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13. ABSTRACT (Maximum 200) Both the growth and the development of the mammary gland are controlled by the female hormones estrogen and progesterone, and by interactions between the epithelial and stromal compartments of the breast. Changes in the regulation of any of these processes may lead to breast cancer. Therefore, we need to understand in detail the mechanisms that control proliferation and development of the mammary gland. We have investigated the role of progesterone in the process of sidebranching and alveologenesis in the mammary gland using mice lacking the progesterone receptor which are defective in these processes. By reconstituting murine mammary glands in vivo, we have shown that the progesterone receptor is required only in epithelial cells for proper sidebranching to occur. Our studies suggest that progesterone acts in a paracrine manner and more recent data suggests that Wnt-4 is a possible mediator of this paracrine effect. In parallel studies, we have observed that breast cancer cells can dramatically downregulate the expression of hepatocyte growth factor in cocultured fibroblasts. These results suggest an important role of paracrine interactions also in breast cancer development. In addition, we have characterized the role of the estrogen receptor in regulating the proliferation of breast cancer cells. We postulate that the ability of estrogen receptor to control cyclin D1 expression and proliferation of breast cancer cells may be acquired during breast cancer development. In conclusion, we have studied the role of female hormones and stroma-epithelial interactions in regulating mammary gland development and tumorigenesis. Understanding how these pathways are altered during breast cancer development may lead to new forms of breast cancer therapy.				
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All references to figures cited below refer to figures presented in papers and manuscripts that are numbered and attached to the text of this Report.

The proposed research was designed to address how two specific hormones - estrogen and progesterone - participate in normal breast morphogenesis and in the deregulated proliferation associated with mammary carcinoma cells. More specifically, the emphases in this research were on the role of estrogen in breast cancer cell proliferation and the role of progesterone in normal breast development. During the period of this supported research, we found that estrogen acts in breast cancer cells through its ability to induce cyclin D1 while progesterone acts in normal breast development through its ability to induce paracrine signals that in turn encourage ductal branching.

Estrogen signaling

While estrogen is involved in ductal elongation in the normal mammary epithelium and in the expression of progesterone receptor, its effect in mammary carcinoma cells is directly mitogenic, driving the proliferation of breast cancer cells. We examined specifically the effects of estrogen exposure on various components of the cell cycle clock apparatus.

We examined the effect of estrogen on MCF-7 mammary carcinoma cells by treating them with tamoxifen to block the actions of the estrogen receptor and used this in turn to synchronize these cells, thereby blocking them in the G1 phase of the cell cycle. We then reversed this block by addition of estrogen, accompanied by the removal of tamoxifen. This switch caused the cells to enter into the active cell cycle and to progress through its G1 phase (Fig.1, Ref.1).

Our examination of the various components of the cell cycle clock revealed that the levels of most components, including cyclin-dependent kinases (CDKs) and CDK inhibitors was unaffected by estrogen and/or tamoxifen. (Fig. 4 of Ref. 1). In contrast, cyclin D1 responded dramatically to estrogen. More specifically, extended exposure to tamoxifen caused a collapse of cyclin D1 levels, and reversal of the tamoxifen block by treatment with estrogen resulted in a 6-10 fold induction in cyclin D1 levels (Fig. 3A of Ref. 1).

The resulting increased levels of cyclin D1 had several consequences. First, it drove the initial phosphorylation of pRB, the retinoblastoma protein, through its ability to form active cyclin D1:CDK4 complexes (Fig. 2B of Ref. 1). The resulting phosphorylation in turn rendered pRB susceptible to further

phosphorylation by cyclin E-CDK2 complexes that led to the functional inactivation of pRB and the liberation of E2F transcription factors from control by pRB.

A second consequence of the induction of cyclin D1 was associated with the cyclin D1:CDK4 complexes. In the presence of tamoxifen, cyclin E:CDK2 complexes were inhibited by association with the p21 CDK inhibitor. Once the cyclin D1:CDK4 complexes formed, they were able to abstract the p21 inhibitor away from cyclin E:CDK2 complexes, liberating the latter and enabling them to complete the phosphorylation and hence functional inactivation of pRB (Fig. 7 of Ref. 1).

This work along with the research of others published at the same time provides a rationale for the ability of estrogen, acting via its receptor, to act in a mitogenic fashion to drive mammary carcinoma cell proliferation. However, it leaves at least two questions unresolved, both of which we continue to be interested in. First, how does estrogen activate the cyclin D1 promoter? Second, does this control of cyclin D1 by the estrogen receptor represent a normal, physiologic control mechanism or the acquisition by breast cancer cells of a signaling pathway that does not normally exist in normal mammary epithelial cells?

In response to the first question, we have found, as have others, that the estrogen receptor response element within the cyclin D1 promoter is maps together with the AP-1 site that is known to be used by Fos/Jun heterodimeric transcription factors to activate cyclin D1 transcription in response to mitogenic signals, specifically those funneled through the Ras-Raf-MAPK signal transduction cascade. Conversely, we were unable to find a canonical estrogen receptor response element within the cyclin D1 promoter. (M. Planas-Silva and R.A. Weinberg, unpublished observations). One response to this finding would be to postulate that the estrogen receptor is able to form transcription factor complexes with members of the AP-1 family of factors, in this way potentiating cyclin D1 transcription. For example, we have observations, still unpublished, that AIB-1, the ER-associated, steroid receptor co-activator that is overexpressed in some human breast cancers, is able to potentiate the ER-mediated activation of the cyclin D1 promoter (Ref. 3). Nonetheless, we have been unable to find direct evidence in support of complexes between the ER and the AP-1 factors, recognizing that they may well exist *in vivo* and not survive the rigors associated with the preparation of transcription factor complexes for *in vitro* analysis. An alternative mechanism, which we would like to explore in the future, is that estrogen, acting via its receptor, is able to activate the Ras-MAP kinase signaling cascade at a point high in this cascade, thereby eliciting all of the downstream responses known to be evoked by this pathway.

A second unresolved question concerns the physiologic nature of this estrogen receptor-mediated activation of the cyclin D1 promoter. Thus, an examination of the literature on estrogen in mammary morphogenesis, as mentioned above, indicates that estrogen plays two well-documented roles in mammary development. First, it causes ductal elongation early in breast development; second, it causes estrogen receptor-positive (ER+) cells to express the progesterone receptor (PR), thereby becoming double-receptor positive (i.e. ER+,PR+). In the first instance, the ER that plays a role in ductal elongation is located largely if not exclusively in the mammary stromal cells, which respond to estrogen by releasing EGF-like mitogens that proceed to drive the epithelial cell proliferation that in turn enables ductal elongation to proceed.

Significantly, in neither of these instances does estrogen act as a direct mitogen on ER+ cells. Indeed, there are reports in the literature that those epithelial cells in the normal human breast that are ER+ are not found among the subset of cells that are actively mitogenic. Together, this logic and these observations converge on the hypothesis that estrogen does not act as a direct mitogen on normal human mammary epithelial cells, and that the inception of ER+ mammary carcinomas depends upon the ability of the founding cells to acquire the ability to exploit estrogen as a direct mitogen. They may do so through their ability to couple the ER to the mitogenic signaling pathway, achieving through the ectopic expression of a signal transducing protein that is not normally expressed in mammary epithelial cells. In support of this, we have recently demonstrated that the presence of a functional ER in human cells is not, on its own, sufficient to enable estrogen-mediated induction of cyclin D1 synthesis (Ref. 2), strongly implying, as argued here, that additional changes are required in order to enable the functional coupling of the ER with the cyclin D1 transcriptional promoter. In the future we would like to determine whether, as speculated above, the ER is indeed able to directly activate the Ras-MAPK signaling pathway, and if so, whether this represents an aberration of normal mammary epithelial cell signal transduction through the ectopic expression in mammary epithelial cells of coupling proteins not normally expressed in these cells.

Progesterone signaling

Progesterone elaborated by the ovary is responsible for important morphogenetic steps that occur during the development of the ductal epithelium. We used progesterone receptor (PR) knockout mice to precisely delineate the steps that are triggered by PR activation. The initial studies on the effects of PR inactivation on breast morphogenesis were confounded by the fact that such mice have additional reproductive defects beyond those directly involved in mammary morphogenesis.

To address this problem, we transplanted PR^{-/-} mammary epithelium into the mammary fat pads of wild type host mice, thereby placing the PR^{-/-} mammary epithelial cells (MECs) in the normal hormonal environment. In doing so, we demonstrated two distinct effects on mammary ductal morphogenesis. First, ductal branching was severely compromised (Fig. 1 of Ref. 4). Second, the subsequent ability of alveoli to form was also strongly reduced (Fig. 3 of Ref. 4). We concluded that the PR acts in a PR-positive MECs, enabling them to respond to ambient progesterone by initiating the topologically related events of ductal side-branching and initiation of alveogenesis. Conversely, other experiments indicated that the PR expression in the stromal cells of the mammary fat pad played virtually no role in ductal morphogenesis (Fig. 2 of Ref. 4).

We asked furthermore whether PR needed to be expressed in the MECs that participated directly in ductal side-branching, or whether alternatively PR expression in nearby MECs sufficed to mediate the side-branching of nearby PR-negative MECs. We did this by creating chimeric mammary epithelia in which we reconstituted breasts by introducing mixtures of PR^{+/+} and PR^{-/-} into a wild type host mammary fat pad. These cells were marked by a beta-galactosidase transgene that enabled us to distinguish histologically the two cell types within the mammary epithelium.

The outcome of this exercise was clear: PR^{+/+} cells were able to help nearby PR^{-/-} to participate in ductal branching (Fig. 4 of Ref. 4). We concluded that the PR, when activated by progesterone, enables side branching by causing the PR^{+/+} cells to release a paracrine factor that proceeded to drive ductal side-branching. This provoked the further question of the paracrine factor that is responsible for ductal side-branching.

Our more recent work has revealed that Wnt-4, a protein of the Wnt signaling family, is a strong candidate for the progesterone-induced paracrine factor that acts as the morphogen inducing ductal side-branching (Ref. 5). We developed several lines of evidence to support this identification. First, we examined the ability of the Wnt-4 related protein, Wnt-1, to override the side-branching defect observed in PR^{-/-} mammary epithelia. We did this by breeding an MMTV (mouse mammary tumor virus)-driven Wnt-1 gene into either PR^{+/+} or PR^{-/-} genetic background. The MECs from the resulting females were then engrafted into wild type fat pads and their behavior upon subsequent pregnancy of the hosts was observed. We found that the expression of the Wnt-1 transgene could overcome the side-branching defect exhibited by the PR^{-/-} MECs (Fig. 1 of Ref. 5). This established that a Wnt protein is capable of eliciting a morphogenetic response that is similar if not

indistinguishable from that normally induced by progesterone. Moreover, since this response was observed even in epithelia that lacked the PR, this strongly suggested that a Wnt protein lies downstream of the PR in a signaling pathway, and that its ectopic expression can obviate the activation of the PR by its progesterone ligand (Fig. 1 of Ref. 5). Hence, the Wnt-1 protein, and by extension similarly acting Wnt proteins, could mimic the actions of the unidentified paracrine factor described above. We proved this by constructing mixed epithelia composed of MECs carrying the MMTV-Int-1 transgene and MECs carrying a transgenic beta-galactosidase marker gene. Indeed, ectopic branching of the latter was caused in the resulting chimeric breasts by the presence of nearby cells expressing the Int-1 transgene (Fig 2 of Ref. 5).

Third, while there are multiple Wnt proteins that in principle could mediate the observed side-branching during normal morphogenesis, we focused our attentions on Wnt-4 specifically, as its pattern of expression during breast morphogenesis rendered it an attractive candidate for the progesterone-induced side-branching morphogen. To assess specifically the role of Wnt-4, we examined breast tissue from Wnt-4^{-/-} mice. This experiment was complicated by the fact that such mice normally die *in utero* because of defects in kidney development. We circumvented this problem by preparing mammary gland anlagen from 14.5 day-old embryos, implanting them into host fat pads as before. These experiments revealed that in the absence of Wnt-4, ductal elongation occurred normally but there was a readily detectable defect in ductal sidebranching (Fig. 3 of Ref. 5).

Fourth, we examined the pattern of Wnt-4 and PR expression in the breast using *in situ* hybridization with probes for the mRNAs of these two proteins. This work revealed that indeed the two proteins had very similar patterns of expression. However, the resolution of this technique did not allow us a definitive proof that the two proteins were expressed in the same cells (Fig. 4 of Ref. 5).

Fifth, we treated MECs in culture with progesterone and observed a specific and reproducible induction of Wnt-4 in response but observed no effects on the expression of the related Wnt-5a, Wnt-5b and Wnt-6 proteins which are known to be induced during pregnancy (Fig. 5 of Ref. 5).

These data leave us with the following model. Early in pregnancy, progesterone induced PR-expressing MECs in the primary mammary ducts to release one or more paracrine factors, the most important of which is Wnt-4. Once released, Wnt-4 is able to act on nearby MECs and/or stromal cells to elicit secondary effects that result ultimately in the branching of the ducts. These effects would appear to be mediated over short distances, as it is known that PR-positive cells tend to be located near to sites of ductal

branching. This paper evoked the writing of a long commentary in *Genes and Development* that appeared together with this paper (Ref. 6).

This work provokes a number of questions that remain unanswered. In the context of normal morphogenesis, the most obvious of these are i) the issue of how progesterone is able biochemically to induce Wnt-4 expression; ii) the cell types on which Wnt-4, once produced, is able to act; and iii) how Wnt-4, by acting on these cell types, is able to elicit side-branching. Resolution of these questions may eventually shed light on breast cancer pathogenesis and on the issue of whether progesterone is a mitogen for breast cancer cells or acts in other ways to promote the progression of MECs into malignant tumor cells.

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Estrogen-Dependent Cyclin E-cdk2 Activation through p21 Redistribution

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In order to elucidate the mechanisms by which estrogens and antiestrogens modulate the growth of breast cancer cells, we have characterized the changes induced by estradiol that occur during the G₁ phase of the cell cycle of MCF-7 human mammary carcinoma cells. Addition of estradiol relieves the cell cycle block created by tamoxifen treatment, leading to marked activation of cyclin E-cdk2 complexes and phosphorylation of the retinoblastoma protein within 6 h. Cyclin D1 levels increase significantly while the levels of cyclin E, cdk2, and the p21 and p27 cdk inhibitors are relatively constant. However, the p21 cdk inhibitor shifts from its association with cyclin E-cdk2 to cyclin D1-cdk4, providing an explanation for the observed activation of the cyclin E-cdk2 complexes. These results support the notion that cyclin D1 has an important role in steroid-dependent cell proliferation and that estrogen, by regulating the activities of G₁ cyclin-dependent kinases, can control the proliferation of breast cancer cells.

A variety of models have been proposed to explain how estrogen drives the proliferation of normal mammary epithelial cells and breast cancer cells (8). By acting through the estrogen receptor (ER), estrogen can regulate the transcription of a cohort of responsive genes and in this way appears to regulate cell cycle progression. Even in the face of still-incomplete mechanistic insight into how estrogen regulates growth, effective antitumor therapies directed against the ER have been developed around the use of antiestrogens such as tamoxifen (20). Many studies of the effects of tamoxifen have indicated that tamoxifen acts in a cytostatic fashion on breast cancer cells, causing them to arrest in the G₀/G₁ phases of their growth cycle (36, 49). For these reasons, it is important to understand how estrogens and antiestrogens control G₁ progression.

The central regulator of this process is the cell cycle clock apparatus, which operates in the cell nucleus and is assembled from an array of cyclins and cyclin-dependent kinases (cdks) (45). The activities of the cdks are positively controlled by their association with cyclins and restrained by cdk inhibitors. Included among the latter are p21, p27, and p57, which can inhibit a wide range of cyclin-cdk complexes, and the INK4 family (p15, p16, p18, and p19), which specifically inhibits cdk4 and cdk6 (46).

Extracellular signals such as those conveyed by growth factors affect the activity of cyclins and cdks largely during the G₁ phase of the cell cycle. The most important components of the cell cycle clock apparatus during this period are (i) the D-type cyclins together with their catalytic partners cdk4 and cdk6 and (ii) cyclin E, which interacts with cdk2. Both classes of G₁ cyclin-cdk complexes are known to drive the phosphorylation of the retinoblastoma protein (pRb) (18, 19, 21, 42). This phosphorylation represents a key event in G₁ progression (53). Hypophosphorylated pRb is active in mediating G₁ arrest while hyperphosphorylated pRb appears to be inactive in blocking cell cycle advance.

Overexpression and amplification of G₁ cyclin genes have

been observed in a number of primary breast cancers and in tumor-derived cell lines (7, 23, 24). For example, amplification of the chromosomal region 11q13 containing the cyclin D1 gene is frequently observed in breast cancer (26). This amplification seems to occur preferentially in ER-positive tumors and has been linked to poor prognosis (1, 44). Consistent with an important causal role in breast cancer, cyclin D1 overexpression can be observed in primary breast cancers, even at early stages of the disease (3, 54). Microinjection of antibodies or antisense to cyclin D1 during G₁ can prevent cell cycle progression of pRb-positive breast cancer cell lines (3). Moreover, ectopic expression of the cyclin D1 gene in the breast cancer cell line T47D shortens G₁ and induces cell cycle progression (33). Overexpression of cyclin D1 in breast cancer cells also reduces their rate of exiting from the cell cycle, allowing cell cycle progression and pRb phosphorylation even in the absence of growth factors (56).

Other research using mouse models has supported the notion that cyclin D1 plays a central role in regulating the proliferation of mammary epithelial cells. Transgenic mice expressing cyclin D1 under the control of the mouse mammary tumor virus promoter develop mammary hyperplasias and carcinomas in a pregnancy-dependent fashion (51). Moreover, the mammary glands of mice lacking the cyclin D1 gene fail to undergo full development during pregnancy while virtually all other tissues in these mice develop normally (12, 47). Since the main extracellular regulators of mammary development are ovarian steroids, the above results strongly support the notion that cyclin D1 is involved in mediating the steroid-dependent growth of mammary epithelial cells.

Indeed, several reports have suggested a role for steroids in regulating cyclin D1 expression. Musgrove et al. (32) were able to associate progesterone-dependent G₁ progression with changes in the expression of cyclin D1. Work from the same laboratory has suggested that the main target of antiestrogen action is cyclin D1 (52). Treatment of ER-positive breast carcinoma cell lines with antiestrogens led to an increase in hypophosphorylated pRb and to G₁ arrest. Decreases in expression of cyclin D1 preceded the antiestrogen-mediated cell cycle arrest. These changes were followed by a decrease in cdk2 kinase activity (52).

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More recently, direct regulation of cyclin D1 transcription by estrogen has been shown by others (2). In these recent studies, estrogen was able to overcome the cell cycle arrest imposed by the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor simvastatin by inducing cyclin D1 expression and pRb phosphorylation. The effects of estrogen were attributed to an estrogen-responsive regulatory region between -934 and -136 bp of the human cyclin D1 promoter. The activation of the cyclin D1 promoter by estrogen was independent of mitogen-activated protein kinase activity, which is inhibited by simvastatin (4).

To characterize more precisely the mechanisms by which estradiol induces cell cycle progression, we have studied MCF-7 cells arrested by tamoxifen. We present evidence that release of the cell cycle block by the addition of estrogen leads to rapid activation of cyclin E-cdk2 kinase and pRb phosphorylation. This occurs via a mechanism that is dependent upon induction of cyclin D1 by estrogen and a shift of the p21 cdk inhibitor from cyclin E-cdk2 to cyclin D1-cdk4 or cyclin D1-cdk6.

MATERIALS AND METHODS

Cell culture and synchronization. MCF-7 cells were obtained from M. Brown (Dana-Farber Cancer Institute, Boston, Mass.). They were routinely cultured in Dulbecco's modified essential medium (DMEM; Gibco-BRL) with 5% heat-inactivated fetal bovine serum (Sigma Chemical Co., St. Louis, Mo.), penicillin (50,000 U/liter), streptomycin sulfate (50,000 µg/liter), and amphotericin B (Fungizone; 125 µg/liter). For tamoxifen synchronization, cells were plated between 5×10^3 and 10×10^3 /cm². After 48 to 60 h, medium was changed to DMEM (phenol red free) with 5% charcoal-stripped serum (CSS) (HyClone, Logan, Utah), antibiotics, and 1 µM tamoxifen for 48 h (10). Cells were released from the arrest by addition of 500 nM 17β-estradiol or by changing the medium to fresh DMEM (phenol red free) with 5% CSS and 5 nM 17β-estradiol. For controls, cells were either given ethanol or changed to similar medium without estradiol. In some cases, the latter control contained 1 µM tamoxifen.

Cell cycle analysis. MCF-7 cells were plated for thymidine analysis on either 6-, 12-, or 24-well plates. Thymidine incorporation was assessed by labelling synchronized MCF-7 cells with 1 µCi of [*methyl*-³H]thymidine per ml for 30 min. At the indicated time points, cells were washed once with phosphate-buffered saline (PBS) and once with 5% ice-cold trichloroacetic acid. Then, they were incubated in 5% trichloroacetic acid for at least 30 min on ice. After this incubation, cells were washed three times with water and lysed with 0.1 N NaOH. An aliquot of each sample was quantified by liquid scintillation counting.

For fluorescence-activated cell sorter (FACS) analysis, MCF-7 cells were harvested by trypsinization, pelleted gently, and resuspended in 2 ml of PBS. Cells were fixed by the gradual addition of 5 ml of 95% ethanol while being vortexed. After 30 min at room temperature, cells were stored at 4°C. Before processing, cells were collected by centrifugation and stained by addition of 1 ml of a 50-µg/ml propidium iodide solution. RNase A was added to these samples at a final concentration of 100 µg/ml, and the samples were incubated at room temperature for 15 min. Cell cycle analysis was carried out with a Becton Dickinson FACScan flow cytometer.

Preparation of cell extracts. Cell pellets were lysed for 20 min on ice in Nondet P-40 (NP-40) lysis buffer (PBS [pH 7.2] containing in addition 250 mM NaCl, 2 mM EDTA, 0.1% NP-40, 1 mM dithiothreitol [DTT], 2 µg of aprotinin per ml, 2 µg of leupeptin per ml, 50 µg of phenylmethylsulfonyl fluoride per ml, 1 mM NaF, 1 mM orthovanadate, 60 mM β-glycerophosphate). Cell debris was pelleted by centrifugation at 14,000 rpm for 15 min on an Eppendorf centrifuge (Brinkmann) at 4°C. The supernatant was assayed for protein content by Bradford analysis (Bio-Rad) and either used immediately or flash-frozen on dry ice and stored at -70°C.

Antibodies. Monoclonal antibodies against cyclin E and polyclonal antibodies against cdk2, cdk4, cdk6, p21, and p27 with their competing peptides when available were obtained from Santa Cruz Biotechnology, Santa Cruz, Calif. Monoclonal anti-pRb and polyclonal anti-cdk4 were purchased from Pharmingen, San Diego, Calif. Mouse monoclonal antibody against p27 was from Transduction Laboratories, Lexington, Ky. Monoclonal antibodies against p21 (CP-68 and CP-36) were a kind gift from B. Dynlacht, Harvard University, Cambridge, Mass. Monoclonal antibodies against human cyclin D1 and cyclin E were kindly provided by E. Harlow, Massachusetts General Hospital, Charlestown. Polyclonal antibody against p57 was a generous gift from S. Elledge, Baylor College of Medicine, Houston, Tex.

Western blot analysis and immunoprecipitation. Equal amounts of protein were processed for Western blot analysis by either sodium dodecyl sulfate (SDS)-7.5% polyacrylamide gel electrophoresis (PAGE) or SDS-12% PAGE. In general, 100 µg of protein per lane was separated by SDS-PAGE and trans-

ferred to Immobilon-P membrane (Millipore) by standard protocols. The membrane was blocked in PBS with 5% nonfat dry milk for 1 h. Subsequently, it was incubated for 3 h with a dilution of the specific antibody in PBS (2.5% nonfat dry milk, 0.05% Tween). After five washes with PBS (0.1% Tween), the filter was incubated for 1 h with a 1:5,000 dilution of horseradish peroxidase-linked secondary antibody (Jackson Laboratories). Immunodetection was achieved with an enhanced chemiluminescence system (Amersham).

For immunoprecipitations followed by Western blotting, lysates were incubated with the desired antibody cross-linked to beads for 3 h at 4°C with rocking. Beads were pelleted briefly on a microcentrifuge and washed twice with 1 ml of lysis buffer before electrophoresis and transfer to membrane. For immunodepletion, three sequential immunoprecipitations were carried out with each sample. An aliquot equivalent to 100 µg was taken from each supernatant for Western blot analysis to assess remaining proteins. Quantification of blots was done with PDI software (Huntington Station, N.Y.). Images were processed with Adobe Photoshop software and a Lacie Silverscanner II.

Kinase assays. For histone H1 phosphorylation, the amount of lysate immunoprecipitated varied with the specific antibody used in order to ensure that the kinase assay was conducted within the linear range. After immunoprecipitation, the beads were washed twice with lysis buffer and once with kinase assay buffer (20 mM Tris [pH 7.5], 5 mM MgCl₂, 2.5 mM MnCl₂, 1 mM DTT). Beads were incubated with 15 µl of kinase mix (kinase buffer containing 10 µM ATP with 2.5 µg of histone H1 [Boehringer Mannheim] and 16 µCi of [*γ*-³²P]ATP per reaction mixture) for 30 min at 30°C and stopped by addition of Laemmli sample buffer. Gels were stained with Coomassie blue, and excised bands were quantified by Cerenkov counting. To determine inhibitory activity present in extracts, equal amounts of protein or amounts as indicated were mixed and incubated at 30°C for 30 min before immunoprecipitation and kinase assay.

