Chapter

Cell Cycle Machinery:
Links with Genesis and Treatment of Breast Cancer

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Abstract

Loss of normal growth control is a hallmark of cancer. Thus, understanding the mechanisms of tissue-specific, normal growth regulation and the changes that occur during tumorigenesis may provide insights of both diagnostic and therapeutic importance. Control of cell proliferation in the normal mammary gland is steroid hormone (estrogen and progestin)-dependent, involves complex interactions with other hormones, growth factors and cytokines and ultimately converges on activation of three proto-oncogenes (c-Myc, cyclin D1 and cyclin E1) that are rate limiting for the G1 to S phase transition during normal cell cycle progression. Mammary epithelial cell-specific overexpression of these genes induces mammary carcinoma in mice, while cyclin D1 null mice have arrested mammary gland development and are resistant to carcinoma induced by the *neu/erbB2* and *ras* oncogenes. Furthermore, c-Myc, cyclins D1, E1 and E2 are commonly overexpressed in primary breast cancer where elevated expression is often associated with a more aggressive disease phenotype and an adverse patient outcome. This may be due in part to overexpression of these genes conferring resistance to endocrine therapies since in vitro studies provide compelling evidence that overexpression of c-Myc and to a lesser extent cyclin D1 and cyclin E1, attenuate the growth inhibitory effects of SERMS, antiestrogens and progestins in breast cancer cells. Thus, abnormal regulation of the expression of cell cycle molecules, involved in the steroidal control of cell proliferation in the mammary gland, are likely to be directly involved in the development, progression and therapeutic responsiveness of breast cancer. Furthermore, a more detailed understanding of these pathways may identify new targets for therapeutic intervention particularly in endocrine-unresponsive and endocrine-resistant disease.

Introduction

Loss of normal growth control, including aberrations in the homeostatic mechanisms that ensure integrity of cell cycle progression, is a hallmark of cancer.[1] A pivotal regulatory pathway determining rates of cell cycle transition from G_1 to S phase is the cyclin/cyclin-dependent kinase (CDK)/$p16^{INK4A}$/retinoblastoma protein (Rb) pathway.[2] Alterations to different components of this pathway through overexpression, mutation and epigenetic gene silencing are almost universal in human cancer.[3] Interestingly, there appears to be a degree of tissue specificity in the particular genetic abnormalities within the Rb pathway in different cancers with aberrations in the expression of cyclins D1, E1 and the CDK inhibitor $p27^{Kip1}$ common in breast cancer.

In the mammary gland the sex steroid hormones, estrogen and progesterone and their cognate receptors, ER and PR, are essential for normal development and physiological function. There is now an expansive literature documenting the molecular mechanisms through which these hormones

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exert their mitogenic effects both in the normal mammary gland and in breast cancer. These data show that estrogen/progestin action converges on a number of molecules with pivotal roles in the regulation of the Rb pathway and thus, in the G_1 to S phase transition of the cell cycle. These include the proto-oncogenes c-Myc, cyclins D1, D3, E1 and E2 and the CDK inhibitors, $p21^{WAF1/Cip1}$ and $p27^{Kip1}$. Furthermore, the expression of several of these molecules changes significantly during breast tumorigenesis and is associated with distinct breast cancer phenotypes and patient outcome. Thus, aberrant expression and/or function of cell cycle regulatory molecules involved in the normal physiological response to sex steroid hormones is a common feature of breast cancer and may be intimately involved mechanistically in the disease process.

This review briefly summarizes contemporary literature addressing the functions of selected cell cycle regulatory genes in mammary epithelial cells and their potential roles in the development and progression of human breast cancer.

Cell Cycle Control Mechanisms and Their Regulation in Breast Cancer Cells

Mechanisms of Cell Cycle Control

Cyclins are the regulatory subunits of holoenzymes whose catalytic subunit is a CDK. Cyclins share a sequence motif termed the 'cyclin box' that mediates binding to a similarly well-conserved region on the CDK.[4] Members of this family of serine/threonine kinases were originally characterized by virtue of their roles in cell cycle control, although more recently identified cyclin-CDK complexes have roles in transcriptional control.[5] In addition, cyclin D1 can act as a transcriptional cofactor, a function which is CDK-independent.[6] As the name suggests, CDKs lack kinase activity in the absence of cyclin association and thus, regulation of cyclin abundance is an important, but not the only, control mechanism for CDK activation.[4]

Progress through the cell cycle is accompanied by sequential accumulation of different cyclins that is correlated with the activation of specific cyclin-CDK complexes: cyclin E-CDK2 at the G_1/S phase boundary, cyclin A-CDK2 during S phase, cyclin A-CDK1 (CDC2) during G_2 and cyclin B-CDK1 during mitosis (Fig. 1). The D-type cyclins (cyclins D1-3) are less profoundly regulated during the cell cycle but are strongly mitogen-dependent. Consequently, the CDKs formed by association of D type cyclins and CDK4 or CDK6 can be viewed as 'mitogen sensors', that act during G_1 phase to link signals from the extracellular environment to other CDKs that comprise the 'core cell cycle machinery'.[7]

Several substrates for the different CDKs have been identified. A prevailing concept has been that each cyclin-CDK complex has a distinct substrate preference and that this specificity is a determinant in ordering cell cycle events. This is supported by several lines of evidence, for example the different spectra of cellular proteins phosphorylated by various recombinant cyclin-CDK complexes[8] and the distinct consensus sequences for phosphorylation by cyclin D1-CDK4 and cyclin E-CDK2 or cyclin A-CDK2.[9] However, the ability of cyclin E and cyclin D2 'knocked-in' to the cyclin D1 locus to complement defects in mice lacking cyclin D1 and the ability of fibroblasts lacking all three D-type cyclins or both E-type cyclins to proliferate, argue for significant functional redundancy between the cyclins.[10] Thus, an alternative view is that the spatial and temporal control of cyclin expression is a major determinant of specificity.[11]

