can no longer stimulate mitosis (130). The effects of estrogen and progesterone on

the chicken oviduct epithelium are summarized in Table 5.

Several later studies addressed the effect of progesterone on various aspects of estrogen-induced DNA synthesis and cell replication. The estrogen-induced increase in ornithine decarboxylase activity, an enzyme involved in polyamine synthesis and cell replication, was inhibited by simultaneous administration of progesterone, while progesterone alone had a modest effect on activity (131). Studies on DNA polymerase α activity illustrated that progesterone initially inhibited estradiol-induced increases in enzyme activity although the effect was relatively short-lived, *i.e.* approximately 6 h. Thereafter, the activity in animals receiving both steroids began to increase at a rate greater than that after treatment with estrogen alone such that, by 12 to 18 h after progesterone treatment, enzyme activity was greater in the oviduct of animals receiving both steroids (132).

The studies discussed in this section illustrate that antagonistic or synergistic effects of progesterone on estrogen-induced proliferation can be obtained depending upon the temporal relationship of administration of the two compounds; this principle further complicates the interpretation of progestin effects on cell proliferation.

III. Effects of Progestins on Cell Proliferation *in Vitro*

The effects of progestins on cell proliferation *in vitro* have been investigated in a number of different experimental systems including organ culture of uterus and mammary gland, cell culture of normal breast and uterine epithelium, benign breast tumors, and established cell lines of breast and endometrial carcinoma.

A. Organ culture

Early studies on human endometrial tissue in organ culture were not extensive but defined the inhibitory effect of progesterone on DNA synthesis in both normal (49) and neoplastic (62) tissue. Subsequent studies on primary cultures of rat (133) and rabbit (134–137) endometrium supported a primary growth- inhibitory effect of progesterone on uterine epithelial cell proliferation *in vitro*. While the data with rat uterine cultures, demonstrating progesterone inhibition of the rate of epithelial cell proliferation with little effect on stromal cells, tended to confirm earlier observations *in vivo,* the inhibitory effect of progesterone on proliferation of rabbit uterine cultures was markedly different from the stimulatory effects observed *in vivo* (*Section II.A.2* and Refs. 42–44). Progesterone antagonized proliferation induced by the synthetic estrogen, diethylstilbestrol, and led to the formation of increased numbers of large, multinucleated cells typical of decidualization (134). Both hormones appeared to have synergistic effects on protein synthesis as had been demonstrated in mouse uterus *in vivo* (31). The same authors suggested that progesterone decreased cell proliferation rates both by increasing the proportion of G_0 cells and increasing the lengths of both G_1 and S phase in the population that continued to cycle (135).

TABLE 5. Cell proliferation in the chicken oviduct epithelium

Developmental state	Treatment					
	E		P		E + P	
	Single	Repeated	Single	Repeated	Single	Repeated
Immature	++++	++++	+	−	++++	+
Differentiated	++++	++++	−	ND	ND	ND

Abbreviations are defined in Table 1.

[a] Adapted with permission from Ref. 130.

In agreement with the *in vivo* effects of progesterone on the mammary gland (*Section II.B*), *in vitro* culture of rat mammary gland explants in the presence of progesterone led to stimulation of the labeling and mitotic indices with marked proliferation of ductal epithelium and moderate development of lobular structures. The concomitant addition of progesterone and PRL induced further hyperplasia of ductal epithelium and considerable lobuloalveolar development (138). Studies with DMBA tumors in organ culture demonstrated that, in the presence of insulin, progesterone significantly increased DNA synthesis in about 50% of tumors. The effect appeared to be synergistic when PRL was also added. In some tumors progesterone, in the absence of PRL, induced considerable cellular hypertrophy, and mitoses were frequently observed; progesterone also appeared to inhibit PRL-induced secretory changes (139). These data are consistent with the proposed stimulatory role of progesterone alone, or in combination with other hormones, *e.g.* estrogen and PRL, in mammary gland epithelial cell proliferation *in vivo.*

B. Cell culture

1. Inhibition of growth by progestins. The majority of data relating to the effects of progestins on cell proliferation *in vitro* have been derived from studies with tumor cell lines. Many of the published studies employing these systems appear to lack an appreciation of the principles of cell proliferation kinetics. Effects of a given agent on cell proliferation are best studied when the growth rate of the control population is in steady state, *i.e.* when cell numbers increase exponentially with time. There are very few published studies in which compliance with this minimal requirement is demonstrated. Many either fail to present growth curves, present such data as linear

(rather than logarithmic) plots of cell number against time, or present cell number relative to control after an unknown number of control doubling times.

While this does not preclude making statements to the effect that the treatment affects cell number, it does preclude any conclusions about relative growth rates, thereby hampering the formulation of rational hypotheses on mechanisms of growth inhibition. Where only a small fraction of the population is actively dividing, extended exposure times may be necessary before changes in growth rate will significantly affect cell number. Furthermore, it is important to appreciate that even when the population growth rate is high, apparent sensitivity is greatly influenced by the duration of the experiment relative to the cell cycle time of control cells, particularly where the agent has cell cycle-specific effects. In the first cell cycle of exposure, much of the population may be effectively insensitive by virtue of their position in the cell cycle, and these cells will continue past mitosis, until they reach the point of sensitivity. In the case of an agent which acts in early G_1, as suggested for progestins by our own data (140), cell numbers may almost double before there is any reduction in the cellular birth rate. This is illustrated in Fig. 2 where the sensitivity of T-47D cells to the synthetic progestin, MPA, is recorded after 1, 2, and 4 cell cycle times of the untreated control population.

Even in these exquisitely sensitive cells, no change in the relative cell number can be detected until the control cells have completed more than one round of replication, and the apparent sensitivity is markedly influenced by the time of exposure. When it is appreciated that in most experiments demonstrating stimulatory effects of progestins on breast cancer cell growth *in vitro*, control cells have been manipulated to grow at very slow rates, *i.e.* with doubling times of 4–7 days, the importance of experimental design to the ultimate interpretation of the data is apparent.

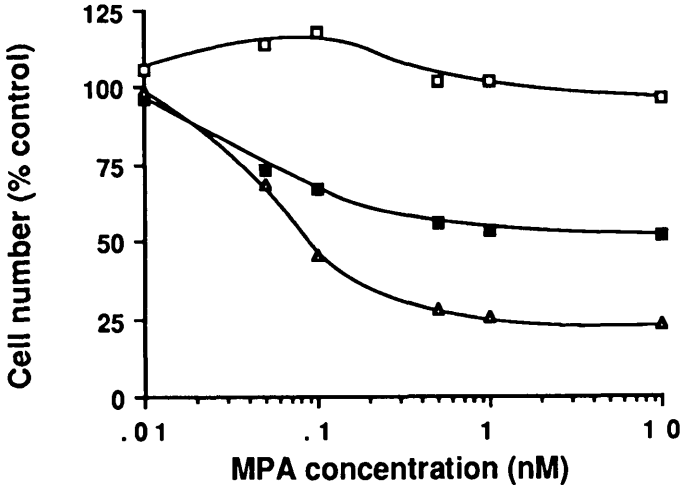

FIG. 2. Effect of duration of exposure of breast cancer cells to progestin on apparent sensitivity of growth regulation. Viable cell counts of T-47D cells treated with the progestin MPA while in exponential growth phase are expressed as a percentage of the cell count in vehicle-treated control flasks. Cells were harvested after approximately 1 (□), 2 (■) or 4 (Δ) population doublings of control cells.

Despite these potential limitations to the interpretation of some published data, there is now overwhelming evidence that progestins can directly influence the growth of target cells *in vitro*. Although the most detailed data come from studies on human mammary carcinoma cell lines, particularly MCF 7 and T-47D (140–153), similar observations have been made using normal breast epithelial cells (112) and endometrial carcinoma cells (154).

The first data on the effects of progestins on breast cancer cell proliferation *in vitro* were obtained with an estrogen-responsive clone of T-47D cells and the antiestrogen resistant variant of MCF 7, R 27, in which the growth-inhibitory effects of the synthetic progestin, R 5020, were apparent only in the presence of growth stimulation by estradiol (141). The effect was not observed with dihydrotestosterone or dexamethasone, suggesting that R 5020 acted on cell proliferation via PR. The authors concluded that R 5020 was acting as an antiestrogen, although the observation that it could inhibit the tamoxifen-resistant R 27 cell line suggested that the two types of antiestrogens, *i.e.* progestins and nonsteroidal antiestrogens, acted through different mechanisms (141). It was also proposed that inhibition of the synthesis and secretion of estrogen-induced growth factors might account for the antiproliferative effect, since the same authors had previously reported on the secretion of specific estrogen-induced proteins by these cells and inhibition of their secretion by progestins (155). These direct growth-inhibitory effects of progestins on human cells in culture have now been confirmed in a number of studies (140, 142–150); others, however, have reported no effect (156–158) or a growth-stimulatory effect of progestins (146, 149, 151–153).

The question of whether or not progestins can only inhibit estrogen-induced cell proliferation has remained controversial since the original report of Vignon *et al.* (141). There is, however, convincing evidence that this is not the case. Horwitz and Freidenberg (144), using the antiestrogen resistant, ER-negative T-47Dco cell line, demonstrated profound growth inhibition by R 5020. Similarly, Terakawa *et al.* (154) showed that an endometrial carcinoma cell line, with undetectable ER but significant quantities of PR, was growth inhibited by R 5020. A likely explanation for the apparent discrepancy relates to the cell growth conditions employed in the first study (141) and perhaps the level of PR, and its regulation, in the different cell lines employed (141, 144, 154). In the data presented by Vignon *et al.* (141) on T-47D

cells in the absence of estrogen, the cell numbers in R 5020-treated cultures were lower than those in control flasks at each concentration above 0.1 nM, although this was not statistically significant. More prolonged treatment may have resulted in more marked suppression of growth. Furthermore, no data were presented on the estrogen inducibility of PR in these cells (141). If, unlike the cell lines employed by Horwitz and Freidenberg (144) and Terakawa *et al.* (154), the cells employed by Vignon (141) required estrogen stimulation to attain significant levels of PR, this could explain the greater sensitivity of estrogen-treated cells to the growth-inhibitory effect of the progestin. Although a number of studies clearly demonstrate that sensitivity to progestin-induced growth inhibition is modulated by estrogens, it is now apparent that as long as PR is present progestins can be active in the absence of estrogen (140, 144, 154). Further convincing evidence of progestin inhibition of cell proliferation in the absence of estrogen is apparent in studies from this laboratory, in which insulin-stimulated growth of T-47D cells in which PR is constitutively expressed, in a chemically defined, serum-free medium, devoid of phenol red, was markedly inhibited in a time- and concentration-dependent manner by the synthetic progestin ORG 2058 (159).