For glutathione S-transferase (GST)-Rb phosphorylation, we followed the conditions used previously to evaluate cdk4 activity from MCF-7 cells (15). In brief, cell pellets were lysed in Tween buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween 20, 10% glycerol, 1 mM DTT, 10 µg of aprotinin per ml, 5 µg of leupeptin per ml, 50 µg of phenylmethylsulfonyl fluoride per ml, 5 mM NaF, 100 µM orthovanadate) for 30 min followed by centrifugation for 15 min at 4°C. A total of 500 µg of each extract was immunoprecipitated with cdk4 polyclonal antibodies (Pharmingen or Santa Cruz). Beads were washed twice with Tween buffer and three times with Rb-kinase buffer (50 mM HEPES [pH 7.5], 5 mM EGTA, 1 mM DTT, 10 mM β-glycerophosphate, 1 mM NaF, 100 µM orthovanadate). The reaction was started by addition of 15 µl of Rb-kinase mix (Rb-kinase buffer with 30 µM ATP, 1 µg of GST-Rb [Santa Cruz], and 16 µCi of [*γ*-³²P]ATP per reaction mixture). After 30 min at 30°C, samples were mixed with Laemmli buffer and analyzed by SDS-PAGE.

cdc25A assay. For cdc25A assays, cyclin E-cdk2 complexes were immunoprecipitated with cyclin E antibodies. The immunoprecipitates were washed with cdc25A wash buffer (50 mM Tris [pH 7.9], 5 mM MgCl₂, 1 mM DTT) before addition of the reaction mix. The cdc25A reaction mix (50 mM Tris [pH 7.9], 5 mM MgCl₂, 10 mM DTT) contained when indicated recombinant GST-cdc25A (generous gift of Michele Pagano, New York University Medical Center, New York, N.Y.) alone or in the presence of 5 mM Na₃VO₄. The cdc25A reaction was carried out at 30°C for 30 min. The reaction was stopped by addition of 1 ml of cold NP-40 lysis buffer and processed for histone H1 kinase assays.

RESULTS

Characterization of estradiol-induced cell cycle reentry. We have used the estrogen-responsive MCF-7 human breast cancer cell line to study the effects of estradiol on the cell cycle. Exponentially growing cultures of MCF-7 cells were arrested in G₀/G₁ by treatment with 1 µM tamoxifen for 48 h in the presence of CSS. Synchronous release of the tamoxifen-arrested cells occurred after removal of tamoxifen and addition of fresh medium containing CSS and 17β-estradiol (5 nM estradiol). The results of a representative experiment are shown in Fig. 1A. Entry into S phase was determined by measuring thymidine incorporation. The peak of DNA synthesis was observed after 22 h of estradiol treatment, at which time the rate of thymidine incorporation increased more than 10-fold over uninduced levels. Tamoxifen-arrested MCF-7 cells that received only fresh medium containing 5% CSS or with 1 µM tamoxifen were not able to enter into S phase. FACS analysis of MCF-7 cells (Fig. 1B) confirmed that the tamoxifen-arrested cells (*T* = 0 h) were mostly in G₀/G₁ with a low percentage (8%) of cells in S phase. Following addition of fresh medium containing estradiol, over 50% of the cells had begun replicating their DNA by the time the peak of thymidine

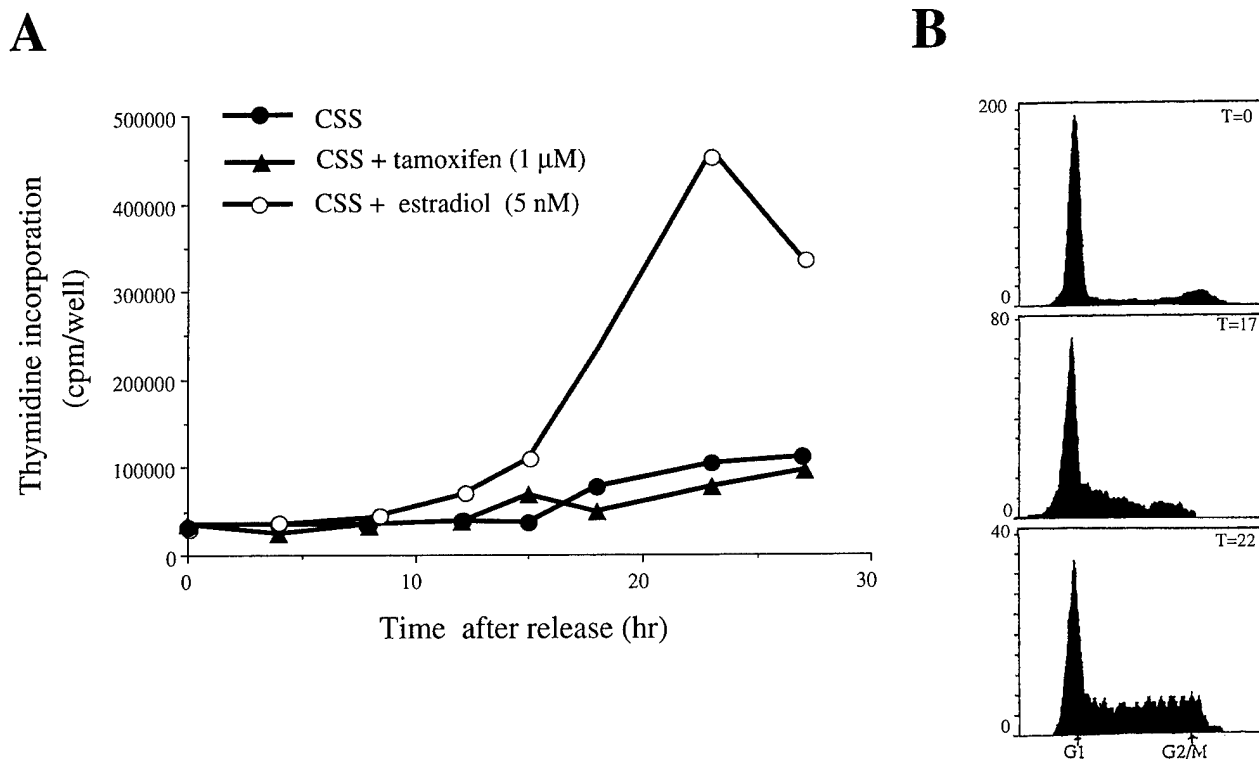


FIG. 1. Estrogen-dependent cell cycle progression. (A) MCF-7 cells were treated with tamoxifen for 48 h in the presence of 5% CSS. At $T = 0$ h, medium was changed to 5% CSS alone or with addition of either 1 μ M tamoxifen or 5 nM estradiol. At the times indicated, cells were pulsed with [*methyl*- 3 H]thymidine for 30 min and thymidine incorporation was determined as described in Materials and Methods. (B) MCF-7 cells treated for 48 h with tamoxifen (0 h) or after change to 5% CSS with 5 nM estradiol for the indicated times (in hours) were fixed, incubated with propidium iodide, and analyzed by FACS. The y axes show the number of cells.

incorporation occurred ($T = 22$ h). Synchronized cell cycle reentry of tamoxifen-blocked cells could also be induced by adding relatively high concentrations of estradiol (500 nM) to cells in the continued presence of the tamoxifen blocking agent (data not shown).

Phosphorylation of the Rb protein and escape from tamoxifen inhibition. Previous work addressing the action of tamoxifen on the cell cycle had indicated that MCF-7 cells are sensitive to the antiestrogen tamoxifen and other similarly acting compounds only in a narrow window of time in the cell cycle in early to mid- G_1 (35, 50). However, these studies did not evaluate the effect of tamoxifen on the cell cycle machinery. The period of responsiveness to tamoxifen in G_1 is reminiscent of the effects of transforming growth factor β (TGF- β) on cell cycle advance, which are known to involve primarily a blockage of pRb phosphorylation (14, 25). Thus, after cells have phosphorylated their complement of pRb in late G_1 , they become nonresponsive to the growth-inhibitory effects of TGF- β .

For these reasons, we determined whether the acquired refractoriness to tamoxifen in late G_1 could be correlated with phosphorylation of pRb. To do so, we arrested cells with tamoxifen for 48 h and then released them by addition of fresh medium containing 5% CSS and 5 nM estradiol. At various time points thereafter, estradiol-containing medium was removed and replaced with tamoxifen-containing medium. Effects on cell cycle advance were ascertained by measuring the subsequent ability of these cells to incorporate thymidine at $T = 22$ h, the time of peak thymidine incorporation by control cells that had not been treated with tamoxifen following the estradiol-induced cell cycle progression.

As shown in Fig. 2A, most of the cells could be prevented

from subsequent S-phase entry if tamoxifen was added back immediately after estradiol addition at $T = 0$ h. However, by 6 h, virtually all the cells were refractory to tamoxifen treatment, achieving levels of thymidine incorporation similar to those of cells that had been exposed continuously to estradiol for 22 h. These data indicated that, within several hours after estradiol addition, tamoxifen (and presumably the ER) no longer exerted control over cell cycle advance.

Western blot analysis of the Rb protein indicated that phosphorylation of pRb correlated closely with the acquisition of refractoriness to tamoxifen (Fig. 2B, top). Densitometric analysis of the different pRb forms revealed the almost complete disappearance of the hypophosphorylated form and its replacement by the hyperphosphorylated form by 6 h. Indeed, the ratio of hypo- to hyperphosphorylated forms dropped dramatically in the first few hours after estradiol stimulation (Fig. 2B, bottom). Surprisingly, the kinetics of pRb phosphorylation preceded by many hours the entry into S phase and differed in this way from the schedule of changes normally seen during cell cycle progression from G_0/G_1 to S phase (6, 9).

Such acquired resistance in mid- to late G_1 to growth inhibition has been observed in a number of other cases besides the aforementioned nonresponsiveness to TGF- β seen in late G_1 . Thus, introduction of low levels of cycloheximide or removal of mitogens has been observed to block G_1 advance when applied early in this phase but not in the last several hours of this phase (29, 37, 38). In each case, the time of acquired resistance has been equated with passage through a restriction point (R point). By extension, the acquired refractoriness to tamoxifen inhibition also represents a restriction point transition. As with the other operationally defined R

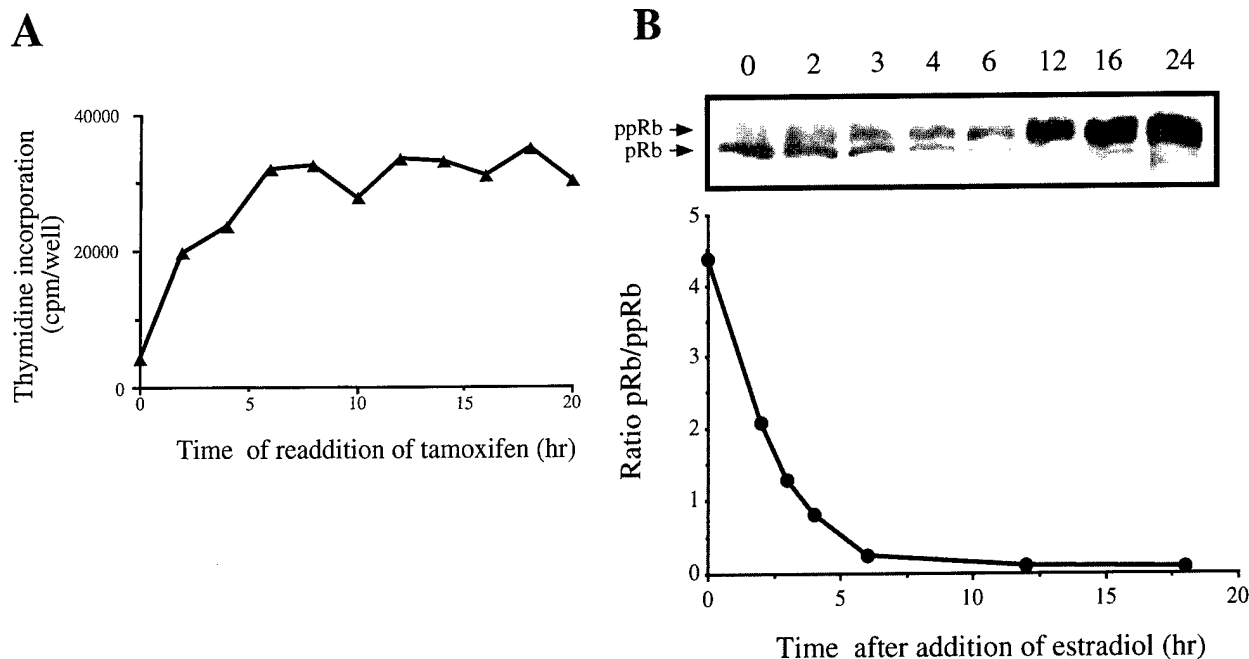


FIG. 2. Refractoriness to tamoxifen and phosphorylation of the retinoblastoma protein (pRb). (A) Tamoxifen-arrested MCF-7 cells were stimulated with 5 nM estradiol at $T = 0$ h. At the indicated times, the medium was replaced with 5% CSS containing 1 μ M tamoxifen. After 22 h, all the cells were pulsed with [3 H]thymidine and processed as described in Materials and Methods. (B) (Top) Total cell extracts of MCF-7 cells (100 μ g) at different time points after release (in hours) from tamoxifen-induced cell cycle block were analyzed for pRb protein by Western blotting. (Bottom) Densitometric analysis of pRb phosphorylation. The ratio of hypophosphorylated pRb to hyperphosphorylated pRb (ppRb) was plotted as a function of time after estradiol addition.

points, this transition is closely contemporaneous with and likely connected with the phosphorylation of pRb.

Regulation of cdk4 by estradiol in MCF-7 cells. Since pRb phosphorylation is known to be driven largely by cdk4, we characterized the effects of estradiol on the activity of the cyclin-cdk complexes implicated in this phosphorylation in mid- to late G_1 . Recent reports have suggested that the cyclin D1-cdk4 complex may be a direct target of estrogen action (see Introduction). For this reason, we first characterized the changes in cyclin D1 protein and cyclin D1-cdk4 kinase activity following estradiol addition to tamoxifen-arrested MCF-7 cells. To avoid the possible confounding effects of freshly added serum, we reversed the cell cycle block by adding only estradiol (500 nM final concentration) to tamoxifen-arrested cells. The control cells received only the solvent vehicle (ethanol).

The results shown in Fig. 3A (top) confirmed that estradiol can induce expression of cyclin D1 protein very rapidly. Thus, an increase of cyclin D1 levels was already apparent within 2 h of treatment. Levels of cyclin D1 continued to increase steadily, achieving fivefold higher levels by 6 h. Taken together with the previously reported work (2), the present results indicated a specific effect of estrogen on cyclin D1 levels.

In parallel with these measurements of cyclin D1 levels, we measured cdk4 activity by using GST-Rb as a substrate and antibodies against a peptide derived from the carboxy terminus of cdk4 to immunoprecipitate cyclin D1-cdk4 kinase complexes. We assumed here that the activity of cdk4 could be used as well as an index of the activity of the similarly regulated cdk6 enzyme. As a negative control, we blocked specific binding by preincubating the antibodies with the antigenic peptide. Figure 3A (bottom) shows the changes of cdk4-dependent kinase following estrogen release of tamoxifen-arrested cells. Considerable cdk4 activity toward the GST-Rb substrate was

apparent in the tamoxifen-arrested cells prior to their release by estradiol compared to the negative control. After addition of estradiol, this activity increased slowly and steadily, starting at 2 h after addition of estradiol and peaking by 6 h, ultimately reaching threefold higher levels. Similar results were obtained when the kinase assay was performed after immunoprecipitation with cyclin D1-specific antibodies (reference 2 and data not shown). Indeed, we anticipated that we would observe similar kinetics of enzyme activation following immunoprecipitation with either anti-cdk4 or anti-cyclin D1 antibody. While the other D-type cyclins, D2 and D3, are equally able to activate cdk4, our work and that of others have shown that cyclin D2 is not detectable in these cells and cyclin D3 is present in low and constant amounts following estradiol treatment (reference 53 and data not shown).

In addition, we also investigated the status of the other important G_1 cyclin-cdk complex, cyclin E-cdk2. In this instance, cyclin E-cdk2 activity was assayed with immunoprecipitates obtained with an anti-human cyclin E monoclonal antibody and histone H1 as substrate. A dramatic induction of this kinase activity that was first apparent within 2 h of estradiol addition was observed (Fig. 3B, top). At 6 h, this activity had already peaked at a level that was ~ 20 times higher than that seen at $T = 0$ h (Fig. 3B, bottom). A similar relative induction was seen when cdk2-specific antibodies were used to prepare the precipitates analyzed in the kinase assay (see Fig. 6A). Analysis of cyclin A-dependent kinase activity in the same extracts showed a delayed induction of this enzyme (Fig. 3B, middle) with respect to that of cyclin E with maximum levels reached by 24 h (Fig. 3B, bottom). This induction correlated well with entrance of the cells into S phase.

The changes in cyclin D1-cdk4 kinase could be explained by the increase in cyclin D1 expression. Possible changes in the levels of INK4A were not considered as MCF-7 cells carry a

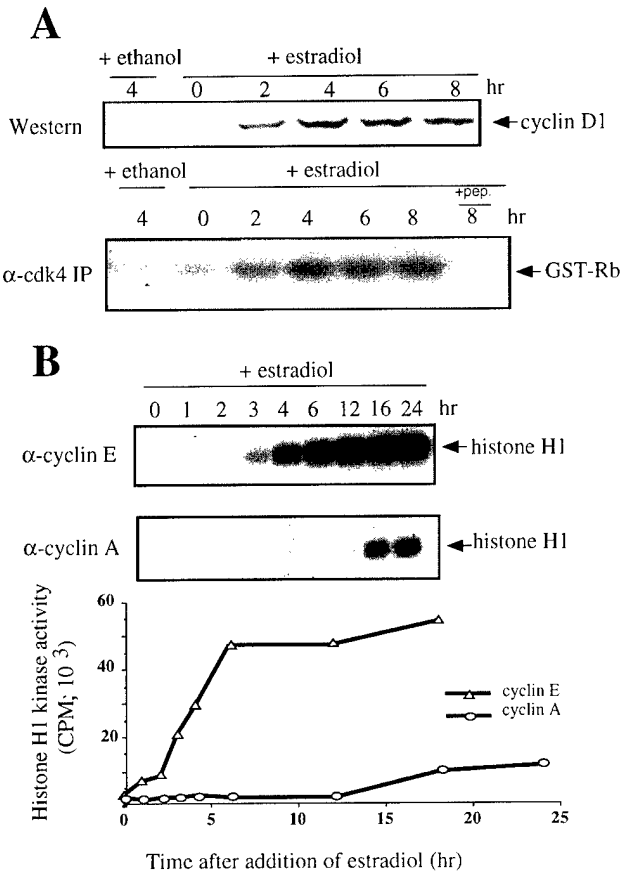


FIG. 3. Effect of estradiol on G₁ cdk. (A) Changes in cyclin D1-cdk4 after estradiol addition. (Top) Western blot analysis of cyclin D1 expression at the indicated time points after the addition of 500 nM estradiol or ethanol to tamoxifen-arrested cells. (Bottom) The same extracts were immunoprecipitated with a polyclonal antibody against the carboxy terminus of cdk4. The kinase activity of these immunocomplexes was measured with GST-Rb as substrate. Background activity (lane + pep.) was determined by blocking cdk4 antibody with a specific antigenic peptide. (B) (Top and middle) Activity of cyclin E (top)- or cyclin A (middle)-associated kinases was assessed with histone H1 as substrate. Tamoxifen-arrested MCF-7 cells were released by a change to 5% CSS with 5 nM estradiol. Cells were harvested and processed for histone H1 kinase assays at the times indicated as described in Materials and Methods. (Bottom) Quantitation of kinase activities. The amount of ³²P incorporated into histone H1 in counts per minute was obtained by determining the Cerenkov counts on the excised histone H1 bands.

deletion of this gene (31). However, the induction of cyclin E-cdk2 kinase activity still required explanation. Thus, we initiated further experiments to determine how cyclin E-dependent kinase activity was being inhibited by tamoxifen treatment and induced by subsequent estradiol treatment.

Expression of cyclin E, cdk2, and cyclin E-cdk2 inhibitors following estradiol release of tamoxifen-arrested MCF-7 cells. The most likely explanation for the observed changes in the activity of the cyclin E-cdk2 complexes was that estrogen, acting through its receptor, modulated the levels of cyclin E, cdk2, or associated regulatory molecules. Indeed, addition of actinomycin or cycloheximide at the time of estradiol treatment prevented cyclin E-cdk2 activation by estradiol, indicating the need for protein synthesis following estradiol addition (data not shown). However, this need for de novo protein synthesis could not be explained by a requirement for increased levels of cyclin E, since Western blot analysis indicated that the levels of a major form of cyclin E were relatively constant in the 6 h

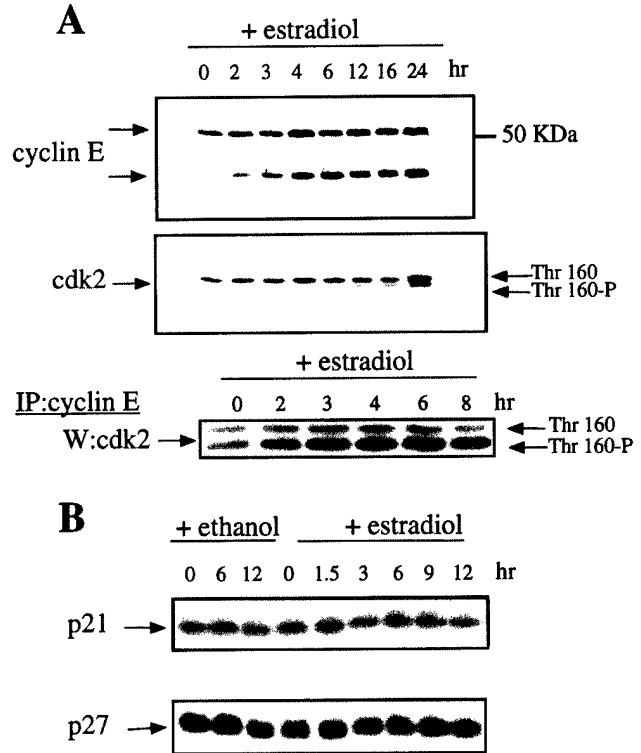


FIG. 4. Effect of estradiol on components of the cyclin E-cdk2 complex. (A) Analysis of cyclin E (top), cdk2 (middle), and cyclin E-associated cdk2 (bottom) from extracts obtained at different time points after release from the tamoxifen block by change to 5% CSS-5 nM estradiol. W, Western blot antibody; IP, immunoprecipitating antibody. (B) Western blot analysis of cell cycle inhibitors, p21 (top) and p27 (bottom), from tamoxifen-arrested cells that received either ethanol or estradiol.

following estradiol addition (Fig. 4A, top). Occasionally, increases in a more rapidly migrating form of cyclin E (22, 23) were apparent during this time period; these increases were not observed reproducibly and could not be correlated with increases in cyclin E-associated kinase activity. The levels of the cdk2 protein also remained essentially unchanged until 24 h (Fig. 4A, middle), long after the functional activation of the cyclin E-cdk2 complexes. A slight increase in the phosphorylated form of cdk2 was seen at 6 h. However, a more remarkable increase in this active form was seen only much later, coinciding with the greatest accumulation of cells in S phase (Fig. 1).

