Cyclin-dependent kinases as downstream targets of oestrogen action: potential prognostic indicators and therapeutic targets

R L Sutherland, O W J Prall, K M Alle, N R C Wilcken, R Hui, J R Ball, B Sarcevic, S M Henshall, E A Musgrove and C K W Watts

Cancer Research Program, Garvan Institute of Medical Research, St Vincent’s Hospital, Sydney, New South Wales 2010, Australia

(Requests for offprints should be addressed to R L Sutherland)

Introduction

The clinical studies of Beatson reporting responses to oophorectomy in women with advanced breast cancer (Beatson 1896) provided the first experimental evidence that an ovarian factor was critical for continued tumour growth in a proportion of breast cancers. It took another 58 years for this factor to be identified as oestrogen (Pearson et al. 1954). Subsequent research employing experimental models of carcinogen-induced mammary carcinoma revealed that oestrogen was essential for both the initiation and progression of breast cancer (Henderson et al. 1988). Studies with human breast cancer cell lines xenografted to athymic nude mice have demonstrated an absolute requirement for oestrogen in tumour formation and growth (Soule & McGrath 1980). Such observations provided the rationale for the introduction of anti-oestrogen therapy, the current treatment of choice for hormone-responsive breast cancer (Jordan & Murphy 1991). The subsequent demonstration of the efficacy of the anti-oestrogen tamoxifen in decreasing both disease progression and the development of contralateral breast cancer (Early Breast Cancer Trialists’ Collaborative Group 1992) provided further compelling evidence for the pivotal role of oestrogen-regulated cell proliferation in breast cancer. Despite this, little is known of the molecular basis of cell proliferation control by oestrogen.

Early studies on cell proliferation in the rodent uterus and mammary gland in vivo demonstrated that oestrogen increases the proportion of cells synthesizing DNA by recruiting non-cycling cells into the cell cycle and reducing the duration of G1 phase in already cycling cells (Sutherland et al. 1983a). Using breast cancer cells synchronized at the G1/S boundary or at G2/M to test the effect of oestrogen added at different stages of the cell cycle, Leung & Potter (1987) concluded that the sensitive cells were in early G1 phase, immediately following mitosis. These data supported observations that both non-steroidal (Sutherland et al. 1983b) and steroidal anti-oestrogens (Wakeling et al. 1991, Watts et al. 1995) arrest oestrogen receptor (ER)-positive breast cancer cells in G1 phase. More precise mapping of the point of anti-oestrogen action within G1 phase using cells synchronized by mitotic selection identified a window of sensitivity in early to mid G1 phase (Taylor et al. 1983). Together these data are
compatible with a model whereby oestrogens and antioestrogens, through their interactions with the ER, control the rate of transcription of genes that regulate key control points in G₁ progression.

Candidate genes that might fulfill this role include ‘immediate early’ and ‘delayed early’ genes with established roles in signal transduction and cell cycle control. Of particular interest are the proto-oncogene c-myc, genes encoding components of the AP-1 transcription complex, c-fos and c-jun, and the more recently described cyclin/cyclin-dependent kinase (CDK) complexes involved in G₁ progression. This report focuses on the latter pathway.

Progress through G₁ phase requires inactivation of the pRB protein, the product of the retinoblastoma susceptibility gene RB-1, by phosphorylation and the consequent release of a number of pRB-bound molecules including the E2F family of transcription factors. These factors then activate transcription of genes whose products are required for S phase progression (Riley et al. 1994, Weinberg 1995). Phosphorylation of pRB is mediated by the action of the G₁ phase CDKs (Cdk4, Cdk6 and Cdk2), which are activated by cyclin binding: Cdk4 and Cdk6 by D-type cyclins, and Cdk2 by cyclin E. Control of G₁ CDK activity is achieved by several mechanisms including: transcriptional activation of D-type cyclins and cyclin E, the rate-limiting regulatory subunits of the G₁ cyclin/CDK complexes; activation and inactivation of the enzyme complexes by phosphorylation/dephosphorylation events; and the abundance and action of two families of CDK inhibitors (Morgan 1994, Sherr & Roberts 1995). Modulation at any of these levels could regulate pRB phosphorylation and hence G₁ phase progression.