That the growth-inhibitory effects of the synthetic progestins, R 5020, MPA, and ORG 2058, are mediated via PR has been supported by several studies (140–142), although it has been suggested that part of the MPA response in MCF 7 cells is mediated via the GR (149). While it is conceivable that the known glucocorticoid activity of some synthetic progestins could lead to some effects being mediated via GR, this is not the case in T-47D cells, which have high levels of PR and in which steroid specificity has been studied in detail. In these cells steroid-induced growth inhibition has been convincingly demonstrated to be confined to progestins and high concentrations of androgens, with glucocorticoids and estrogens having no effect (140). Furthermore, ER- human breast cancer cell lines, which express higher levels of GR than ER+, PR+ lines (121), fail to respond to the growth-inhibitory effects of MPA (140) while ER−, PR− but AR+, GR+ NMU rat mammary tumor cells were insensitive to growth inhibition by R 5020 (141).

While these *in vitro* data provide good evidence that the majority of growth-inhibitory effects of progestins are mediated via PR, the presence of PR in human breast cancer cell lines does not ensure a growth-inhibitory response to MPA (140). Investigation of the sensitivity of 5 PR-positive cell lines to MPA demonstrated that there was a greater than 2,000-fold range in sensitivity despite only a 20-fold difference in PR levels and furthermore, sensitivity was not in the same hierarchy as PR concentrations. One possible interpretation of this anomaly is that there are aberrations in receptor function or postreceptor events in some of these cell lines. Another interesting observation to emanate from this study was that the maximal level of growth inhibition induced by MPA varied from 70% for T-47D cells to 20% in ZR 75–1 cells (140). These data provide evidence that progestin-controlled pathways account for only part of the overall control of proliferation of these cells since proliferation continues, albeit at a reduced rate, in the presence of progestins. Furthermore, they confirm the view that antiestrogens and progestins inhibit growth by different mechanisms (11, 140), since in all the cell lines studied, with the exception of T-47D, maximal responses to antiestrogens were significantly greater than maximal inhibitory responses to progestins (140, 160).

There is only one published study reporting the cell kinetic basis of progestin-mediated growth inhibition *in vitro* (140). Studies on changes in cell cycle phase distribution and G_1 exit kinetics after treatment with MPA were undertaken with both T-47D and MCF 7 cells. Treatment of exponentially growing T-47D cells resulted in a redistribution of cells between the G_0/G_1 and S phases of the cell cycle. At a maximal growth-inhibitory concentration, *i.e.* 10 nM in T-47D cells, no effect was observed during the first 12 h. The redistribution between cell cycle phases was half-maximal at 18 h and maximal at 24 h, with an apparent slight recovery at 48 h (Fig. 3). A half-maximal effect at 18 h indicated that the earliest mean point of action of the progestin was 18 h before cells enter S phase. The point of action of the hormone, however, must be within G_1 phase since progression through S and G_2 + M phases was unaltered during the first 12 h of treatment as evidenced by constant cell cycle phase distribution over this period. That the effect is early in G_1 phase is supported by data on cell growth which showed insignificant differences between control and drug-treated cell numbers after 24 h, indicating that almost all cells could complete the first replication cycle after administration of the drug (Fig. 2). Since the mean length of G_1 phase in these cells was 13.5 h, there must have been a delay of at least 4.5 h between addition of progestin and the mean point of action on cell cycle progression (140). This may represent the time required for PR-mediated modulation of gene transcription to be reflected in the production or depletion of specific gene products controlling cell cycle progression in this cell line. In this regard, it is interesting to note that progestin inhibition of the transcription of its own receptor is not apparent until 3 h after treatment (161).

After the initial redistribution of cells between G_0/G_1 and S phases of the cell cycle and the decline in growth rate, a concentration-dependent recovery was apparent.

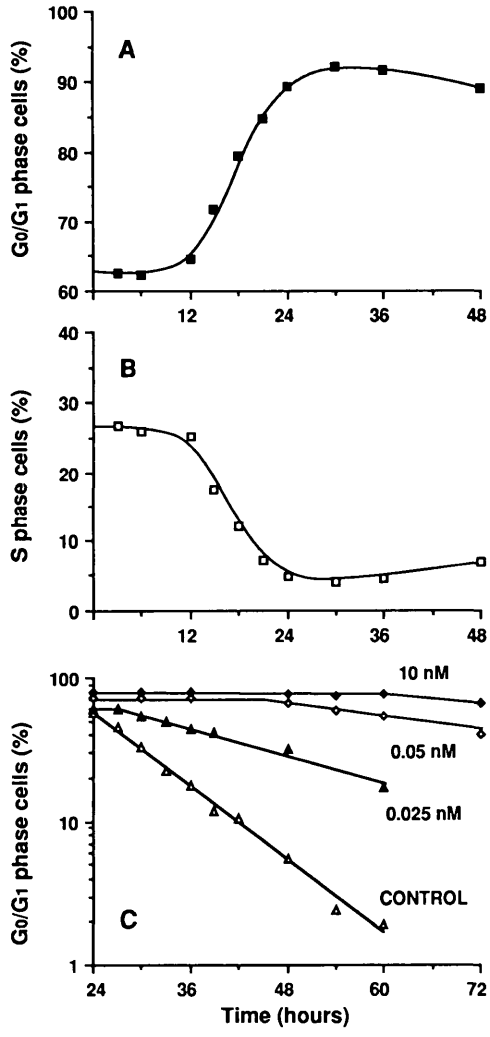

FIG. 3. Effect of the progestin treatment of breast cancer cells on cell cycle kinetic parameters. T-47D cells treated with MPA were harvested at the indicated times and cell cycle phase distribution determined by analytical DNA flow cytometry. A and B, Changes in G_0/G_1 and S phases after treatment with 10 nM MPA. C, After 24 h pretreatment with MPA, T-47D cells were additionally exposed to ICRF 159, an inhibitor of cytokinesis. The subsequent rate of decrease in percent G_0/G_1 phase cells is shown and indicates the rate of efflux of cells from G_1 phase. [Data are redrawn with permission from R. L. Sutherland et al.: Cancer Res 48:5084–5091, 1988 (140).]

This was most convincingly illustrated in studies on G_1 exit kinetics of T-47D cells after treatment with various concentrations of MPA, in which the drug was shown to cause a transient but complete arrest of cell cycle progression, the duration of which varied with the concentration of MPA. After cell cycle arrest there was a resumption of cell cycle progression, the rate of which was dependent upon the concentration of MPA (Fig. 3). These experiments were conducted in the continuous presence of the drug, MPA having been administered once at 0 h with no subsequent medium change, and gave a somewhat different result (140) to that observed after transient exposure to R 5020 (144). The latter experiment involved treatment of T-47Dco cells with a 1 h pulse of 100 nM R 5020 followed by washing of the monolayer and reincubation in hormone-free medium. While such a treatment might not be expected to completely deplete the cells of R 5020 due to its high affinity for PR and its low level of metabolism, it is likely that the majority of drug was removed. Under these circumstances cell growth was initially arrested, but thereafter the growth rates of treated and untreated cultures were indistinguishable (144). Such data imply that the decreased cell cycle progression observed in our experiments after recovery from the initial arrest by MPA was due to the persistence of MPA and its action after continuous exposure to the drug. Further evidence that short term exposure of T-47D cells to progestins can result in marked effects on progestin responses, *e.g.* growth rate and expression of the epidermal growth factor receptor (EGF-R), comes from our published (162) and unpublished data with the rapidly metabolized natural progestin, progesterone.

Although in all the other progestin-responsive cell lines studied (BT-474, MCF 7, MDA-MB-361 and ZR-75–1) the changes in cell cycle phase distribution were similar to those reported for T-47D, relatively detailed studies were only undertaken with MCF 7 in which changes in cell cycle phase distribution were not complete within the first cell cycle of drug exposure but increased with increased time of exposure. Since growth experiments showed that MCF 7 cells were not only significantly less sensitive to progestins but that the maximal response was also diminished, the results might be explained by the fact that a much smaller proportion of the total population was sensitive to MPA and that the effect was maximized by multiple exposure at the point of sensitivity (140).

2. Stimulation of growth by progestins. In agreement with the known stimulatory effects of progesterone on normal and neoplastic rodent mammary gland in organ culture, similar responses have been observed in cell culture. When 13762NF rat mammary tumors were cultured in collagen gels under serum-free conditions, but in the continuous presence of insulin, progesterone synergized with both EGF and PRL to promote growth. Administration of progesterone alone had a modest effect on growth (163). Similar effects were noted when NMU-induced tumors were grown in a soft agar clonogenic assay. Both progesterone and R 5020 were stimulatory, and part of this effect was attributed to progestin-induced autocrine growth factor production by these tumor cells (164, 165).

