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EXPRESSION AND REGULATION OF CYCLIN GENES IN BREAST CANCER

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Cyclins, the regulatory subunits of cyclin-dependent kinases, control passage through key checkpoints within the cell cycle. Since dysregulated expression and function of cyclins can lead to loss of normal growth control some of these genes are oncogenes. We have studied cyclin gene expression, regulation and function in breast cancers. Induction of cyclin D1 is an early event in mitogenic stimulation of breast cancer cells by growth factors and steroids. Furthermore, inhibition of cyclin D1 expression is an early response to growth inhibition by antioestrogens. Ectopic expression of cyclin D1 in T-47D breast cancer cells demonstrated that cyclin D1 is rate-limiting for progression through G_1 phase and is sufficient for growth arrested cells to complete the cell cycle. Since this gene is frequently overexpressed in human breast cancers it may contribute to the development and progression of some breast carcinomas.

The loss of normal regulatory control, leading to unrestrained cell proliferation, is a hallmark of cancer. This loss of regulatory constraints is due to both gain of function mutations in proto-oncogenes and/or deletions or loss of function mutations in tumour suppressor genes (1, 2). Understanding the roles of these genes in normal growth regulation is therefore fundamental to an understanding of the molecular basis of cancer. Emerging evidence indicates that some oncogenes and tumour suppressor genes are directly involved in the cell cycle machinery responsible for cell division. In human breast cancer a number of known and potential oncogenes and tumour suppressor genes have been identified. These include genes encoding growth factor receptors (e.g., the *erb*B family), signalling molecules involved in transmitting signals from receptors on the cell surface to the nucleus (e.g., *src*, *ras*, Grb 2 and Brb7) and genes with known roles in cell cycle regulation (e.g., c-*myc*, cyclin D1, p53 and RB). Since members of the cyclin gene family mediate increased rates of cell proliferation in response to diverse mitogens, by determining rates of progression through checkpoints within the cell cycle, and are oncogenes in some tumour types (3, 4), recent studies in this laboratory have focussed on the expression, regulation and function of members of the cyclin gene family in human breast cancer (5–8). These data are reviewed below.

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Cell cycle control

Cell division in higher eukaryotes is regulated by the activity of the cyclin-dependent kinases (CDKs). Active CDKs consist of a serine/threonine kinase subunit whose catalytic activity is dependent on association with a regulatory cyclin subunit (9). The abundance of the cyclin subunit oscillates in a cell cycle-dependent manner, thereby regulating the activity of the kinase subunit at specific points in the cell cycle. Rapidly expanding numbers of both the kinase and cyclin subunits have been identified in mammalian cells. The cyclin family includes cyclins A, B, C, D and E while at least 10 different kinases related to the prototypic cyclin-dependent protein kinase, CDC2, have been identified (9). The association of different kinase subunits with different cyclin subunits is thought to control different stages of cell division. Cyclins D and E which

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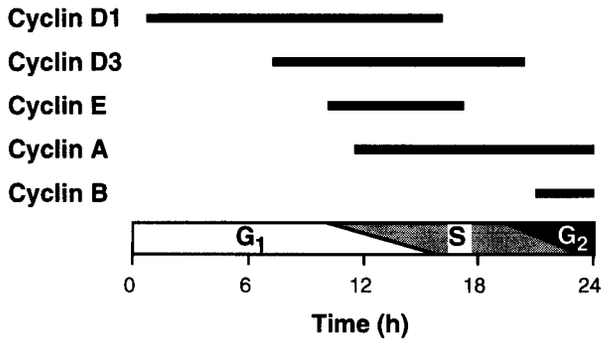

Fig. 1. Sequential induction of cyclin mRNA following growth factor stimulation of breast cancer cells. Bars indicate approximate times of maximum expression of individual cyclins after growth factor stimulation of growth-arrested cells at 0 h.