The best-understood CDK substrate is Rb, the product of the retinoblastoma susceptibility gene. The importance of Rb as a CDK substrate is illustrated by the observation that cyclin D1 is not required for G_1 phase progression in cells lacking Rb.[12] However, cyclin D1-associated CDKs are not the only Rb kinases; there are 16 possible consensus sites for CDK phosphorylation within Rb and the protein is progressively phosphorylated by different CDKs during cell cycle progression.[2] Phosphorylation of Rb by cyclin D-CDK4 and/or cyclin D-CDK6 early in G_1 phase displaces histone deacetylases from Rb and allows subsequent phosphorylation of Rb by cyclin E-CDK2 and cyclin A-CDK2.[13] Phosphorylation by both sets of CDKs is necessary to completely overcome the growth inhibitory effects of Rb, release E2F transcription factors and allow initiation

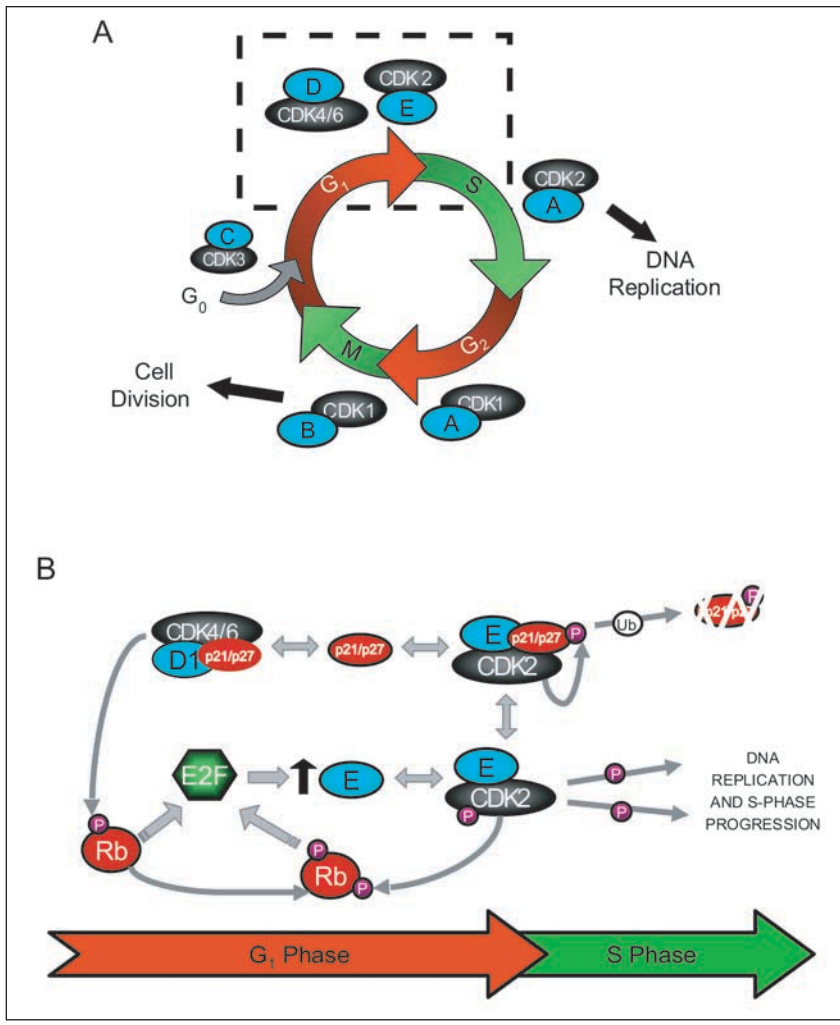

Figure 1. The eukaryotic cell cycle and phase-specific activation of cyclin-CDK complexes. A) the eukaryotic cell cycle involves the sequential action of cyclin-CDK complexes to move between the distinct phases of the cell cycle. The letters A, B, C, D and E denote each respective cyclin. B) The main features of G_1 to S phase progression. Briefly, sequential phosphorylation of Rb by cyclin D1-CDK4/6 and cyclin E-CDK2 allows E2F-mediated transcription of target genes including cyclin E and consequent progression into S phase. The distribution of the CDK inhibitors $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ between these complexes provides an additional level of control over their activity. The levels of these CDK inhibitors are in part regulated by their ubiquitin-mediated degradation. D1: cyclin D1; E: cyclin E; Ub: ubiquitin; P: phosphorylation.

of DNA synthesis (Fig. 1).[13,14] Recent data also implicate another CDK, cyclin C-CDK3, in the phosphorylation of Rb during the transition from quiescence (G_0) to G_1.[15]

In addition to regulation of cyclin abundance there exist several other levels of regulation for CDK activity including a network of regulatory kinases and phosphatases,[4] and two families of endogenous small molecular weight CDK inhibitory proteins.[7] The INK4 family of CDK inhibi-

tors ($p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, $p19^{INK4D}$) specifically target CDK4 and CDK6.[7] The Cip/Kip family inhibitors ($p21^{WAF1/Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$) target a wider spectrum of CDKs. They profoundly inhibit the activity of cyclin E-CDK2 and cyclin A-CDK2, but also function as assembly factors for cyclin D-CDK complexes.[16] Like the cyclins, these inhibitors are mitogen-responsive. For example, $p27^{Kip1}$ expression provides a 'threshhold' that must be exceeded to allow CDK activation during mitogenic stimulation. One function of cyclin D1 appears to be sequestration of $p27^{Kip1}$: alterations in cyclin D1 abundance not only directly affect the activity of CDK4 and CDK6 but can indirectly influence the activation of cyclin E-CDK2 by altering the availability of $p27^{Kip1}$.[7]

Steroid Regulation of Cell Cycle Progression

In the mammary gland the majority of development occurs postnatally under the influence of the ovarian sex steroid hormones, estrogen and progesterone. Although several other hormones, growth factors and cytokines regulate normal mammary gland physiology, the sex steroid hormones are required for mammary gland development, playing a pivotal role in side-branching and lobulo-alveolar development. These roles of the sex steroids carry over to breast cancer where estrogen action is essential for the development and maintenance of the majority of breast cancers[17] and the synthetic analogs of progesterone, progestins, exert both growth stimulatory and inhibitory effects depending on the stage of the disease process and the cellular phenotype.[18] Furthermore, progestins increase breast cancer risk when administered in HRT regimens and a PR allele that leads to the preferential expression of PR-B is associated with increased breast cancer risk.[19] Detailed analyses of the effects of sex steroids on breast cancer cell proliferation identify that both estrogens and progestins control this process by regulating the G_1 to S phase transition in the cell cycle.[20,21]