It was also possible that estradiol affected the assembly of cyclin E-cdk2 complexes. To assess this possibility, we analyzed the changes in the levels of the cyclin E-associated cdk2 following estradiol addition to tamoxifen-arrested cells. Cell lysates prepared at different times after estradiol addition were immunoprecipitated with cyclin E-specific antibodies followed by Western blot analysis using anti-cdk2 antibody as probe. This analysis revealed that cyclin E-cdk2 complexes were already present during the tamoxifen arrest and that their levels did not change substantially following estradiol treatment (Fig. 4A, bottom). There was a small increase in the phosphorylated, active cdk2 form following estradiol addition. However, no changes were seen in the cdk-activating kinase activity following treatment with estradiol (data not shown). Together, these data indicated that changes in cyclin E and cdk2 levels or their association with one another could not explain the marked increases in the activity of the cyclin E-cdk2 complexes. This

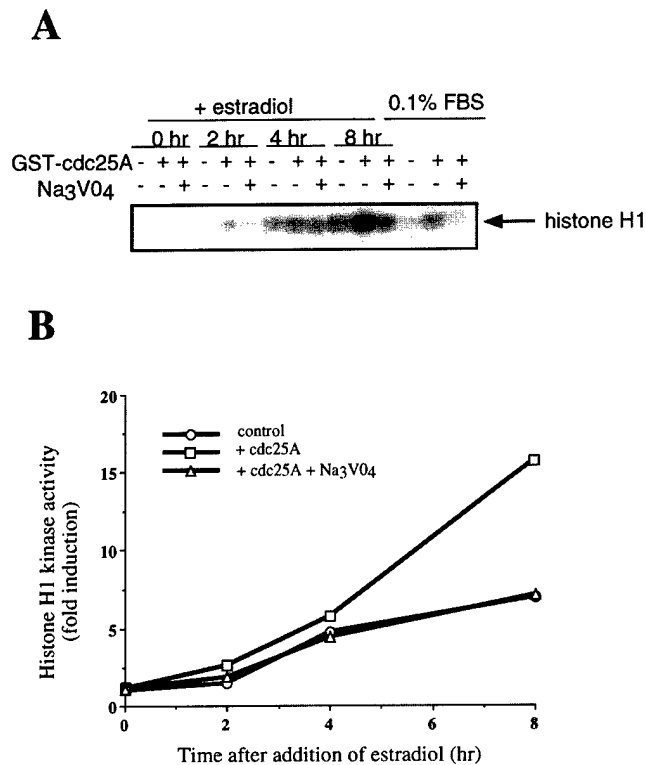


FIG. 5. Effect of recombinant cdc25A on cyclin E-cdk2 activity. (A) Asynchronous MCF-7 cells were treated with either 5% CSS with 1 μ M tamoxifen or 0.1% fetal bovine serum (FBS) for 48 h. Tamoxifen-arrested cells were then treated with estradiol for the times shown. Cyclin E-cdk2 complexes were immunoprecipitated from the indicated cell extracts with antibodies specific for cyclin E. The immunocomplexes obtained were split into three and incubated in cdc25A reaction mix in either the presence or the absence of recombinant GST-cdc25A and sodium orthovanadate. (B) Graphic representation of cyclin E-associated histone H1 kinase activity obtained from results shown in panel A.

suggested in turn that other regulators of cyclin-cdk activity were responsible for modulating cyclin E-associated kinase activity following estradiol treatment. More specifically, cdk inhibitors were attractive candidates for such a role. Hence, we determined whether expression of any of the known cdk2 inhibitors was affected by estrogen.

As shown in Fig. 4B, levels of the p21 (top) or p27 (bottom) cdk inhibitors did not change substantially during the hours preceding cyclin E-cdk2 activation. The p57 cdk inhibitor was not detectable in these cells (data not shown). Levels of the INK4 class of inhibitors were not monitored here, as these inhibitors affect only cdk4 and cdk6. Therefore, we concluded that the observed changes in cyclin E-cdk2 activity following estradiol treatment could not be the result of changes in the overall levels of the known cdk2 inhibitors.

Role of cdc25A in cyclin E-cdk2 activation. Cyclin E-cdk2 complexes can also be activated by dephosphorylation mediated by the cdc25A phosphatase (30). Thus, it was possible that the cyclin E-cdk2 complexes from tamoxifen-arrested cells were held inactive by the inhibitory phosphorylation. To address this possibility, we tested the ability of recombinant GST-cdc25A to activate cyclin E-cdk2 complexes immunoprecipitated from cell extracts prepared at different times after estradiol addition. The results shown in Fig. 5A indicated that cdc25A was not able to activate the complexes obtained from tamoxifen-arrested cells. Nevertheless, there was a gradual increase in the ability of cyclin E-cdk2 complexes to be activated

by cdc25A in complexes obtained from cells treated with estradiol. This activation was abolished in the presence of the tyrosine phosphatase inhibitor Na₃VO₄ (Fig. 5A). Quantitation of the activation induced by cdc25A indicated that, while cyclin E-cdk2 complexes obtained from tamoxifen-arrested cells were not significantly activated, complexes from cells treated with estradiol for 8 h were activated more than twofold (Fig. 5B). A similar fold activation was also seen when cyclin E-cdk2 complexes obtained from serum-starved MCF-7 cells were used (Fig. 5A, last three lanes). Taken together, these results suggested that the absence of cyclin E-cdk2 kinase activity during tamoxifen-mediated cell cycle arrest was not due to inhibitory phosphorylation of cdk2 attributable in turn to the lack of cdc25A activity.

Analysis of cdk-inhibitory activity in tamoxifen-arrested cells. The absence of substantial changes in the levels or functioning of the various molecules that contribute to cdk2 activity caused us to undertake direct biochemical analysis of cdk2 complexes prepared from cells treated with tamoxifen or estradiol. In particular, prior to immunoprecipitation we mixed extracts from tamoxifen-blocked cells (0 h) with those prepared from cells that had been released from the tamoxifen block by 8 h of estradiol treatment (8 h). In doing this, we hoped to determine whether the tamoxifen-blocked cells contained a soluble inhibitor of cdk2 activity.

The results of these *in vitro* assays, shown in Fig. 6A, indicated that tamoxifen-arrested cells did indeed contain an activity capable of reducing the activity of cdk2 to levels as low as those observed with extracts from tamoxifen-arrested cells. This inhibition of estradiol-treated extracts was seen when either cyclin E- or cdk2-specific antibodies were used to immunoprecipitate cyclin E-cdk2 complexes prior to assay for kinase activity (Fig. 6A). Moreover, this inhibition was seen only when the extract mixtures were preincubated at 30°C, not when they were preincubated at 4°C (Fig. 6A, last two lanes).

These findings pointed to the presence of a soluble inhibitory substance in tamoxifen-arrested cells capable of abolishing the activity of cyclin E-cdk2 complexes. We assumed tentatively that this inhibitory substance detected *in vitro* was responsible for the observed inhibition of cyclin E-cdk2 activity in tamoxifen-arrested cells.

Changes in inhibitory activity after estradiol-induced release of the cell cycle block. We wished to monitor the fate of this inhibitory substance following the release of cells from the tamoxifen block. To do so, we tested the inhibitory activity from cells prepared at different time points after estradiol addition. In parallel, we also measured the activity of cdk2-dependent kinase from the same extracts. Figure 6B (left) shows high levels of cyclin E-cdk2 activity at 8 h after addition of estradiol. Mixing of this extract ($T = 8$ h) with an extract prepared from tamoxifen-arrested cells led to complete inactivation of the induced activity (Fig. 6B, right) in confirmation of the results reported above. The inhibitory activities obtained by mixing cell extracts prepared from cells at various times after estradiol treatment were then compared with the inhibitory activity observed in this 0-h extract (100%). Extracts prepared from cells that had been treated for 2 h with estradiol had only 40% of the inhibitory activity of the tamoxifen-arrested extract, and this activity was reduced further at 4 h, being undetectable by 8 h of estradiol treatment.

These results indicated that estradiol treatment caused the rapid loss of the inhibitory activity that had accumulated during the tamoxifen-imposed cell cycle block. However, the fact that the disappearance of the inhibitory activity did not lead immediately to a reciprocal increase in cdk2 kinase activity suggested that other cdk regulators such as the cdk-activating

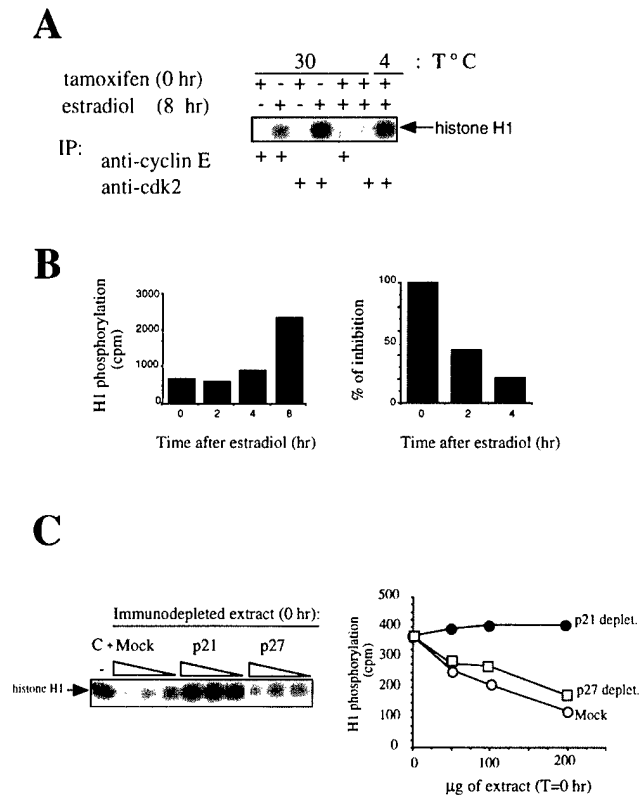


FIG. 6. Characterization of cdk2-inhibitory activity present in tamoxifen-arrested cells. (A) Temperature-dependent inhibition of cyclin E- or cdk2-associated kinase activity. Extracts from tamoxifen-arrested cells and estradiol-stimulated cells alone or mixed as indicated were subjected to immunoprecipitation with cyclin E- or cdk2-specific antibodies. The immunocomplexes were then assayed for kinase activity against histone H1. (B) Changes in cdk2-dependent kinase activity (left) and inhibitory activity (right) after addition of 0.5 μ M estradiol to tamoxifen-arrested cells. (C) Immunodepletion of inhibitory activity against cyclin E-cdk2 kinase. (Left) Extracts from tamoxifen-arrested cells were immunodepleted with antibodies against p21 (CP-68) and p27 (SC-528) or irrelevant antibodies (mock). The inhibitory activity remaining after immunodepletion was evaluated by mixing the respective supernatants with extracts from estradiol-treated samples (8 h) as for panel A. The mixture was incubated for 30 min at 30°C followed by a standard immunoprecipitation and kinase assay against histone H1. The use of other antibodies against either cdk inhibitor gave similar results. (Right) Quantitation of the results from immunodepletion (left). The level of histone H1 phosphorylation was plotted against the amount of extract from each immunodepletion that was mixed with 100 μ g of extracts from cells at 8 h poststimulation.

kinase may be required to achieve full activation of cyclin E-cdk2.

Depletion of cdk2 inhibitors in tamoxifen-arrested cells. Obvious candidates for the cyclin E-cdk2 inhibitory activity described above were the cdk inhibitors, specifically, p21 and p27. While their overall levels in the cell did not change following estradiol treatment, it was possible that they underwent relocalization in the cell in response to estradiol. Accordingly, we attempted to associate the observed inhibitory activity with specific cdk inhibitors, doing so by treating the inhibitory extract with antibodies reactive with one or another of these proteins in order to deplete these molecules. Following antibody treatment, we tested the remaining supernatants for any inhibitory activity that survived immunodepletion by mixing them with the 8-h extract.

We immunodepleted either p21 or p27 from the tamoxifen-treated (0 h) extract with anti-p21 or anti-p27 specific antibodies, respectively. Immunodepletion was performed by three

sequential immunoprecipitations to ensure greater than 99% removal of the inhibitor (see Fig. 7B or Fig. 8). Different amounts of extract ($T = 0$ h) were used to quantify more precisely any resulting changes in inhibitory activity. As shown in Fig. 6C (left), immunodepletion with anti-p21 antibody efficiently abolished the inhibitory activity of the extract prepared from tamoxifen-arrested cells compared to mock-depleted extracts. In contrast, immunodepletion of p27 led to only a slight reduction of the inhibitory activity of the tamoxifen-treated cell extracts (Fig. 6C, right). These observations suggested that the great bulk of the cyclin E-cdk2 inhibitory activity present in the tamoxifen-treated cell extracts was due to p21 molecules present in these extracts while a minor component was due to p27.

Other evidence indicated that p27 was indeed present in these extracts although not in a configuration that permitted it to inhibit cyclin E-cdk2 activity under our mixing conditions. Thus, when extracts from tamoxifen-treated cells were heated to 100°C prior to assay of cdk2-inhibitory activity, greater than 60% of the inhibitory activity present was due to p27 as judged by immunodepletion with anti-p27 antibodies (data not shown). This also indicated that in tamoxifen-arrested MCF-7 cells the levels of p27 as determined by this functional assay were actually greater than those of p21. Hence, in tamoxifen-treated cell extracts, the great bulk, and perhaps all, of the available, active cdk2-inhibitory substance was derived from p21 molecules. p27 molecules, though present in the extracts, were sequestered in heat-labile complexes from which they could be liberated by brief heating. Moreover, as shown in Fig. 6B, this soluble p21-associated inhibitory activity declined dramatically during the hours following estradiol treatment.

Characterization of complexes between p21 and G₁ cyclin-cdks. As shown above (Fig. 3A), estrogen treatment of the MCF-7 cells causes them to express increased levels of cyclin D1. The resulting cyclin D1-cdk4 complexes might act to bind increasing proportions of the cell's pool of p21 and p27 molecules, thereby abstracting them from cyclin E-cdk2. Indeed, just such a model of cdk inhibitor action has also been proposed to operate in other cell types (46). With this model in mind, we determined the changes in the association of p21 with cyclin D1-cdk4 and cyclin E-cdk2 following estradiol addition with the same extracts analyzed previously to test cdc25A activation.

As expected from the results of earlier experiments, levels of cyclin D1 increased very rapidly, reaching levels fourfold higher than those of control by 6 h (Fig. 7A, top). Moreover, immunoprecipitation of p21 followed by Western blot analysis for cyclin D1 revealed an increase in p21-associated cyclin D1 after addition of estradiol (Fig. 7A, bottom). This increase was first seen within 2 h of estradiol treatment and peaked by 6 h with fourfold higher levels than those that were seen in tamoxifen-blocked cells. Therefore, these results indicated that increases in total levels of cyclin D1 by estradiol correlated in time and magnitude with the changes in levels of p21 associated with cyclin D1. The activation of cyclin E-cdk2 complexes occurred at approximately the same time as these changes, suggesting a possible link between the events (Fig. 5).

The observed increases in p21 associated with cyclin D1 following estradiol treatment were compatible with a model in which D1-cdk4 complexes compete with cyclin E-cdk2 complexes for a limited pool of p21 and abstract increasing amounts of p21 from cyclin E-cdk2 complexes following estradiol treatment. However, the above data did not prove this point, as they did not reveal the proportion of total cellular p21 that was present in these two types of cyclin-cdk complexes at various times after estradiol treatment.

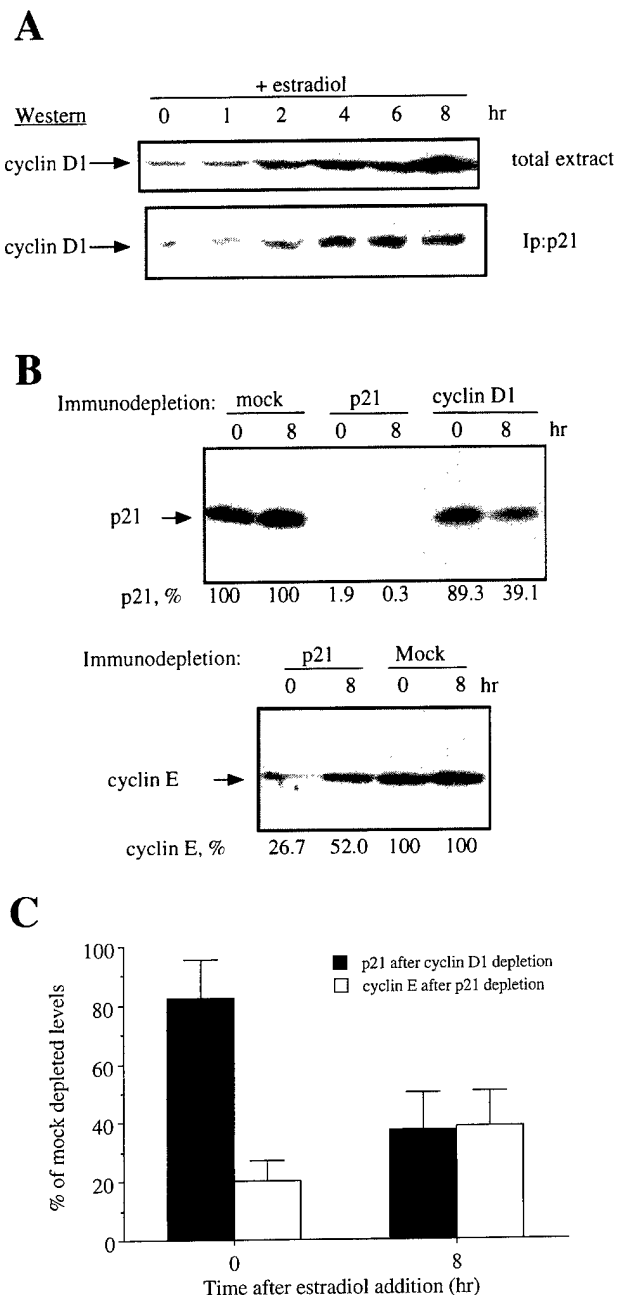


FIG. 7. Changes in p21 distribution among G_1 cyclin-cdk complexes (A). (Top) Cyclin D1 expression from MCF-7 cells after estradiol addition was determined by Western blot analysis of total cell extracts at the time points indicated. (Bottom) Levels of cyclin D1 associated with p21. Cell extracts obtained from tamoxifen-arrested cells or at different times after stimulation with estradiol were subjected to immunoprecipitation with p21 antibodies. The immunoprecipitated complexes were then analyzed for the presence of cyclin D1 by Western blot analysis. (B) (Top) Cell extracts from $T = 0$ and $T = 8$ h after addition of estradiol were immunodepleted by three sequential immunodepletions with non-relevant antibodies (mock) or with antibodies against either p21 (SC-397) or cyclin D1 (HD-33). The amount of p21 surviving immunodepletion was evaluated by Western blot analysis of 100 μ g of total protein with p21-specific antibodies. Numbers at the bottom of each lane represent the amount of specific protein remaining in the supernatant after immunodepletion as determined by densitometric quantitation of images. (Bottom) Levels of cyclin E surviving mock or p21 immunodepletion. The amount of cyclin E remaining in supernatants from mock-depleted extracts was taken as 100%. (C) Quantitation of three independent experiments similar to those described for panel B in which levels of p21 and cyclin E were monitored simultaneously after cyclin D1 and p21 immunodepletion, respectively. Error bars indicate the standard deviations of the samples.

To approach this question directly, we undertook immunodepletion studies with cyclin D1- and p21-specific antibodies. The immunodepleted extracts were then examined for the levels of p21 remaining by Western blot analysis (Fig. 7B, top). Quantitation of p21 levels indicated that p21 immunodepletion removed over 99% of p21 levels from extracts obtained at $T = 0$ h or $T = 8$ h. Anti-cyclin D1 antibodies were similarly successful in removing cyclin D1 from cell extracts (data not shown). When anti-cyclin D1 antibodies were used for immunodepletion, about 10% of the cells' complement of p21 could be removed from extracts prepared at $T = 0$ h while more than 60% of the p21 pool could be immunodepleted from estradiol-treated extracts ($T = 8$ h). Therefore, within 8 h of estradiol treatment, the bulk of cellular p21 associated with cyclin D1-cdk4. We concluded that much and perhaps all of the observed decrease in cdk2-inhibitory activity observed in cell extracts from estradiol-treated cells could be ascribed to this redistribution of p21 to cyclin D1-cdk4 complexes.

To address whether the increase in association of p21 with cyclin D1 coincided with a decrease in the amount of cyclin E complexed with p21, we performed the following experiment. Cell extracts from tamoxifen-arrested cells or from cells treated with estradiol for 8 h were subjected to immunodepletion with p21 antibodies. As shown in Fig. 7B (bottom), the percentage of cyclin E that was not bound by p21 increased from 27% ($T = 0$ h) to 52% after 8 h of estradiol treatment. These results indicated that the activation of cyclin E-cdk2 complexes (Fig. 3B and Fig. 5) correlated with the increase of the amount of cyclin E not complexed to p21. To further strengthen the correlation between the increase in cyclin D1 binding to p21 and the release of p21 from cyclin E-cdk2, we repeated the immunodepletion experiment with several independently obtained samples. The results of three of these experiments are shown in Fig. 7C. The levels of p21 associated with cyclin D1 increased on average from ~20 to ~60% while the levels of cyclin E associated with p21 decreased from ~80 to ~60% following estradiol treatment.

The experiments described above measured the exchange of p21 between cyclin E-cdk2 and cyclin D1 after 8 h of estradiol treatment. However, the induction of cyclin D1 protein levels already occurred between 2 and 4 h after estradiol addition (Fig. 3A and 7A). In order to determine the amount of cyclin E associated with p21 during the period when cyclin D1 expression was induced, we performed an immunodepletion with anti-p21 antibodies at different times poststimulation with estradiol, measuring the amounts of residual cyclin E in cell lysates that survived immunodepletion and were therefore not complexed with p21. An increase in the levels of cyclin E that was not associated in complexes with p21 was evident in lysates prepared from cells at 2 h after estradiol treatment and reached a maximum in lysates prepared after 6 h of estradiol treatment (data not shown). These results correlated with the increase in p21 association with cyclin D1 as shown in Fig. 7A (bottom) and, more importantly, with the increase in cyclin E-cdk2-associated kinase activity (Fig. 5). Taken together, these results indicated that the activation of the cyclin E-cdk2 complex after estradiol stimulation is the result of the redistribution of p21 from cyclin E to cyclin D1.

Characterization of active cyclin E-cdk2 complexes. The data described above suggested a strong correlation between increases in cyclin E-cdk2 complexes devoid of p21 and increases in cyclin E-cdk2 activity. This correlation argued that the pool of cyclin E-cdk2 free of p21 was responsible for the cyclin E-associated histone H1 kinase. However, cyclin E-cdk2 complexes can also be regulated by bound p27 molecules, making it possible that p27 was binding and regulating cyclin

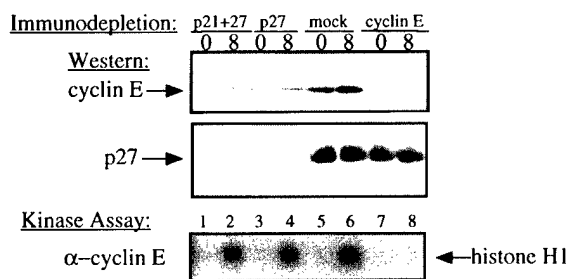


FIG. 8. Characterization of the presence of p27 in cyclin E-cdk2 complexes and formation of active cyclin E-cdk2 complex. Cell extracts from tamoxifen-arrested cells and at 8 h after release by estradiol (times are indicated in hours at the top of the figure) were immunodepleted with nonspecific antibodies (mock) or the indicated specific antibodies. For double immunodepletion of p21 and p27 (p21+p27), a mixture of both specific antibodies was used in the three sequential immunodepletions. (Top) Western blot analysis of cyclin E remaining in the supernatant after immunodepletion. A total of 100 μ g of total protein from each supernatant was analyzed. (Middle) Western blot analysis of p27 remaining in the supernatant after immunodepletion. (Bottom) Cyclin E-dependent kinase activity against histone H1 was assayed in supernatants after respective immunodepletions.

E-cdk2 activity in a manner similar to that of p21. For this reason, we determined if the association between p27 and cyclin E-cdk2 complexes was affected by estradiol. Immunodepletions with anti-p27 antibodies were performed on extracts obtained from cells either arrested by tamoxifen or released by estradiol for 8 h with anti-p27 antibodies. p27 antibodies were able to immunodeplete similar levels of cyclin E (>80%) at both time points (Fig. 8, top; compare lanes 3 and 4). In this experiment, p27 was efficiently immunodepleted by the specific antibodies where no effect on p27 was observed in mock-depleted extracts (Fig. 8, middle). To our surprise, either the anti-p21 or the anti-p27 antibody could deplete the majority (over 60%) of cyclin E. One explanation for this apparent discrepancy is that some cyclin E-cdk2 complexes bind p21 and p27 molecules simultaneously. Nonetheless, these results did indicate that changes in p27 association with cyclin E-cdk2 could not explain the observed increases in cyclin E-cdk2 activity following estradiol treatment.

These conclusions were based on the notion that association of cyclin E-cdk2 complexes with either p21 or p27 resulted in the functional inactivation of the cyclin-cdk complex. To validate this directly, we immunodepleted lysates with p21 and p27 antibodies simultaneously. An initial experiment indicated that such immunodepletion was able to completely remove cyclin E (~99%) from cells arrested with tamoxifen (Fig. 8, top, lane 1). The same immunodepletion of p21 and p27 from extracts of estradiol-treated cells containing active cyclin E-cdk2 complexes still immunodepleted the bulk (90%) of cyclin E (lane 2). These results indicated that, even at the peak of activity, the pool of cyclin E-cdk2 complexes free of cdk inhibitors represented only a minor proportion of the total cyclin E-cdk2 complexes present in the MCF-7 cells.

To test if the observed cyclin E-cdk2 activity was associated exclusively with the cyclin E-cdk2 complexes that were free of p21 and p27, we evaluated the cyclin E-dependent kinase activity remaining in the supernatants of the extracts that were previously immunodepleted with p27 antibodies either alone or in conjunction with p21 antibodies (Fig. 8, top). As control, we assayed the cyclin E-dependent kinase activity left in the supernatant fractions after cyclin E immunodepletion. The results of histone H1 kinase assays after immunoprecipitation of these immunodepleted extracts with cyclin E antibodies are shown in Fig. 8 (bottom). As expected, cyclin E immunodeple-

tion completely removed all cyclin E-dependent kinase activity from the extracts (lanes 7 and 8). However, neither p27 immunodepletion nor simultaneous immunodepletion of p21 and p27 decreased the cyclin E-dependent kinase activity of the extracts significantly compared to mock-depleted extracts (lanes 1 to 6). Thus, we concluded that the active cyclin E-cdk2 complexes formed after estradiol-induced cell cycle progression are devoid of these two cdk inhibitors.