There are also recent reports demonstrating increases in cell number after progestin treatment of breast cancer cells (146, 149, 151–153). In several cases the cells under

study had previously demonstrated resistance to the growth inhibitory effects of progestins *in vivo* or *in vitro* (146, 149, 151). However, similar data were derived from the T-47D cell line which has been the source of much of the data on the growth-inhibitory effects of progestins. One of the most interesting aspects of the data presented to date is that, while the stimulatory effect on T-47D cells is clearly time- and progestin concentration-dependent, the maximal increase in cell number is never greater than 2-fold, which is markedly less than the 7-fold increase observed with estradiol (153). Interestingly, R 5020 partially inhibited the estrogen-induced proliferative response under the same experimental conditions, and this inhibitory effect was augmented by the simultaneous addition of both R 5020 and hydroxytamoxifen (153). These data confirm both a predominantly inhibitory role for progestins in estrogen-induced proliferation and the independent actions of progestins and nonsteroidal antiestrogens on estrogen-induced growth of breast cancer cells.

Although no data have been presented that provide a mechanistic explanation for the progestin-stimulatory effect, Moore and associates (152, 153) have attributed the ability to detect this phenomenon to the absence of phenol red, a known estrogen agonist, from the culture medium with the concurrent loss of progestin antagonism of estrogen-induced proliferation. Another likely contributing factor is the ability of these investigators to decrease the growth rate of their control cells to a level markedly below that observed under normal culture condition. This was probably attained by a combination of removal of phenol red from the medium, the use of charcoal-treated fetal calf serum devoid of steroids and some growth factors, inoculation of experimental cultures at relatively high cell number from confluent stock flasks, and frequent medium changes, which would deplete the cultures of autocrine growth factors produced by the cells themselves (152, 153). A likely consequence of these manipulations is an increase in the proportion of noncycling or G_0 cells.

Recent unpublished data from our laboratory may shed further light on this stimulatory effect. When experiments of the type described in Fig. 3 were undertaken in a serum-free, phenol red-free, chemically defined medium and the T-47D cells stimulated to grow with insulin, ORG 2058 inhibited cell growth in an apparently analogous manner to that observed with MPA in medium supplemented with fetal calf serum and phenol red. However, unlike the situation depicted in Fig. 3, the progestin caused an initial depletion of G_0/G_1 phase cells and a transient increase in S phase cells (Fig. 4), a result compatible with a progestin-induced increase in the rate of progression through G_1 phase. This synchronous peak of cells, which reached a maximum in S phase at 12 h,

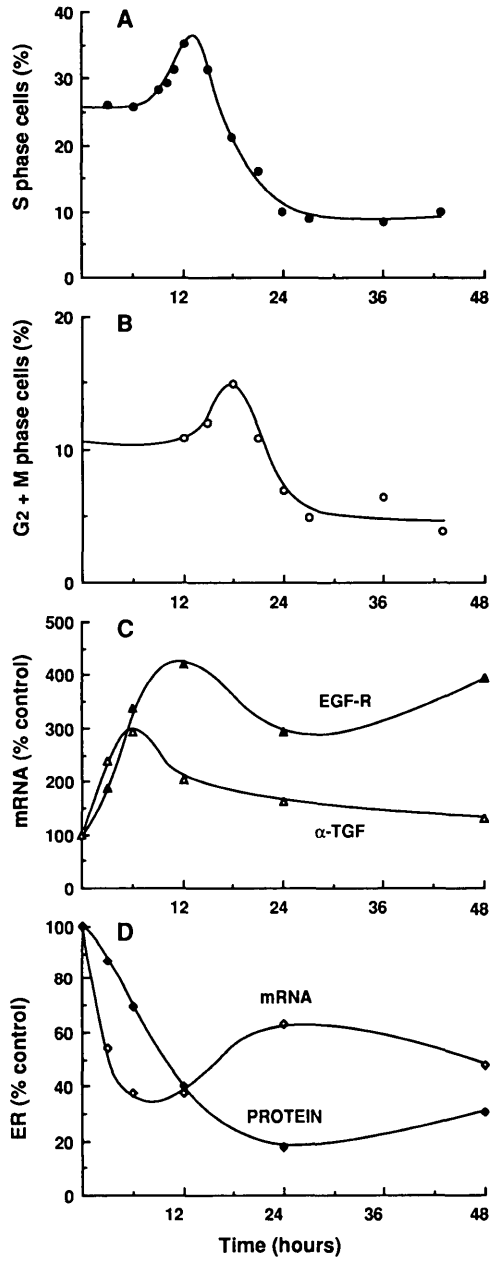

FIG. 4. Changes in cell cycle phase distribution and the expression of α-TGF, EGF-R, and ER after progestin treatment. T-47D cells were treated with 10 nM ORG 2058 and harvested at the times indicated. A and B, Changes in S and G_2 + M phases after ORG 2058 treatment of T-47D cells in insulin-supplemented serum-free medium. C, α-TGF and EGF-R mRNA levels measured by Northern analysis. D, ER mRNA and ER protein levels assessed by Northern blot and radioligand binding analyses.

progressed through the cell cycle being apparent in G_2 + M phase at 18 h after treatment (Fig. 4B). After 12 h, the proportion of cells in S phase fell rapidly as the previously reported inhibitory effect was manifested. Thus, under experimental conditions in which the growth rate of control cells was slightly slower than in the presence of fetal calf serum, progestin appears to

have two distinct effects in cell cycle progression. These are an initial stimulatory effect which appeared to result in a transient shortening of G_1 phase, allowing a cohort of cells to transverse the cell cycle at a greater than normal rate, and a later inhibitory effect resulting from arrest and/or delayed progression through G_1 phase. The potential mechanisms responsible for these two effects will be discussed in *Section V.B.*

IV. Progesterone Receptor and Molecular Mechanisms of Progestin Action

Irrespective of the final molecular mechanisms through which progestins control proliferation, the first event in progestin action involves its interaction with the intracellular PR. With the recent cloning and identification of functional domains within the steroid/thyroid hormone family of transcriptional activators, the structure of the PR is now being defined as are the mechanisms by which it controls gene transcription. Since these processes are fundamental to an understanding of progestin action on any cellular parameter the current status of this field is summarized.

A. PR: structure and regulation

The PR is a member of a gene superfamily of transacting transcriptional factors now known to contain receptors for steroid hormones, thyroid hormones, and retinoic acid as well as other known transcription factors and proteins of unknown function. Members of this family share several common features which include a distinct structure characterized by a poorly conserved N-terminal region, a highly conserved cysteine-rich DNA binding domain, and a conserved hormone binding C-terminal domain. The structure of the gene superfamily has been the subject of several recent reviews (166–168). PR has been cloned from the chick oviduct (169, 170), the rabbit uterus (171), and human breast cancer cells (172). The chromosomal PR gene structure has been elucidated in the chicken to be a single copy 38 kilobasepair gene composed of 8 exons, which code for the functional domains of the receptor molecule (173). The human PR gene has been mapped to chromosome 11 (174, 175).

The PR is unusual among steroid hormone receptors in being detectable as 2 proteins of dissimilar mol wts (designated B, 100–120 K; and A, 79–94 K). The smaller protein arises in the chicken when translation is initiated from an internal translation initiation site (176–179). The functional consequences of N-terminally truncated chicken PR proteins have been examined, and the N-terminal region of chicken PR has been implicated in specifying both transactivational efficiency (176) and specificity (180). Both the rabbit and human PR sequences also contain a second methionine residue, 165–166 residues from the initiation site (171, 172), although there is yet no evidence that internal initiation is responsible for the formation of the A protein in the human and rabbit. Transcription/translation *in vitro* of complementary DNAs (cDNAs) containing the coding region of rabbit or human PR resulted in the detection of the B protein as the predominant product (181). This supported previous evidence that posttranslational proteolysis was responsible for A formation in the rabbit (182, 183) but did not explain the consistent detection of both B and A in the human (184–187). In relation to this, it has been shown that the 5′ untranslated sequence of the cDNA directs the relative amounts of the chicken B and A proteins synthesized (178). Presence of the 5′ leader sequence resulted in the detection of approximately equal levels of B and A proteins, whereas deletion of this sequence resulted in the B protein being the predominant product detected. It is perhaps interesting to note that the constructs used to demonstrate that the B protein was the predominant product of *in vitro* transcription/translation of rabbit and human PR cDNA contained the coding region but did not contain the entire 5′ untranslated sequence (181).

The PR is under the dual control of estrogen and progestin, which act sequentially to regulate the cellular concentration of PR and therefore the likely cellular responsiveness to progestins. The receptor is increased by estrogen in most target cells and tissues, due to an estrogen-mediated increase in PR mRNA levels (169–171, 188) and increased PR protein synthesis (189). The higher mol wt B protein is detectable on denaturing analysis as a series of 3 closely migrating mol wt isoforms, 2–4 K apart. These isoforms appeared sequentially in the nascent PR protein upon exposure to estrogen (190, 191) due to posttranslational modification by phosphorylation (185, 191–196). This initial phosphorylation is accompanied by a second round of phosphorylation upon progestin binding. Although the second, ligand-mediated, phosphorylation step has been correlated with the onset of progestin action, the role of the initial phosphorylation step is still unclear. However, recent studies have shown that PR postulated to lack the initial phosphorylation is able to bind to its progestin ligand and to specific hormone response elements (191, 197), but whether the initial phosphorylation is necessary for PR to activate transcription has yet to be determined.

Many physiological situations in which serum progesterone levels are elevated, such as during the luteal phase of the human menstrual cycle or during progestin treatment of postmenopausal patients with carcinoma of the uterus or breast, are associated with markedly reduced levels of cellular PR. The decrease in the progestin binding capacity measured after progestin exposure has

been shown to result from a decrease in the PR protein concentration and half-life, and a down-regulation in PR mRNA concentration (188, 189, 198, 199). Progestin treatment of T-47D cells results in a rapid loss of PR protein and mRNA, which both reach their nadir 12 h after treatment (161). While the effect of progestins on PR mRNA levels was not detectable until 3 h after exposure to this agent, effects on the PR protein were evident much sooner than this (161). The evidence that progestin effects on the PR protein precede any detectable decrease in the level of PR mRNA suggests that the protein may participate directly in the regulation of its own mRNA levels, either by binding directly to negative response elements in the promoter region of the PR gene or by stimulating the synthesis of an unknown regulatory protein that would inhibit transcription of the PR gene.