The effects of estrogen and progestins are mediated through ligand-activated transcription factors belonging to the nuclear receptor superfamily. Two ERs have been characterized, ERα and ERβ. Studies using ER knockout models have shown that ERα is the predominant mediator of the mitogenic effects of estrogen in the mammary gland,[22] while ERα appears to mediate the drive to proliferation in breast cancer cells but ERβ is growth inhibitory.[23] Although only one PR gene has been identified, there are 2 distinct isoforms of the receptors, PR-B and PR-A, that are generated from different transcriptional start sites. These isoforms have differential effects on mammary gland development and the regulation of breast cancer cell proliferation and differentiated function in vitro.[18]

Since both estrogens and progestins control G_1 to S phase progression much work has focused on the links between steroid hormone receptor signaling and the cell cycle machinery. This is most developed in the case of estrogen stimulation of breast cancer cell proliferation. One of the earliest transcriptional responses in the mitogenic response to estrogen is increased *MYC* expression, which occurs within 15 min of estrogen stimulation.[24] Similarly, acute downregulation of *MYC* expression is an early event in antiestrogen inhibition of breast cancer cells while downregulation of c-Myc with antisense oligonucleotides mimics the effect of antiestrogens on breast cancer cell cycle progression.[25] The DNA binding region of ERα is required for *MYC* induction and the P2 promoter region of the *MYC* gene contains an atypical ERE region.[26] Recently a strongly estrogen-inducible ER binding site 67 kb upstream of *MYC* has been identified which may also contribute to estrogen regulation of *MYC*, although its functional significance is yet to be characterized.[27]

The c-Myc protein is a nuclear transcription factor that has profound mitogenic effects on breast cancer cells through its ability to modulate regulators of cell cycle progression.[28] Inhibition of c-Myc expression abrogates estrogen-stimulated breast cancer cell proliferation and blocks cell cycle progression leading to a G_1 arrest.[29] Furthermore, induction of c-Myc can mimic the effects of estrogen and induce antiestrogen-arrested cells to reinitiate cell cycle progression,[30] implicating c-Myc as a prominent mediator of estrogen action in breast cancer cells. Numerous genetic targets of c-Myc activation and repression have been identified, including many cell cycle regulators (reviewed in ref. 31). Thus, a major mechanism governing c-Myc's effects on cell cycle progression in breast cancer cells is the activation of cyclin E-CDK2 via repression of the CDK inhibitor, $p21^{WAF1/Cip1}$.[30,32]

In this respect, c-Myc's actions closely mimic those of estrogen,[33] again emphasizing its potential role as a major mediator of estrogen action in breast cancer cells.

The effects of estrogen on cell cycle progression are also tightly linked to increased expression of cyclin D1. Cyclin D1 induction in breast cancer cells shortens G_1 and can rescue growth factor-deprived and antiestrogen-arrested cells enabling them to complete the cell cycle.[34] While estrogen rapidly induces cyclin D1 expresssion, antiestrogens have a converse acute inhibitory effect.[33,35,36] Furthermore, abrogation of cyclin D1 activity by cyclin D1 antibodies or the Cdk4 inhibitor $p16^{INK4A}$ blocks estrogen-induced G_1-S phase progression,[37] indicating that estrogen acts, at least in part, through upregulation of cyclin D1 expression. Like c-Myc, inducible cyclin D1 expression can mimic the effects of estrogen allowing cell cycle re-entry in antiestrogen-arrested breast cancer cells.[30,36]

Estrogen also elicits rapid activation of cyclin E-CDK2 in breast cancer cells.[33,38,39] The mechanism governing this action is not fully elucidated, although it is known to involve estrogen-mediated inhibition of the CDK inhibitor, $p21^{WAF1/Cip1}$.[33,38] Overall, estrogen activation of cyclin D1 expression increases cyclin D1-CDK4 complex formation and sequestration of $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ at the expense of cyclin E-CDK2 complexes, thus activating the latter enzyme. The cyclin E-CDK2 complex binds hyperphosphorylated p130 in the absence of $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ binding, which may prevent reassociation with CDK inhibitors.[33] The activity of the cyclin E-CDK2 complex is further enhanced through upregulation of Cdc25A, which removes inhibitory phosphatases from the cyclin E-CDK2 complexes. Finally, $p27^{Kip1}$ is relocalized from the nucleus to the cytoplasm by estrogen-induced ERK activation and simultaneously the degradation of $p27^{Kip1}$ is increased through estrogen-mediated induction of Skp2.[40] Cyclin D1 expression also elicits effects on the activation of cyclin E-CDK2 similar to those of c-Myc.[30] However, in our MCF-7 model system overexpression of cyclin D1 did not induce c-Myc expression or vice versa, consistent with evidence that both *MYC* and *CCND1* are direct targets of the ER,[27,41] and further, suggesting that estrogen-stimulated cell cycle progression is mediated initially by distinct c-Myc and cyclin D1 pathways that converge on the activation of cyclin E-CDK2.[30] A summary of estrogen regulation of breast cancer cell cycle progression is presented in Figure 2.

In contrast to the stimulatory actions of estrogen in breast cancer cells in vitro, progesterone has a biphasic effect on cell proliferation, where it initially accelerates cells from G_1 to S phase but subsequently arrests cells in early G_1 following mitosis.[42] Progestins induce a similar effect to estrogen in the stimulatory phase of their action in that c-Myc and cyclin D1 are induced transiently within 2-3 hours of progestin treatment.[20] After the transient induction of S phase, cell proliferation is inhibited following a reduction in cyclin E-CDK2 and cyclin D-CDK4 activity.[20] This is mediated, in part, by a reduction in levels of cyclin D1 and cyclin E1, as well as increased expression of $p18^{INK4c}$, which disrupts cyclin D-CDK4/6 binding and hence, contributes to inactivation of CDK4/6.[43,44] The proportion of inactive cyclin E-CDK2 complexes bound by the CDK inhibitors $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ increases, due to both the upregulation of the CDK inhibitors and their redistribution from cyclin D-CDK4/6 complexes.[20,44] Inducible overexpression of cyclin D1 in progestin-pretreated cells restores the activity of cyclin E-CDK2 complexes,[44] emphasising the role of cyclin D1 abundance in regulating the availability of CDK inhibitors. A summary of these effects of progestins on the cell cycle machinery is presented in Figure 3.