Taken together, these results argue that estradiol was able to cause a reduction in the levels of cdk-inhibitory activity associated with cyclin E-cdk2 complexes during the tamoxifen arrest. This reduction was achieved through the induction of cyclin D1 expression, which allowed the formation of cyclin D1-cdk4-cdk6 complexes; these complexes, in turn, served to abstract p21 associated with cyclin E-cdk2, giving rise to active cyclin E-cdk2 complexes.

DISCUSSION

The studies presented here describe the effects that estrogen has on the growth of estrogen-sensitive MCF-7 cells. We conducted these experiments with the aim of determining the specific mechanisms by which estrogen affects the cell cycle clock and leads thereby to the proliferation of mammary epithelial cells. Our data indicate that estrogen can induce rapid and strong activation of cyclin E-cdk2 complexes through its ability to increase cyclin D1 expression. The observed rapid response of the cell cycle clock apparatus to estrogen stimulation argues for a direct effect of estrogen on one or more components of this apparatus. Conversely, it provides evidence against an indirect mechanism involving induction by estrogen of growth factors that in turn act in an autocrine fashion to elicit the observed responses.

The upregulation of cyclin E-cdk2 activity took place as a consequence of the increased cyclin D1 expression induced by estrogen. The increased levels of cyclin D1 led to increased cyclin D1-cdk4 complexes (2, 40), a resulting increase in the amount of the p21 cdk inhibitor associated with this cyclin-cdk complex, and a corresponding reduction in the amounts of p21 bound to cyclin E-cdk2 complexes. This redistribution of the p21 inhibitor then permitted activation of the cyclin E-cdk2 complexes. Similar models have been proposed to explain the mechanism of action of growth inhibitors. For example, TGF- β , by inducing expression of p15 or by downregulating cdk4, can lead to a redistribution of the p27 cdk inhibitor, from cyclin D1-cdk4 to cyclin E-cdk2 complexes (11, 16, 43).

Role of cyclin D1 protein in proliferation of ER-positive cells. Cyclin D1 gene amplification is observed in 15 to 30% of breast cancers (39). A strong correlation of increased levels of cyclin D1 mRNA with ER overexpression has also been noted elsewhere (7). Moreover, this amplification has been associated with a poor prognosis for ER- and progesterone receptor-positive breast tumors (5). Our results indicate that estrogen can induce expression of cyclin D1 in the first hours after its addition to tamoxifen-arrested cells (Fig. 3A and 7A). Thus, these results support the view that estrogen affects expression of cyclin D1 directly.

The model presented here suggests that the ER and cyclin D1 conspire to drive human mammary carcinoma cells through the G₁ phase of their cell cycle. High levels of the ER can cause high expression of cyclin D1 and the resulting removal of p21 from cyclin E-cdk2 complexes. Alternatively, high levels of cyclin D1, which may result from amplification of the cyclin D1 gene or other ER-independent mechanisms, may achieve the same end. Moreover, tumors expressing high levels of either the ER or cyclin D1 may be able to overcome the inhibitory

effects of concomitantly expressed p21 by sequestering the latter in cyclin D1-cdk4 or cyclin D1-cdk6 complexes. Our model is further supported by the work of Musgrove et al. (34). These authors have developed an inducible cyclin D1 system using another ER-positive breast cancer cell line (T47D). Induction of cyclin D1 expression in these cells leads to activation of cyclin E-cdk2 kinase, pRb hyperphosphorylation, and cell cycle progression.

Induction of cyclin D1 by estrogen recruits p21. The present data indicate that the cdk inhibitor p21 serves to couple cyclin D1 levels with the activity of cyclin E-cdk2. We conclude that p21 is involved in the observed cyclin E-cdk2 activation based on the following facts. (i) Extracts obtained from tamoxifen-arrested cells contain a readily detected cdk2-inhibitory activity (Fig. 6A). (ii) This activity can be immunodepleted by p21-specific antibodies but not by anti-p27 antibodies (Fig. 6C). (iii) The ability of p21 antibodies to immunodeplete cyclin E decreases after estradiol treatment of tamoxifen-arrested cells (Fig. 7B, bottom). (iv) Immunodepletion of cyclin D1 removes most of the p21 from extracts of estradiol-stimulated cells but not when extracts from tamoxifen-arrested cells are used (Fig. 7B, top). (v) Levels of cyclin D1 that are associated with p21 increase substantially following estrogen-mediated release of the tamoxifen block (Fig. 7A).

While this work was in progress, Foster and Wimalasena (13), using methionine-glutamine-deprived MCF-7 cells, also observed increased synthesis of cyclin D1, cyclin E-cdk2 activity, and pRb hyperphosphorylation after estradiol-induced cell cycle progression. However, these authors propose that the regulation of cyclin E-cdk2 activity is due to a decrease in p27. The slower kinetics of cyclin E-cdk2 activation and the changes in p27 levels observed by these authors may be due to their use of amino acid starvation to synchronize MCF-7 cells.

Our model of cyclin D1-mediated p21 redistribution is further supported by biochemical measurements that gauge the relative affinities of the p21 cdk inhibitor for association with the cyclin E-cdk2 and cyclin D2-cdk4 complexes. Others have shown elsewhere (17) that the K_i value for inhibition of cyclin E-cdk2 by p21 is 3.7 nM while that for cyclin D2-cdk4 is 0.6 nM. We presume that these inhibitory concentrations reflect the relative affinities of p21 for these two types of complexes and that the affinity of p21 for cyclin D2-cdk4 is similar to that for cyclin D1-cdk4. These relative affinities of p21 would therefore explain the ability of cyclin D1-cdk4 or cyclin D1-cdk6 complexes to abstract p21 from cyclin E-cdk2 complexes. Consistent with this notion is the recent demonstration that cyclin D1 is more effective than cyclin E in rescuing p21-dependent growth suppression (28).

Yet other lines of evidence point to a role played by cyclin D-cdk4 or cyclin D-cdk6 complexes as a reservoir of cdk inhibitors (41, 46, 48). For example, the levels of different cdk inhibitors relative to those of cyclin D-cdk4 or cyclin D-cdk6 complexes may determine a signal threshold level that determines the timing in G_1 of cyclin E-cdk2 activation and consequent pRb phosphorylation. Consistent with this reservoir model, others have shown that p53-mediated growth arrest through induction of p21 can be overcome by ectopically expressed, inactive forms of cdk4 and cdk6 (27).

The dramatic increase of cyclin E-cdk2 catalytic activity following estradiol treatment is due to the translocation of p21 from the cyclin E-cdk2 complexes to cyclin D1-cdk4 complexes. Perhaps surprisingly, this marked increase in the functioning of the cyclin E-cdk2 complexes is attributable to the activity of only a small minority of the complexes present in the estrogen-treated cells. Thus, after estradiol treatment, the entirety of the cyclin E-cdk2 catalytic activity is traceable to a

small proportion of the cyclin E-cdk2 complexes that have been freed of p21; the vast majority of these complexes remain associated with cdk inhibitors and in a functionally inactive state. Therefore, in contrast with previous reports (17, 55), we find that the dissociation of p21 from cyclin E-cdk2 complexes is essential for their functional activity. A similar requirement for the removal of p21 from cdk2 complexes has been noted recently, arguing that p21 interaction is strictly inhibitory for cyclin E-cdk2 complexes present in certain cells (27).

In summary, we conclude that estrogen, by regulating cyclin D1 expression and p21 distribution, can control cyclin E-cdk2 activity and pRb phosphorylation in breast cancer-derived MCF-7 cells. This major role of estrogen in controlling levels and activities of G_1 cyclin and the associated kinases fits well with its essential role in driving mammary epithelial cell proliferation.

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Functional Activity of Ectopically Expressed Estrogen Receptor Is Not Sufficient for Estrogen-mediated Cyclin D1 Expression¹

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Abstract

Estrogen receptor function can drive cyclin D1 expression and proliferation in human breast cancer cells (MCF-7). Recent studies showing that estrogen receptor-positive epithelial cells in the human mammary gland are nonproliferative suggest that the direct mitogenic effect of estrogen on mammary epithelial cells may be acquired during breast cancer development. Because estrogen-dependent cyclin D1 expression has been linked to its mitogenicity, we characterized the ability of estrogen to regulate cyclin D1 expression in estrogen receptor-negative breast cancer cells (MDA-MB-231) and nontransformed human keratinocytes (HaCaT) stably expressing the estrogen receptor. In both cases, estrogen receptor function did not induce cyclin D1 expression. Although MCF-7 cells respond to estrogen by inducing the AP-1 family components c-Fos and c-Jun, HaCaT cells expressing estrogen receptor do not. These results may explain the lack of estrogen-dependent cyclin D1 expression and proliferation in cells ectopically expressing the estrogen receptor. Therefore, estrogen receptor function alone is not sufficient for estrogen-dependent cyclin D1 expression and proliferation. Other transcriptional cofactors that allow estrogen receptor to induce expression of AP-1 may be required for estrogen to act as a mitogen.

Introduction

Estrogen is an essential hormone that controls the normal physiology of the mammary gland and breast cancer development. To determine how estrogen regulates growth of breast cancer cells, we and others have characterized the effects of estrogen on the cell cycle of MCF-7 breast cancer cells (1-4). Such studies have indicated that the induction of cyclin D1 by estrogen may be a key to understanding estrogen-dependent proliferation. This estrogen-dependent expression of cyclin D1 is essential for estrogen-induced proliferation of MCF-7 cells (5) and is the earliest estrogen-mediated effect on the cell cycle machinery (3, 4). Ectopic expression of cyclin D1 in MCF-7 cells mimics estrogen effects on the cell cycle (6). In addition, inducible overexpression of cyclin D1 in these cells reverses the growth-inhibitory effects of antiestrogen (7). Together, these results suggest that the ability of estrogen to drive cyclin D1 expression is crucial for the proliferation of ER⁺-positive breast tumors.

The precise molecular mechanism by which estrogen and its receptor control cyclin D1 expression is at present poorly defined. Cyclin D1 does not represent a classical ER target gene, because the cyclin D1 promoter lacks an ERE. Altucci *et al.* (1) mapped the estrogen-responsive region to a fragment between -944 and -136 of the cyclin D1 promoter. Several potential binding sites for known tran-

scription factors can be found in this region of the promoter, such as a site for the AP-1 transcription factor. This suggested the possibility that estrogen regulates cyclin D1 expression through modulation of AP-1 activity. However, no conclusive evidence exists at present to validate this hypothesis. It is unclear whether the presence of ER is sufficient to confer estrogen-mediated cyclin D1 expression.

Several investigators have stably introduced ER into ER-negative cells and have demonstrated estrogen-dependent expression of classical ERE-containing genes such as *cathepsin D* and *transforming growth factor- α* (8-10). However, estrogen did not stimulate proliferation of these cells, and in many cases, estrogen caused growth inhibition (10, 11). In addition, a recent histopathological study demonstrated that normal ER-positive cells in the human mammary gland *in vivo* are nonproliferating, whereas ER-positive breast cancer cells are actively proliferating (12). These two observations together suggest that the presence of ER *per se* is not sufficient for estrogen-induced proliferation of the ER-positive cell.

A possible explanation for the lack of estrogen-stimulated proliferation in cells ectopically expressing ER is that estrogen is unable to induce cyclin D1 expression in these cells. To test this hypothesis, we determined whether MDA-MB-231 and HaCaT cells engineered to express ER can up-regulate cyclin D1 in response to estrogen. Our results suggest that expression of ER alone is not sufficient to confer estrogen-inducible cyclin D1 expression. The absence of cyclin D1 induction by estrogen and the ER may be related to the inability of this receptor to regulate the expression of components of the AP-1 transcription factor complex.

Materials and Methods

Cell Culture, DNA, and Transfection. S30 cells were generously provided by Dr. V. Craig Jordan (Northwestern University Medical School, Chicago, IL). The plasmid pCMV-ER was a gift of Dr. Myles Brown (Dana-Farber Cancer Institute, Boston, MA). The ERE-driven luciferase constructs ERE-SV40-luc and ERE2-109-A3-luc were kindly given to us by Dr. Barry Gehm (Northwestern University Medical School, Chicago, IL). ER-expressing clones were obtained after calcium phosphate-mediated transfection of MDA-MB-231 and HaCaT cells with pCMV-ER and selection with G-418. Transient transfections of HaCaT cells were performed using Tfx-50 reagent (Promega Corp., Madison, WI), following the manufacturer's guidelines.

Antibodies and Western Analysis. Preparation of cell extracts and Western blot analysis was carried out as described previously (3). Monoclonal antibody against cyclin D1 (HD-45) was a gift from Ed Harlow (Massachusetts General Hospital, Charlestown, MA). Rabbit polyclonal antibodies used to detect ER (SC-543), c-Fos (SC-52), and c-Jun (SC-44) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Results

Cyclin D1 Regulation in ER-containing MDA-MB-231 Cells.

Several studies have shown that estrogen responsiveness of target genes can be obtained by stably expressing ER in ER-negative cells (8-10). However, estrogen does not have the ability to induce proliferation of these ER-containing cells. We wanted to understand this

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³ The abbreviations used are: ER, estrogen receptor; ERE, estrogen receptor element; CSS, charcoal-stripped fetal bovine serum; FBS, fetal bovine serum.

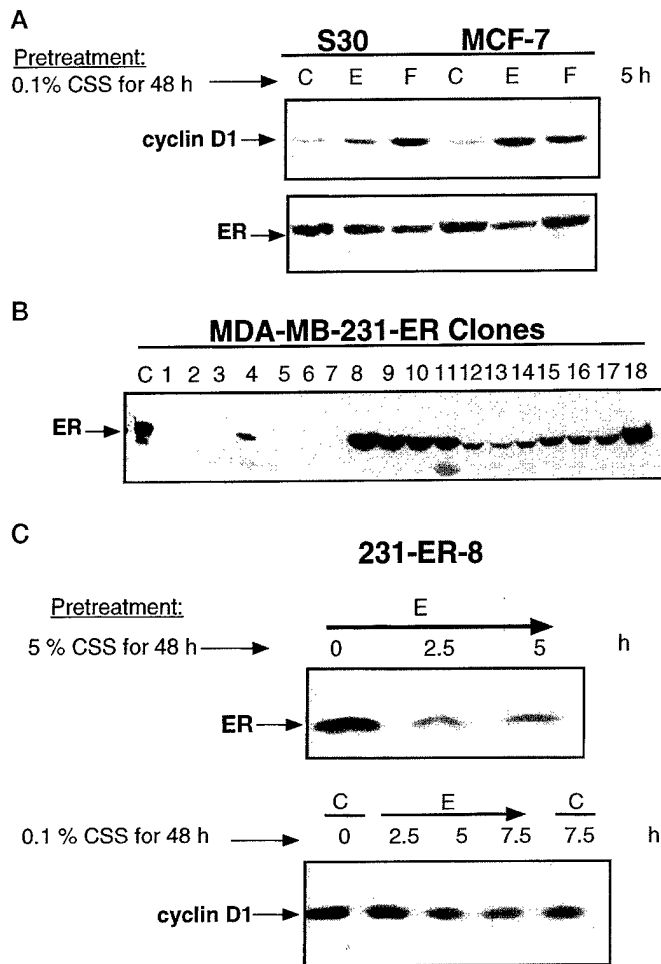


Fig. 1. Characterization of 231-ER cells. **A**, S30 cells and control MCF-7 cells were estrogen and serum deprived by incubating them in phenol red-free medium containing 0.1% CSS for 48 h. After this period, cells were treated with 5 nm of 17 β -estradiol (E), 5% FBS (F), or remained untreated (C). Five h later, all samples were harvested and processed for Western blot analysis. *Top*, analysis of cyclin D1 expression. *Bottom*, detection of ER expression. **B**, G418-resistant clones were isolated after transfection of MDA-MB-231 cells with pCMV-ER. Cell extracts were prepared and analyzed for ER expression by Western blot analysis. **C**: *top*, ER expression after estrogen treatment of steroid-deprived 231-ER-8 cells; *bottom*, cyclin D1 expression after addition of 5 nm 17 β -estradiol to 231-ER-8 cells maintained in 0.1% CSS for 48 h.

phenomenon in greater detail by determining whether the lack of proliferation was attributable to the inability of estrogen to induce cyclin D1. For this purpose, we used S30 cells, a derivative of MDA-MB-231, an ER-negative breast cancer cell line, which stably express ER and show estrogen-dependent expression of the progesterone receptor (10).

We wished to determine the ability of the ectopically expressed ER to induce cyclin D1 protein in the ER-expressing S30 cells. For this analysis, we first reduced the basal level of cyclin D1 protein in S30 cells by culturing these cells in phenol red-free medium containing 0.1% CSS. In this way, we also minimized exposure to residual ER-activating agents. After 48 h of serum and estrogen deprivation, S30 cells and MCF-7 cells received either 5 nm 17 β -estradiol or 5% FBS as a positive control. Another set of dishes remained untreated to determine the basal levels of cyclin D1. All of the samples were harvested after 5 h of treatment and processed for Western blot analysis.

Estrogen was unable to induce cyclin D1 expression significantly, whereas exposure of these cells to FBS resulted in strong induction of cyclin D1 (Fig. 1A, *top*). In contrast, in MCF-7 cells, both estrogen

and FBS were capable of strongly inducing cyclin D1 expression (Fig. 1A, *top*). This suggested that the presence of the ER in mammary carcinoma cells, although necessary for the estrogen-mediated stimulation of cyclin D1 synthesis in MCF-7 cells, was not sufficient in other cell types.

To confirm that the ectopically expressed ER was functional in our assay, the same Western blot was analyzed for changes in ER protein levels after estrogen treatment. Estrogen can down-regulate expression of its own receptor in MCF-7 cells or in other cells when ER is ectopically expressed (13, 14). This decrease in ER occurs at the protein and mRNA levels and depends on a functional ER. Indeed, addition of estrogen to serum-starved MCF-7 cells caused down-regulation of ER protein expression (Fig. 1A, *bottom*), as has been reported previously (13). In a similar way, S30 cells were able to modulate ER expression after estrogen addition (Fig. 1A, *bottom*), indicating that the ER is functional in these experiments.

To rule out the possibility that the results obtained were specific to the S30 cell clone, we generated a new series of ER-expressing MDA-MB-231 cells. Eighteen cell clones were isolated after transfection of MDA-MB-231 with pCMV-ER. Twelve of the clones obtained, termed 231-ER, expressed detectable levels of ER as assayed by Western immunoblot analysis (Fig. 1B). MCF-7 cells (*left lane*) were used as positive control. Four clones that displayed different levels of ER were further characterized to assess ER function and estrogen-dependent cyclin D1 expression. The results obtained with one of these clones (231-ER-8) are shown in Fig. 1C. Expression of ER protein was down-regulated to 30% of the initial level after 2.5 h of estrogen addition to estrogen-deprived 231-ER-8 cells (Fig. 1C, *top*). This indicated that ER was functional in these cells. However, cyclin D1 expression was not affected when estrogen was given to serum-starved 231-ER-8 cells (Fig. 1C, *bottom*). The other three clones analyzed were also not able to induce cyclin D1 expression after treatment with estrogen (data not shown). We concluded that the inability of estrogen to up-regulate cyclin D1 levels may be due to some specific features of these breast cancer cells (MDA-MB-231) that are extraneous to the ER itself.

Cyclin D1 Regulation in ER-containing HaCaT Cells. One explanation for the inability of the ectopic ER to regulate cyclin D1 expression in the MDA-MB-231 cells is that these cells are derived from tumors, and that some of the changes that they have undergone during tumor progression prevent the ER from interacting functionally with the cyclin D1 promoter. To address this possibility, we expressed the ER in a quite distinct type of human epithelial cell, the HaCaT nontransformed human keratinocyte cell.

Thirty-eight HaCaT clones stably transfected with the pCMV-ER vector were obtained and analyzed for expression of ER. Twelve of these clones expressed ER at levels comparable with those seen in MCF-7 cells. Eight of them, termed HaCaT-ER clones, expressed functional ER as determined by the ability of estrogen to down-regulate its own receptor (data not shown).

To obtain a more quantitative estimate of ER function in these HaCaT-ER cells, we also determined their ability to mediate estrogen-dependent transcription. For this purpose, we measured estrogen-dependent luciferase expression after transient transfection with two distinct promoter constructs that contained either one (ERE-SV40-luc) or two copies (ERE2-109-A3-luc) of the ERE (15). Fig. 2A shows the results obtained with the cell clone (HaCaT-ER-38) that gave consistently the highest estrogen-dependent transcriptional induction. The amount of induction obtained (5-fold with ERE-SV40-luc and 10-fold with ERE2-109-A3-luc) is comparable with the response seen with MCF-7 cells (data not shown), thereby confirming the presence of functional ER in HaCaT-ER-38 cells.

We then proceeded to evaluate the ability of the ectopically ex-

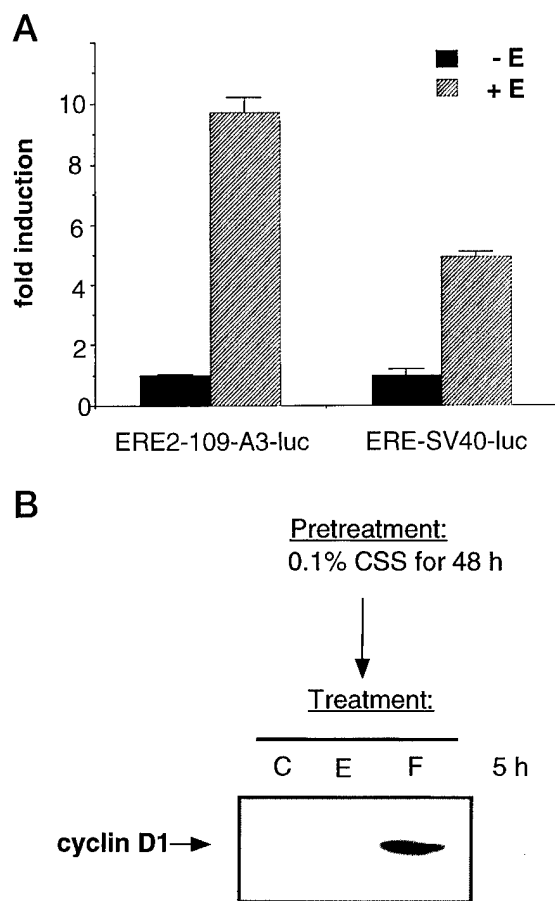


Fig. 2. Analysis of HaCaT-ER-38 cells. *A*, ER function was assessed by transient transfection of HaCaT-ER-38 cells with ERE-driven luciferase constructs. The amount of relative luciferase units obtained in the absence of estrogen was taken as one. *B*, cyclin D1 regulation in HaCaT-ER-38 cells. Asynchronously growing HaCaT-ER-38 cells were placed 0.1% CSS. After 48 h, cells were either treated with 5 nM 17 β -estradiol (*E*) or 5% FBS (*F*). Control cells (*C*) received no treatment. All samples were harvested after 5 h, and cyclin D1 expression was determined by Western analysis.

pressed ER in HaCaT-ER-38 cells to activate *cyclin D1* gene expression. To do so, we starved these cells for 48 h to reduce the basal level of cyclin D1 expression. Similar to what we observed with 231-ER cells, estrogen addition was unable to up-regulate cyclin D1 expression, whereas 5% FBS was able to induce cyclin D1 levels dramatically (Fig. 2*B*). Comparable analyses were carried out with eight other ER-expressing HaCaT cell clones, and none of these was able to up-regulate cyclin D1 expression in response to added estrogen (data not shown). Thus, as was seen previously with the MDA-MB-231 cells, the presence of a functional ER did not suffice to allow estrogen to induce expression of cyclin D1.

Changes in AP-1 Components after Estrogen Treatment. These data suggested that the regulation of cyclin D1 expression by the ER is complex and may involve the mediation of other proteins that convey signals between the ER and the cyclin D1 promoter. Such proteins might hypothetically be absent or functionally inactive in the MDA-MB-231 cells and the HaCaT cells, explaining the inability of the ER to activate cyclin D1 expression in these cells.

As mentioned earlier, an attractive candidate for regulating cyclin D1 expression is the AP-1 transcription factor. Estrogen can affect AP-1 either by regulating the synthesis of AP-1 family members (16–18) or by modulating its transcriptional activity (19). Moreover, both the Fos and Jun proteins, two common component subunits of the AP-1 factor, are known to be involved in the regulation of *cyclin D1* gene expression (20, 21). For these reasons, we decided to evaluate

whether there was a differential regulation of these genes by estrogen in MCF-7 and HaCaT-ER cells.

To approach this question, we tested whether estrogen could modulate expression of *c-Fos* and *c-Jun* levels in either MCF-7 or HaCaT-ER-38 cells. Asynchronously growing MCF-7 and HaCaT-ER-38 cells were serum-starved in 0.1% CSS for 48 h. Cells were treated with either 5 nM 17 β -estradiol or 5% FBS and harvested for Western analysis at 1 or 3 h. To begin, we determined the expression of the cyclin D1 protein. Cyclin D1 levels were induced in MCF-7 cells after 3 h of either estrogen or FBS treatment. As before, we observed that although estrogen and serum each were able to induce cyclin D1 expression in MCF-7 cells, only serum succeeded in doing so in HaCaT-ER-38 cells (Fig. 3, *top*).