The generally accepted dogma that progestins reduce cellular levels of PR is likely to be an oversimplification however. The advent of monoclonal antibodies against PR enabled cell-specific PR distribution to be determined. Immunohistochemical evidence shows that stromal and myometrial PR levels in the human uterus persist in the face of increasing luteal progesterone concentrations (56, 200, 201), consistent with the observation that these cells maintain sensitivity to progestins. There are also tissue-specific differences in the ability of progesterone to reduce PR concentrations (202, 203), and there is evidence that the levels of mouse mammary gland PR decrease with increasing tissue differentiation (204). Furthermore, sustained progesterone exposure has been associated with maintenance or even increases in cellular PR levels (205–207). Clearly, progestin regulation of PR levels is likely to vary among cell types, between species, and more importantly in different physiological situations in order to regulate cellular sensitivity to progestins.

B. Molecular mechanisms of progestin action

Steroid hormone receptors bind to specific upstream sequences [hormone response elements (HRE)] in order to regulate transcription of hormone-sensitive genes. The present state of knowledge on how PR interacts with its cognate HRE to mediate progestin action has been recently reviewed (208, 209) and will be summarized briefly. The PR was first shown to stimulate RNA synthesis in the chick oviduct, in which the stimulatory effects of progesterone on the synthesis of egg white proteins such as ovalbumin, conalbumin, and ovomucoid were well known, and binding sites for PR in several genes including chicken lysozyme were defined (210–214). It has been postulated that the function of ligand binding to PR is to increase the affinity of human PR for its HRE, and thereby to increase the specificity of transcriptional activation (215, 216). These data followed earlier studies which showed that PR was able to bind in a sequence-specific manner to the uteroglobin gene independently of the presence of progestin (217), and that PR bound to the anti-progestin RU 38486 (RU 486) was able to inhibit progestin-mediated transcriptional activation by putative competition at the HRE (218). In contrast, *in vitro* measurement of chicken PR affinity for its HRE showed only a minor hormone-induced alteration, insufficient to explain the profound effect of PR on gene transcription (219). Recent data have shown that PR mutants truncated in the ligand binding domain possess ligand-independent transactivational capacity, but that the magnitude of transactivation was dependent on the amount of mutant expressed and on the cell type transfected, and that its effectiveness never matched that of the wild type receptor (220, 221).

Recent studies have shown that the same 15 base pair HRE sequence can mediate both progestin and glucocorticoid induction of gene expression (222), suggesting that progestins and glucocorticoids acting through their respective receptors could activate gene expression by interacting with the same DNA sequences. This was demonstrated when transfection of the PR gene into previously PR-negative cells conferred progestin inducibility on endogenous glucocorticoid-regulated genes (223). "Cross-talk" of receptors at the HRE level has now been widely demonstrated for progestin-, glucocorticoid-, and androgen-responsive elements, and there is evidence of close similarity between these HREs and estrogen-responsive elements (224, 225). The way in which steroid-specific gene activation takes place in the face of such close similarity between HRE is one of the unanswered questions in this area, but there is evidence that the same HRE behaves differently in different cells with regard to its ability to modulate promoter function (220, 226) and presumably that cell-specific factors, as yet undefined, may play a part in directing steroid modulation of gene transcription in hormone-sensitive cells. Receptors may compete with one another for access to functionally limiting transcription factors, and this competition may determine the transcriptional outcome of cell treatment with steroid hormones (226). There is also mounting evidence that PR and GR interact as dimers with their HREs, and that HREs can interact synergistically with one another and with other transcription factors to regulate gene expression (228–231).

V. Potential Mechanisms of Progestin Effects on Proliferation

A. Effects on ER levels

In the mammalian uterus, high circulating levels of progesterone are generally associated with a decrease in

tissue growth and inhibition of estrogen actions. Recognition of this effect was coupled with the realization that cellular ER levels tended to be lower in progestin-dominated physiological states such as the luteal phase of the menstrual cycle. For instance, in the human uterus or the monkey oviduct, the concentration of ER is higher in the follicular phase than during the luteal phase of the cycle. The implications of these observations were that rising progesterone levels during the luteal phase inhibited the action of estrogen on the tissue, and did so by mechanisms that possibly included reducing the cellular concentration of ER.

Experimental support for the view that progestins regulated the concentration of ER began to emerge in the early 1970s, in studies on the human, primate, and rodent. Artificial menstrual cycles were induced in castrated Rhesus monkeys, in which constant estradiol administration was supplemented with cyclical progesterone treatment every 2 weeks for 2 weeks. Interestingly, the onset of progesterone administration was accompanied by a rapid and dramatic decrease in the concentration of ER measured in low salt extracts of the monkey oviduct. ER levels remained depressed for the duration of progesterone exposure (232). In the rat uterus, progesterone or MPA decreased ER (233–235) and was able to prevent estradiol-mediated increases in ER in the myometrium (236). It was also shown that *in vivo* administration of the synthetic progestin MPA to women in the follicular phase of the menstrual cycle resulted in a decrease in cellular ER, to levels normally found in secretory tissue (237).

In these early studies, ER measurements were made in both cytosolic and nuclear extracts from the cell, and it was generally agreed that estradiol initially caused depletion of cytosolic ER with a concomitant increase in nuclear receptor. The decline in cytoplasmic ER was transitory and was followed by an estradiol-mediated recovery to levels higher than control. In the rat uterus it was shown that administration of progesterone in combination with estradiol resulted in the cytosolic ER depletion seen with estradiol alone, but in the presence of progesterone the cytosolic recovery of ER levels was inhibited (238). The effects of progesterone on the recovery of cytosolic ER were mimicked by the effects on ER of actinomycin D or cycloheximide given *in vivo,* which suggested that recovery of ER was transcriptionally and translationally mediated and that progesterone inhibited the process at both levels. These effects were demonstrable when progesterone was given either in conjunction with or preceded by estradiol exposure, and were absent if progesterone alone was injected into the immature rat uterus. An effect of progesterone on nuclear ER was also noted in the cat and hamster uterus (239, 240). In the hamster and rat uterus progestins selectively decreased only ER that was bound to its ligand (241, 242), and this decrease in the hamster was mediated by an alkaline phosphatase activity that was stimulated by progestins (243). The hamster uterus model was used to show that the continued presence of progestins suppressed ER levels, which recovered progressively when the influence of progestins was removed (244). Recent studies confirming this observation in the rat uterus have shown furthermore that nuclear ER levels decreased as serum progesterone levels increased (245). The suppressive effect of progestins on ER was confirmed with the advent of monoclonal antibodies to ER, which were used to demonstrate recovery of immunoreactive ER in the monkey uterus after progesterone withdrawal (246). Monoclonal antibodies were also used to show in the human uterus that immunoreactive ER levels decreased equally in all the cell types of the uterus in the luteal, progesterone-dominated, phase of the menstrual cycle (56).

The largely inhibitory action of progesterone on ER in the mammalian uterus was in contrast to the effect seen in the chick oviduct, where progesterone was shown to have both antagonistic and synergistic effects on ER (132, 247). Injection of progesterone alone to the estradiol-primed then withdrawn chicken resulted in a marked decrease in total (cytoplasmic + nuclear) ER levels 3–6 h after treatment, which was followed by a recovery of ER to levels above control. This was similar to the effect seen when estradiol was administered alone. However, when progesterone was administered in conjunction with estradiol, progestins maintained a reduced ER level for at least 6 h below the level achieved with estradiol alone, then allowed ER recovery at a rate and to an extent often greater than that seen with estradiol alone. This synergistic action of progesterone and estradiol was also noted on ovalbumin and conalbumin synthesis. Removal of progesterone resulted in a gradual recovery of ER levels in the chick oviduct.

It has only been in the last 3–4 yr that detailed mechanistic studies have been carried out on the progestin regulation of ER. Density shift analysis was used to demonstrate in hamster decidual cells that progestins decreased the half-life of ligand-occupied ER from 4 to 2 h, suggesting that progestins increased the degradation rate of the receptor (244, 248). Progestins also decreased the rate at which new receptor molecules were synthesized, so it became apparent that cellular levels of ER fell after progestin treatment due to a combination of reduced ER synthesis and increased ER degradation. The mechanisms by which progestins down-regulate ER in the uterus are being elucidated as a consequence of these studies. However, there is presently no direct evidence that progestins regulate the concentration of ER in normal breast cells, despite the fact that progestin regulation of ER in human breast cancer cells has been

demonstrated. The reduction in the synthesis of ER has been shown recently in human breast cancer cells to result from progestin-mediated decreases in the steady state levels of ER mRNA (249–252). ER down-regulation by androgens has also been described in the ZR 75–1 human breast cancer cell line (251). Progestin treatment of T-47D cells resulted in a rapid decline in the cellular levels of ER mRNA, which was detectable as early as 1 h after and was maximal 6 h after treatment (252) (Fig. 4). Progestins did not affect the half-life of ER mRNA, which suggested that the decline in mRNA levels was due to a progestin-mediated decrease in the transcription of the ER gene (252). The rapidity with which the effects of progestins on ER mRNA can be detected suggests further that the mechanism is likely to involve PR binding directly to a putative progestin responsive element in the promoter region of the ER gene.

The demonstration that progestins reduce cellular ER has generated the hypothesis that this reduction is a means by which progestins remove the influence of estradiol from the cell and thereby antagonize estrogen-mediated events including cell proliferation. Preliminary evidence suggests that progestin treatment alters cell sensitivity to estradiol (252) and it is noteworthy that reduction in ER levels preceded progestin mediated inhibition of proliferation (Fig. 4). In immature rat uterus, progesterone pretreatment was able to blunt but not eliminate the ability of subsequently administered estradiol to increase uterine wet weight (238), which supports the hypothesis that progestins attenuate cell sensitivity to estradiol by decreasing ER levels. However, more detailed analysis of this question awaits future experimentation.