Progestins regulate both proliferation and differentiation in breast cancer cells and there has been much interest in identifying progestin targets that may contribute to the co-ordination of these processes. One candidate is the HLH protein Id1, which is progestin-regulated and has roles in both the proliferation and differentiation of mammary epithelial cells. More recently, we have demonstrated a role for Wilms Tumor Protein 1 (Wt1) in mediating the growth inhibitory/differentiation-inducing effects of progestin action in breast cancer cells.[45] Progestin treatment of breast cancer cells leads to a rapid downregulation of Wt1 mRNA and protein. Conversely, overexpression of Wt1 attenuates progestin-mediated growth inhibition and activation of lipogenesis, a marker of differentiation in these cells. This is accompanied by the sustained expression of cyclin D1 despite progestin treatment and increased levels of Rb phosphorylation at sites targeted by

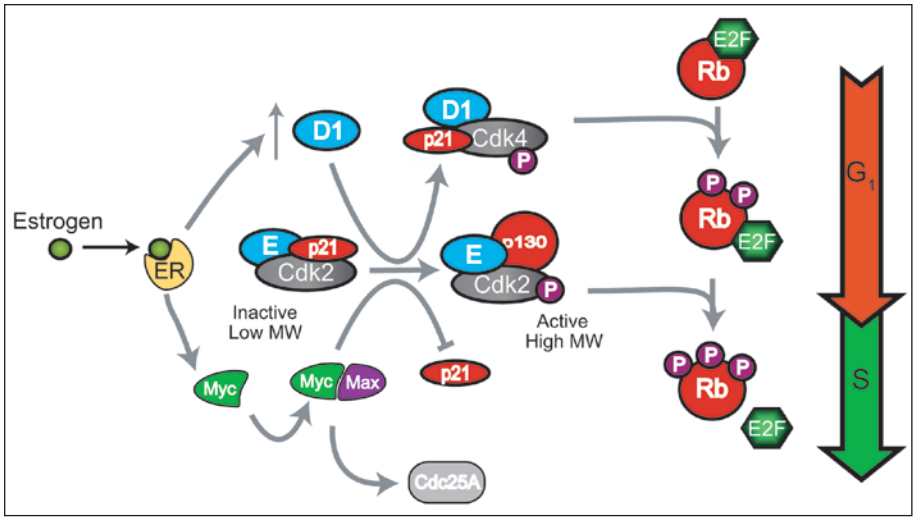

Figure 2. Estrogen action on the cell cycle machinery. Estrogen binding to the estrogen receptor activates parallel pathways through c-Myc and cyclin D1, resulting in the inhibition of $p21^{WAF1/Cip1}$. This leads to the activation of cyclin D1-CDK4 and cyclin E-CDK2 complexes and the subsequent phosphorylation of Rb, releasing E2F and allowing progression from G_1 to S phase. ER: estrogen receptor; D1: cyclin D1; E: cyclin E; P: phosphorylation.

cyclin D1-CDK4 (Ser249/Thr252). Furthermore, Wt1 overexpression only modulates the effects of progestins and not either antiestrogens or androgens. These results indicate that Wt1 is an important early target of progestins that may co-ordinate proliferation and differentiation in breast cancer cells.

Cell Cycle Control Genes as Putative Breast Cancer Oncogenes/Tumor Suppressor Genes

Evidence that c-Myc and the cyclins are potential oncogenes and that the CDK inhibitors are potential tumor suppressor genes in breast cancer comes from both experimental model systems and studies of human breast cancer tissue.

Animal Models

c-Myc was one of the earliest characterised proto-oncogenes and the first oncogene demonstrated to induce mammary carcinoma in transgenic mouse models.[46] However, subsequent studies of various *MYC* transgenic mammary tumor models have demonstrated extended latencies and insufficiency of aberrant *MYC* expression alone to induce mammary tumorigenesis and have given support to the hypothesis that the acquisition of additional genetic lesions is a critical step in c-Myc-induced carcinogenesis. This may result from c-Myc-induced genomic destabilization through a dominant mutator phenotype and center upon suppression of the intrinsic apoptotic function of c-Myc. Indeed, transgene-mediated suppression of c-Myc-induced apoptosis (via expression of *ras, neu/erbB2, bcl-2* or *tgf-*α) in bitransgenic mouse models, leads to a potent accentuation of mammary tumorigenesis (reviewed in ref. 47).

Overexpression of cyclin D1 in the mammary gland leads to hyperplasia and eventually to carcinoma.[48] Similarly, cyclin E1 overexpression in mammary epithelium promotes tumor formation, but with low penetrance and long latency.[49] Thus, cyclin D1 and cyclin E1 are oncogenic in mice, although weakly so and it is likely that they co-operate with other oncogenes to mediate this effect. Given its role as a target of mitogenic signaling, it is not surprising that cyclin D1 is implicated