The same Western blot used to detect cyclin D1 above was subsequently probed for the presence of Fos. The antibody used by us is specific for *c-Fos* and does not detect FosB, Fra-1, or Fra-2. Because of changes in phosphorylation, *c-Fos* is present as several distinctly migrating electrophoretic species (22). In MCF-7 cells, the levels of most of these isoforms increased by 3–4-fold after addition of either estrogen or FBS (Fig. 3, *middle*). HaCaT-ER-38 cells showed high basal levels of Fos expression when compared with MCF-7, and no induction of Fos occurred after addition of estrogen. However, FBS was able to augment Fos expression in these cells, suggesting that the lack of response to estrogen is not due to the presence of a *c-fos* gene in these cells that is refractory to further induction. Thus, the inability of the ER to induce cyclin D1 in HaCaT-ER-38 cells was paralleled by its inability to induce Fos synthesis.

A very similar outcome was noticed when *c-Jun* expression was evaluated (Fig. 3, *bottom*). This molecule is also subject to modification and can be seen as a series of bands. In the absence of serum, MCF-7 cells expressed low levels of the Jun protein. However, addition of estrogen or FBS to serum-starved MCF-7 cells caused a transient induction of Jun protein that parallels the changes detected for Fos. Again, as was the case with Fos, HaCaT-ER-38 cells had high basal levels of Jun that could be superinduced by FBS but not by estrogen. If anything, estrogen caused a decrease in Jun expression. Thus, the ability of either estrogen or FBS to induce cyclin D1 correlated well with their ability to increase Fos and Jun expression. Because induction of AP-1 members occurs within 1 h of addition of estrogen, before any cyclin D1 is observed, this suggests that induction of these genes anticipates and may be required for the proper regulation of cyclin D1 synthesis. Together, these results suggest that the lack of cyclin D1 induction by ectopically expressed ER may be explained by its inability to modulate expression of the *c-fos* and *c-jun* genes.

Discussion

An elucidation of the molecular mechanism of cyclin D1 regulation by the ER is of central importance to our understanding of the molecular pathogenesis of human breast cancers. If estrogen-dependent cyclin D1 expression is necessary for tumor growth, antiestrogens may be able to inhibit cancer growth by preventing estrogen-mediated cyclin D1 expression. Thus, development of antiestrogen resistance may be associated with changes in hormonal regulation of cyclin D1. It is plausible that alterations in the expression or function of specific molecules will allow cyclin D1 expression in the presence of tamoxifen. It is also possible that the ability of estrogen to induce cyclin D1 and proliferation is acquired as a pathological trait during the course of breast cancer development. For this reason, it is important to determine the mechanisms by which cancer cells display ER-dependent cyclin D1 transcription.

In the studies presented here, we have investigated the ability of ER

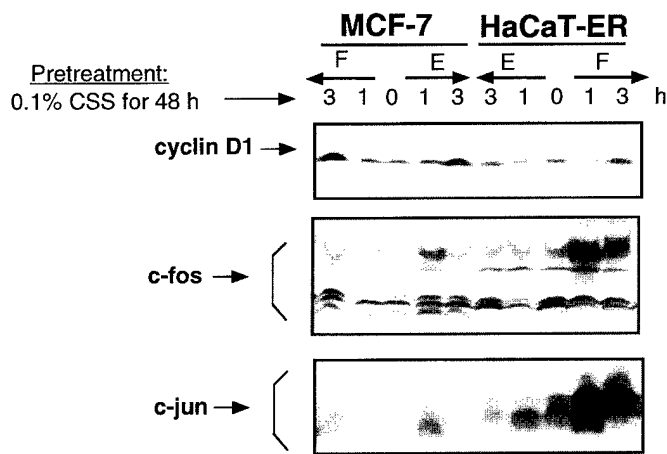


Fig. 3. Expression of AP-1 members after estrogen treatment of MCF-7 and HaCaT-ER cells. MCF-7 and HaCaT-ER-38 cells were estrogen and serum deprived by incubating them in 0.1% CSS for 48 h. At $T = 0$ h, cells received either 5 nM 17 β -estradiol (E) or 5% FBS (F). Samples were harvested at the indicated times and processed for Western analysis of cyclin D1 (top), c-Fos (middle), or c-Jun (bottom).

to drive expression of cyclin D1 after its stable introduction into two distinct, previously ER-negative human cell types. Our aim was to evaluate whether the inability of ectopically expressed ER to confer estrogen-dependent mitogenesis was related to the lack of cyclin D1 induction in ER-negative cells forced to express ER. Our findings indicate that in all of the clones ectopically expressing ER, cyclin D1 expression cannot be augmented by addition of estrogen. Nevertheless, these clones were competent to down-regulate ER expression or drive transcription of an ERE-luciferase construct after addition of estrogen. Moreover, we found that the inability of ER to drive cyclin D1 may be related to the lack of induction of AP-1 components by estrogen. Consequently, these data support the idea that estrogen may control proliferation of breast cancer cells by its ability to induce AP-1 members and cyclin D1.

Cyclin D1 as a Mediator of ER-stimulated Proliferation. The primary cell cycle target of estrogen in MCF-7 cells appears to be cyclin D1. This is supported by the fact that inducible expression of cyclin D1 overcomes growth-arrest mediated by antiestrogens (7). More recently, an extension of this earlier work demonstrated that high levels of cyclin D1 expression led to p21 redistribution, cyclin E-cdk2 activation, and retinoblastoma hyperphosphorylation of the pRB, the retinoblastoma protein, in antiestrogen-arrested MCF-7 cells (6). Thus, the increases of cyclin D1 protein by estrogen may be necessary and sufficient for proliferation of breast cancer cells.

In breast cancer tumors, the expression of cyclin D1 has been correlated with the expression of ER (23). The amplification of cyclin D1 occurs preferentially in ER-positive tumors, and the levels of cyclin D1 parallel in many cases the levels of ER present in the tumors (23). These observations suggest that the functional connections between the ER and cyclin D1 observed in MCF-7 cells *in vitro* correctly model what is seen in breast carcinomas *in vivo*. If this is the case, why does ER expression in ER-negative cells not confer estrogen sensitivity to the *cyclin D1* gene?

In many cases where ER has been stably introduced into ER-negative cells, different endogenous genes can be turned on, depending on the cell type used. One recent report, which appeared while our studies were ongoing, analyzed cyclin D1 expression in cells engineered to ectopically express ER (24). These authors analyzed cell proliferation and cyclin D1 after estrogen treatment of ER-transfected MCF-10AE^{w15}. Similar to our results, they could not detect any increases in cyclin D1 expression after estrogen treatment of the

transfected cells. They did not determine whether ER was transcriptionally active in MCF-10AE^{w15} cells. However, they found that ER from MCF-10AE^{w15} had altered ligand-binding affinity when compared with ER derived from MCF-7 cells by sucrose gradient sedimentation.

One reason for the inability of estrogen to drive cyclin D1 in ER-transfected cells could be that the *cyclin D1* locus is not responsive to transcriptional activation in these cells. However, the ability of FBS to rapidly yield increases in cyclin D1 levels argues against this possibility. Thus, the lack of estrogen-mediated cyclin D1 expression is most likely due to the presence or absence of other factors that mediate estrogen-dependent cyclin D1 transcription.

The recent reports that normal estrogen receptor-positive human mammary epithelial cells are nonproliferative suggest that estrogen is unlikely to act as a direct mitogen of normal ER-positive cells in the mammary gland (12, 25). Accordingly, the ability of estrogen to act as a direct mitogen and induce expression of cyclin D1 would appear to represent a pathological aberration acquired during the process of breast cancer progression. This aberration in signaling may occur early during tumor progression, because overexpression of cyclin D1 mRNA has been observed at early stages of breast cancer (26).

AP-1 Members as Potential Mediators of Estrogen-dependent Cyclin D1 Expression. In the search for possible molecular intermediaries between estrogen and cyclin D1 transcription, components of the AP-1 transcription factors emerge as highly attractive candidates. Studies of cells from mice bearing germ-line inactivations of the *c-jun*, *c-fos*, or *c-fosB* genes have revealed that c-Jun and either c-Fos or FosB are necessary for normal transcription of cyclin D1 (20, 21). Moreover, previous mapping of an estrogen-responsive region in the cyclin D1 promoter identified a region of the promoter that contained an AP-1 site but no ERE elements (1). Together, these observations suggest that the ability of estrogen to modulate AP-1 activity may be required for cyclin D1 expression and therefore proliferation.

Several possible scenarios can be considered to explain how estrogen interacts with AP-1: (a) the ER protein, like other steroid receptors, may bind physically to the AP-1 factor to activate transcription from AP-1 sites; or (b) alternatively, estrogen may act indirectly to drive synthesis of the component subunits of the AP-1 factor, which then proceed to assemble and drive the transcriptional activation of the *cyclin D1* gene locus (27); or (c) a third scenario could be that both mechanisms may operate in MCF-7 cells. Our results revealed that there is a differential regulation by estrogen in the synthesis of AP-1 members between MCF-7 cells and HaCaT-ER cells. There is a clear induction of c-Jun and c-Fos by estrogen in MCF-7 but not in HaCaT-ER. However, both gene products are induced by FBS, indicating that, as was the case with the cyclin D1 promoter, the defect in estrogen signaling is not due to promoter silencing. This suggests that the lack of cyclin D1 induction by estrogen may be traced to the inability of estrogen to modulate c-Jun and c-Fos expression in HaCaT-ER cells.

The mechanisms by which estrogen promotes synthesis of AP-1 components in MCF-7 cells are not clear. Several investigators have tried to map estrogen-inducible sequences in the *c-fos* gene (28–30) or *c-jun* gene (31). These studies have led to the identification of a variety of candidate regulatory sequences in the promoters of these genes. For example, a recent study using MCF-7 was able to map the estrogen-responsive region to an imperfect Sp1-binding site (28). The authors claim that induction of *c-fos* expression depends on the formation of a transcriptionally active ER/Sp1 complex. Thus, the inability of estrogen to drive cyclin D1 expression in HaCaT-ER cells may be linked to a deficient interaction between ER and Sp1 in these cells.

In summary, we propose that the ability of ER to drive ERE-

mediated transcription can be dissociated from its role as a mitogen. The competence of estrogen to drive proliferation may be linked to the regulation of cyclin D1 transcription, which in turn may be mediated by the actions of the AP-1 transcription factor. This points to the importance of determining how estrogen modulates AP-1 function and what the specific changes in breast cancer cells are that allow estrogen to affect AP-1, cyclin D1, and proliferation. Understanding these mechanisms may provide clues on how ER-positive breast tumors develop and how they become refractory to antiestrogen treatment.

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Responsiveness of cyclin D1 promoter constructs to ectopically expressed AIB1 protein.

- a. Comparison of ERE-luc and Cyclin D1-luc reporter construct responsiveness to co-expressed AIB1. AIB1 enhancement of estrogen-dependent transcription of each promoter was calculated as the ratio between fold induction by estrogen after transfection of 250 ng. of an AIB1 expression plasmid and in the absence of the transfected AIB1 plasmid. The values observed in the absence of additional AIB1 were normalized to 100%.
- b. Specificity of the transcription-enhancing effect of co-expressed AIB1. HaCaT-ER cells were transfected with pGL3-luc vector lacking any promoter activity, cyclin E-luc or cyclin D1-luc reporter constructs. . Estrogen-dependent expression was determined at different doses of AIB1.

FIGURE 3A

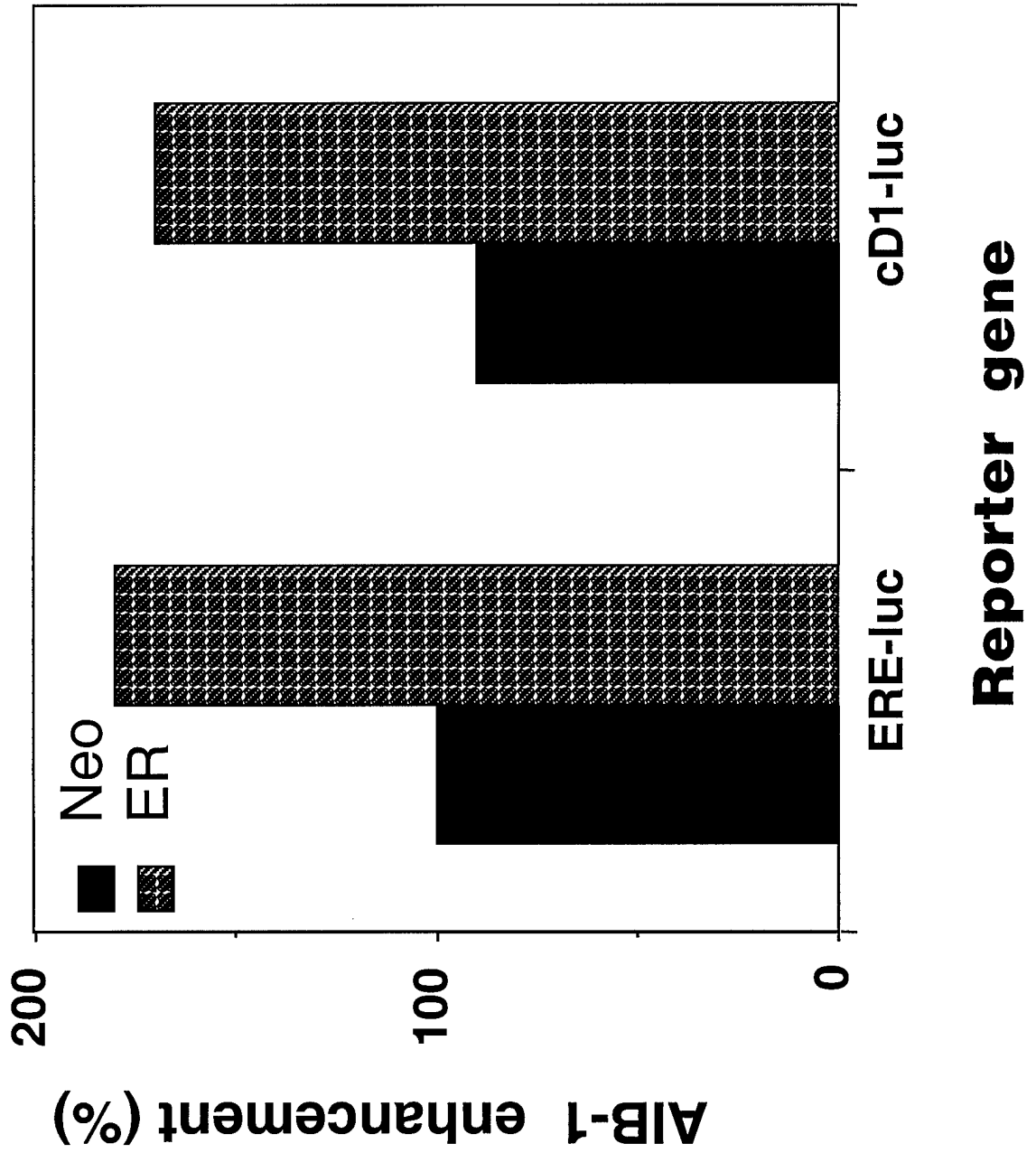
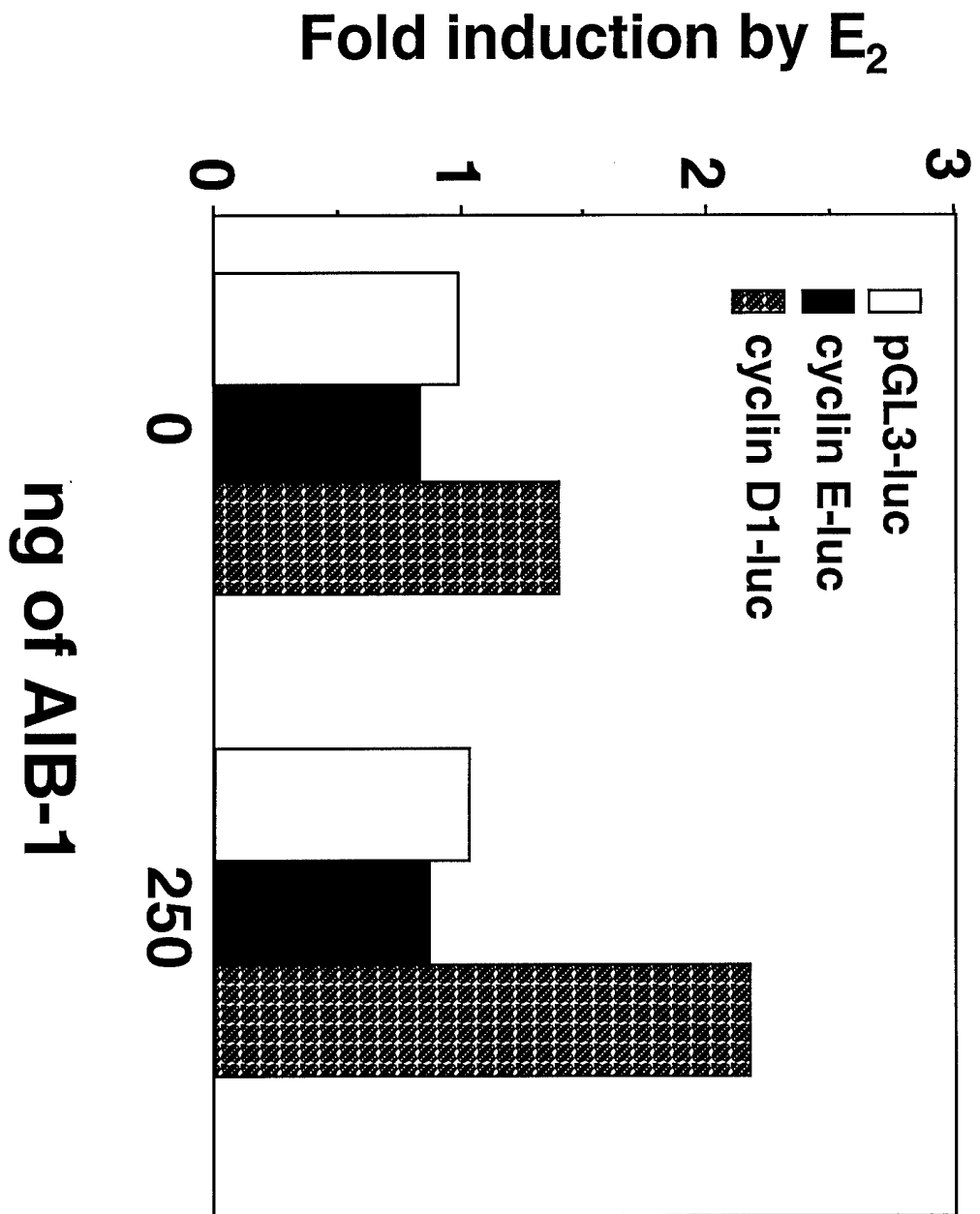


FIGURE 3B



A paracrine role for the epithelial progesterone receptor in mammary gland development

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Contributed by Robert A. Weinberg, February 13, 1998

ABSTRACT Recently generated progesterone receptor (PR)-negative (PR^{-/-}) mice provide an excellent model for dissecting the role of progesterone in the development of the mammary gland during puberty and pregnancy. However, the full extent of the mammary gland defect in these mice caused by the absence of the PR cannot be assessed, because PR^{-/-} mice do not exhibit estrous cycles and fail to become pregnant. To circumvent this difficulty, we have transplanted PR^{-/-} breasts into wild-type mice, and we have demonstrated that the development of the mammary gland in the absence of the PR is arrested at the stage of the simple ductal system found in the young virgin mouse. Mammary transplants lacking the PR in the stromal compartment give rise to normal alveolar growth, whereas transplants containing PR^{-/-} epithelium conserve the abnormal phenotype. Chimeric epithelia in which PR^{-/-} cells are in close vicinity to PR wild-type cells go through complete alveolar development to which the PR^{-/-} cells contribute. Together, these results indicate that progesterone acts by a paracrine mechanism on a subset of mammary epithelial cells to allow for alveolar growth and that expression of the PR is not required in all the cells of the mammary epithelium in order for alveolar development to proceed normally.

The mouse provides a useful model to study mammary gland development. At the onset of puberty, a simple system of branching ducts begins growing out from the nipple area into a pad of fatty connective tissue that underlies the skin. During the luteal phase of the estrous cycles, the ductal system becomes more complex through the growth of side branches. Ductal side-branching becomes more extensive during early pregnancy, and subsequently alveolar bodies develop from these ducts, fill up the fat pad, and differentiate to become the sites of milk production.

The serum levels of the sex steroid progesterone are elevated during diestrus, the phase of luteal activity of the estrous cycle, and pregnancy. Moreover, experimental manipulation of the hormonal system has implicated this hormone as an essential stimulus required for the induction of ductal branching and for alveologenesis (1). However, the elucidation of the role of progesterone is complicated by the fact that, in the mammary epithelium, synthesis of the progesterone receptor (PR) depends on estrogen, the serum levels of which are also elevated during puberty and pregnancy. This has made it difficult to assess which developmental effects can be attributed to progesterone alone.

To dissect the role of progesterone from that played by estrogen, we generated mice lacking the PR by targeted inactivation of the PR gene in the mouse germ line (2). The

mammary glands of the resulting young virgin PR^{-/-} females show the same extent of ductal development as is seen in wild-type (wt) female mice (2). However, when wt and PR^{-/-} virgin females were exposed to estradiol and progesterone, the wt breast tissue responded with side-branching and lobuloalveolar development, whereas the mammary glands of PR^{-/-} females remained essentially unchanged. This suggested that PR is not required for initial ductal growth but is essential for subsequent side-branching and alveologenesis.

The administration of exogenous estrogen and progesterone, as was done in the above-described experiments and in a subsequent study extending this work (3), did not permit us to properly gauge the full spectrum of complex hormonal changes that occur during a normal pregnancy. During this period, the serum levels of a wide array of other hormones, including growth hormone, prolactin, placental lactogen, and adrenal steroids, are elevated. Moreover, the secretion of each of these hormones follows specific diurnal rhythms, and it is unlikely that injections of exogenous hormones achieve physiologic serum levels and correct local concentrations.

For these reasons, we resorted to transplanting PR^{-/-} mammary tissues into wt animals that were subsequently impregnated. This allowed us to study the morphogenesis of the breast tissue in a hormonal environment that faithfully recapitulated that seen in pregnant, unmanipulated, wt animals. The results of previous research did not provide us with clear predictions of the outcomes of these transplantation experiments. For example, the PR is expressed in both stromal and epithelial compartments of the mammary gland (4). Within the epithelium, the distribution of the PR is variegated (5). Together, such observations provided no clear indication of the contributions of various subtypes of stromal and epithelial cells to mammary epithelial morphogenesis occurring in the presence or absence of the PR.

By grafting PR^{-/-} epithelium or stroma in combination with PR wt stroma or epithelium, we have found that the primary target for progesterone is the mammary epithelium, while a direct response of the mammary stroma is not required in order for side-branching and lobuloalveolar development to occur. Furthermore, PR^{-/-} mammary epithelial cells can give rise to alveoli when placed in close vicinity to PR wt epithelial cells, indicating that progesterone does not need to act directly on the alveolar cells and instead can orchestrate the morphogenetic and proliferative events of alveologenesis by affecting nearby cells in the mammary epithelium.

MATERIALS AND METHODS

Mice. ROSA26 and RAG1^{-/-} mice were purchased from The Jackson Laboratory. The PR mutant mice were described

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Abbreviations: PR, progesterone receptor; wt, wild-type; X-Gal, 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside; MEC, mammary epithelial cell.

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elsewhere (2); transcription of both A and B forms of the PR was disrupted. All mice were bred in 129SV/C57BL6 genetic background.

For PR genotyping, genomic DNA was isolated from tails and analyzed by PCR. PCR was performed by denaturing the DNA at 94°C for 1 min, followed by 30 cycles of amplification: 94°C for 1 min, 60°C for 2 min, 72°C for 1 min, and a final extension step at 72°C for 5 min. The following PR-specific primers were used: P1 (5'-TAG ACA GTG TCT TAG ACT CGT TGT TG-3'), P2 (5'-AGC AGA AAA CCG TGA ATC TTC-3'), and a *neo* gene-specific primer, N2 (5'-GCA TGC TCC AGA CTG CCT TGG GAA A-3').

Presence of the β -galactosidase transgene was tested for by subjecting a piece of tail to the 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-Gal) staining procedure described below.

Whole-Breast Transplant. Four- to 6-week-old PR^{+/+} or PR^{-/-} female mice were sacrificed and their inguinal mammary glands were dissected. RAG1^{-/-} females of the same age were anesthetized with Avertin i.p. (6). The ventral skin was incised and the abdominal muscle wall was exposed. A PR^{-/-} and a PR^{+/+} mammary gland were placed onto the abdominal wall and the incision was closed with surgical staples. Three weeks after surgery the recipients were mated. They were sacrificed at parturition. The two transplanted glands and an endogenous mammary gland were analyzed by whole-mount microscopy.

Fat-Pad Transplant. Three-week-old PR^{+/+}, PR^{+/-}, and PR^{-/-} females were sacrificed and their inguinal mammary glands were exposed. The nipple-near region was removed. Into the remaining empty fat pad we injected primary mammary epithelial cells derived from ROSA26 females. The engrafted fat pads were placed onto the abdominal muscle wall of virgin RAG1^{-/-} females.