B. Effects on growth factors and growth factor receptors

1. Effects on growth factor expression. In human breast cancer cells in culture and rat uterus *in vivo,* the proliferative response to estrogen is accompanied by increased expression of a number of growth factor genes that are thought to control proliferation via autocrine and paracrine mechanisms. Growth factors implicated in these types of responses include EGF, α-and β-transforming growth factors (α-and β-TGF), insulin-like growth factors I and II (IGF-I and -II), platelet derived growth factor, and fibroblast growth factor. There is accumulating evidence that α-TGF and IGF-I promote growth in these systems while β-TGF is thought to be a growth inhibitor (4).

In view of this recent emphasis on the involvement of autocrine and paracrine growth factors in the proliferative response to steroids (4, 10, 11), it is perhaps surprising that these phenomena have not been investigated more thoroughly in well characterized *in vivo* models. Although mouse uteri have been shown to express mRNA for prepro-EGF, and expression increased after estradiol treatment suggesting an autocrine role for EGF in the uterus, the effects of progestin treatment were not reported (6). An earlier study in young ovariectomized rats showed significant induction of IGF-I mRNA and protein in the uterus after estrogen administration, but again no data were presented on the effect of progestins alone or in combination with estrogen (5). Interestingly, the well-characterized hemopoetic growth factor, colony stimulating factor-I (CSF-1), is found in high concentrations in the pregnant mouse uterus, and it has recently been demonstrated that it is synthesized in the luminal and glandular epithelium under the regulation of estradiol and progesterone. It has been postulated that uterine CSF-1 regulates placental trophoblast proliferation and differentiation via a paracrine mechanism (253). More recently another cytokine, γ-interferon, has been shown to inhibit the proliferation of both normal and neoplastic human endometrial cells *in vitro.* However, it is unclear at this stage how interferon interacts with estrogen and progesterone to control endometrial proliferation (254).

Murphy and co-workers (255) were the first to examine steroidal regulation of EGF gene expression in human breast cancer cells. Using a cDNA probe to the human EGF precursor they demonstrated EGF mRNA in T-47D, ZR-75, and MDA-MB-468 but not in BT 20, MDA-MB-231, or HBL 100 cells. Furthermore, it was shown in T-47D and ZR-75 cells that EGF expression was regulated by progestins in a steroid-specific, time- and concentration-dependent manner. Progestin induction of EGF mRNA was inhibited by the antiprogestin, RU 486. Although this increased gene expression was associated with the presence of high mol wt 40 and 18 K peptides in the conditioned medium of T-47D cells that could be immunoprecipitated with antibodies to human EGF and its precursor, fully processed 6 K EGF was not detected either in the conditioned medium or the cell lysate. The authors stated that, under the experimental conditions employed, the known growth-inhibitory effects of progestins on T-47D cells were difficult to reconcile with progestin stimulation of growth factor synthesis. The possibility was raised that such a phenomenon could be a compensatory response to growth inhibition by progestins. If this were so, it is not a mechanism shared with other growth inhibitors of T-47D cells, *e.g.* antiestrogens and RU 486, and would appear inconsistent with the known ability of exogenous EGF to partially reverse the growth-inhibitory effects of both progestins and antiestrogens in these cells (256–259). Since the biological activity of the high mol wt forms of EGF produced by the T-47D cells is unknown, it is not yet possible to determine whether this interesting phenomenon is im-

portant in progestin control of human breast cancer cell proliferation.

The same group also investigated the effects of progestins on α- and β-TGF gene expression in T-47D cells (260). Like EGF, the expression of α-TGF in this cell line was increased in a time- and concentration-dependent manner by progestins and was inhibited by RU 486. Other steroid hormones were without effect. In contrast, the abundance of β-TGF mRNA was decreased in a time- and concentration-dependent manner by progestins, but the relevance of this to any autocrine role is unclear, in view of an inability to detect high affinity β-TGF receptors on T-47D cells. The authors concluded that these data are not consistent with α- and β-TGF functioning directly as autocrine modulators of progestin-induced growth inhibition (260) as had been previously suggested for antiestrogens (4). However, these data need to be reinterpreted in the light of new information on progestin stimulation of growth (152, 153) and cell cycle progression in human breast cancer cells.

Similar experiments have been undertaken in this laboratory except that cells were treated with progestins while still in exponential growth; in the studies of Murphy *et al.* (255, 260) regulation experiments were conducted with confluent cultures. In agreement with the previous findings (260) we observed a progestin-specific induction of α-TGF mRNA in T-47D cells; the effect, however, was much more rapid, being first apparent at 30 min, maximal at 6 h, and declining thereafter (261). Such a result raises the question of the relationship, if any, of this transient increase in α-TGF mRNA to the rapid progression of a proportion of T-47D cells into S phase, which was observed 12 h after progestin treatment (Fig. 4).

There are a number of problems in attempting to draw conclusions on how these data on progestin regulation of growth factor gene expression (255, 260) relate to potential autocrine mechanisms of progestin-mediated growth responses. Clearly, a number of regulatory steps occur between mRNA production and the appearance of biologically active growth factor, *e.g.* RNA processing, translation, posttranslational modification, secretion of propeptides, and cleavage to the active product. Thus progestins could modulate the level of gene transcription without a comparable effect on the biologically active and available peptide. Until such data are available the relationship between progestin control of growth factor gene expression and autocrine regulation of growth will remain unclear. However, what is clear from the limited data currently available is that the situation for progestins is much more complex than that for estrogens and antiestrogens, where growth stimulation by estrogen is associated with increased concentrations of biologically active IGF-I and α-TGF, which appears to reflect transcriptional activation of these genes. Antiestrogens appear to have the opposite effect (4).

2. Effects on growth factor receptor expression. Two of the most potent mitogens for human breast cancer cells *in vitro* are insulin and EGF (262–265). Since responsiveness to growth factors can be potentially modulated by both the concentration of the growth factor and its receptor, progestin regulation of growth factor receptor levels may be important in progestin-mediated growth responses.

Horwitz and Freidenberg (144) reported that growth inhibition of the T-47Dco cell line by progestins was associated with an increase in insulin receptor levels as assessed by radioligand binding. Investigation of the relationship between insulin receptor levels and growth in untreated and R 5020-treated cells demonstrated that elevation in insulin binding was most marked (4- to 5-fold) during the first 3 days of treatment, when R 5020-treated cells were completely growth arrested, but thereafter levels gradually declined to about twice control as growth resumed at a new steady state. The implications of these data are unclear, although the authors raised the possibility that an increase in insulin receptors was a generalized effect of a decreased growth rate (144).

Similar increases in cell surface receptor binding after progestin treatment were noted in MCF 7 and T-47D cells when lactogenic and EGF receptors were studied (162, 266, 267). It was subsequently shown that this effect of progestins could be accounted for by a progestin-induced increase in EGF-R gene expression (268, 269). As with the effect on insulin receptor, the induction of EGF-R levels was not sustained (257). The initial suggestion (162) that progestin increases in EGF-R and insulin receptor number could sensitize the cells to growth factor action appears unlikely, in view of the recent observation of the lack of shift in the dose-response to insulin and EGF in progestin-treated cells (257).

Increases in EGF-R levels in the immature rat uterus have been reported after estrogen administration (270), and, in the mature animal, changes in EGF-R levels parallel changes in plasma estrogen levels and the amount of nuclear ER (271). Thus, in different experimental systems, *i.e.* human breast cancer cells and rat uterus, increases in EGF-R are accompanied by either steroid-induced decreases or increases in cell proliferation. Such data indicate divergent regulation of growth and EGF-R expression by sex steroids in different species and/or target tissues.

3. Modulation of progestin effects by growth factors. Further evidence for an intimate association between progestins and growth factors in the control of breast cancer cell proliferation comes from a series of recent papers

demonstrating modulation of the antiproliferative effects of progestins by insulin and EGF (257–260, 272, 273). These studies build upon earlier observations that the sensitivity of breast cancer cells to the antiproliferative effects of antiestrogens could be reduced by simultaneous treatment with insulin or EGF (256, 258). Based on the knowledge that PR is a substrate for EGF-R and insulin receptor tyrosine kinases *in vitro* (274, 275), Sarup *et al.* (257) investigated the effects of EGF on PR levels in breast cancer cells. These studies provided evidence for an EGF-mediated decrease in PR binding that may account for the EGF attenuation of progestin-induced inhibition of cell growth. Insulin was also shown to decrease PR binding (257). To date there are no published data describing the mechanism of this down-regulation. A similar EGF-induced down-regulation of GR in HBL 100 breast cancer cells was associated with phosphorylation of GR, suggesting a direct action of EGF-R kinase on steroid receptors (276).

When T-47D cells were stimulated to proliferate in serum-free medium with either EGF or insulin, both peptides were able to attenuate progestin-induced growth inhibition in a concentration-dependent fashion, with complete attenuation of the inhibitory effect of R5020 at 0.1 nM and 1 μM EGF and insulin, respectively (257). It should be noted, however, that in these experiments cells were harvested after only 1–1.5 population doublings of control cells. The observation that nanomolar concentrations of insulin caused a half-maximal decrease in PR binding, while micromolar concentrations were required to antagonize progestin-induced inhibition of cell growth, argues against a causal relationship between these effects. The finding that insulin reversal of progestin-induced cell growth inhibition was only seen in serum-free medium, while the comparable EGF effect was apparent either in the presence or absence of serum, suggests that the two factors antagonize progestin action via different mechanisms (257). The latter suggestion agrees with the studies of Koga *et al.* (258), where the effects of insulin and EGF alone, or in combination, on progestin-induced growth inhibition were investigated in medium supplemented with fetal calf serum. When EGF and ORG 2058 were added simultaneously to exponentially growing cultures, EGF attenuated the growth-inhibitory effect of ORG 2058 in a concentration-dependent manner over a concentration range that indicated that the effect was EGF-R mediated. At maximal effective concentrations EGF restored the growth rate to midway between control and ORG 2058-treated cultures. Insulin failed to modulate ORG 2058-induced inhibition but acted synergistically with EGF to attenuate the reduced growth rate. These data support the view of Sarup *et al.* (257) that insulin and EGF modulate sensitivity to progestins by different mechanisms, a conclusion that is further supported by the differential effects of insulin alone or in combination with EGF on the attenuation of the response to antiestrogens in the same cells (258). These data are interpreted as being compatible with the autocrine hypothesis of breast cancer cell replication, in that if progestins inhibited breast cancer cell cycle progression in G_1 phase by inhibiting autocrine growth factor production and action, then the effect would be expected to be attenuated by addition of exogenous growth factor, which was indeed the case. However, alternative mechanisms, particularly those involving growth factor modulation of steroid sensitivity via changes in steroid receptors and vice versa, are also likely (258). Further data on the interactions between growth factors and steroid hormones in the control of breast cancer cell replication are urgently required.