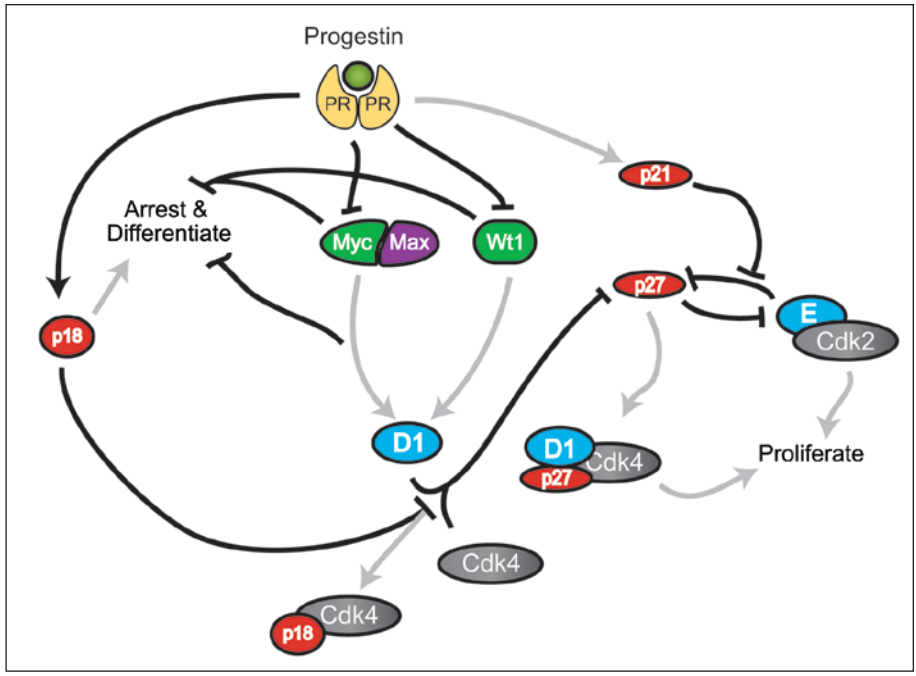

Figure 3. Progestin action on the cell cycle machinery. Progestin-mediated cell cycle arrest requires repression of c-Myc and upregulation of the CDK inhibitors $p21^{WAF1/Cip1}$ and $p18^{INK4C}$. Downregulation of c-Myc then regulates the expression of numerous targets, including cyclin D1. Reduced cyclin D1 and increased $p18^{INK4C}$ expression then cooperate to inhibit the formation of cyclin D-CDK complexes. The loss of these complexes liberates sequestered $p27^{Kip1}$ which then cooperates with the induced $p21^{WAF1/Cip1}$ to inhibit cyclin E-CDK2 complexes. Grey lines indicate positive regulation, black lines represent repression. Dotted lines signify proposed pathways. PR: progesterone receptor; D1: cyclin D1; E: cyclin E.

in the oncogenic actions of *ras* and *neu/erbB2*. In the mouse mammary gland, tumors induced by either oncogene display increased expression of cyclin D1.[50,51] Conversely, decreased cyclin D1 expression blocks the growth of tumors formed by mammary cells expressing activated *neu/erbB2*[50] and cyclin D1-null mice are resistant to tumor formation resulting from mammary-specific expression of *ras* or *neu/erbB2*.[51] Interestingly, although cyclin D1 has also been implicated as a target of Wnt signaling, *wnt*-stimulated oncogenesis was not impaired in cyclin D1-null mice.[51] In support of conclusions drawn from in vitro studies c-*myc* also induces mammary carcinoma independent of cyclin D1.

The observations that overexpression of $p15^{INK4b}$ and $p16^{INK4A}$, which target the cyclin D1-associated CDKs, can suppress *ras*-mediated transformation in vitro,[52] and that $p16^{INK4A}$ expression blocks *neu/erbB2*-induced mammary tumor formation in mice,[53] all indicate that the dependence on cyclin D1 is likely to be mediated by the ability of cyclin D1 to increase CDK activity, either by direct activation of CDK4 or by indirect activation of CDK2 through sequestration of CDK inhibitors. Although the formation of mammary tumors after expression of activated *neu/erbB2* is impaired in cyclin D1-null mice, some tumors do develop and these are characterized by increased cyclin E expression.[54] Similarly, mice that have cyclin E1 'knocked-in' to the cyclin D1 locus develop *neu/erbB2*-induced mammary tumors at a rate similar to wild-type, indicating that cyclin E1 expression can compensate for the absence of cyclin D1 during oncogenesis.[55] This

is consistent with the idea that the requirement for cyclin D1 in mammary carcinoma reflects a need for CDK activity, or at least cell proliferation.

More recent experiments have addressed this issue directly. Development of *neu/erbB2*-induced mammary cancers is significantly impaired both in CDK4-null mice[56] and in knock-in mice where endogenous cyclin D1 is replaced with a 'kinase-dead' cyclin D1 point mutant that binds CDK4 and sequesters CDK inhibitors but is unable to activate the CDK4 kinase.[56,57] This does not simply result from failed mammary epithelial cell proliferation, since virgin CDK4-null mice display retarded mammary development but normal alveolar proliferation and differentiation occur during pregnancy and the 'kinase dead' cyclin D1 mutant is able to rescue the defects in pregnancy-associated mammary gland development in mice lacking cyclin D1. It is therefore clear that *neu/erbB2*-induced mammary oncogenesis requires active cyclin D1-CDK4, in contrast with mammary development, which is 'CDK-independent'. The ability of cyclin E1 to substitute for the function of cyclin D1 in mammary development as well as oncogenesis[55] suggests, however, that there is no absolute requirement for cyclin D1 and that the CDK independent function required for mammary development is likely to be sequestration of $p27^{Kip1}$ rather than the ability of cyclin D1 to regulate transcription.

The necessity for cyclin E1 in transformation has not been tested in vivo. However, in in vitro assays, fibroblasts lacking both cyclin E1 and E2 do not form foci in response to c-Myc or to Ras in combination with either c-Myc or dominant-negative p53. Although these fibroblasts display defects in cell cycle re-entry from quiescence, once proliferation is initiated it is only modestly impaired compared to controls with wild-type cyclin E, suggesting a specific requirement for cyclin E in oncogenic proliferation.[58] Thus, there is an emerging body of evidence, which is perhaps the most compelling for cyclin D1, that c-Myc and cyclins D1 and E1 are important for mammary tumorigenesis.

Deregulation in Breast Cancer

Studies of gene expression in human breast cancer tissue have provided substantial evidence for aberrant expression of c-Myc, several cyclins and $p27^{Kip1}$ in human breast cancer (Table 1). In clinical cohorts, *MYC* gene amplification is associated with the transition from in situ to invasive carcinoma, markers of an aggressive disease phenotype and poor prognosis in general.[59-61] *MYC* gene amplification occurs in approximately 15-20% of patients with breast cancer[60] but overexpression of *MYC* mRNA and c-Myc protein occurs more frequently, generally 30-50%, particularly in high-grade tumors.[60,62,63] Immunohistochemical studies have generally failed to demonstrate an association between c-Myc protein expression and outcome[64] but this may be due, in part, to difficulties in assessing c-Myc expression by immunohistochemistry with currently available antibodies. While some studies show an association between c-Myc overexpression and negative prognostic factors such as poor differentiation and high proliferation index,[62] at present it is difficult to draw definite conclusions regarding the prognostic significance of c-Myc protein overexpression in breast cancer.