D-type cyclins are induced as delayed early response genes by a variety of mitogens in many cell types and removal of growth factors in G₁ phase leads to their rapid down-regulation (Matsushima et al. 1991), consistent with the notion that these cyclins act as mitogenic sensors linking extracellular signals with cell cycle progression (Sherr 1993). An essential role for cyclin D1 in mammary gland development is demonstrated by the absence of lobulo-alveolar structures in transgenic mice with disruption of the cyclin D1 gene (Fanti et al. 1995, Sicinski et al. 1995). There is also accumulating evidence that D-type cyclins play a role in mediating the effects of growth factors and steroid hormones on breast cancer cell cycle progression by binding and activating Cdk4 (Musgrove et al. 1993, 1996a, Sweeney et al. 1996). The abundance of cyclin D1 increases following growth factor and progestin stimulation of breast cancer cell proliferation (Musgrove et al. 1993) and declines rapidly following exposure to growth-inhibitory antioestrogens (Musgrove et al. 1993, Watts et al. 1995). Similarly cyclin E expression also increases following growth factor stimulation of breast cancer cells (Musgrove et al. 1993), but at times later than for cyclin D1 and compatible with its established role in the control of the G₁ to S phase transition. Finally, ectopic expression of cyclin D1 in T-47D breast cancer cells is sufficient for Cdk4 and Cdk2 activation, pRB phosphorylation and G₁-S phase progression (Musgrove et al. 1996b). Thus G₁ cyclins and their associated kinases are potential downstream targets of oestrogen-induced mitogenesis.

This paper summarizes recent studies from this laboratory in which we developed an in vitro model of oestrogen-induced synchronous progression of human breast cancer cells through the cell cycle. This model was employed to investigate the effects of oestrogen on cyclin/CDK expression and function. These data demonstrated that induction of cyclin D1 gene expression with consequent activation of Cdk4 and Cdk2 activity were major consequences of oestrogen action in breast cancer cells. Further studies were initiated to ascertain the frequency of dysregulated expression of cyclin D1 in clinical breast cancer and its likely functional consequences.

A model of oestrogen-induced cell cycle progression

To establish a sensitive experimental system for the study of specific oestrogen-regulated events associated with cell cycle progression we exploited the unique properties of a new class of steroidal anti-oestrogens which, unlike their non-steroidal counterparts, are pure antagonists devoid of oestrogen agonist activity (Thompson et al. 1989, Wakeling et al. 1991). Thus exposure of oestrogen-dependent cells to these compounds will block oestrogen-induced gene expression with consequent inhibition of cell proliferation. This was attained by treating exponentially growing MCF-7 cells with the pure oestrogen antagonist ICI 182780 and then ‘rescuing’ this effect by the addition of oestradiol.
A representative time-course for changes in cell cycle phase distribution following this treatment strategy is shown in Fig. 1. In exponentially growing cultures the percentages of cells in G1, S and G2+M phases were 50, 40 and 10 respectively. With anti-oestrogen pretreatment for 48 h the S phase fraction declined markedly and cells accumulated in G1 phase such that the cell cycle phase distribution changed to 85, 10 and 5% respectively. Following oestradiol treatment there was little change in cell cycle phase distribution over the first 10 h but thereafter the proportion of cells in S phase increased about 6-fold from 10% to 60% (Fig. 1), indicating a synchronized population of cells progressing from G1 to S phase. The subsequent decline in the proportion of S phase cells after 24 h was accompanied by an increase in the proportion of cells in G2+M phases from 5% prior to 21 h to a maximum of 25% at 27 h. Oestrogen treatment, therefore, was sufficient for cells to progress from G1 to S phase and then to G2+M phases as indicated by the 5- to 6-fold increase of cells in S phase and then cells in G2+M phases. The subsequent decline of the proportion of cells in S and G2+M phases was accompanied by semi-synchronous re-entry into G1 phase after 24 h, indicating that cell division had occurred (Prall et al. 1997). Thus oestradiol treatment induced synchronous progression through at least one cell cycle.

**Effects of oestrogen on cyclin/CDK expression and function**

Since current models of cell cycle progression propose that mitogenic activation is followed sequentially by transcriptional activation of G1 phase cyclins, formation and activation of cyclin/CDK complexes, and consequent phosphorylation and inactivation of pRB (Morgan 1994, Sherr 1995, Weinberg 1995), we measured the effect of oestradiol on each of these parameters using the experimental paradigm described in Fig. 1.

The earliest and most pronounced changes were seen in the expression of G1 cyclins. Cyclin D1 protein levels increased 2.6-fold at 4 h (Fig. 2), reached maximum levels at 8 h (5- to 7-fold), prior to any change in % S phase, and then decreased towards control at 24 h. In contrast, cyclin D3 levels increased as the proportion of cells in S phase

![Figure 1](image1) Changes in cell cycle phase distribution following rescue of anti-oestrogen-pretreated cells with oestradiol. MCF-7 cells were growth-arrested for 48 h with 10 nM ICI 182780 and treated at time 0 with 100 nM oestradiol (O) or vehicle (●). Redrawn from Prall et al. (1997) with permission.