C. Effects on estrogen synthesis and metabolism

A potential mechanism for progestins to modulate the effect of estrogens on cellular replication is by regulating the concentration of the active estrogen, 17β-estradiol, thereby reducing the cellular concentration of this ligand available for high affinity interaction with the ER. This could be accomplished by progestin inhibition of the rate of estradiol synthesis and/or stimulation of the rate of estradiol inactivation to less potent metabolites. The principal mechanism by which estrogen inactivation takes place is by conversion to estrogen metabolites with reduced or absent affinity for ER, and evidence discussed below supports a role for progestins in this process. Oxidation of estradiol to estrone, which has an affinity for ER only 30% of that of estradiol, is the major route of estradiol metabolism, particularly in the uterus, and is usually accompanied by sulfurylation to form water-soluble estrogen sulfates, which have no affinity for ER and are rapidly excreted from the cell. Dehydrogenase and sulfotransferase activities are accompanied by glucuronidation reactions in tissues involved with serum detoxification such as the liver, and in these cases the latter two activities are predominant. However, in target tissues for sex steroid hormones such as the uterus and breast, glucuronidation is a minor pathway of estrogen metabolism and will not be discussed further. Inhibition of estrogen synthesis is thought to be a major effect of treatment of postmenopausal breast cancer patients with MPA. Evidence that high dose MPA suppresses the hypothalamo-pituitary-adrenal axis and, in turn, the synthesis of adrenal androgens, supports this conclusion (277). A further mechanism that is likely to be important in regulating estrogen concentration is the activity of the enzyme aromatase, which catalyzes estrogen formation from androgen precursors, and this is discussed briefly.

1. Estradiol 17β -hydroxysteroid dehydrogenase. Estradiol

17β-hydroxysteroid dehydrogenase activity was demonstrated in the mammalian uterus in the 1960s. It was shown that its activity in this tissue was low during the proliferative phase of the menstrual cycle, when serum estradiol levels were high, and increased dramatically when serum progesterone levels increased during the luteal phase of the cycle (13, 278, 281). This suggested that estradiol oxidation to the less active estrone may have a role in the conversion of proliferative to secretory endometrium, and further implicated progesterone in this process. This hypothesis was tested *in vitro* in the mid-1970s, when it was demonstrated in perfusion experiments that estradiol was rapidly converted to estrone in secretory endometrium (280). The involvement of progestins in the oxidation of estradiol was first demonstrated at that time, when it was shown that culture of human proliferative endometrial explants with progesterone or the synthetic progestin norgestrel resulted in a dramatic increase in estradiol dehydrogenase activity (282). This increase was noted as early as 7 h after treatment, and was shown to be dependent on ongoing transcription and translation. The location of estradiol dehydrogenase in the endometrium was investigated by ultrastructural and biochemical methods, which showed that the highest concentration of the enzyme was found in epithelial cells (283, 284). It was further shown that the ability of progestins to increase estradiol dehydrogenase was restricted to the glandular epithelium of the endometrium, and was not evident in stromal tissue, and this was attributed to the higher concentration of PR in epithelial *vs.* stromal cells (285, 286).

The demonstration *in vitro* of the ability of progestins to increase the activity of estradiol dehydrogenase in the normal proliferative endometrium was followed by an examination of the activity of this enzyme in endometrial carcinoma tissue (124, 287, 288). Estradiol dehydrogenase was shown to be present in biopsy material, and its activity was markedly higher in patients who had received progestin therapy (such as MPA). The increase in estradiol dehydrogenase after therapy was related also to the PR status of tumors.

The role of estradiol dehydrogenase in estradiol oxidation, and the action of progestins in stimulating this activity in the uterus, became widely acknowledged. It was also shown that estradiol dehydrogenase activity was highest in normal breast tissue in the early secretory phase of the cycle (289). The implication that progestins increased the enzyme activity in the breast was not tested directly until the development of *in vitro* culture systems for normal mammary cells, and it was shown that progestins stimulated the activity of the enzyme in the epithelial but not the fibroblast, or stromal, components of the breast (290), confirming the observations made in the uterus. The progestin-mediated increase in estradiol dehydrogenase could be prevented 19 h after treatment by both transcriptional and translational inhibitors, but only by translational inhibitors 6 h after treatment (112), perhaps suggesting that although the progestin effect was mediated transcriptionally, the half-life of the estradiol dehydrogenase mRNA was long.

Estradiol dehydrogenase activity was shown to be higher in breast carcinoma tissue from premenopausal patients in the luteal phase of the menstrual cycle, and in postmenopausal patients who had been treated with progestins such as lynestrenol (291). The ability of progestins to increase estradiol dehydrogenase was restricted to patients whose tumors were PR positive.

The necessity for prior tissue/cell exposure to estradiol before progestins were able to increase estradiol dehydrogenase activity has been established in a number of studies and presumably reflects the necessity for PR synthesis to take place before progestin responsiveness can be manifested. In the endometrium it has not been shown directly that estradiol pretreatment is required, but there is evidence that very early in the cycle, *i.e.* before maximal estradiol effects are apparent, proliferative tissue in normal endometrium responds poorly to progestins in increasing estradiol dehydrogenase (282). Furthermore, endometrial carcinoma tissue almost universally contains ER, yet only those tumors in which PR is also present (presumably reflecting estrogen influence) respond to progestins with an increase in estradiol dehydrogenase (287). In the breast it has been shown directly in culture that estradiol pretreatment of normal mammary cells was required before progestin-induced increase of estradiol dehydrogenase could be observed (112); moreover as mentioned above, progestin increase of estradiol dehydrogenase was apparent in breast tumors that contained PR. Taken together, the available evidence in the uterus and breast support the necessity for estrogen pretreatment before the stimulatory effects of progestins on estradiol dehydrogenase activity can be manifested.

Estradiol dehydrogenase is now known to consist of a number of isoenzyme forms (292) which may be regulated differently in various tissues and, indeed, within the same tissue or cell in various metabolic states. It has also been demonstrated in a number of studies that the tissue concentrations of estrogens do not mirror the serum concentrations, particularly in postmenopausal women (293, 294). Despite the predominance of estrone in postmenopausal serum, estradiol is the principal estrogen measured in endometrial and in breast cancer tissue, suggesting either that a concentration gradient of estradiol was maintained against the serum, or that serum estrone was metabolized to estradiol by estradiol dehydrogenase in these tissues. This has resulted in a reexamination of the activity of estradiol dehydrogenase and

its significance in the regulation of active estrogen levels. In breast tumor tissue, where the activity of estradiol dehydrogenase was measured under identical assay conditions, the estradiol oxidative pathway was more rapid than the reductive pathway, and the affinity of the enzyme for estradiol and estrone was the same, suggesting that under optimal conditions, the formation of estrone would predominate (294). In studies examining the effect of progestins on estradiol dehydrogenase, the oxidative pathway has usually been examined. However, more recent studies using the human breast cancer cell line MCF 7 have shown that progestins are able to stimulate both oxidative and reductive activities equally (150). The inference is then that the fate of tissue estrogens is acutely dependent on the substrate and cofactor milieu within the cell, presumably favoring the oxidative pathway under some conditions and the reductive in others. The implications of this in determining the role of estradiol dehydrogenase in regulating tissue availability of estrogens in general, and the part played by progestins in particular, need further examination in the light of these observations. Nevertheless, oxidation of estradiol to estrone, when it occurs in well-characterized instances such as in the endometrium, appears effective in reducing the concentration of estrogen available for binding to the estrogen receptor (295), a process that is augmented greatly by progestins.

2. Estrogen sulfotransferase. Sulfated metabolites of estrogen are increased in the human uterus and in the serum in the secretory phase of the cycle (278), in parallel with the formation of estrone by estradiol dehydrogenase, and these water-soluble metabolites fail to bind to ER and are rapidly excreted from the cell. Sulfurylation is therefore a most effective mechanism whereby estrogen-mediated effects on proliferation can be attenuated. Organ culture experiments have shown that estrogen sulfotransferase activity, like estradiol dehydrogenase, is increased by progestins in the human endometrium (296, 297). Unlike estradiol dehydrogenase, however, the concentration of estrogen sulfotransferase in the proliferative phase of the cycle is undetectable, and this enzyme is therefore under much tighter control by progestins. Very little if anything is known about the activity of this enzyme in the breast, and most of the present information has been derived from studies in the mammalian uterus. The relative importance of the sulfurylation *vs.* oxidative/reductive pathways of estrogen metabolism in determining the ultimate fate of cellular estrogens is presently unclear, although a recent study *in vivo* using inhibitors of estrogen sulfotransferase suggested that sulfurylation of estrogens reduced the pool of available estrogens for binding to the ER (298).