Cyclin D1 protein is overexpressed in ~45 % of breast cancers, predominantly in the ER-positive phenotypes.[65] The expression of cyclin D1 protein mirrors stages in the progression model of breast cancer, being expressed at low levels in normal breast, then at increasing levels in hyperplasia and ductal carcinoma in situ.[65] Amplification of the *CCND1* gene, as part of the 11q13 locus, partially accounts for the observed overexpression being present in ~13% of breast cancers. The overexpression of cyclin D1 protein in the remaining ~30% of breast cancer cases is probably due to alterations in transcriptional regulation and/or protein stabilisation that may in turn be due to deregulation of upstream mitogenic signaling pathways.

In contrast to cyclin D1 and cyclin D3 that are highly expressed,[66] cyclin D2 is not expressed in most cultured breast cancer cell lines or in breast cancer due to the cyclin D2 promoter being highly methylated.[67] The relationship of cyclin D3 expression to clinicopathological parameters has only been examined in a small series of studies. These indicate that cyclin D3 is overexpressed

Table 1. Aberrations of cell cycle regulators in breast cancer

	Frequency range (%)	Mean (%)
MYC amplification	4-52	19
c-Myc overexpression	11-70	38
11q13 amplification	9-17	13
Cyclin D1 overexpression	28-81	45
Cyclin E overexpression	28-35	32
Decreased $p27^{Kip1}$ expression	50-63	57

in ~10% of breast cancers,[68] is not associated with gene amplification or ER status but often correlates with cyclin D1 overexpression.

Cyclin E1 is overexpressed in ~30% of breast cancers,[69] predominantly the ER-negative phenotype and is correlated with disease stage and markers of proliferation, i.e., Ki67, PCNA and mitotic index.[70,71] Low molecular weight forms of cyclin E1 have been detected in breast cancer and were proposed as indicators of poor patient outcome.[72] Functionally, these isoforms may act through increased binding to CDK2 and decreased affinity for $p21^{WAF1/Cip1}$ and $p27^{Kip1}$,[73] as well as differential regulation by full-length cyclin E1. However, recently the relevance of these low molecular weight forms has been questioned since these isoforms were also identified in normal mammary epithelial cells in a similar ratio to that found in breast cancer tissue.[74]

Data concerning the role of the more recently described cyclin E2 in breast cancer, is less evolved. Some of the earliest publications on cyclin E2 documented its overexpression in breast cancers, but these were restricted to small numbers of samples with limited clinicopathological data. Transcript profiles of larger series of breast cancers have identified cyclin E2 as a component of several gene expression signatures associated with reduced survival.[75-77] Cyclin E2 is the only gene present in all three prognostic signatures and was among 60 genes associated with poor outcome in ER-positive patients.[76] These data prompted two recent qRT-PCR studies of the potential role of cyclin E2 as an individual prognostic marker compared with cyclin E1.[78,79] Although cyclin E2 levels were similar in ER-positive and -negative cancers, cyclin E1 was more highly expressed in ER-negative cancers while cyclin E2 was significantly associated with both grade and ER-positivity.

Of the Cip/Kip family of CDK inhibitors, $p27^{Kip1}$ has the strongest association with the disease process while there is conflicting evidence on the importance of $p21^{WAF1/Cip1}$ expression in breast tumorigenesis. $p27^{Kip1}$ is normally expressed at high levels in epithelial cells, but undergoes profound downregulation in breast cancer where it is strongly correlated with ER-negativity, high tumor grade and poor outcome. The downregulation of $p27^{Kip1}$ does not appear to occur through genetic mutation or loss of heterozygosity. Instead $p27^{Kip1}$ is downregulated through a combination of mechanisms including decreased stability of nuclear $p27^{Kip1}$ through the amplification of processes responsible for its degradation.[80] Both Skp2 and Cks1, which form part of the SCF^{Skp2} complex that targets nuclear $p27^{Kip1}$ for degradation, are amplified or overexpressed in breast cancer,[81] and Skp2 overexpression correlates with low $p27^{Kip1}$ expression.[82]

Dysregulated signaling through growth factor pathways also decreases nuclear $p27^{Kip1}$ levels via cytoplasmic relocalisation and degradation. $p27^{Kip1}$ is targeted for phosphorylation and subsequent degradation by the ErbB2 and EGFR MEK/MAPK and Ras signaling pathways, leading to degradation.[83] Since these pathways are frequently altered in breast cancer, they are also likely to affect $p27^{Kip1}$ activity through upregulation of c-Myc and cyclin D1[83] leading in turn to decreased $p27^{Kip1}$ expression and increased levels of cyclin D-CDK4 complexes that sequester $p27^{Kip1}$ with resultant increased cyclin E-CDK2 activity. Finally, the PI3K/PKB pathway, which is also activated via ErbB2 and Ras, targets $p27^{Kip1}$ for cytoplasmic relocalization from the nucleus through phosphorylation of T157.[83] The activation of PI3K is opposed by PTEN, which also downregulates Skp2. PTEN is downregulated in breast cancer and is associated with low $p27^{Kip1}$ levels.[84]

While cytoplasmic $p27^{Kip1}$ is often degraded, it has been suggested that the presence of low levels of undegraded cytoplasmic $p27^{Kip1}$ may also provide an oncogenic feedback loop. Wu et al have identified that cytoplasmic $p27^{Kip1}$ enhances the assembly of cyclin D1-CDK4 complexes, as well as increasing AKT kinase levels.[85]

Of the INK4 family of inhibitors, only $p16^{INK4A}$ is altered in breast cancer predominantly through promoter hypermethylation in ~20-30% of cases.[86] $p16^{INK4A}$ inhibits cell cycle progression by disrupting cyclin D-CDK 4/6 complexes such that Rb phosphorylation is inhibited. Given that Rb is not usually directly mutated in breast cancer, the inactivation of $p16^{INK4A}$ may be important to overcome cell cycle arrest. Several recent reports have identified $p16^{INK4A}$ promoter methylation in normal breast and in early benign lesions, suggesting that $p16^{INK4A}$ downregulation may not associate with breast carcinogenesis.[86-88] However, there is compelling evidence that it is a subpopulation of normal breast cells that have $p16^{INK4A}$ methylation.[86] When cultured in vitro, this population escapes senescence and bear other characteristics of early carcinoma that are dependent on the $p16^{INK4A}$ methylation status, including upregulation of further methylation events and downregulation of p53.