Transplantation of Mammary Epithelium. The fat pads of 3-week-old RAG1^{-/-} females were cleared (see above). Pieces of mammary tissue of 1-mm diameter were removed from the nipple region of PR^{+/+} and PR^{-/-} females and implanted as described before (7). Alternatively, the cleared fat pads were injected with PR^{+/+} and PR^{-/-} primary cells, cultured as described in ref. 8.

Mammary Gland Whole Mounts. The inguinal mammary glands were dissected, spread onto a glass slide, fixed in a 1:3 mixture of glacial acetic acid/100% ethanol, hydrated, stained overnight in 0.2% carmine (Sigma) and 0.5% AlK(SO₄)₂, dehydrated in graded solutions of ethanol, and cleared in 1:2 benzyl alcohol/benzyl benzoate (Sigma) as described previously (9).

Pictures were taken on a Leica MZ12 stereoscope with Kodak Ektachrome 160T.

X-Gal Staining. The transplanted mammary glands were dissected, fixed for 1 hr in 4% formaldehyde in phosphate-buffered saline (PBS), washed three times over 3 hr with rinse buffer (2 mM MgCl₂/0.1% sodium deoxycholate/0.2% Nonidet P-40 in PBS) and rotated in X-Gal staining solution (1 mg/ml X-Gal, 5 mM potassium ferricyanide, and 5 mM potassium ferrocyanide in rinse buffer) at 37°C for 18 hr, washed in PBS, and processed for whole-mounting as described above.

Histological Examination and Immunohistochemistry. For histological examination of the alveolar structures the whole-mounted mammary glands were washed in 100% ethanol prior to paraffin embedment. Sections were cut at 10 μ m. Anti- β -casein antiserum (10) was diluted 1:500 and applied overnight at 4°C. Biotinylated secondary antibodies were detected with a Vectastain ABC kit (Vector Laboratories).

RESULTS

Development of the Mammary Gland During Pregnancy in the Absence of the PR.

To analyze the role progesterone plays in the mammary gland during normal pregnancy, entire mammary glands from PR^{-/-} female mice and their wt littermates were transplanted onto the abdominal muscle wall of PR wt females. The transplanted glands included both epithelial and stromal compartments. The recipient females were of the same 129SV/C57BL6 genetic background and were homozygous for the inactivated RAG1 allele (11). Females of this genotype are immunocompromised and therefore able to accept allografts. The engrafted females were mated 3 weeks after surgery and sacrificed immediately after a completed pregnancy. In all cases, the implants along with an endogenous mammary gland were analyzed by whole-mount microscopy.

While the wt implants and endogenous glands (Fig. 1 *Center* and *Right*, respectively) showed full alveolar development at parturition, the PR^{-/-} grafts developed only a simple ductal system (Fig. 1 *Left*). These observations validated the transplantation procedure. More significantly, they demonstrated, as suggested by previous reports (1, 12), that progesterone is essential for side-branching and lobuloalveolar growth and showed that, in the absence of the PR, the mammary gland fails to undergo substantial proliferation in the presence of the full array of pregnancy-associated hormones.

Involvement of the Stromal and the Epithelial Compartments in PR-Mediated Responses. To address the question of whether progesterone acts on the mammary stroma or epithelium, engrafted animals were created in which either the mammary epithelium or the fat pad lacked PR because of inactivation of the PR gene. The development of the mammary gland in response to physiological hormonal stimulation was then followed.

In the mouse, the mammary epithelium grows out from the nipple into a fat pad that underlies the skin. At three weeks after birth, the epithelium of the gland has not yet penetrated extensively into the stroma and can be eliminated by removing the nipple region of the mammary gland (7). Mammary epithelial cells (MECs) that are introduced into the remaining "cleared" fat pad will give rise to a new ductal system. They can grow out from a piece of breast tissue that is placed into the fat pad (7, 13), or from single-cell suspensions that are injected into the fat pad (14).

We adapted these surgical procedures to create mammary glands that specifically lacked the PR in their stromal cells. Briefly, the nipple regions containing the mammary epithelium were removed from the fourth mammary glands of 3-week-old PR^{-/-} females and their wt littermates. The resulting cleared fat pads were then implanted with mammary epithelium derived from a wt donor. Subsequently, the resulting reconstituted mammary glands were dissected and transplanted onto the abdominal muscle wall of RAG1^{-/-} females.

We validated this transplantation procedure by implanting PR wt epithelium into PR wt fat pads. The resulting engrafted glands developed like the endogenous mammary glands in virgin as well as postpartum recipients, demonstrating that the

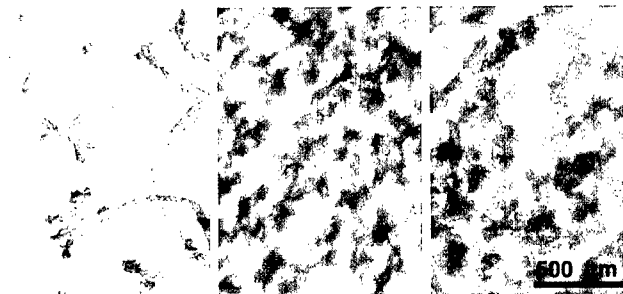


FIG. 1. Whole breast transplantation. Whole-mount preparations of the PR^{-/-} (*Left*) and PR^{+/+} (*Center*) whole breast implant and endogenous mammary gland (*Right*) derived from RAG1^{-/-} recipient mouse after parturition.

engrafted fat pad had become fully vascularized when transplanted in this fashion.

The interpretation of these experiments depended upon our ability to distinguish implanted mammary epithelium from any residual endogenous epithelium that inadvertently had not been removed during the preparation of the cleared mammary fat pads. In fact, in the virgin gland, it is easy to distinguish ducts arising from implanted epithelium from those that are endogenous to this gland because of the distinctive orientations of ductal growth. Thus, the endogenous epithelium grows unidirectionally from the nipple into the fat pad, whereas the ducts arising from the implant, which we place into the center of the cleared fat pad, grow centrifugally. At parturition, however, when the fat pad is filled with alveoli, it is difficult to distinguish the two ductal systems, making it impossible to rule out that the observed epithelial structures derive from residual endogenous epithelium.

To address this difficulty, mammary epithelium derived from ROSA26 female mice was exploited (15). Mice of this transgenic strain express the β -galactosidase gene in virtually all their tissues. The mammary epithelium of these ROSA26 mice was implanted into the cleared fat pads of wt mice. When these reconstituted fat pads were subjected to an X-Gal staining procedure, the implanted ROSA26-derived epithelium turned blue and could thus be unequivocally distinguished from any endogenous epithelium, which was visualized by the red color of the carmine/alum counterstain. Together, the above-described preliminary experiments and the use of ROSA26 cells validated our transplantation procedures and our ability to study engrafted tissues without the confounding effects of residual tissue originating from the recipient breast.

The above procedures were utilized to resolve the respective roles of stroma- and epithelium-derived PR populations in mammary gland proliferation and differentiation. First, ROSA26.PR^{+/+} epithelium was transplanted into cleared PR^{-/-} fat pads; the resulting reconstituted mammary glands were then placed onto the abdominal muscle wall of a RAG1^{-/-} recipient female. Four weeks later, the engrafted RAG1^{-/-} recipients were mated. After they had given birth, the transplanted mammary gland and an endogenous mammary gland were analyzed by whole-mount microscopy. As can be seen in Fig. 2, the injected ROSA26-derived mammary epithelial cells grew equally well in transplanted fat pads from wt (Fig. 2 *Right*) and PR^{-/-} (Fig. 2 *Left*) donors. This result demonstrated that the presence of the PR in the mammary stroma was not essential for the pregnancy-induced side-branching and lobuloalveolar development.

Next, we assessed the role of the PR in the epithelium independent of its function in the stroma. To do this, mammary epithelial cells derived from either PR^{-/-} or wt donors were transplanted into the cleared mammary fat pads of wt recip-

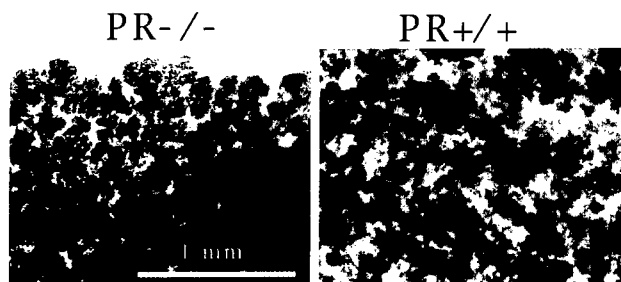


FIG. 2. Transplantation of engrafted fat pads. Whole-mount preparations of transplanted reconstituted breasts. Fat pads from PR^{-/-} or PR^{+/+} mice were engrafted with ROSA26 (β -galactosidase⁺) PR^{+/+} primary mammary epithelial cells and transplanted onto the abdominal muscle wall of PR^{+/+}.RAG1^{-/-} recipients. The reconstituted mammary glands were removed from the recipients after parturition and stained with X-Gal before whole-mounting.

ients. The engrafted recipients were mated and their mammary glands were analyzed at parturition. The results of these experiments are shown in Fig. 3. Whereas the wt implant gave rise to a fully developed mammary tree, the epithelium lacking the PR grew into only a simple ductal tree (Fig. 3 *Left*). Similarly, when we analyzed the mammary glands of engrafted virgin females 2 months after surgery, the wt implant as well as the endogenous breasts showed side-branching, whereas the PR^{-/-} breast had only a simple ductal system (Fig. 3 *Right*). Table 1 summarizes the results of these transplantation experiments. These results allowed us to conclude that the mammary epithelium is the prime target of progesterone both before and during pregnancy, and that a direct response of the mammary stroma to progesterone does not play an essential role.

Role of the PR in the Development of Alveoli. The experiments above indicated that the absence of the PR from all cells of the mammary epithelium resulted in a failure of side-branching and lobuloalveolar growth. However, they did not address the question of whether the presence of PR was required in all cells of the ductal epithelium or in only a subset of MECs in order for these morphogenetic processes to proceed normally.

To distinguish between these possibilities, we created mosaic mammary epithelia containing both PR^{-/-} and PR^{+/+} MECs. The latter cells were derived from ROSA26 mice. In this case, tissue structures composed of PR^{+/+} cells would turn blue upon X-Gal staining when analyzed by whole-mount microscopy. Structures composed of PR^{-/-} cells would turn red, being stained only by the carmine/alum counterstain.

Mixtures of PR^{+/+} and PR^{-/-} MECs in different ratios were injected into the cleared mammary fat pads of RAG1^{-/-} females. These mixtures were obtained either by combining single-cell suspensions derived from PR^{-/-} and PR^{+/+}.ROSA26 primary cultures or by mixing finely minced mammary tissues dissected from females of these two strains. Two months later, the engrafted recipients were mated, and the engrafted breasts were analyzed toward the end of pregnancy.

Depending on the degree of homogeneity of the injected mixture and the ratio in which the cells of the different genotypes were mixed, we found two types of chimerism. In the

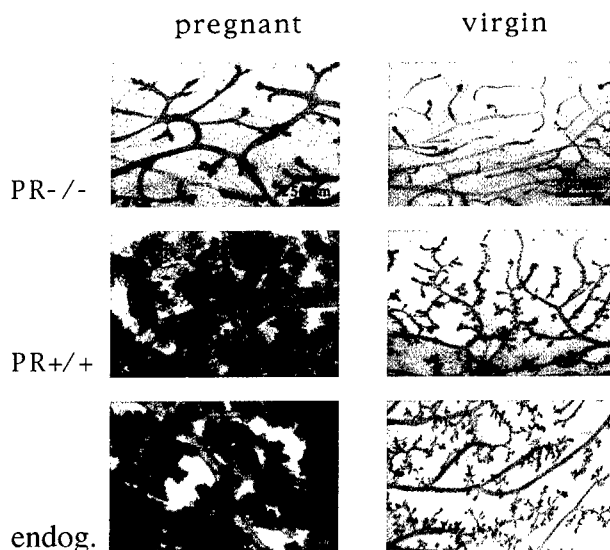


FIG. 3. Transplantation of epithelium. Whole-mount preparations of mammary glands from PR^{+/+}.RAG1^{-/-} recipients. (*Left*) Preparation derived from a recipient after parturition. (*Right*) Preparation derived from a virgin mouse. (*Top*) Transplanted PR^{-/-} epithelium. (*Middle*) Transplanted PR^{+/+} epithelium. (*Bottom*) Endogenous mammary gland.

Table 1. Requirement of the PR in the stroma and/or the epithelium for alveolar development in mammary transplants analyzed post partum

Transplant	No. samples with alveolar growth/no. successful transplants
Mammary glands <i>in toto</i>	
Stroma PR ^{+/+} /epithelium PR ^{+/+}	8/8
Stroma PR ^{-/-} /epithelium PR ^{-/-}	0/8
Fat pad injected with	
PR ^{+/+} .ROSA26 epithelium cells	
Stroma PR ^{+/+} /injected epithelium PR ^{+/+}	6/6
Stroma PR ^{+/-} /injected epithelium PR ^{+/+}	8/8
Stroma PR ^{-/-} /injected epithelium PR ^{+/+}	6/6
Epithelium	
Stroma (host) PR ^{+/+} /epithelial transplant PR ^{+/+}	13/13
Stroma (host) PR ^{+/+} /epithelial transplant PR ^{-/-}	0/13

first type, the mammary glands showed discrete sectors having distinct phenotypes. An example, representative of 17 samples of this type of chimerism, is shown in Fig. 4. One half of the epithelial component of the mammary gland stained red while the other half stained blue; this indicated the origins of these two sectors from PR^{-/-} and ROSA26 engrafted cells respectively. The sector composed of the PR^{-/-} cells represents a simple ductal tree, whereas the sector composed of the PR^{+/+}.ROSA26 cells shows extensive lobuloalveolar growth. This result demonstrated that the coexistence of MECs of PR^{+/+} and PR^{-/-} in one fat pad is not sufficient to rescue the morphogenetic defect intrinsic to the PR^{-/-} cells.

Most of the chimeric epithelia that arose from single-cell suspensions in which the wt cells were in 10-fold excess over PR^{-/-} cells showed complete lobuloalveolar development. However, at higher magnification distinct red alveoli and blue alveoli could be identified. This observation suggested but did

not prove that PR^{-/-} cells could participate in alveolar formation if they were in close proximity with wt MECs.

Any conclusions concerning the ability of the PR^{-/-} MECs to form alveoli were clouded by the possibility that certain PR^{+/+}.ROSA26 cells that participated in alveologenesis had failed to stain blue, thereby taking on the appearance of the PR^{-/-} cells in the same mixed grafts. To address this issue, we crossed the β -galactosidase transgene into the PR^{-/-} genetic background. By transplanting PR^{-/-}.ROSA26 mammary epithelium into wt recipients and analyzing the transplanted glands after birth we were assured that the transgene did not affect the PR^{-/-} phenotype (data not shown). Subsequently, suspensions of PR^{-/-}.ROSA26 MECs were mixed with PR^{+/+} MECs lacking the β -galactosidase transgene to generate chimeric breasts. On this occasion, we looked for a result opposite to that seen previously—alveolar cells that stained blue. Indeed, as shown in Fig. 4 *Center*, a representative of 26 independent grafts, the mammary glands obtained from pregnant engrafted females showed areas with blue alveoli, proving conclusively that PR^{-/-} cells can participate in the formation of alveoli if they are in close vicinity to wt epithelial cells.

To determine whether the alveolar structures constituted by PR^{-/-} cells are functional we assessed their morphology on histological sections. As shown in Fig. 4 *Right*, the lumina of the blue PR^{-/-} alveoli compare with those of wt alveoli, indicating the presence of secreted material. Similarly, secretory vacuoles are present. Immunostaining with anti- β -casein antibody revealed the expression of the milk protein (arrow, Fig. 4 *Upper Right*). Together these results indicate that the PR^{-/-} alveoli are fully differentiated. Thus, the presence of the PR is required in only a portion of the MECs in order for lobuloalveolar development to occur. Moreover, these findings suggest that progesterone activates a paracrine signaling route that operates between distinct subtypes of MECs, permitting PR^{-/-} MECs to participate directly in lobuloalveolar proliferation and differentiation.

DISCUSSION

Hormonal ablation/reconstitution experiments (1) have suggested that progesterone plays an important role in the changes that the mammary gland undergoes during early pregnancy,

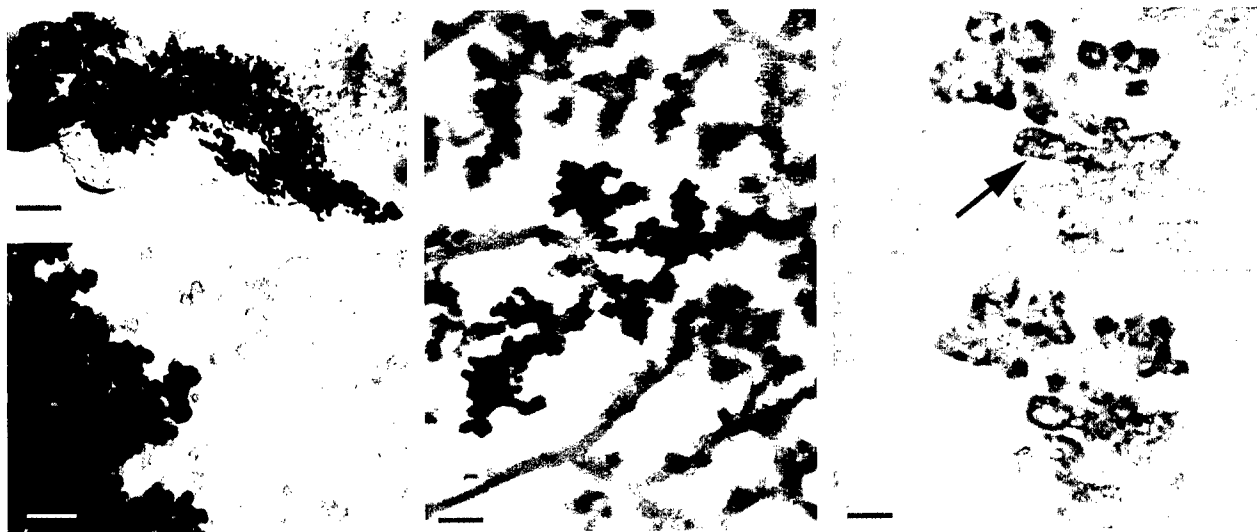


FIG. 4. Rescue of the PR^{-/-} phenotype in PR^{-/-} and PR^{+/+} chimeric epithelia. (*Left*) Whole-mount preparation of cleared PR^{+/+}.RAG1^{-/-} fat pad implanted with a mixture of PR^{-/-} (red) epithelium and ROSA26.PR^{+/+} epithelium (blue) in a 1:1 ratio. The engrafted mammary gland was removed after the recipient had given birth, subjected to X-Gal staining, and whole-mounted. (Bar in *Upper* corresponds to 2 mm; bar in *Lower*, to 200 μ m). (*Center*) Whole-mount preparation of cleared PR^{+/+}.RAG1^{-/-} fat pad injected with a mixture of PR^{-/-}.ROSA26 (blue) epithelium and PR^{+/+} epithelium (red) injected in a 1:10 ratio, treated as for *Left*. (Bar corresponds to 200 μ m.) (*Right*) Adjacent histological sections of an area with PR^{-/-}.ROSA26 alveolar structures. (*Upper*) Expression of β -casein in wt and PR^{-/-}.ROSA26 alveoli. (*Lower*) Control without primary antibody. Arrow indicates PR^{-/-}.ROSA26 alveolus expressing β -casein. (Bar corresponds to 50 μ m.)

namely side-branching and initial alveolar growth. To determine the extent to which progesterone signaling is limiting in development, we generated mice lacking the PR gene (2). However, because the PR^{-/-} females have multiple impairments in their reproductive functions, the specific consequences of PR inactivation on mammary gland development could not be assessed in these mice.

To circumvent this difficulty, we have used various transplantation techniques to elucidate the role of progesterone in the development of the mammary gland. In particular, we have made use of cells derived from mice carrying the β -galactosidase transgene. These cells turn blue upon X-Gal staining, making it possible to distinguish these cells histochemically from neighboring β -galactosidase-negative cells. In one experiment, this allowed us to distinguish the β -galactosidase-positive implanted MECs from the β -galactosidase-negative endogenous cells of an engrafted breast; in another setting, this procedure made it possible for us to distinguish MECs carrying two functional PR alleles from those lacking the PR.

Most transplantation experiments involving nonsyngeneic grafts have exploited nude mice as recipients. We note here in passing the utility of the RAG1^{-/-} mice used for transplantation experiments designed to elucidate mammary gland physiology. Because nude mice have low estrogen levels, they do not represent good recipients in transplantation experiments designed specifically to gauge mammary function. In contrast, the RAG1^{-/-} mice used here exhibit developmental defects that are strictly limited to B and T cell development (11).

Our initial experiments involving the transplantation of PR^{-/-} mammary glands into PR^{+/+}.RAG1^{-/-} females were motivated by the need to assess the role of the PR in an *in vivo* physiologic environment in which the full array of pregnancy-associated hormonal signals was present. PR^{-/-} mammary glands grafted into a PR^{+/+}.RAG1^{-/-} recipient developed only a simple ductal system, even when the host went through a series of estrous cycles and a normal pregnancy. This indicated that side-branching and lobuloalveolar growth rely on the presence of the PR, and that other signaling mechanisms operating in the breast tissue cannot compensate for the absence of the PR to allow these processes to proceed normally.

These initial results left us with two distinct scenarios. In one, both side-branching and lobuloalveolar proliferation, each in its own right, depends on the presence of progesterone. In the other, side-branching depends on progesterone, whereas lobuloalveolar growth depends on prior side-branching and is therefore only indirectly dependent on progesterone. Our analysis of a series of whole mounts of mammary glands from wt pregnant mice showed that alveoli sprouted not only from side branches (secondary ducts) but also from the primary ducts (data not shown). This finding indicated that side-branching is not an absolute prerequisite for alveolar growth. For this reason, we concluded that the PR is required for lobuloalveolar proliferation *per se* in addition to its demonstrated role in side-branching.

We next addressed the issue of whether progesterone needs to act on the mammary stroma, the epithelium, or both. One important clue for resolving this puzzle appeared to come from the longstanding observation that morphogenesis in many epithelial-mesenchymal organs such as the mammary gland is controlled by inductive events (16) that require cross-talk between epithelial and stromal components. In the breast in particular, the embryonic mammary mesenchyme induces the overlying epithelium to develop into the mammary bud (17). Moreover, in male embryos of various mouse strains, androgens act on the stroma to induce the involution of the mammary anlage (18, 19). The estrogen receptor is required in the mammary stroma for ductal growth to occur (20).

The role of the stroma in mediating progesterone-dependent processes in the breast has been less clear. For example, ligand-binding studies have shown that 80% of the progesterone receptors in the mouse mammary gland localize to the epithelium, while the remaining 20% are found in the stroma (4). Such observations have been compatible with models in which the epithelial cells, the stromal cells, or both cell types are required to mediate the direct responses to progesterone.

More recently, epithelial/stromal reciprocal transplantations between wt and estrogen receptor (ER)^{-/-} and wt and PR^{-/-} tissues have demonstrated that stromal derived ER and PR exert paracrine effects on the epithelium both in the uterus (21) and in the vagina (G. R. Cunha and B.W.O., unpublished observations). We show here that mammary glands lacking PR in the stroma undergo normal development, whereas the absence of the PR from the epithelium confers the PR^{-/-} phenotype, indicating that the target cells of progesterone in the mammary gland are in the epithelium. While effects of progesterone on the mammary stroma cannot be excluded, they do not appear to contribute in any obvious way to the development of the ductal tree and alveoli.

Recently reported experiments in which we participated (3) yielded results that are in conflict with one aspect of the present work. These previous experiments appeared to indicate that the PR that functions within the stromal compartment exerts an effect on epithelial ductal growth, contrary to the present results, which indicate the opposite. We find the present results more compelling for several reasons. The number of transplanted animals examined here was much larger. Moreover, we have analyzed the behavior of mammary glands in a situation in which the only PR-negative tissue in engrafted animals was the mammary stroma; the earlier work, in contrast, examined the behavior of wt epithelium transplanted into the cleared PR^{-/-} fat pad of a PR^{-/-} host. In concordance with our conclusion, a recent immunostaining failed to detect any PR protein in the fat pad (22).

The present work together with previous observations of others (1, 12) indicates that progesterone is required for two distinct morphogenetic processes in the breast—side-branching and preparation of ductal cells for subsequent lobuloalveolar development. The precise mechanisms by which progesterone enables ductal MECs to participate in alveologenesis has been unclear. The pattern of PR expression in the mammary epithelium is inhomogeneous (5), suggesting the involvement of only a subset of ductal cells in progesterone-triggered processes. The connected issue of whether the PR-expressing cells represent the precursors of the alveolar outgrowths is addressed here.

Our observation that PR^{-/-} cells can give rise to alveolar structures if they are in close vicinity to PR^{+/+} cells indicates that progesterone does not need to act directly on a ductal epithelial cell for it to participate in alveolar formation. Instead, it appears that progesterone acts on a subtype of ductal cell, causing it to release paracrine signals that permit other nearby epithelial cells to participate directly in lobuloalveolar proliferation.