3. Aromatase. The ability of breast tumors to aromatize androgen precursors to estrogens has been well established and has been proposed as a mechanism that contributes to the etiology of breast cancer in postmenopausal women. It may also provide another explanation for the previously mentioned high concentration of estradiol in tissues from these women. The effect of progestins on aromatase activity has been examined and there is evidence that progestins stimulate this activity in endometrial stromal cells without having any effect on the activity in epithelial cells (299). There is also evidence that aromatase activity measured in normal endometrium was lower in tissue from women in the secretory phase of the cycle (300, 301), which may suggest that aromatase activity is inhibited by progestins. Progestins also appear able to inhibit aromatase activity modestly in cultured breast carcinoma cells (302). Clearly, the role of progestins in inhibiting estrogen formation by the aromatase enzyme requires further clarification.

D. Effects on cellular differentiation

The acquisition of specialized functions and the inability to proliferate are among the marks of terminally differentiated cells, and the induction of cellular differentiation is an acknowledged mechanism through which progestins act on estrogen-treated cells. The ability of progestins to differentiate cells after estrogen treatment is therefore one of a series of likely pathways through which progestins inhibit the stimulatory effects of estrogens on cellular replication. There is a vast literature on the effects of progestins on a variety of metabolic processes, including progestin stimulation of triglyceride biosynthesis and fatty acid synthetase in human breast cancer cells (157, 303), but the evidence that these effects lead to cellular differentiation is not clearcut. However, the differentiating effects of progesterone on human uterine physiology in preparation for implantation of the fertilized ovum are well known. In the human uterus, progestins induce glandular epithelial secretory activity and decidual transformation of stromal fibroblasts. The differentiating action of progestins on the human endometrium is terminal: if implantation does not occur, the tissue is shed and endometrial renewal from the basal portion of the endometrium takes place.

1. Progestin effects on endometrial secretory activity. Endometrial secretory products produced in response to progestins are important in the conversion of the endometrium into an hospitable environment for the implanting blastocyst. The production of secretory products by epithelial cells represents a well defined circumstance in which progestins induce differentiation of epithelial cells. Secretory proteins represent a major epithelial product in progestin-treated endometria, and progestin control

of such proteins has been demonstrated directly in rabbit (uteroglobin) and porcine (uteroferrin) uteri. In the pig, in particular, which has noninvasive placentation, uteroferrin is thought to play a key role during pregnancy in nourishing the developing conceptus. Uteroglobin from the rabbit uterus has been cloned and sequenced, and progestins have been shown to regulate its concentration by a transcriptional mechanism [(304); reviewed in Ref. 14]. Pig uteroferrin has recently been cloned, and progestins have been shown to increase the steady state concentration of its mRNA (305). In the rabbit and the pig therefore, it is clear that progestins act directly to promote secretory capacity of the uterus.

In the human, histochemical analysis has shown that glycogen production and secretion by epithelial cells was high in the secretory phase of the cycle. Progestin involvement in transcriptional regulation of this event has yet to be demonstrated. However, organ culture of human endometrial explants from women in the proliferative phase of the cycle showed that progesterone treatment consistently increased tissue glycogen content (306). The addition of estradiol in combination with progesterone did not enhance the effect further, suggesting that the tissue was fully estrogen primed at the time of progesterone treatment. The increase in tissue glycogen content after progesterone exposure in the secretory phase of the cycle has been shown to be due to increases in the activities of glycogen synthetase and glycogen phosphorylase enzymes, which reached their maximal activity in the midsecretory phase (307).

2. Progestin induction of stromal decidualization in the human uterus. Decidualization is described as the progesterone-mediated differentiation of small fibroblast-like stromal cells into large epithelioid decidual cells. This process begins late in the menstrual cycle, on or around day 23, and is accompanied in fertile cycles by the implantation event. Decidualization is accompanied by an increase in the uterine production of decidual PRL (dPRL), which has been shown to be identical to pituitary PRL and is a marker of progestin-induced tissue differentiation. PRL produced in decidual cells is found in high concentration in amniotic fluid, presumably after traversing the chorion, and is thought to be involved in osmoregulation and preservation of amniotic fluid volume.

In order to study the involvement of progesterone in stromal decidualization and dPRL production, organ cultures of human endometrium were employed. That dPRL was a product of secretory tissue was shown in 2 ways. First, proliferative tissue alone was incapable of dPRL synthesis but could be induced to produce dPRL after 2 days of culture in the presence of progesterone (308, 309). Second, secretory tissue, even when cultured in the absence of progesterone, produced dPRL although exogenous progesterone was required to maintain dPRL secretion beyond 3 days of culture. Production of dPRL from tissue grown in organ culture in the presence of progesterone was accompanied by morphological indications of stromal decidualization (308, 309). PRL production was maintained during several weeks of continuous progesterone exposure after which the tissue was composed almost entirely of decidual cells, the glandular epithelial elements having largely disappeared (309). Continued secretion of dPRL was dependent on the continued presence of progesterone; withdrawal of the steroid from organ cultures of secretory endometrium resulted in continued synthesis of dPRL for several days, due presumably to the long half-life of the dPRL mRNA, followed by a progressive decline in synthesis to unstimulated levels. Readministration of progesterone at this point resulted in a second wave of PRL synthesis (308). The influence of estrogen on the stromal decidualization process has not been closely examined, but there is evidence that concomitant administration of estradiol and progesterone delays and even inhibits decidualization (310), and inappropriately high estradiol levels in the secretory phase have been implicated in the dysharmonic luteal phase syndromes and resultant reproductive disorders.

VI. Conclusions

A. Effects of progesterone on cellular proliferation in vivo

It is abundantly clear that the effects of progesterone on cell proliferation in general, and on estrogen-mediated cell proliferation in particular, are diverse. They vary among the different cell types of the uterus and mammary gland within a single animal, in the same cell types in different species, and even between species in the various physiological states characterizing the female reproductive cycle.

In the uterus, there are marked differences in proliferation in epithelial and stromal elements. However, the growth-inhibitory actions of progesterone in this organ are largely consistent both with the known distribution and regulation of ER in the cell types comprising the uterus, and with the likely mechanisms, discussed in *Section V*, by which progestins modulate cell proliferation. Progestins have been shown in the human to down-regulate ER in epithelium, stroma, and myometrium, in agreement with the ability of progestins to inhibit estrogen-mediated proliferation in all the cell types of the uterus. Furthermore, progestins increase estradiol dehydrogenase and estrogen sulfotransferase activities which, in the cellular microenvironment, would be expected to reduce the concentration of estradiol available for binding to ER. Progestin induction of epithelial secretory

activity followed by secretory exhaustion combines with these effects on ER and estrogen metabolizing activities to completely remove the influence of estrogen from the epithelium and induce terminal differentiation. In this regard both the action of progesterone in opposing estrogen-mediated events, and the likely mechanisms by which this action takes place, are easily explained by the available experimental and biological evidence. The known effectiveness of progestin therapy on a proportion of endometrial carcinomas can also be explained by invoking progestin action via the same regulatory mechanisms, particularly in well-differentiated malignancies.

In contrast with the epithelium, the uterine stroma has a unique response to estrogen and progesterone, due in no small measure to its specialized role in the development and maintenance of pregnancy. In the adult animal the stroma, like the epithelium, proliferates in response to estrogen, but in the rodent the response to estrogen can only occur after progesterone priming. The zona basalis of the monkey endometrium proliferates under the influence of progesterone, and it is postulated that this causes multiplication of stem cells responsible for endometrial regeneration. Furthermore, in the human the stroma proliferates during the secretory, progesterone-dominated, phase of the cycle in preparation for blastocyst implantation and eventual transformation of the stroma into the maternal compartment of the placenta.

It is important to note that where progesterone stimulates proliferation in the uterus or oviduct, with the notable exception of the rabbit uterus in which progesterone is clearly the main drive to proliferation, the proliferation that ensues is usually transient. This proliferation differs from the sustained proliferative action of estradiol, and may be required to "prime" cells in some way to respond to the differentiating effects of progesterone, PRL and/or other hormones. In this way progesterone, by inhibiting estradiol-mediated proliferation and either priming cells for differentiation or acting directly as a differentiating agent, is likely to be the main factor that commits the uterus to prepare for pregnancy.

The growth-stimulatory action of progesterone in the stroma of the human uterus is consistent with the known regulation of PR. Growth stimulation is observed in the face of high circulating progesterone concentrations, and it has been shown that although PR levels are decreased markedly by progesterone in epithelial cells, the reduction observed in the stroma and myometrium is transient and modest in magnitude. There is also growing evidence that PR is not down-regulated in a variety of situations in which sustained progesterone exposure may be associated with continued responsiveness to progesterone. This demonstrates that PR is maintained in cells that maintain progesterone responsiveness.

In the mammary gland, estradiol and progesterone both stimulate proliferation but in different morphological elements. While estradiol is responsible for the proliferation of the epithelial end bud and ductal systems, progesterone causes ductal side branching and lobuloalveolar development. Because lobuloalveolar development is required for milk production, progesterone can be considered to have the same role in the mammary gland as in the uterus, namely the commitment of the organ to its differentiated function. It is also apparent from studies in the mouse that the proliferative effect of progesterone in the mammary gland is transient, as appears to be the case in the uterus, although the effect of sustained progestin exposure on long term proliferative activity in the human breast has yet to be determined. In contrast with the uterus, however, progesterone does not have a dramatic inhibitory effect on estrogen-mediated proliferation and may even potentiate the action of estrogen. Accordingly, the generally accepted view that progesterone acts by opposing estrogen action is not easily reconciled with the effects of progesterone on the mammary gland in general, but may be of importance in particular cell types such as ductal epithelium and thus perhaps ductal carcinoma of the breast.