Despite the importance of $p16^{INK4A}$ promoter methylation, it is actually the overexpression of $p16^{INK4A}$ that has been reported to be of prognostic significance in breast cancer. This has been examined in only a small series of studies, where overexpression of both $p16^{INK4A}$ mRNA and protein is associated with poor outcome.[89,90] In two studies, the high levels of $p16^{INK4A}$ protein have been observed to be primarily cytoplasmic, perhaps indicating functional inactivation through cytoplasmic sequestration, or oncogenic cytoplasmic functions of $p16^{INK4A}$.[87,91]

Thus, aberrant expression of several cell cycle regulatory genes is a common feature of breast cancer and often cosegregates with features of the pathophysiology of the disease e.g., disease phenotype and patient outcome. However, further work is required to determine if any of these will become biomarkers with clinical utility in the routine management of breast cancer.

Relationship of Cell Cycle Deregulation to Patient Outcome and Response to Endocrine Therapy

While there have been many studies in which archival tissue from breast cancer cohorts has been analyzed for expression of various cyclins, their relationship to response to endocrine therapy is not well defined. Thus, it is in cell culture systems that the evidence for the involvement of c-Myc and cyclins in the response to endocrine therapy, predominantly antiestrogens, is most compellingly demonstrated (Fig. 4).

The role of c-Myc in the proliferative response to estrogens is discussed above and importantly, provides evidence that c-Myc may play a role in the development of antiestrogen resistance. Inhibition of ER by estrogen withdrawal, aromatase inhibition, or treatment with tamoxifen or faslodex (ICI 182780), all downregulate *MYC* mRNA, which in turn induces cell cycle arrest.[25] Conversely, the acquisition of estrogen independence in MCF-7 cells maintained in estrogen-deprived medium is associated with the upregulation of selected estrogen-regulated genes including ER and *MYC*.[92] Furthermore, overexpression of c-Myc alone is capable of partially reversing the growth suppressive effects of antiestrogens in MCF-7 cells.[30,93]

The amplification of growth factor receptor signaling cascades can also converge on activation of c-Myc, thus potentially influencing endocrine responsiveness. High levels of ErbB2/ErbB3 signaling are frequently observed in breast cancer and lead to persistent Ras and Akt activity via amplification of the MAPK and PI-3 kinase signaling pathways. Ras phosphorylates c-Myc at serine-62 leading to protein stabilization and activation of the PI-3 kinase pathway stimulates translation of *MYC* mRNA and protein stabilization.[94,95] Furthermore, c-Myc protein levels are reduced by an ErbB2 inhibitor (PD153035) and this effect is reversed by ectopic expression of *MYC*.[96] This is consistent with the clinically observed antiestrogen resistance seen in breast cancers overexpressing ErbB2. A synergistic interaction between deregulated c-Myc and EGFR signaling has also been seen in mammary carcinomas in transgenic mice.[97] It is notable that co-amplification

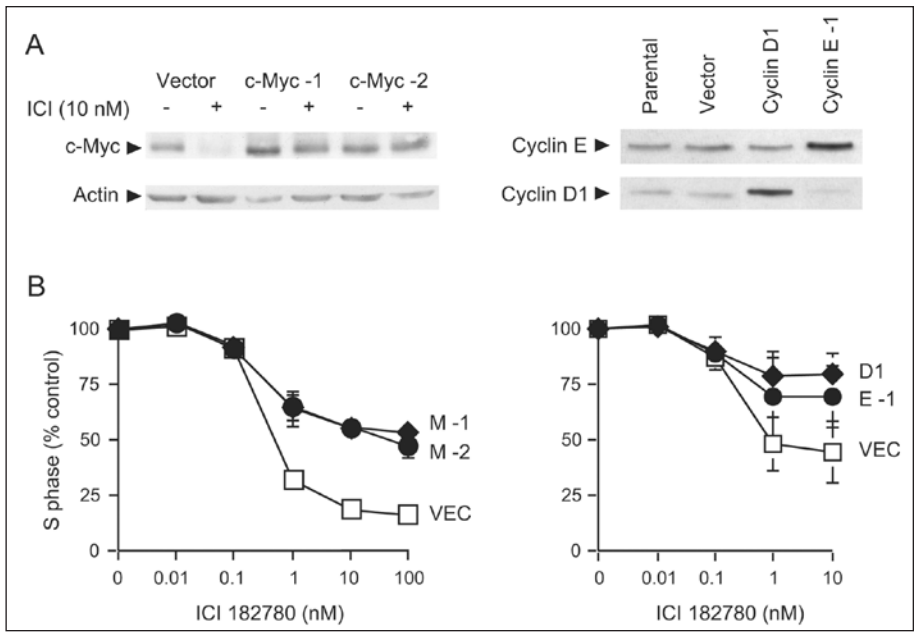

Figure 4. Overexpression of c-Myc, cyclin D1 or cyclin E1 modulates the response to antiestrogens. A), Western analysis of c-Myc, cyclin D1 and cyclin E1 expression in breast cancer cells stably transfected with empty vector, or human cDNAs for c-Myc, cyclin D1 or cyclin E1. B), Acute effects of c-Myc, cyclin D1 and cyclin E overexpression on the response to the pure antiestrogen, ICI 182780. After treatment of proliferating cells with ICI 182780 at the concentrations shown, cells were harvested and stained with ethidium bromide. The S phase fraction was determined by flow cytometry and represented relative to vehicle treated controls. Data points indicate mean of duplicate experiments ± S.D.

of *NEU/ERBB2* and *MYC* is associated with poorer survival in several clinical cohorts[98] although data are conflicting in this regard.[61]

At a clinical level, the impact of *MYC* amplification and expression on response to endocrine therapy is less clear than might be expected from in vitro studies and there are few data evaluating the relationship between *MYC* amplification and response to endocrine therapy. In a cohort of 181 patients with node-negative disease *MYC* amplification predicted recurrence but no differences were detected in the response to tamoxifen treatment among patients with and without gene amplification.[61] In another study, those patients with *MYC* amplification tended to have a slightly longer progression-free survival on endocrine therapy.[99] However, it is difficult to draw conclusions regarding the role of c-Myc from these small cohorts particularly because of the discrepancy between *MYC* amplification (~19%) and c-Myc protein overexpression (~38%). Further elucidation of the relationship between c-Myc overexpression and response to endocrine therapy must await more reliable immunohistochemical assessment in large cohorts of patients treated in the context of randomized treatment trials.