The present work provides no indication about the nature of the paracrine signal released by the progesterone-activated ductal cell. However, the observation that close apposition of PR-positive with PR-negative cells is required to rescue the PR^{-/-} phenotype indicates that the signal, whatever its biochemical nature, is transmitted only over short intercellular distances. Factors that are tightly associated with the extracellular matrix such as wnt proteins and fibroblast growth factors, which are differentially expressed during mammary gland development (23, 24), are attractive candidates for conveying such paracrine signals.

Our data provide no indication whether or not these paracrine signals communicate directly between the progesterone-

activated ductal cells and closely apposed alveolar precursor cells. It remains equally possible that the progesterone-activated ductal cell communicates with the stroma; the latter, in turn, may pass on a signal directly to the alveolar precursor cells as suggested by others (25). The use of tissue reconstitution techniques and genetically altered cells should allow the further dissection of the molecular mechanisms of mammary morphogenesis over the next several years.

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RESEARCH COMMUNICATION

Essential function of *Wnt-4* in mammary gland development downstream of progesterone signaling

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Female reproductive hormones control mammary gland morphogenesis. In the absence of the progesterone receptor (PR) from the mammary epithelium, ductal side-branching fails to occur. We can overcome this defect by ectopic expression of the protooncogene *Wnt-1*. Transplantation of mammary epithelia from *Wnt-4*^{-/-} mice shows that *Wnt-4* has an essential role in side-branching early in pregnancy. *PR* and *Wnt-4* mRNAs colocalize to the luminal compartment of the ductal epithelium. Progesterone induces *Wnt-4* in mammary epithelial cells and is required for increased *Wnt-4* expression during pregnancy. Thus, Wnt signaling is essential in mediating progesterone function during mammary gland morphogenesis.

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Development of the mammary gland occurs largely postnatally under the control of the female reproductive hormones estrogen, progesterone, and prolactin (Nandi 1958). A system of ducts grows outward from the nipple into the mammary fat pad that lies under the skin. The ducts then elongate and bifurcate during puberty until they reach the edges of the fat pad (Daniel and Silberstein 1987). Subsequently, with recurrent estrous cycles and during early pregnancy the ductal system increases in complexity through the addition of sidebranches that sprout from the preexisting ducts (Daniel and Silberstein 1987). The mechanisms that enable the systemic factors to control locally acting factors involved in these morphogenetic events remain largely unknown. Recently, we and others have shown that progesterone acts via the progesterone receptor (PR) in the mammary epithelium to induce side-branching (Lydon et al. 1995; Humphreys

et al. 1997; Brisken et al. 1998) and that it does so by a paracrine mechanism (Brisken et al. 1998).

We speculated that Wnt proteins might function as the paracrine factors that operate downstream of progesterone and the PR to mediate the process of side-branching. Wnt proteins have important roles in the development of various vertebrate and invertebrate tissues (Nusse and Varmus 1992; Cadigan and Nusse 1997). These factors are secreted glycoproteins that bind to members of the Frizzled family of seven-transmembrane-domain receptors. Several *Wnt* genes can function as oncogenes in the mouse breast when their transcription is activated by insertion of the provirus mouse mammary tumor virus (MMTV) (Nusse and Varmus 1982; Roelink et al. 1990; Lee et al. 1995) or when they are expressed ectopically (Tsukamoto et al. 1988).

Results and Discussion

To test whether a Wnt factor might function downstream of progesterone signaling in triggering ductal side-branching in the breast, we crossed mice carrying an MMTV LTR-driven *Wnt-1* transgene (Tsukamoto et al. 1988) with mice heterozygous for a previously described inactivating mutation at the *PR* locus (Lydon et al. 1995), to generate *Wnt-1* transgenic females that were either *PR*^{-/-} or *PR*^{+/+}. We then sought to test whether the ectopically expressed *Wnt-1* protein might restore the side-branching that is lacking in *PR*^{-/-} mammary ducts (Fig. 1A).

Mammary epithelia were removed from mice of both genotypes and transplanted into the inguinal fat pads of 3-week-old *PR*^{+/+} females. These fat pads previously had been surgically cleared of endogenous epithelium. When epithelial tissue (DeOme et al. 1959) or primary cells (Daniel and DeOme 1965) are engrafted into such cleared fat pads, they are able to form a new ductal system. These recipient females were also mutant at the *RAG1* locus (*RAG1*^{-/-}), as these mice are immunocompromised and therefore able to accept allografts (Mombaerts et al. 1992; Brisken et al. 1998).

Ten weeks after grafting, control unmanipulated mammary glands in these recipient females showed a simple ductal system characteristic of a 13-week-old virgin mouse. However, the fat pads carrying implanted *PR*^{+/+} MMTV *Wnt-1*^{tg} and *PR*^{-/-} MMTV *Wnt-1*^{tg} epithelia showed increased side-branching (Fig. 1B). Thus, ectopic expression of *Wnt-1* can induce side-branching in a *PR*^{-/-} epithelium in which side-branching is defective, suggesting that Wnt signaling can mimic this progesterone-induced response and may therefore act downstream of the PR.

We reported previously that in chimeric epithelia derived from mixed wild-type and *PR*^{-/-} mammary epithelial cells (MECs), the branching defect of the mutant MECs could be rescued if these cells grew in close proximity to their wild-type counterparts (Brisken et al. 1998). This suggests that progesterone elicits its morpho-

[Key Words: Mammary gland; morphogenesis; *Wnt-4* signaling; side-branching, progesterone function]

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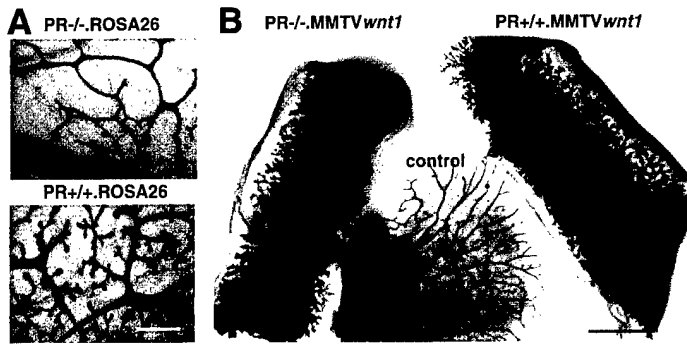


Figure 1. Side-branching in the presence of *Wnt-1* and absence of the PR. (A) Intrinsic side-branching defect in *PR*^{-/-} mammary epithelium. Mammary epithelium was harvested from *PR*^{-/-} *ROSA26* (top) and *PR*^{+/+} *ROSA26* (bottom) 10-week-old female mice and engrafted to the cleared fat pads of 3-week-old F₁ (129SV/C57B16) recipients. Shown are whole-mount preparations of mammary glands subjected to X-gal stain, from a recipient at day 12 of pregnancy (10 weeks after surgery). Results were similar to those reported previously. Bar, 400 μ m. (B) Constitutive side-branching of MMTV *Wnt-1*^{ts} irrespective of the PR status. Mammary epithelium was harvested from *PR*^{-/-} MMTV *Wnt-1*^{ts} and *PR*^{+/+} MMTV *Wnt-1*^{ts} 10-week-old female mice and engrafted to the cleared fat pads of 3-week-old recipients. Shown are whole-mount preparations of mammary gland from a virgin *RAG1*^{-/-} recipient 10 weeks after surgery. (Left) Inguinal fat pad engrafted with *PR*^{-/-} MMTV *Wnt-1*^{ts} mammary epithelium; (right) inguinal fat pad engrafted with *PR*^{+/+} MMTV *Wnt-1*^{ts} mammary epithelium; (center) thoracic mammary gland, as an ungrafted endogenous control. Identical results were obtained in 16 independent grafts of *PR*^{-/-} MMTV *Wnt-1*^{ts} and control mammary epithelium. Bar, 5 mm.

genetic effects, at least in part, by causing PR-positive MECs to release a factor that acts over short distances on other cells within the breast.

To test whether Wnt-1 also acts in a paracrine fashion to induce side-branching, we mixed MMTV *Wnt-1*^{ts} MECs with MECs derived from *ROSA26* mice (Friedrich and Soriano 1993). These latter cells carry a *lacZ* transgene, which makes their identification possible upon whole mount analysis of breast tissue. As expected, the MMTV *Wnt-1*^{ts} cells, stained in red, showed increased side-branching (Fig. 2). In addition, the blue wild-type cells carrying the *lacZ* transgene, located adjacent to these MMTV *Wnt-1*^{ts} MECs, also showed increased side-branching. This indicates that secreted Wnt-1 is sufficient to cause side-branching and that Wnt-1, like the factor released by PR-positive cells, acts in a paracrine fashion to induce side-branching. When wild-type MECs were mixed with MECs derived from *ROSA26* mice, ductal branching was not affected (data not shown), indicating that the increased branching is not induced by experimental manipulation.

Although these experiments indicate that a Wnt protein was sufficient for side-branching, being able to mimic the morphogenetic response normally elicited by progesterone, they did not resolve whether a Wnt factor has an essential role in the normal morphogenetic process. *Wnt-1* itself is not normally expressed in the mammary gland, but the related gene *Wnt-4* (Munsterberg et al. 1995; Kispert et al. 1998), which acts similarly to

Wnt-1 when ectopically expressed in the mammary epithelium (Bradbury et al. 1995), is expressed during the period when side-branching occurs in early to mid-pregnancy (Gavin and McMahon 1992; Weber-Hall et al. 1994). To evaluate the specific role of *Wnt-4* in mammary morphogenesis, we analyzed mammary epithelium from mice lacking both copies of the *Wnt-4* gene (Stark et al. 1994). These mice die perinatally due to kidney failure (Stark et al. 1994), precluding analysis of subsequent mammary development. Responding to this, we harvested the mammary buds from 14.5-day-old *Wnt-4*^{-/-} and wild-type embryos and engrafted them into the cleared mammary fat pads of wild-type hosts. Both types of implants initially gave rise to normal ductal systems in virgin recipients (Fig. 3, left). However, at day 12 of pregnancy *Wnt-4*^{-/-} implants showed substantially less ductal branching than their wild-type counterparts (Fig. 3, middle). Later in pregnancy, engrafted *Wnt-4*^{-/-} epithelia began to resemble wild-type epithelial grafts, exhibiting a more normal pattern of arborization (Fig. 3, right). This may be explained by the actions of other members of the Wnt family of factors that are known to be expressed late in pregnancy, such as *Wnt-5a*, *Wnt-5b*, and *Wnt-6*.

In situ hybridization with PR- and *Wnt-4*-specific cRNA probes on sections of mammary glands from virgin mice and during early pregnancy (days 4 and 8) reveal that both molecules are expressed at low levels in the virgin and induced during pregnancy (Fig. 4, top). Higher magnifications illustrate that as reported

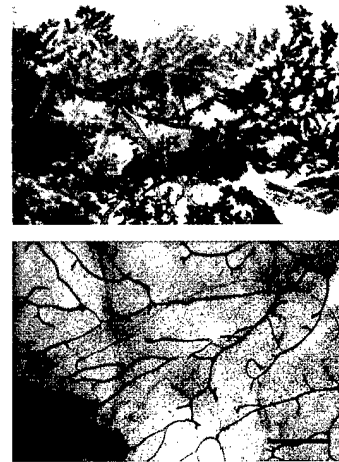


Figure 2. Paracrine induction of side-branching by Wnt-1. Primary mammary epithelial cells were derived from 10-week-old MMTV *Wnt-1*^{ts} and *ROSA26* females. After 5 days in vitro culture, the cells were trypsinized, mixed in a 1:1 ratio, and injected into cleared fat pads of 3-week-old *RAG1*^{-/-} recipients. Mammary glands from *RAG1*^{-/-} recipients at 10 weeks after surgery were subjected to X-gal stain, carmine alum counterstain, and mounted whole. (Top) Cleared fat pad reconstituted with a mixture of *ROSA26* (blue) and MMTV *Wnt1* (red) mammary epithelial cells; (bottom) ungrafted control, thoracic mammary gland. Bar, 500 μ m. *Wnt-1* overexpressing cells (red) induce premature side-branching in wild-type cells (blue).

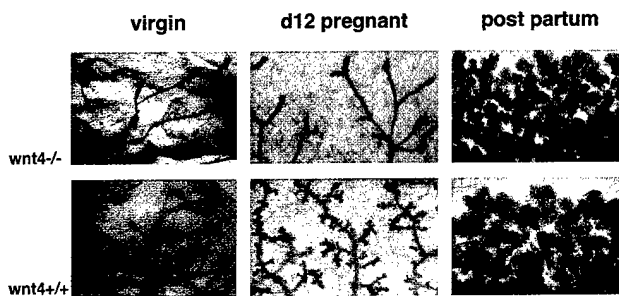


Figure 3. Function of *Wnt-4* in the mammary epithelium at mid-pregnancy. Mammary buds were prepared from *Wnt-4*^{-/-} and wild-type littermates (129SV/C57B16 mixed genetic background) at E14.5 and engrafted to the cleared fat pads of 3-week-old F₁ (129SV/C57B16) recipients. At 10 weeks after surgery the mammary glands from virgin and impregnated recipients were analyzed by whole-mount preparation. (Top) Mutant grafts; (bottom) wild-type control grafts. Shown are mammary glands derived from a grafted virgin mouse (left), a mouse at day 12 of pregnancy (center), or at parturition (right). *Wnt-4*^{-/-} epithelium fails to initiate side-branching at day 12 of pregnancy. Results were obtained with 4 virgin, 10 mid-pregnant, and 5 recipients at parturition. Bar 500 µm.

previously (Silberstein et al. 1996), the PR is not expressed in the myoepithelium but is restricted instead to the luminal epithelium (see arrows in Fig. 4, bottom) and that the same is true for *Wnt-4*. The same colocalization was observed on sections from the murine uterus during early pregnancy (data not shown). These observations of colocalized expression are consistent with a model that progesterone signaling induces *Wnt-4* expression.

To test whether *Wnt-4* expression is under the control of progesterone, we injected groups of ovariectomized mice with either 17- β -estradiol, 17- β -estradiol and progesterone, or the vehicle alone for 20 days as described (Said et al. 1997). 17- β -Estradiol injections were required to induce expression of the PR in MECs (Said et al. 1997). At the end of these treatments, one mammary gland from each mouse was analyzed by whole-mount microscopy to assess the morphology of the ductal system, enabling us to control for adequate gonadectomy in the vehicle-treated mice and to assess the efficacy of hormone replacement in the stimulated mice. RNA was extracted from a second mammary gland of each mouse and assayed by RT-PCR for levels of *GAPDH* and *Wnt-4* mRNA expression. We found a slight increase in the expression of *Wnt-4* mRNA in response to 17- β -estradiol treatment alone, but a three- to fivefold increase of *Wnt-4* mRNA following 17- β -estradiol and progesterone treatment (Fig. 5A).

The above results suggest that increased *Wnt-4* expression during pregnancy is under progesterone control. To test this possibility

further, we assayed *Wnt-4* expression in the mammary glands of pregnant mice that had been engrafted with *PR*^{-/-} epithelium in one fat pad and *PR*^{+/+} epithelium in the contralateral fat pad. In both cases, the transplanted epithelial cells also carried a *lacZ* gene, enabling us to use RT-PCR analysis to gauge the level of RNA recovered from the engrafted epithelium of each reconstituted gland. At day 12 of pregnancy, a threefold difference between the levels of *Wnt-4* mRNA was consistently observed between the *PR*^{-/-} implants and their wild-type counterparts (Fig. 5B), which compares to the induction of endogenous *Wnt-4* expression normally seen during pregnancy (Gavin and McMahon 1992; Weber-Hall et al. 1994; data not shown). The levels of *lacZ* mRNA were comparable between the two grafts. Thus, progesterone signaling is required within the grafted mammary epithelium for the induction of *Wnt-4* expression that is normally seen during pregnancy.

To test whether the induction of *Wnt-4* by progesterone is a direct effect of PR action on mammary epithelial cells, we treated primary MECs in culture with progesterone. As shown in Figure 5C, representative of eight independent experiments, *Wnt-4* RNA expression was significantly induced as early as 4–8 hr after progesterone

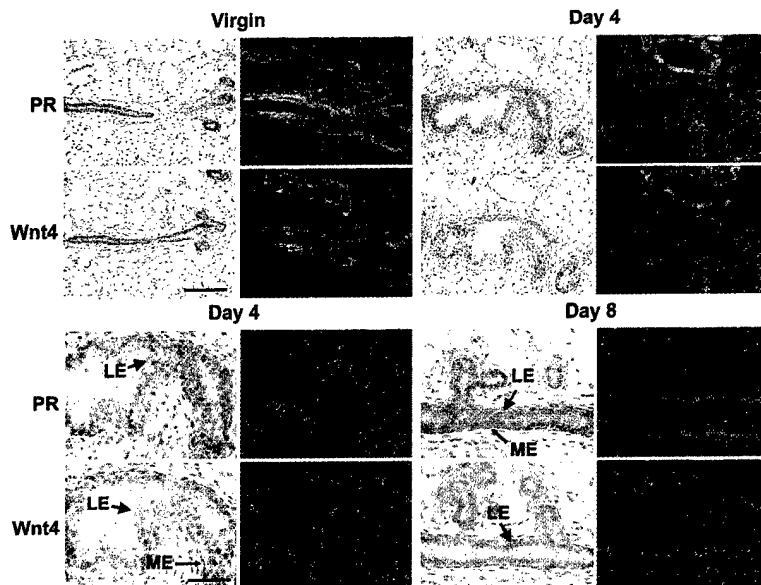


Figure 4. Coexpression of *PR* and *Wnt-4* mRNAs in the luminal mammary epithelium. Mammary glands were harvested from a virgin adult female mouse and from mice at days 4 and 8 of pregnancy. The glands were processed for in situ hybridization; adjacent sections were hybridized with ³⁵S-labeled antisense cRNA probes for *PR* or *Wnt-4* and exposed for 7 days. Hematoxylin- and eosin-stained sections are shown next to the corresponding dark-field exposures. (Top) Coordinated induction of *PR* and *Wnt-4* mRNAs during pregnancy. Low magnification (bar, 150 µm) of mammary gland showing increased signal intensity for both *PR* and *Wnt-4* mRNAs in the ductal epithelium at 4 days of pregnancy vs. virgin. (Bottom) Colocalization of *PR* and *Wnt-4* mRNA expression in the mammary luminal epithelium. High magnification (bar, 75 µm) of selections from mammary glands at days 4 and 8 of pregnancy showing that both *PR* and *Wnt-4* mRNA expression localizes to the luminal epithelium (LE) and is absent from the myoepithelium (ME). Light green areas represent the dense fibrous stroma surrounding the mammary ducts.

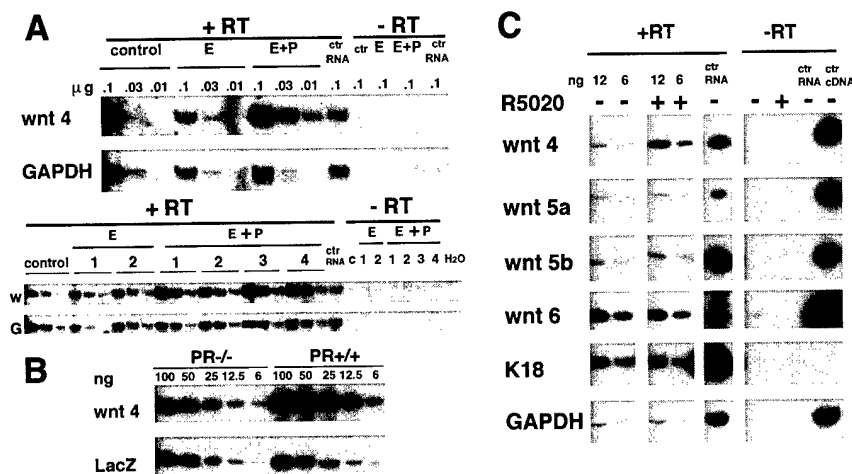


Figure 5. Induction of *Wnt-4* expression in the mammary epithelium *in vivo* and *in vitro* by progesterone. **(A)** Quantification of *Wnt-4* mRNA expression by semiquantitative PCR in mammary glands after 20 days of hormone treatment. Ten week-old virgin mice were ovariectomized. After 3 weeks they were injected for 20 days either with vehicle only (control), 10 μ g of 17- β -estradiol (E)/day or 10 μ g of estradiol and 100 μ g of progesterone (E+P)/day. Total RNA was prepared from individual mammary glands, and samples in three serial dilutions, to ensure a linear signal response, were subjected to RT-PCR with primers specific for *Wnt-4* or *GAPDH*. The same amounts of RNA in three serial dilutions were analyzed in each case. The undiluted RNA subjected to PCR amplification yielded no signal. Shown are two independent experiments, one comprising three mice (top) and one comprising seven mice (bottom). The products were quantified by densitometric scanning. The ratio of *Wnt-4*/*GAPDH* of the progesterone-treated samples was three- to fivefold higher than the 17- β -estradiol-treated samples. **(B)** *Wnt-4* mRNA expression in mammary glands engrafted with *PR*^{-/-} or *PR*^{+/+} mammary epithelium. Mammary epithelium was harvested from *PR*^{-/-} *ROSA26* and *PR*^{+/+} *ROSA26* 10-week-old female mice and engrafted to the cleared fat pads of 3-week-old F₁ (129SV/C57B16) recipients. Six weeks after surgery the recipients were mated and the engrafted mammary glands were harvested at day 12 of pregnancy. RNA samples in five serial dilutions were subjected to RT-PCR with primers specific for *Wnt-4* as in A. In parallel, RT-PCR was performed with *lacZ*-specific primers allowing normalization of the amount of transplanted epithelium. Densitometry reveals that the *Wnt-4* signal is increased threefold in the *PR*^{-/-} *ROSA26* vs. the *PR*^{+/+} *ROSA26* transplant. The same results were obtained in three independent experiments. **(C)** *Wnt-4* mRNA expression in cultured primary mammary epithelial cells after progesterone exposure. Primary mammary epithelial cells were cultured on collagen-coated dishes for 3 days. RNA was harvested from untreated cells and cells after 8 hr of stimulation with the progesterone agonist R5020 (20 nM) (P). Shown are two out of five serial dilutions of RNA subjected to RT-PCR with primers specific for *Wnt-4*, *Wnt-5a*, *Wnt-6*, *keratin-18*, and *GAPDH*. In each case, the undiluted RNA subjected to PCR amplification without reverse transcription yielded no signal. Although the levels of *Wnt-5a*, *Wnt-5b*, *Wnt-6*, *keratin-18*, and *GAPDH* mRNA were unaffected by the treatment with R5020, the levels of *Wnt-4* mRNA increased two- to threefold within 4–8 hours as confirmed in eight independent experiments.

exposure. However, the expression levels of *Wnt-5a*, *Wnt-5b*, and *Wnt-6*, which are also increased during pregnancy, were unaffected by progesterone treatment. Further studies to determine whether *Wnt-4* induction by progesterone could occur in the presence of the protein synthesis inhibitor cycloheximide, were hampered by increased basal *Wnt-4* mRNA levels induced by the cycloheximide treatment, possibly reflecting cycloheximide-induced stabilization of *Wnt-4* mRNA (data not shown).

Together, our findings indicate that Wnt signaling is centrally important to progesterone-induced side-

branching of the mammary ductal epithelium. In contrast, a second major morphogenetic process in the mammary gland—ductal elongation—does not appear to be mediated by Wnt signaling. In support of this, the work of others has demonstrated that the defect in ductal elongation observed in epithelia lacking the estrogen receptor is not reversed in the presence of the MMTV-driven *Wnt-1* transgene (Lubahn et al. 1993; Bocchinfuso et al. 1999).

Although we find that *Wnt-4* is the only *Wnt* gene directly induced by progesterone, it is not unique in its ability to trigger side-branching, as late in pregnancy, the ductal epithelium of *Wnt-4*^{-/-} shows normal side-branching. We speculate that this compensation is due to the expression of other Wnt proteins later in pregnancy (Gavin and McMahon 1992; Weber-Hall et al. 1994), consistent with the notion that various Wnt proteins trigger similar biochemical responses and that their different biological functions are due to differences in their patterns of expression.

Materials and methods

Mice

ROSA26, *RAG1*^{-/-}, *Wnt-4*^{-/-}, and *PR*^{-/-} mice were maintained on a C57Bl/6 \times 129SV background. Genotyping for the β -galactosidase transgene was tested by X-gal-staining tail biopsies, PR, and MMTV *Wnt-1*^{tr} genotyping as described (Lydon et al. 1995; Bocchinfuso et al. 1999).

Mammary glands

E14.5 embryos were harvested from crosses of *Wnt-4*^{-/-} parents and phenotyped. The phenotyping was subsequently confirmed by PCR-based genotyping (Stark et al. 1994). The mammary anlagen were dissected and subsequently engrafted to cleared inguinal fat pads of 3-week-old recipients.

Mammary gland whole mounts, X-gal stain, and cell culture are as described previously (Briskin et al. 1998). For progesterone stimulation

cells were plated on collagen-coated dishes and maintained in DMEM/F12 with prolactin (5 μ g/ml) and insulin (5 μ g/ml) for 3 days prior to treatment with 20 nmoles of R5020.