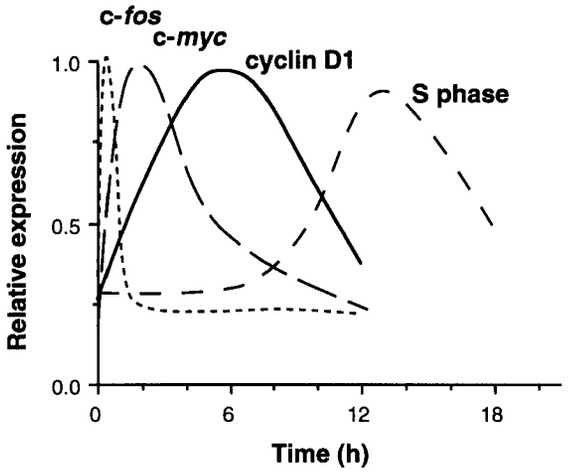

Fig. 2. Effects of progestin stimulation on cell cycle regulatory genes. Temporal changes in c-*fos*, c-*myc* and cyclin D1 mRNA following progestin stimulation of T-47D breast cancer cells are shown, based on data from ref. (6).

were isolated by their ability to complement yeast cells deficient in G_1 cyclins are mammalian G_1 cyclins, i.e. they regulate progress through G_1 checkpoints (9).

In steroid-responsive tissues, including normal and neoplastic breast epithelium, the regulation of proliferation by steroids and steroid antagonists occurs by cell cycle-specific actions on cells in G_1 phase (10–12). The central mechanism of steroid hormone action is the regulation of transcription by ligand-activated steroid hormone receptors binding to specific response elements in the regulatory regions of target genes (13). Steroid antagonists bind to the ligand-binding domain of steroid hormone receptors and interfere with their transactivation of gene expression. Steroid and steroid antagonist effects on proliferation are thus likely to be mediated by modulation of the expression of specific genes intimately involved in the control of cell cycle progression. The transcriptional regulation of cyclins suggested that they might be part of the mechanism by which steroids and steroid antagonists regulate cell cycle progression. Thus, our initial experiments investigated the relationship between changes in cell cycle progression induced by growth factors, steroids and steroid antagonists and specific cyclin gene expression.

Steroid and growth factor control of cyclin expression

The relationship between cell cycle position and cyclin gene expression in breast cancer cells was examined using the T-47D cell line. In serum-free medium these cells become growth-arrested in early G_1 phase but re-initiate proliferation upon the addition of a single growth factor (e.g., insulin, IGF-I, bFGF, EGF), progressing synchronously through the cell cycle from early G_1 (6). Sequential induction of cyclin gene expression was observed following stimulation of growth-arrested T-47D cells with insulin (Fig. 1). Cyclin D1 mRNA levels were increased within 2 h of insulin addition while increased cyclin D3 mRNA levels were observed as cells progressed through G_1. Increased expression of cyclins E and A coincides with entry into S-phase. Sequential induction of cyclins D1, D3 and E during G_1 progression in T-47D cells was also observed after treatment with other potent breast cancer mitogens, i.e. IGF-I, fetal calf serum (6) and bFGF (unpublished data). Furthermore, the proportion of cells which later entered S-phase was correlated with the degree of induction of cyclins D1, D3 and E (6).

Steroid hormones, oestrogens and progestins, are also major regulators of cell cycle progression in breast cancer cells. Progestins induce a transient increase in the rate of cell cycle progression (12) which is accompanied by a transient induction of cyclin D1 mRNA (Fig. 2). The time-course of cyclin D1 induction was delayed compared with that of the immediate-early proto-oncogenes c-fos and c-myc (Fig. 2) (12) and maximum induction was reached at 3–6 h. Progestin-stimulated cells began to enter S-phase after approximately 8 h of treatment (Fig. 2) (12).