![Figure 2](image2) Effects of oestradiol on cyclin expression. Cells were treated as outlined in Fig. 1, harvested at the times shown for preparation of lysates and Western blotted with specific antibodies. Redrawn from Prall et al. (1997) with permission.
increased, i.e. after 12 h (Fig. 2) to reach a maximum 3.3-fold above control at 24 h. Cyclin D2 is expressed at very low levels in these cells (Buckley et al. 1993) with no detectable change in expression following oestradiol treatment. Surprisingly, antioestrogen-induced growth inhibition was associated with an approximate 4-fold increase in cyclin E levels above those observed in exponentially growing cells. Following oestradiol treatment there was no significant change in the expression of cyclin E from 2 to 16 h relative to controls (Fig. 2), but levels declined slightly at 24 h as cells progressed through S phase. The S phase cyclin, cyclin A, was essentially undetectable until 16 h after oestradiol rescue, when the proportion of cells in S phase significantly increased and was further increased when the proportion of cells in S phase was maximal (Fig. 2). Similarly, increased expression of cyclin B1 protein was detected at 24 h when the proportion of cells in G2+M phases had increased 5-fold, consistent with the established role for cyclin B1 in G2+M phases (Fig. 2).

To test if these early changes in cyclin D1 gene expression were reflected in increased amounts of cyclin D1/CDK complexes, whole cell lysates were immunoprecipitated with anti-cyclin D1 antiserum and Western blotted for various components of the complexes. Antioestrogen pretreatment decreased the levels of both cyclin D1 and Cdk4 in cyclin D1 immunoprecipitates consistent with the decrease in total cyclin D1 protein. Following oestradiol rescue the levels of immunoprecipitated cyclin D1 increased approximately 3-fold at 4 h and remained elevated until 16 h, paralleling the temporal changes in total cyclin D1 protein, although the relative changes were of lower magnitude. The relative levels of Cdk4 in the complexes at early time-points paralleled the levels of immunoprecipitated cyclin D1 protein and were maximal at 8 h (3.4-fold). Subsequently, cyclin D1-associated Cdk4 declined, reaching control levels at 24 h. Since these data demonstrate a major increase in cyclin D1/Cdk4 complex formation as early as 4 h after oestradiol treatment the effects of oestradiol on Cdk4 activity were then investigated. Cdk4 activity (GST-pRB773-923 substrate) was elevated 4.6-fold at 3 h after oestradiol treatment (Fig. 3), maximally elevated at 6 h (6.6-fold) and thereafter declined. The initial changes in Cdk4 activity were temporally similar to those in expression for cyclin D1 protein (Fig. 2) and consequent cyclin D1/Cdk4 association, suggesting that an important activating mechanism for Cdk4 was increased cyclin D1 expression.

After antioestrogen pretreatment cyclin E-associated kinase activity (histone H1 substrate) was approximately 60% of that in exponentially growing cells. Oestradiol treatment restored this activity by 1.5-fold at 4 h and 3-fold at 6 h (Fig. 3). Cyclin E/Cdk2 activity continued to increase, reaching a maximum at 16 h, approximately 7-fold relative to control levels and equivalent to about 4-fold the activity in exponentially growing cells.

The substantial and early changes in both Cdk4 activity and cyclin E/Cdk2 activity between 4 and 6 h indicated that both kinases were likely to contribute to increased cell cycle progression following oestradiol treatment, presumably by phosphorylation of pRB. After 48 h of ICI 182780 pretreatment, almost all pRB was hypophosphorylated (time 0, Fig. 4). Following oestradiol treatment an increase in more slowly migrating, phosphorylated forms of pRB was first apparent at 6 h. The proportion of phosphorylated pRB increased at subsequent time-points with a corresponding decrease in hypophosphorylated pRB, such that after 12 h, when cells commenced their synchronous entry into S phase (Fig. 1), little or no hypophosphorylated pRB remained. These later
changes in pRB phosphorylation were accompanied by an increase in the total amount of pRB protein; at 24 h total pRB levels were increased approximately 4-fold relative to cells treated with ICI 182780 alone.

In summary, these data are compatible with a model in which oestrogen stimulation of cell cycle progression is preceded by an oestrogen-induced increase in cyclin D1 gene expression which, in turn, leads to the accumulation of active cyclin D1/Cdk4 enzyme complexes that phosphorylate and thus deactivate the cell cycle inhibitory effects of pRB. Interestingly, activation of cyclin E/Cdk2 complexes also occurred as a relatively early event following oestrogen stimulation by a novel mechanism which was not associated with changes in the level of cyclin E, Cdk2 or the CDK inhibitors p21 or p27 (Prall et al. 1997).