RT-PCR

Total RNA (1 μ g) was reverse transcribed (GIBCO BRL) using random hexamers (Boehringer). Amplification was carried out by touchdown PCR using the following primers: mouse *GAPDH* (Clontech), 20 cycles; *lacZ* (Bjornson et al. 1999), 27 cycles; *keratin-18* (Schroeder and Lee 1998), 20 cycles; *Wnt-4F*, AGGAGTGCCAATACCAGTTCC; *Wnt-4R*, TGTGAGAAGGCTACGCCATA, 27 cycles; *Wnt-5aF*, ACAGGCATCAAGGAATGCCAGTA; *Wnt-5aR*, AACGGGTGACCATAGTCGATGT, 25 cycles; *Wnt-5bF*, CAGAGAGTGCCAACACCAGTTT; *Wnt-5bR*, TACTCCACGTTGTCTCCACA, 22 cycles; *Wnt-6F*, CTAG-

GATGGTCGTAGACGTCCT, *Wnt-6R*, CGTTTGTGCTTTTCGACAG-AG; 30 cycles.

In situ hybridization

In situ hybridization was performed as described previously [Das et al. 1994]. In brief, frozen sections (14 μ m) were mounted onto poly-L-lysine-coated slides and fixed in 4% paraformaldehyde in PBS for 15 min at 4°C. The sections were prehybridized followed by hybridization with ³⁵S-labeled antisense or sense cRNA probes for *Wnt4* [Stark et al. 1994] or *PR* [Tan et al. 1999] for 4 hr at 45°C. After hybridization and washing, the sections were incubated with RNase A (20 μ g/ml) at 37°C for 20 min. RNase-A-resistant hybrids were detected by autoradiography using Kodak NTB-2 liquid emulsion [Eastman Kodak, Rochester, NY]. The autoradiographic exposures were from 7 to 12 days. The slides were post-stained with hematoxylin and eosin. The reddish brown grains indicate the sites of mRNA accumulation. This color is the result of lateral light scattering from the eosin staining under dark-field microscopy. Day 8 uterine sections hybridized with the *Wnt-4* or *PR* antisense probe served as positive controls, whereas sections hybridized with the sense probes served as negative controls (data not shown).

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Side-branching in the mammary gland: the progesterone–Wnt connection

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The mammary gland is a derivative of the ectoderm whose development begins in the embryo and progresses after birth. The major part of development occurs in the adolescent and adult animal. Hormones produced by the pituitary, the ovaries, the uterus, the placenta, and the mammary gland itself control this process. Over the past century, surgical, biological, and genetic tools have been used to gain insight into physiological and pathological processes in the mammary gland. Originally, endocrine ablation and reconstitution experiments provided a descriptive framework of the role of ovarian and pituitary hormones (Halban 1900; Nandi 1958). These experiments demonstrated a clear requirement for the ovarian steroids estrogen and progesterone for ductal growth and alveolar development (Topper and Freeman 1980). In pre-pubescent mice the gland consists of a small ductal tree that emanates from the nipple into the proximal part of the fatty stroma, the mammary fat pad (Fig. 1). Upon initiation of ovarian hormone secretion, the mammary epithelium enters an accelerated growth phase that leads to extension and branching of the ducts until they reach the limits of the fat pad. In response to changing levels of estrogens and progestins during each estrous cycle, alveolar buds are formed from the lateral walls of the ducts and lost again. At the onset of pregnancy extensive epithelial cell proliferation occurs, leading to formation of lobulo-alveolar structures and secretory epithelial differentiation. These morphogenetic and cellular responses are controlled by signaling cascades initiated by progesterone (Lydon et al. 1995), placental lactogens, and prolactin (Horseman et al. 1997; Ormandy et al. 1997).

More recently, the availability of transgenic and gene knockout mice has provided genetic handles to investigate the contribution of the different hormones to the regulation of cell growth, differentiation, and death in the gland, and to dissect the corresponding signaling pathways. Using such gene knockout mice Brisken and colleagues (Brisken et al. 2000) have now been able to link progesterone and Wnt signaling to the branching of

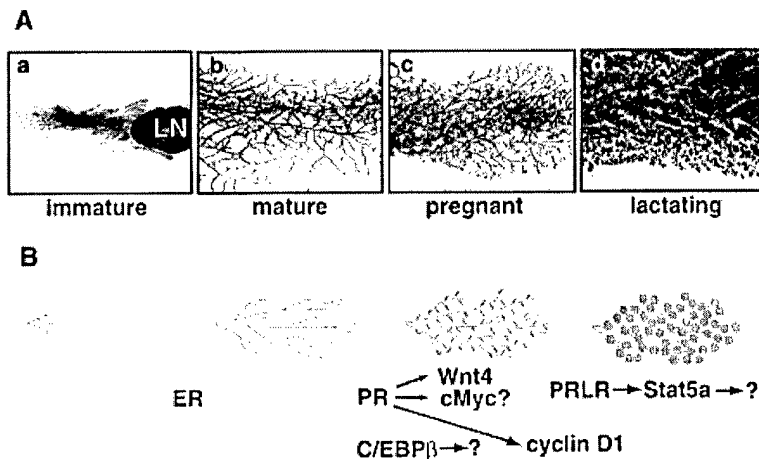
mammary ducts during puberty and pregnancy. Their studies demonstrate that a nuclear signal is converted into a secreted signal that can control the fate of adjacent cells in a paracrine fashion. A genetic understanding of this and other signaling pathways regulating cell growth in the mammary gland will improve our ability to manipulate these processes and thus design strategies for prevention and treatment of breast cancer.

Tools to investigate signaling pathways

Several features of the mammary gland provide unique opportunities for experimental manipulations to integrate systemic, local and cell-specific signaling pathways. Mice with deletions of different genes have been a powerful tool to investigate the respective pathways (Hennighausen and Robinson 1998). However, although the analysis of null mutants created by standard embryonic stem cell-based homologous recombination can provide valuable information on signaling pathways, this approach may fail to address the complexities underlying development of the mammary gland. It is now possible to delete genes in specific cell types and thus define their roles in different compartments (Xu et al. 1999). This technique also bypasses problems encountered with pre- and perinatal lethality and infertility. The power of genetically engineered mice is further enhanced through the use of sophisticated surgical techniques that permit the separation of stromal and epithelial signals. Removal of the epithelium-containing proximal portion of the gland at 3 weeks of age renders an epithelium-free fat pad, which can be implanted with exogenous epithelial cells, resulting in a chimeric gland composed of tissues of different origins (DeOme et al. 1959). In this manner the signaling between epithelium and stroma, a defining aspect of organogenesis, can be evaluated. This approach also circumvents fertility problems and allows one to distinguish primary, cell autonomous defects of a mutation from secondary, systemic hormone effects. This is particularly important in cases where a mutation also affects the ovary in addition to the mammary gland (Robinson et al. 1998). Another unique feature of the mammary epithelium, which can be applied to investigate

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Figure 1. (A) Whole-mount analysis of mammary tissue from 4-week-old virgin (a), 10-week-old virgin (b), day 11 pregnant (c), and lactating (d) mice. (LN) Lymph node. All pictures are taken at the same magnification. (B) Schematic drawings of the mammary fat pad and ductal and alveolar development. A simple ductal tree occupies the fat pad in the immature virgin. The fat pad is filled in mature virgins and substantial side-branching has taken place. Alveolar development occurs during pregnancy and is completed during lactation. Proteins that control defined stages of mammary development (as determined in gene knockout mice) are shown below the corresponding developmental stage. The arrows indicate likely genetic pathways. Although *c-myc* expression is induced by progesterone, it is not clear whether this occurs in MECs. Downstream targets of Stat5a and C/EBP β in mammary tissue are not known. (ER) Estrogen receptor; (PR) Progesterone receptor; (PRLR) Prolactin receptor. The images are deposited at the NIH HistoBank (<http://histology.nih.gov>) and can be viewed under the following accession nos.: mammary tissue from an immature virgin, 1345; mammary tissue from a mature virgin, 1346; mammary tissue from day 11 of pregnancy, 1347; mammary tissue from lactation, 1348.



cell-cell interactions and paracrine signaling, is its large capacity for regeneration. Each portion of the ductal system contains stem cells that are capable of regenerating an entire epithelial tree upon transplantation (Kordon and Smith 1998). By mixing epithelial cells of different origins prior to transplantation, it is possible to create chimeric secretory epithelia in mice. Introducing a tag that can be visualized, such as the *lacZ* gene, into one of the epithelial components makes it possible to identify the origin of individual cells in such mixed epithelial transplants. This is of particular importance in the investigation of paracrine interactions between individual epithelial cells. Finally, the ability of embryonic and neonatal mammary tissue to develop a full mammary gland upon transplantation into cleared fat pads of adult mice provides the opportunity to investigate gene function in lethal knockout mice (Robinson et al. 2000). Unlike the *Cre/LoxP* approach, in which a gene is deleted in the epithelium at a time determined by the transgene, this approach evaluates the outgrowth and development of mutant epithelium.

The progesterone-Wnt connection

More than 100 years ago, Halban observed that ovaries are required for functional development of the mammary gland (Halban 1900). As we know now, estrogen and progesterone are the controlling factors. Whereas estrogen is required for development of the primary ductal system, progesterone controls alveolar budding and development during puberty and pregnancy. Progesterone had been implicated as a mitogen for mammary epithelial cells (MECs), which was confirmed in progesterone receptor (PR)-null mice that exhibited impaired branching and alveolar budding (Lydon et al. 1995). Two PR isoforms (A and B) derived from the same gene by different promoters have been identified, but their specific functions are not understood. Perturbation of their ratio by overexpression

of the A form causes aberrant ductal morphology, more extensive lateral branching, and hyperplasia, an indication of the involvement of PR in mammary epithelial proliferation (Shyamala et al. 1998).

Although it was clear for some time that the PR is required for branching and budding (Lydon et al. 1995), little was known about downstream mediators. The first tip-off that secreted signals conveyed the steroid information came from cell mixing experiments. Briskin, Weinberg, and their colleagues demonstrated that PR-null MECs (tagged with the ROSA26 *lacZ* marker) could form alveolar-like structures after grafting into cleared fat pads when mixed with PR-containing MECs (Briskin et al. 1998). Tissue recombination experiments further demonstrated that the stroma did not contribute to the growth signaling. Based on these results, a paracrine mechanism of PR action confined to epithelial cells was proposed. A recent paper by Briskin and colleagues now solves part of this puzzle and identifies Wnt-4 as a likely mediator of this signal (Briskin et al. 2000).

Signaling through Wnt

Wnts are a family of secreted, cysteine-rich glycoproteins that function as short-range signaling factors. Wnt proteins are associated with the cell surface and extracellular matrix (Parkin et al. 1993; Schryver et al. 1996) and, as such, their effects tend to be spatially localized. The first mammalian *Wnt* gene, originally termed *Int-1*, was identified at a site of murine mammary tumor virus (MMTV) integration in mammary tumors (Nusse and Varmus 1982). The *Int-1* gene exhibited homology to *wingless (wg)*, a *Drosophila* segment polarity gene, and subsequently the name *Wnt* was chosen for members of this family. At least sixteen mammalian *Wnt* genes have been identified, several of which are expressed in mammary tissue (see below). Wnt proteins elicit a variety of cellular responses, including proliferation, differentia-

tion, and morphogenesis, and control developmental decisions such as axis formation in *Xenopus* embryos (Moon et al. 1997). Indeed, Wnts are now recognized as one of the major classes of signaling proteins that regulate development and cell fate in multicellular organisms. Several excellent reviews have been written on the subject and readers are referred to these articles for more details (Nusse and Varmus 1992; Cadigan and Nusse 1997; Moon et al. 1997; Dale 1998; Nusse 1999). Here we shall only briefly summarize the major Wnt signaling pathway that has been proposed from genetic studies in *Drosophila* and *Caenorhabditis elegans*, and molecular experiments performed in vertebrate systems.

The action of Wnts on target cells is mediated by binding to the frizzled (Fz) group of transmembrane receptors, of which there are at least eight members in mammals. The immediate downstream component of the signal transduction pathway is disheveled (Dsh), an intracellular protein that may directly interact with Fz receptors. The target of Dsh is glycogen synthase kinase-3 (GSK-3), which is homologous to the *Drosophila* protein encoded by *zeste-white3*. GSK-3 is believed to be constitutively active and functions to inhibit the Wnt pathway in the absence of Wnt signals. GSK-3 phosphorylates β -catenin, which enhances β -catenin turnover by a ubiquitin-mediated degradation pathway. In response to Wnt signals, GSK-3 activity is inhibited, leading to stabilization of the β -catenin protein. One of the targets of β -catenin is the transcription factor TCF, an HMG-box DNA-binding protein. Multiple TCF family members exist in mammals, including TCF-1, LEF-1, TCF-3, and TCF-4. β -Catenin forms a complex with TCF, converting it from a transcriptional repressor to an activator and thereby stimulating the expression of target genes. Although the genes in mammalian cells that are activated by TCFs in response to Wnt signaling are not well characterized, the *c-myc* proto-oncogene is one target that may mediate proliferative responses to Wnt signals (He et al. 1998).

Wnts and mammary gland development

The observation that *Wnt-1* and *Wnt-3* were activated by a MMTV provirus in virus-induced mammary carcinomas suggested that Wnt signaling can control the growth and differentiation of MECs. Although neither of these genes turned out to be expressed in the normal mammary tissue, other *Wnt* genes are expressed and differentially regulated during mouse mammary gland development (Gavin and McMahon 1992; Buhler et al. 1993; Huguette et al. 1994; Olson and Papkoff 1994; Weber-Hall et al. 1994; Lane and Leder 1997). *Wnt-4*, *Wnt-5b*, *Wnt-6*, *Wnt-7b*, and *Wnt-10b* transcripts are detected in the epithelial compartment at various stages of development. *Wnt-4*, *Wnt-5b*, and *Wnt-6* mRNAs are induced during pregnancy and decrease after lactation has commenced. *Wnt-10b* expression occurs beginning at very early stages in the mammary rudiment and continues into puberty (Lane and Leder 1997). *Wnt-2*, *Wnt-5a*, and *Wnt-6* are detected in stroma at a stage preceding ductal outgrowth (Weber-Hall et al. 1994) raising the possibility that one

or more of these *Wnt* family members are candidates for mediating epithelial induction by the stroma. At present, however, specific functions in mammary development have yet to be demonstrated.

Experiments to address Wnt function in MECs have involved primarily ectopic expression of *Wnt* genes, either in transgenic mice or by making use of recombinant retroviruses to infect cultured epithelial cells followed by transplantation into cleared fat pads of recipient females. Transgenic mice containing the *Wnt-1* gene under control of the MMTV-LTR exhibited a hyperplastic epithelial phenotype (Tsukamoto et al. 1988). The ducts of virgin animals displayed side-branching that had some resemblance to that of early to midpregnant females. The effects on mammary epithelium were also observed in males, indicating that *Wnt-1* can override the female hormonal requirements for at least some aspects of ductal morphogenesis. The role of another *Wnt* gene, *Wnt-4*, in promoting ductal morphogenesis was examined using retroviral gene transfer into MECs and tissue reconstitution from the modified cells (Bradbury et al. 1995; Edwards 1998). Implanted epithelial cells expressing ectopic *Wnt-4* formed ducts with extensive side-branching that was reminiscent of the branching seen in glands of pregnant animals. Indeed, *Wnt-4* caused ductal arborization that more closely mimicked the structures in glands of normal pregnant mice than did *Wnt-1*-expressing tissue, supporting the notion that *Wnt-4* may be the physiological signal that stimulates ductal morphogenesis during pregnancy.

Progesterone activates paracrine Wnt signaling

Although the aforementioned properties of *Wnt-4* indicated that this factor may be involved in hormonally induced morphogenesis of the mammary gland during pregnancy, direct proof of this hypothesis required genetic analysis of mice carrying a targeted disruption of the *Wnt-4* gene. This approach, however, was complicated by the fact that *Wnt-4*-deficient mice die perinatally as a result of renal failure (Stark et al. 1994), which precluded any direct assessment of *Wnt-4* function in postnatal development of the mammary gland. The experiments by Brisken et al. (2000) now demonstrate convincingly the contribution of Wnt signaling in mediating the morphogenetic response of mammary epithelium to hormonal cues received by the mammary gland during pregnancy. Specifically, their studies link the progesterone signal, acting via its nuclear PR in epithelial cells, with expression of the *Wnt-4* gene, whose product then serves as a paracrine signal to induce ductal side-branching.

To elucidate a connection between Wnt and progesterone in ductal side-branching, mice were generated that either contained or lacked a functional PR gene but expressed the MMTV *Wnt-1* transgene. To ensure normal PR function in other tissues, mammary epithelium from these mice was transplanted into cleared fat pads of *PR^{+/-}* recipients. Compared to control transplants, increased side-branching was observed in the ducts of

MMTV *Wnt-1^{tg}* transplants, irrespective of whether the graft expressed a functional PR protein. These findings demonstrated that Wnt signaling functions downstream of PR. Wnt-1 acts in a paracrine fashion to induce branching, as shown by transplanting MECs derived from MMTV *Wnt-1^{tg}* mice, together with MECs from ROSA 26 mice, which express a *lacZ* transgene. Blue-stained wild-type cells that were adjacent to unstained (i.e., Wnt-1-expressing) tissue in the chimeric transplants also exhibited increased side-branching, demonstrating that the Wnt-1 protein can act over short distances to elicit morphogenetic effects in nonexpressing cells.

The key experiment to assess the role of Wnt-4 involved transplanting mammary buds from *Wnt-4^{-/-}* embryos into wild-type recipients and evaluating the ductal structures that developed in virgin and pregnant animals. No defects were apparent in transplants analyzed in virgin mice, demonstrating that Wnt-4 is dispensable for development of the ductal tree preceding pregnancy. However, when analyzed at day 12 of pregnancy, the mutant implants displayed reduced branching in comparison to wild-type grafts, as would be expected if Wnt-4 functions as the morphogenetic signal mediating the response to pregnancy hormones. The link between hormonal signals and Wnt-4 expression came from *in situ* hybridization experiments that colocalized PR and *Wnt-4* transcripts in luminal epithelial cells. Furthermore, *Wnt-4* expression was induced by progesterone in ovariectomized animals, and *Wnt-4* RNA levels were reduced in *PR^{-/-}* epithelium. These experiments show that progesterone/PR activates expression of the *Wnt-4* gene, although it remains to be established whether this occurs by a direct or indirect mechanism. In this regard, it will be important to determine whether the *Wnt-4* promoter contains binding sites for PR that mediate progesterone-induced transcriptional activation.

The Brisken study resolves some previous questions, while at the same time raises other important issues. For instance, what are the roles of other *Wnt* genes that are expressed in mammary epithelium? *Wnt-5b* and *Wnt-6*, which are induced in epithelium during pregnancy, apparently do not substitute for *Wnt-4* in eliciting ductal side-branching at day 12 of pregnancy. However, at later stages of pregnancy branching was observed in *Wnt-4*-deficient glands, suggesting that other Wnts may become expressed and mimic the morphogenetic effects of Wnt-4. Whether *Wnt-5b* and *Wnt-6* are likely candidates for this activity is unclear, since neither factor significantly induced branching morphogenesis in retroviral gene transfer/transplantation experiments (Bradbury et al. 1995). Many of the transgenic and retroviral studies merely evaluate mammary structures on the level of whole mounts, and it is necessary to assess functional differentiation using molecular markers, such as milk proteins. Another issue concerns the functions of *Wnt* genes expressed in the stromal compartment. It is not known whether they have a role in inducing ductal outgrowth or branching. Answers to these questions should

be forthcoming as knockout mice are generated for additional members of the *Wnt* family.

Although Wnt-1 can elicit ductal side-branching, its morphogenetic effects are not identical to those of Wnt-4, the natural inductive signal in mammary epithelium. Moreover, *Wnt* genes differ in their capacity to transform C57MG MECs (Wong et al. 1994). Wnt-1, Wnt-3a, and Wnt-7 are strongly transforming, Wnt-5b, Wnt-6, and Wnt-7b display low but detectable transforming activity, and Wnt-4 and Wnt-5a, which are normally expressed in these cells, do not induce transformation. Similarly, different responses to individual Wnts were observed in stably transfected and transplanted HC11 cells, a clonal mammary epithelial cell line derived from a mid-pregnant mouse mammary gland (Humphreys and Rosen 1997). Thus, the various Wnt family members can exert differential effects on the growth and morphogenesis of MECs. These observations prompt questions about the basis of the Wnt signaling specificity. It will be instructive to determine which Fz receptors are expressed on MECs, as different Wnts might bind to distinct receptors. Alternatively, Wnts could activate different signaling pathways through the same receptor, perhaps by differential interactions with coreceptor proteins, and thereby elicit unique cellular responses.

The genetics of budding and branching

The progesterone-Wnt pathway is only one of the parallel, and perhaps interconnected, signaling cascades that control the formation of the ductal tree and lobulo-alveolar compartment. In the absence of prolactin or its receptor, reduced ductal side-branching has been noted. Other genes have been identified whose elimination also affects mammary gland development, in particular, the prolactin receptor (Ormandy et al. 1997) and cyclin D1 (Fantl et al. 1995; Sicinski et al. 1995). The transcription factor C/EBP β controls fundamental aspects of cell fate and ductal branching in the mammary gland, and its function is autonomous to the epithelium (Robinson et al. 1998; Seagroves et al. 1998). C/EBP β -null mice display little ductal outgrowth, branching and alveolar budding, and expression of milk protein genes is almost entirely lacking. A recent paper by Seagroves and colleagues (Seagroves et al. 2000) now makes a connection between C/EBP β and the PR. These authors showed that the rather uniform expression pattern of PR in the immature virgin switches to a scattered expression pattern as branching and budding is initiated. Interestingly, in their study only PR-negative but not PR-positive cells underwent cell division during this inductive process. In C/EBP β -deficient glands PR expression was not extinguished and proliferation and branching was impaired. These results suggest that a selective, C/EBP β dependent loss of PR enables a specific population of cells to respond to paracrine signals and enter the proliferative phase. At this point it is not clear whether the forced expression of Wnt-1 could bypass this block, or whether a lack of C/EBP β prevents a response to Wnt signals. In addition, it is now important to establish whether ex-

pression of Wnt genes, especially Wnt-4, is impaired in the C/EBP β null mutant.

Based on the available evidence we can predict a network of signaling pathways that first determines the fate of epithelial cells and subsequently induces branching and budding through the actions of nuclear and secreted signals (Fig. 2). Although there is no doubt that progesterone signaling is mandatory for ductal branching, there is a continuing debate about which cells divide to form side-branches. Seagroves and colleagues suggest that PR is present uniformly in ductal cells prior to puberty and that selective loss of this protein occurs at puberty, followed by proliferation of PR-negative cells. In this model, Wnt would be a paracrine cell proliferation signal. Other investigators have shown that PR-positive MECs are clustered in terminal end buds and points of lateral branching, the sites of highest cell proliferation (Silberstein et al. 1996; Zeps et al. 1999). To obtain a clearer picture it will be necessary to perform precise colocalization experiments at different developmental stages and in the available models with impaired mammary development. Clearly PR-positive cells proliferate in hormone dependent tumors and demonstrate that Wnt is probably an autocrine signal. It is possible that distinct signaling mechanisms exist and that at some stage of tumor progression a transition occurs from paracrine to autocrine signaling.

The family of Wnt proteins probably composes only one arm of proliferative signals. Others include epider-

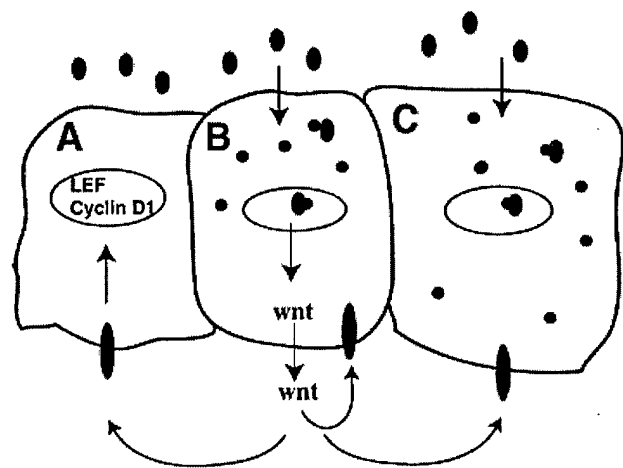


Figure 2. Progesterone-Wnt-induced paracrine/autocrine signaling in MECs. Progesterone (green ovals) activates Wnt-4 gene expression in PR-positive (black dots) cells. Wnt-4 is secreted, binds to the frizzled (Fz) receptor (red ovals) and activates the β -catenin pathway, which culminates in the activation of the transcription factor LEF. The *cyclin D1* gene is a direct target of progesterone. Two distinct models of progesterone-mediated cell proliferation have been proposed. In one model (A) progesterone will promote cell proliferation through Wnts and other growth signals in PR-negative (A) and PR-positive (B and C) cells. In the Seagroves model (Seagroves et al. 2000), progesterone-induced cell proliferation during normal ductal development is limited to PR-negative cells (A). However, it is clear that tumor cells will proliferate independently of their PR status.

mal growth factor (EGF) family members (Luetteke et al. 1999) and signals mediated by the prolactin receptor (Ormany et al. 1997; Brisken et al. 1999) and Stat5a (Liu et al. 1997) as evidenced by the phenotype of their respective gene knockout. In vitro results with mammary cell lines also suggest that progesterone synergizes with other growth factor signaling pathways to promote cell growth (for review, see Lange et al. 1999). We anticipate that the rapid accumulation of new knockout models will help to dissect and integrate the contribution of these different pathways to normal development and carcinogenesis.

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