Similarly, in clinical cohorts the role of the c-Myc target gene and cell cycle regulator $p21^{WAF1/Cip1}$ in predicting overall outcome and response to antiestrogen therapy remains the subject of debate. Some investigators show that $p21^{WAF1/Cip1}$ expression predicts responsiveness to antiestrogens,[100] while others have shown no prognostic benefit in multivariate analyses.[101,102] In contrast, other investigators have shown a negative association between cytoplasmic $p21^{WAF1/Cip1}$ expression and outcome.[103,104] These conflicting data may reflect the fact that $p21^{WAF1/Cip1}$ function in breast cancer

can also be influenced by p53 status, titration by cyclin-CDK complexes and intracellular localization which were not accounted for in these studies.

Like c-Myc, evidence demonstrating the role of cyclins in mediating the proliferative effects of estrogen, suggest they may also be involved in the development of endocrine resistance. Sustained expression of cyclin D1 is seen in breast cancer cells during their acquisition of tamoxifen-resistance.[105] In these cells ER expression and function remained intact and the pure antiestrogen, ICI 164,384 retained its anti-proliferative effects via suppression of cyclin D1. This is consistent with the clinical observation that patients with tamoxifen-resistant disease are able to derive benefit from second line therapy with ER downregulators.[106] Interestingly, overexpression of cyclin D1 confers complete resistance to the growth inhibitory effects of progestins.[107] Cyclin D1 can also potentiate the transcriptional activity of the ER independently of estradiol, with some evidence that this effect is not inhibited by antiestrogens.[108,109] This suggests a further mechanism by which the overexpression of cyclin D1 in breast cancers could lead to sustained ER signaling and endocrine resistance.

The situation is less clear when in vitro hypotheses derived from in vitro experiments are tested in a clinical setting. A large number of studies have examined the prognostic influence of changes in cyclin D1 expression and several show that a poor outcome is associated with amplification at the 11q13 locus.[110] Subsequent studies demonstrated a shortening of relapse-free survival in association with *CCND1* amplification.[111] However, many other studies have reported conflicting relationships between cyclin D1 overexpression and clinical outcome. Variation in methodologies, adjuvant treatment, ER assessment, size of the study cohort and the heterogeneity inherent in human populations may account for some of this variability. Certainly it is difficult to draw definitive conclusions about the relationship between cyclin D1 expression and prognosis from these studies.

When the more specific question of the potential role of cyclin D1 in endocrine responsiveness in the clinical setting is addressed, the data are again conflicting. There are reports of increased expression of cyclin D1 mRNA associated with a reduced response to tamoxifen treatment.[112,113] However, others have shown a trend towards superior response to tamoxifen in metastatic ER-positive tumors that overexpress cyclin D1.[114] Thus, the true impact of cyclin D1 on the response and resistance to antiestrogens in a clinical setting remains the subject of debate and is urgently in need of further study in large cohorts of known therapeutic responsiveness.

It is clear from the earlier discussion that cyclin E-CDK2 complexes are also crucial in mediating estrogen-induced progression through the G_1-S phase of the cell cycle and, as is the case for c-Myc and cyclin D1, there exist in vitro data supporting a role for cyclin E1 in the development of antiestrogen resistance. Studies in MCF-7 cells demonstrate that a three-fold overexpression of cyclin E1 can abrogate tamoxifen-mediated growth arrest.[115] Cyclin E1 overexpression also confers partial resistance to the acute, inhibitory effects of ICI 182,780, although to a lesser extent than that observed with cyclin D1 (Fig. 4).[116] Nonetheless, in clonogenic survival assays overexpression of both cyclin D1 and cyclin E1 confer significant resistance to the growth inhibitory effects of ICI 182,780.[116]

Cyclin E1 is overexpressed in ~30% of breast cancers (Table 1) and studies of protein expression in breast cancer tissue show that cyclin E1 levels correlate strongly with disease-specific and overall survival. In addition, the production of low-molecular weight isoforms of cyclin E1 confers resistance to the effects of the CDK inhibitors $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ and to the effects of antiestrogens in MCF-7 cells.[117] It has also been noted that in experimental systems, overexpression of the low molecular weight isoforms of cyclin E1 is associated with a defect in progression through S phase with concomitant accumulation of chromosomal instability.[117] Importantly, the study on the role of full-length and low molecular weight isoforms of cyclin E1 demonstrated that cyclin E1 outperformed other independent clinical and pathological risk factors of recurrence and death and is consistent with the data from several other clinical studies showing adverse outcome in association with cyclin E1 overexpression.[72] However, on multivariate analysis a number of other clinical studies have failed to show any association between cyclin E1 expression and outcome.[70,118] There is

some evidence that cyclin E1 expression is associated with poor relapse-free survival specifically in patients treated with endocrine therapy.[119] Other studies have shown that antiestrogen treatment has no influence on disease-specific survival among ER-positive cyclin E1 overexpressors, suggestive that cyclin E1 confers resistance to antiestrogens.[72] Again, more definitive conclusions on the role of cyclin E1 in endocrine resistance must await data from large, randomized treatment trials.

Conclusion

Female sex steroid hormones are essential for normal mammary gland development and physiological function through their regulation of cell proliferation, cell differentiation and cell death. These effects, which are retained in neoplastic breast tissue, are mediated, in part, by regulation of cell cycle regulatory molecules including cyclins, CDKs and CDK inhibition. There is compelling evidence that aberrant expression and regulation of these molecules accompanies the oncogenic process in breast tissue and may have a causative role in breast cancer development and progression. This, in turn, raises the possibility that cell cycle regulatory molecules may provide useful markers of disease progression and response to therapy and be targets for future therapeutic intervention. While there is strong preliminary data to support these concepts, more research is required to determine whether such goals are a likely clinical reality.

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