Cyclin D1 overexpression in breast cancer

The data presented here for oestrogens/antioestrogens support our previously published studies that implicate cyclin D1 as a key mediator of steroid- and growth factor-mediated increases in breast cancer cell cycle progression (Musgrove et al. 1993). Furthermore, in studies where the functional consequences of inducible ectopic cyclin D1 expression were investigated we demonstrated that cyclin D1 was able to stimulate growth-arrested cells to progress through the cell cycle in the absence of exogenous growth factors (Musgrove et al. 1994). Together these data imply that overexpression of cyclin D1 could alleviate the requirement for external growth stimuli in maintaining cell proliferation, at least in some breast epithelial cells, and raise the possibility that overexpression of cyclin D1 can confer a growth advantage to such cells by allowing autonomous growth. If this were the case, cyclin D1 overexpression might contribute to the development of breast cancer.

This issue has been addressed previously in transgenic mouse models where dysregulated expression of cyclin D1 in the mammary gland resulted in premature lobulo-alveolar development, hyperplasia and, after a long latency period, a low incidence of adenocarcinoma (Wang et al. 1994). Thus cyclin D1 is a weak oncogene in the mammary gland as it is in other experimental systems where it requires co-operation with other oncogenes to induce transformation (Hinds et al. 1994, Lovec et al. 1994a) and lymphomagenesis (Bodrug et al. 1994, Lovec et al. 1994b). To develop further insight into the possible role of cyclin D1 in the evolution of human breast cancer we examined the relative expression of cyclin D1 in samples of normal, pre-neoplastic and neoplastic breast tissue using immunohistochemistry (K M Alle, S M Henshall, A S Field & R L Sutherland, unpublished observations).

Invasive duct cancers stained positively, as defined by >5% positive nuclei, in 80 of 177 cases (45%). A positive staining pattern was also seen in one of 30 normal samples from reduction mammoplasties (3%), five of 28 epithelial hyperplasias without atypia (18%), six of 19 atypical ductal hyperplasias (32%), six of 23 low grade (26%) and ten of 29 high grade ductal carcinomas in situ (34%) (Fig. 5). The percentage of positive specimens with expression in greater than 25% of nuclei increased.
from zero in the normal samples to approximately 50% of invasive carcinomas (K M Alle, S M Henshall, A S Field & R L Sutherland, unpublished observations).

Thus, increments in cyclin D1 protein levels were observed with progression from normal epithelium through hyperplasias to intraduct and invasive carcinomas. In agreement with these data, Bartkova et al. (1994) have previously shown that cyclin D1 protein levels in intraduct carcinomas correlated with levels in the associated invasive tumours in 98% of 96 cases. Similarly, Weinstat-Saslow et al. (1996) found no difference in the levels of cyclin D1 mRNA expression in intraduct and invasive carcinomas. However, the latter study did not find an increase in cyclin D1 mRNA in hyperplasias, but noted a significant increase in expression at the transition from atypical ductal hyperplasias to intraduct lesions. This finding led the authors to conclude that cyclin D1 expression may define a major oncogenic event, distinguishing benign or premalignant breast lesions from any form of breast carcinoma. In contrast, our data demonstrate cyclin D1 protein overexpression in a substantial proportion of epithelial hyperplasias, with and without atypia - a finding more in keeping with the known effects of cyclin D1 on cell cycle progression (Musgrove et al. 1994), the phenotype of transgenic mice (Wang et al. 1994) and data from experimental models of mammary carcinogenesis (Said et al. 1996).

These discrepant findings may reflect the relatively small sizes and different origins of the sample sets or may be related to differing contributions of transcriptional and translational mechanisms to the regulation of cyclin D1 gene expression in different epithelial phenotypes (Rosenwald et al. 1993). Mechanisms involving increased translational efficiency and/or protein stability of cyclin D1 in preneoplastic disease with increased mRNA levels arising as a result of genetic instability in carcinoma are compatible with both sets of data. Thus our study supports the hypothesis that if epithelial alterations accurately reflect progressive stages in breast oncogenesis, then cyclin D1 is important in breast cancer development at its earliest, premalignant stage, implying a potential causative role. Further research is therefore required to define more precisely the association of aberrant cyclin D1 expression with the

Table 1 Cyclin D1 expression in breast cancer.