It is difficult to reconcile the proliferative effects of progesterone on the breast and mammary gland *in vivo* with the known effectiveness of progestins in the treatment of patients with PR-positive breast cancer without invoking such cell type specificity of action. Furthermore, the mechanisms by which progesterone exerts its effects in the mammary gland are not clearcut, and there is little immunohistochemical evidence either that ER and PR are present in the epithelial cells that respond to estrogen and progesterone or that the regulation of these receptors in normal cells takes place within the accepted model defined in the uterus. There is also no strong evidence that potential mechanisms of progestin action discussed in *Section V* pertain to the mammary gland or to the normal human breast. It is clear therefore that the mechanisms by which progestins affect cell proliferation in the breast are not evident from the *in vivo* literature and must be deduced from an analysis of the effects of progestins *in vitro*.

B. Effects of progestins on cellular proliferation in vitro

In agreement with studies conducted *in vivo*, treatment of progesterone target tissues with progestins *in vitro* has resulted in both growth-inhibitory and growth-stimulatory effects. In concordance with studies *in vivo* the response is cell type specific. The effects on the uterus are the most straightforward, in that progestin treatment of normal and neoplastic tissue from a number of different species in both organ and cell culture confirmed the

predominant inhibitory effect of progestin on uterine epithelial cell proliferation. The limited studies on uterine stromal cells *in vitro* tend to support observations made *in vivo*.

Progestin enhancement of proliferation has been observed most consistently in organ culture of normal and neoplastic rodent mammary gland, cell culture of rat mammary carcinoma, and, less consistently, in normal and neoplastic breast cells and human breast cancer cell lines growing in an estrogen-free environment. Potential species differences in the cellular origin of mammary carcinoma and endocrine control mechanisms make it difficult to formulate rational hypotheses on the molecular basis of these stimulatory effects in most systems studied. However evidence for a direct stimulatory effect comes from studies on T-47D cells, where, in an estrogen-free environment, progestin induces a transient increase in cell cycle progression. Under such circumstances, a significant proportion of the cells traverse the cell cycle at an accelerated rate and complete a round of replication, before succumbing to the ultimate growth-inhibitory effects of the agent in this experimental system (Fig. 4). This mechanism may have parallels with the transient stimulation of a single round of replication after progestin treatment in a number of different target tissues *in vivo*. The molecular basis for this effect is not clear at this time, but probably involves the progestin-induced transcription of specific genes involved directly or indirectly in the control of cell cycle progression (Fig. 5). The recent demonstration that progestins increase expression of α-TGF, a proposed autocrine growth factor for human breast cancer, adds support to such an hypothesis. Whether this increased production of α-TGF is the stimulus for accelerated cell cycle progression, or the consequence of an earlier progestin-induced effect on the cell cycle, remains to be elucidated. It is also unclear whether this *in vitro* phenomenon accounts for the proposed stimulatory effects of progesterone on human lobuloalveolar cell proliferation, particularly that which occurs at the end of each menstrual cycle.

Interestingly, the direct growth-stimulatory effects of progestins on human breast cancer cells have only recently been documented due, presumably, to the use of estrogen-free culture media. When the same cell lines are grown in the presence of estrogen or under other culture conditions that give near maximal growth rates, the stimulatory effects of progestins are masked, due probably to maximal stimulation of cell cycle progression, and the well documented growth-inhibitory effects of progestins predominate. This interpretation is compatible with an emerging view that cell cycle progression in human breast cancer cells is regulated by multiple redundant pathways (Fig. 5).

The inhibition of cell proliferation by progestins is accompanied by specific changes in cell cycle kinetic parameters that have been clearly defined both *in vivo* and *in vitro* and point to specific inhibition/arrest of cell cycle progression at a point early in G_1 phase. A hypothetical model to explain the known effects of progestins in both stimulating and inhibiting cell cycle progression in human breast cancer cells in culture is presented in Fig. 5. In this model the rate of cell cycle progression is controlled by the product of a hypothetical gene termed "START"[1], the transcription of which is positively regulated by a number of known mitogens for breast cancer cells including estrogens, serum, insulin, EGF and α-TGF. Transcription of this gene may be regulated directly by these factors or indirectly by the products of other specifically induced genes. A direct effect of progestin via PR and a progestin response element (PRE) is favored because of the more rapid onset of accelerated cell cycle progression observed after progestin treatment when compared with estrogen or serum (Musgrove and R. L. Sutherland, unpublished observations). The net effect of increased levels of the "START" gene product is to increase the rate of progression through the cell cycle due to a marked reduction in the G_1 transit time. These cells, however, complete only one round of replication because, as they reenter G_1 phase, their further progression is arrested by the action of another progestin-induced gene product termed "STOP" which acts early in G_1 phase to inhibit further cell cycle progression.

This model accommodates most known effects of steroids, steroid antagonists, and growth factors on breast cancer cell proliferation *in vitro* and many of the known effects of progestins *in vivo*. It proposes that stimulation by a number of diverse agents is mediated by multiple redundant pathways that converge on the transcription of START. The inhibitory effects of antiestrogens and antiprogestins would then be mediated via inhibition of the synthesis of START in contrast to progestin action via STOP. This would account for the lack of a transient increase in cell cycle progression before growth arrest and the earlier accumulation of cells in G_1 phase (Musgrove E. A., and R. L. Sutherland, unpublished observations) of these antihormonal agents.

In contrast to the control of START by multiple factors, the STOP gene is postulated to be under the direct and specific control of progestins. Since it acts very early in G_1, it will counteract the potential influence of any signal that occurs later in the cell cycle to stimulate progression, *e.g.* START. This would explain the ability of progestins to inhibit/arrest proliferation that had previously been initiated by other factors including

[1] This gene has functions in common with the 'cell division cycle' genes involved in the regulation of the event in the yeast cell cycle described as 'start' but is not necessarily homologous with any of these yeast genes.

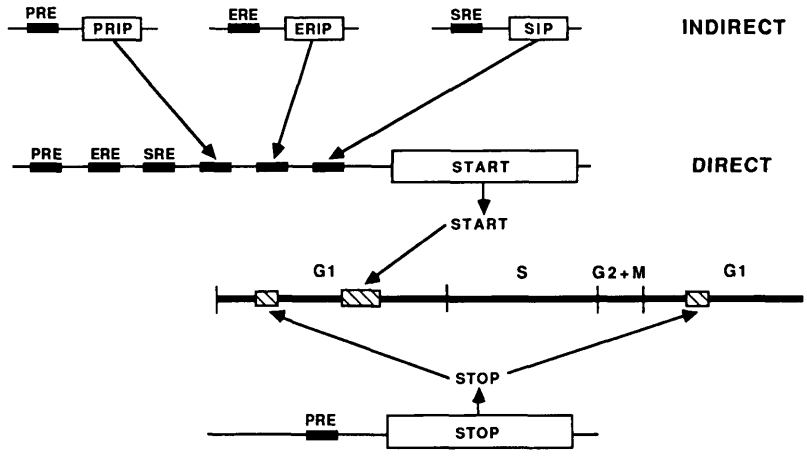

FIG. 5. A hypothetical model of the effects of progestins on cell cycle progression in human breast cancer cells *in vitro*. The rate of cell cycle progression is controlled by the product of a hypothetical gene termed "START" whose promoter activity is regulated directly (PRE, ERE, SRE) and/or indirectly (PRIP, ERIP, SIP) by progestins, estrogens and other serum factors including growth factors. The point of action of START within the cell cycle is beyond the point early in G_1 phase where the hypothetical STOP signal arrests or inhibits cell cycle progression. The activity of the STOP gene product is regulated transcriptionally by a PRE in the promoter region of this gene. PRE, Progestin response element; ERE, estrogen response element; SRE, serum response element; PRIP, ERIP, and SIP are the products of genes induced by PRE, ERE, and SRE, respectively, and are able to bind to, and regulate the activity of the START promoter.

estrogen, insulin, and serum. It appears likely that the STOP gene encodes a differentiation factor that removes the cells from the cell cycle.

It is envisaged that the progestin control of START and STOP is independent, although it is possible that in some cell types both genes are not active, and only one of these mechanisms is operative. Thus, in some cell types *in vivo* progestin could induce a sustained proliferative effect via continued synthesis of START without a concurrent signal to differentiate, while in other cell types the progestin-induction of STOP would invoke growth arrest and differentiation irrespective of the stimulus to proliferate.

C. Future directions

The ability of progestins to both stimulate and inhibit cell proliferation *in vivo* has important implications in the long-term use of these agents in the human for therapy or prophylaxis. It is critical therefore to determine exactly which cells in the breast are stimulated to grow by progestins and, more importantly, whether progestins are able to cause sustained proliferation in these cells. It is also essential to an understanding of progestin action to determine whether the transient proliferation induced by progestins is a prerequisite ("priming" effect) for full expression of the differentiating capacity of progestins.

The presence or absence of ER and PR, particularly in the various cell types of the breast, urgently needs to be evaluated as a first step in determining whether the effects of progestins on the cells that proliferate are likely to be direct or mediated by paracrine mechanisms.

The formulation of a model for the control of cell cycle progression by progestins *in vitro* necessitates the further critical evaluation of its predictions and detailed experimental analysis of its universal applicability to a range of progestin-sensitive cell types. Identification of a cell cycle control gene, with the properties of START and under the control of the regulators proposed in Fig. 5, is an essential part of evaluating this model. Recent major advances in our understanding of the molecular mechanisms of cell cycle control help to identify potential candidate genes, which include the cell cycle division genes (cdc genes) and their regulators, proto-oncogenes, and autocrine growth factor genes. Similarly, the identification of the STOP signal, which may well be a signal for terminal differentiation, is of critical importance. At this time, there are no obvious candidates for this function.

Recent major advances in cell culture techniques have facilitated the study of growth control and differentiation in a wide range of normal and neoplastic tissues in a chemically defined, serum-free environment. The use of such techniques to evaluate the effects of progestins on a variety of cell types, which are known to have diverse responses to progestins *in vivo*, will determine whether or not the stimulatory and inhibitory effects of progestins are always expressed together. Differential expression of the stimulatory and inihibitory pathways in different cell types may help to explain the apparently paradoxical effects of progestins on cell proliferation in different target tissues.

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