<table>
<thead>
<tr>
<th>Publication</th>
<th>Method</th>
<th>Number of high expressors (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lammie et al. (1991)</td>
<td>Northern analysis</td>
<td>5/10 (50%)</td>
</tr>
<tr>
<td>Buckley et al. (1993)</td>
<td>Northern analysis</td>
<td>56/124 (45%)</td>
</tr>
<tr>
<td>Bartkova et al. (1994)</td>
<td>IHC⁹</td>
<td>96/167 (58%)</td>
</tr>
<tr>
<td>Gillett et al. (1994)</td>
<td>IHC</td>
<td>27/63 (43%)</td>
</tr>
<tr>
<td>Bartkova (1994)</td>
<td>IHC</td>
<td>13/35 (37%)</td>
</tr>
<tr>
<td>Zhang et al. (1994)</td>
<td>IHC</td>
<td>35/43 (81%)</td>
</tr>
<tr>
<td>Zuberberg et al. (1995)</td>
<td>IHC</td>
<td>17/48 (35%)</td>
</tr>
<tr>
<td>Bartkova et al. (1995)</td>
<td>IHC</td>
<td>46/83 (56%)</td>
</tr>
<tr>
<td>McIntosh et al. (1995)</td>
<td>IHC</td>
<td>26/93 (28%)</td>
</tr>
<tr>
<td>Gillett et al. (1996)</td>
<td>IHC</td>
<td>183/345 (53%)</td>
</tr>
<tr>
<td>Michalides et al. (1996)</td>
<td>IHC</td>
<td>85/248 (34%)</td>
</tr>
<tr>
<td>Weinstat-Saslow et al. (1996)</td>
<td>ISH⁶</td>
<td>10/12 (83%)</td>
</tr>
<tr>
<td>Alle et al. (unpublished)</td>
<td>IHC</td>
<td>80/177 (45.2%)</td>
</tr>
</tbody>
</table>

⁹IHC=immunohistochemistry; ¹ten chosen on basis of known 11q13 amplification; ²ISH=in situ hybridization (using riboprobe); ³infiltrating duct carcinomas; ⁴ductal carcinoma in situ (DCIS) - comedo forms; ⁵DCIS - non-comedo forms; ⁶invasive carcinomas; ⁷DCIS.
initiation and progression of breast cancer and its mechanistic significance since little information is currently available.

Much of our knowledge of the effects of cyclin D1 overexpression in primary breast cancer has been inferred from data on the frequency of chromosome 11q13 amplification, the genomic locus of the CCND1 gene that encodes cyclin D1. There are a large number of published studies relating 11q13 amplification to various clinicopathological parameters in breast cancer and these are discussed in more detail below. Such data have been recently reviewed (Fantl et al. 1993, Dickson et al. 1995, Peters et al. 1995) and indicate that the 11q13 locus is amplified in about 15% of primary breast cancers. It was therefore somewhat surprising when we first observed overexpression at the mRNA level in about 45% of primary breast cancers (Buckley et al. 1993), leading to the view that cyclin D1 overexpression was more frequent than had been appreciated from studies of gene amplification. The subsequent availability of antibodies suitable for immunohistochemical detection of cyclin D1 in archival, paraffin-embedded material has substantiated this view. Indeed, as is evident from the summary of published data on cyclin D1 expression in breast cancer reported in Table 1, the frequency of overexpression in primary breast cancer is of the order of 50%, making cyclin D1 one of the most commonly overexpressed oncogenes yet defined in breast cancer. The functional consequences of this overexpression in terms of phenotype, disease progression and responsiveness to therapy are major unanswered questions.

**Cyclin D1 amplification and overexpression as markers of prognosis**

In the absence of a large number of published studies on the relationship between cyclin D1 overexpression and breast cancer phenotype, some insight can be gained from data where 11q13 amplification was measured as a surrogate for overexpression of genes at this locus, including cyclin D1.

There are at least 15 published studies addressing potential associations between 11q13 amplification, clinicopathological features of breast cancer and patient outcome (Fantl et al. 1993, Peters et al. 1995, Seshadri et al. 1996, Sweeney et al. 1996). Since many of these studies have involved small numbers of patients it is perhaps not surprising that several of the proposed relationships are not consistent between studies. For this reason, we investigated the clinical significance of CCND1 amplification and its association with established clinicopathologic features of prognosis in 1014 primary breast cancer patients. Amplification of both the CCND1 gene and the INT-2/FGF-3 gene, which maps within 150 kb of CCND1 at 11q13, were studied. There were no associations between CCND1 or INT-2 amplification and patient age, tumour size, tumour grade, axillary lymph node status, HER/neu (c-erbB2) amplification, or p53 expression. CCND1 amplification was predominantly observed in hormone receptor-positive tumours; at a copy number >3, CCND1 amplification was significantly correlated with both ER and progesterone receptor (PgR) positivity. After a median follow-up period of 66 months, CCND1 or INT-2 amplification was not associated with significant increases in relapse or death from breast cancer. However, in the node-negative and ER-positive subgroups, there was a trend for an increased relapse rate in patients with INT-2 or CCND1 amplification (Fig. 6). Other studies (Borg et al. 1991, Berns et al. 1995) have also noted a tendency for early relapse in these two subgroups of breast cancer patients with tumours carrying 11q13 amplification. Thus assessment of CCND1 or INT-2 amplification at 11q13 has a potential role in identifying a subset of poor prognosis patients within the node-negative and ER-positive good prognosis groups. Since, as noted above, the prevalence of CCND1 amplification is much lower than the reported prevalence of cyclin D1 overexpression, further studies are now required to determine the effect of altered cyclin D1 expression on phenotype and prognosis in breast cancer.

Several such studies have recently appeared in the literature (McIntosh et al. 1995, Gillett et al. 1996, Michalides et al. 1996) allowing some tentative conclusions to be drawn. A relationship between cyclin D1 overexpression and ER positivity was observed as predicted from the amplification data and studies on mRNA levels (Buckley et al. 1993, Hui et al. 1996). Two studies examined an association between cyclin D1 overexpression and patient survival; one found no prognostic significance in a series of 248 patients (Michalides et al. 1996) while the other found that cyclin D1 overexpression was associated with a survival benefit in a population of 406 patients with long-term follow-up (Gillett et al.)
levels of cyclin D1 mRNA, these levels were significantly higher in the ER-positive group and ER positivity increased from 58% in the lowest quartile of cyclin D1 expression to 87% in the highest quartile. Analysis by simple linear regression revealed a significant positive correlation between cyclin D1 and ER mRNA levels in the total population ($P=0.0001$), raising the possibility of a functional relationship between the expression of these genes. Given the data described above showing oestrogen induction of cyclin D1 gene expression in breast cancer cells, a likely explanation for this relationship in primary breast cancer is that cyclin D1 is an oestrogen-induced protein, although it is clear from the significant expression in ER-negative tumours that oestrogen is not required for constitutive expression.

Thus tumours with functional ER may synthesize more cyclin D1 and, if this were the case, expression of cyclin D1 in ER-positive tumours might be predicted to more accurately identify the hormone-responsive phenotype, as is the situation in ER-positive/PgR-positive tumours. Conversely, overexpression of cyclin D1 in ER-positive tumours may confer insensitivity to some therapies, e.g. antioestrogen therapy, since decreased cyclin D1 expression is an early and potentially causative event in antioestrogen-mediated growth inhibition (Watts et al. 1995).

To test the latter possibility we constructed clonal T-47D and MCF-7 cell lines in which cyclin D1 could be expressed under the control of the metal-inducible, metallothionein promoter. Cells were then growth arrested with antioestrogens and the effects of ectopic expression of cyclin D1 assessed. Following induction of cyclin D1 gene expression, first evident at 3 h, antioestrogen-arrested cells began to progress into S phase by 9 h (Fig. 7). A greater than 3-fold increase in S phase was apparent at 15 h as a significant proportion of the arrested population progressed semi-synchronously through the cell cycle to reach mitosis at about 21 h (Fig. 7). Treatment with increasing concentrations of zinc resulted in a concentration-dependent increase in cyclin D1 protein and S phase fraction as described previously (Musgrove et al. 1994). Thus ectopic overexpression of cyclin D1 reverses the growth-inhibitory effect of antioestrogens in ER-positive breast cancer cells, providing a potential mechanism for clinical antioestrogen resistance.

Figure 6 Kaplan-Meier survival analysis of relapse-free survival of (A) lymph node-negative and (B) ER-positive subgroups of primary breast cancer patients with or without amplification of the cyclin D1 gene. Normal (○) and amplified (□) refer to relative gene copy numbers of <2 and >2 respectively. Reproduced from Seshadi et al. (1996) with permission.
The only published study to address the relationship between cyclin D1 expression and response to tamoxifen was conducted on a group of 158 patients who received tamoxifen therapy for metastatic disease (Gillett et al. 1996). There was a survival advantage in ER-positive patients expressing cyclin D1 when compared with ER-positive, cyclin D1-negative tumours. Such data are more compatible with cyclin D1 being a marker of functional ER and thus a predictor of responsiveness to endocrine therapy rather than a marker of endocrine resistance. Studies are currently underway to further define the relationship between cyclin D1 expression and responsiveness to tamoxifen therapy in early breast cancer.

**CDKs as potential therapeutic targets**

Since cyclin D1 is frequently overexpressed in carcinoma of the breast and several other human cancers, this molecule and its downstream effectors, particularly the CDKs, provide potential therapeutic targets for the development of new classes of cancer therapeutics. In the context of breast cancer, our preliminary evidence that cyclin D1 overexpression is manifest early in the evolution of breast oncogenesis (KM Alle, SM Henshall, AS Field & RL Sutherland, unpublished observations), is an important downstream target of steroid- and growth factor-mediated mitogenesis (Musgrove et al. 1993, 1996a, Sweeney et al. 1996) and may confer resistance to some forms of endocrine treatment (Wilcken et al. 1997) suggest that agents targeting cyclin D1/CDK function may be useful in the prevention and treatment of breast cancer. Although there are a number of potential strategies to achieve such a goal, including targeted inhibition of cyclin gene expression, gene therapy to increase expression of the naturally occurring endogenous protein inhibitors, e.g. p16, inhibition of molecular interactions essential for enzyme complex formation and activation, perhaps the most significant progress has been made in the area of specific chemical inhibitors of CDK enzymatic activity (Abraham et al. 1995).

Several specific chemical CDK inhibitors have now been identified although none of these are specific for Cdk4, the major kinase partner of cyclin D1 in breast cancer cells. However, since Cdk2 is a downstream target of both oestrogen (Foster & Wimalasena 1996, Prall et al. 1997) and cyclin D1 (Musgrove et al. 1996b) we tested the principle of this approach in our experimental model. Thus to specifically address if Cdk2 inhibitors could inhibit oestrogen-induced cell cycle progression we treated MCF-7 cells, which had been growth arrested with antioestrogen, with oestradiol in the presence or absence of olomoucine, an antimitotic purine derivative that selectively inhibits Cdk2 and Cdc2 by competitive inhibition of ATP binding (Abraham et al. 1995). As can be seen by the data presented in Fig. 8, olomoucine at the concentration tested was...

---

**Figure 7** Changes in cell cycle progression associated with ectopic overexpression of cyclin D1 in antioestrogen-arrested cells. T47D ΔMT cyclin D1-3 cells (Musgrove et al. 1994) were growth arrested as described in Fig. 1 and treated at time 0 with 50 μM zinc sulphate to induce cyclin D1 expression. (A) The percentages of cells in S and G2+M phases of the cell cycle were determined by DNA flow cytometry. (B) Cell lysates were harvested concurrently and Western blotted for cyclin D1. Redrawn from Wilcken et al. (1997) with permission.
capable of inhibiting oestrogen-induced cell cycle progression. The more specific and potent inhibitor roscovitine had the same effects but at lower concentrations. Together these data provide evidence that oestrogen-induced cell cycle progression requires a functional Cdk2 enzyme complex and that inhibition of this enzyme can abrogate the effects of cyclin D1 overexpression. While preliminary, these data suggest that inhibition of G₁ cyclin function may be a fruitful target for new anticancer drug development.

**Conclusions**

Recent data from this laboratory (Prall et al. 1997) and others (Altucci et al. 1996, Foster & Wimalasena 1996) have identified increased cyclin D1 gene expression as an early event in oestrogen-induced mitogenesis. Similar changes in cyclin D1 expression accompany mitogenic stimulation by growth factors and progestins (Musgrove et al. 1993), indicating that cyclin D1 plays a pivotal role in mitogenic signalling in breast epithelial cells, a conclusion supported by studies employing inducible cyclin D1 expression (Musgrove et al. 1994, 1996b). However, transcriptional activation of c-myc (Dubik & Shiu 1988) and the activation of the cyclin E/cdk2 complex (Foster & Wimalasena 1996, Prall et al. 1997) have also been identified as early events accompanying oestrogen-induced cell cycle progression. These data raise important unanswered questions relating to the interactions between c-myc, cyclin D1/cdk4 and cyclin E/cdk2 signalling and the possibility that parallel and perhaps independent pathways of activation of pRB phosphorylation and G₁ progression exist, as might be inferred from the apparent lack of effect on oestrogen-induced ductal development in the mammary gland of cyclin D1 knockout mice (Fantl et al. 1995, Siciński et al. 1995).

The evidence that cyclin D1 is a major downstream target of mitogenic stimulation in breast epithelial cells, together with data demonstrating that it is rate-limiting, sufficient and necessary for G₁ progression in breast epithelial cells (Bartkova et al. 1994, Musgrove et al. 1994, 1996b), raise the possibility that dysregulated expression of this gene could contribute to the development and progression of human breast cancer. Preliminary data summarized herein provide strong evidence that cyclin D1 is overexpressed in up to 50% of primary breast cancers. Amplification of the CCND1 locus at 11q13 appears to account for only about 30% of this effect, although more accurate assessment of gene amplification using fluorescence in situ hybridization may identify a significantly higher frequency. Nonethe-
less, studies with breast cancer cell lines clearly define overexpression in the absence of gene amplification (Buckley et al. 1993) and the mechanisms underlying this aberrant regulation need to be more clearly defined.

Our data on cyclin D1 expression in premalignant lesions and preinvasive cancers provide evidence that overexpression of cyclin D1 is an early event in the evolution of breast cancer. Thus cyclin D1 may have a causative role in the development of sporadic breast cancer in humans, a property previously inferred from animal models of mammary carcinogenesis (Wang et al. 1994, Said et al. 1996). However, considerably more evidence will be required before definitive conclusions can be drawn on this subject.

Further preliminary data relating cyclin D1 expression to phenotype and disease outcome in primary breast cancer confirm a relationship between cyclin D1 expression and ER positivity (Hui et al. 1996), suggesting that oestrogen is a major regulator of cyclin D1 gene expression in vivo. However, data on the relationship between cyclin D1 expression and disease outcome vary widely between the small numbers of published studies and with the method of assessment of cyclin D1 expression, i.e. 11q13 amplification versus cyclin D1 immunohistochemistry. Consequently, more detailed studies on large cohorts of breast cancer patients with long-term follow-up are urgently required to determine whether assessment of cyclin D1 expression is likely to be of any clinical utility in the management of breast cancer.

Since the development of resistance to tamoxifen is a major limiting factor in the effective management of hormone-responsive breast cancer (Katzmannenbogen 1991) the observation that ectopic cyclin D1 expression can overcome oestrogen-induced growth inhibition is of significance in providing another potential mechanism of oestrogen resistance. Further experimental and clinical studies are required to determine the relevance of these findings to clinical tamoxifen resistance in breast cancer.

The identification of cyclin D1/Cdk4 and cyclin E/Cdk2 as major downstream targets of oestrogen action, together with the possibility that aberrant expression of the cyclins and constitutive activation of these enzyme complexes can confer autonomous growth to breast cancer cells, including loss of endocrine responsiveness, identifies cyclin/CDK complexes as potential targets for new anticancer therapeutic development. Data presented in Fig. 8 where a Cdk2 inhibitor olomoucine was able to abrogate oestrogen-induced cell cycle progression support such a view and data on Cdk4 inhibitors are eagerly awaited.

Finally, 100 years after Beatson’s original discovery of the dependence of some clinical breast cancers on ovarian factors, papers describing molecular links to the cell cycle machinery are appearing (Musgrove et al. 1993, Watts et al. 1995, Altucci et al. 1996, Foster & Wimalasena 1996, Prall et al. 1997). Such data identify new mechanisms involved in oestrogen regulation of cell proliferation and thus provide new insights into the development and potential management of breast cancer.

**Acknowledgements**

This work was supported by grants from the National Health and Medical Research Council of Australia and the New South Wales State Cancer Council. O W J P, K M A, N R C W and R H are recipients of Medical Postgraduate Scholarships from the National Health and Medical Research Council. Many of the clinical studies were made possible through active collaboration with a number of other laboratories, particularly those of Dr Ram Seshadri, Flinders Medical Centre, Bedford Park, South Australia, Dr Rob Nicholson, Breast Cancer Laboratory, Tenovus Cancer Research Centre, Cardiff, and Professor Roger Blamey and Dr John Robertson, Department of Surgery, City Hospital Nottingham; their continued collaboration and support is gratefully acknowledged.

**References**


Katzzenellenbogen BS 1991 Antioestrogen resistance: Mechanisms by which breast cancer cells undermine the effectiveness of endocrine therapy. *Journal of the National Cancer Institute* 83 1434-1435.


Matsushima H, Roussel MF, Ashmun RA & Sherr CJ 1991 Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. Cell 65 701-713.


Musgrove EA, Lee CSL, Buckley MF & Sutherland RL 1994 Cyclin D1 induction in breast cancer cells shortens G1 and is sufficient for cells arrested in G1 to complete the cell cycle. Proceedings of the National Academy of Sciences of the USA 91 8022-8026.


Prall OWJ, Sarcevic B, Musgrove EA, Watts CKW & Sutherland RL 1997 Estrogen-induced activation of Cdk4 and Cdk2 during G1-S phase progression is accompanied by increased cyclin D1 expression and decreased cyclin-dependent kinase inhibitor association with cyclin E-Cdk-2. Journal of Biological Chemistry 272 10882-10894.


Rosenwald IB, Lazaris-Karatzas A, Sonenberg N & Schmidt EV 1993 Elevated levels of cyclin D1 protein in response to increased expression of eukaryotic initiation factor 4E. Molecular and Cellular Biology 13 7358-7363.