The induction of cyclin D1 gene expression within 2 h of mitogenic stimulation is compatible with a role for this gene in early G_1 phase, a time when breast cancer cells are sensitive to the growth-inhibitory effects of antioestrogens and antiprogestins (14–16). Examination of cyclin expression after treatment with the antioestrogen ICI 164384, a potent inhibitor of breast cancer cell cycle progression (for example, see (6)) showed time-dependent decreases in the level of cyclin D1 mRNA (Fig. 3). Cyclin D1 expression began to decrease within 4 h of antioestrogen treatment, substantially preceding any decline in DNA synthesis (Fig. 3). Thus, the regulation of cyclin D1 expression by ICI 164384 is not merely a consequence of growth arrest.

Changes in cell cycle phase distribution occur over a similar time-frame after antioestrogen or antiprogestin treat-

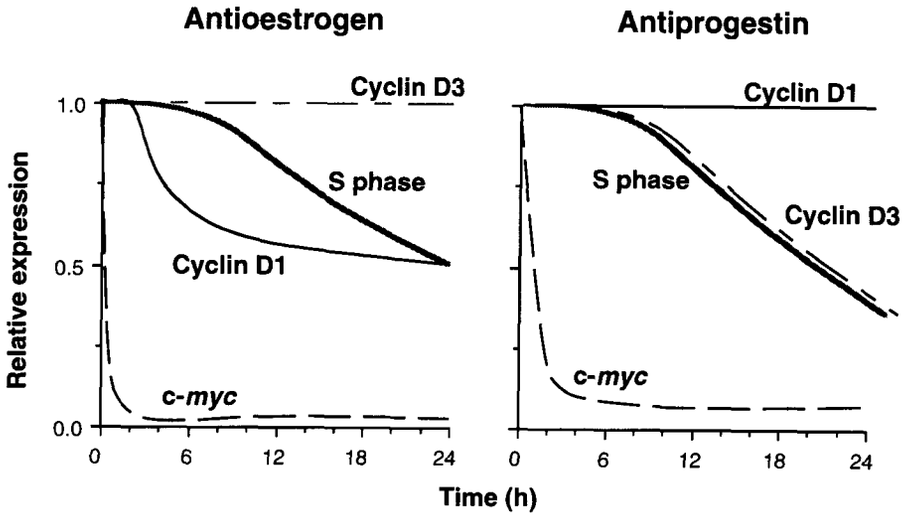

Fig. 3. Effect of antioestrogen or antiprogestin treatment on cell cycle regulatory genes. Changes in the expression of c-*myc*, cyclin D1 and cyclin D3 mRNAs in breast cancer cells following antioestrogen (ICI 164384) or antiprogestin (RU 486) treatment are presented schematically, based on data from ref. (6).

ment (16), and both inhibitors rapidly and profoundly decrease c-*myc* expression (Fig. 3). However, while cyclin D1 expression decreased after antioestrogen treatment, the antiprogestin failed to affect the expression of this gene. These data indicate that cyclin D1 is one of several genes responsible for regulation of cell cycle progression in breast cancer cells, and provide evidence for a role for c-*myc* (6, 12).

In conclusion, these data demonstrate the sequential induction of cyclin genes following mitogenic stimulation of breast cancer cells, a phenomenon shared by many other cell types. More importantly, cyclin D1 was the first G_1 cyclin to be induced and the level of induction correlated closely with subsequent entry into S-phase, supporting a key role for cyclin D1 in determining the rate and magnitude of cell cycle progression. Several lines of evidence from other cell systems support this hypothesis. Inhibition of cyclin D1 expression or function (e.g. by the use of antibodies or antisense techniques) inhibits entry into S-phase (17, 18), showing that cyclin D1 is necessary for completion of G_1. Inhibition of cyclin D1 expression accompanies inhibition of cell proliferation by a variety of agents, including tumour necrosis factor α, interferon γ, 8-Br-cAMP and antioestrogens (6, 8, 19), suggesting that mechanisms for the regulation of proliferation often converge on this gene. The hypothesis that cyclin D1 abundance controls progress through G_1 phase was therefore tested directly in a further set of experiments described below.

Cyclin D1 is rate-limiting and sufficient for cell cycle progression

T-47D breast cancer cells expressing ectopic human cyclin D1 under the control of the metal-inducible metallothionein promoter were generated along with control cell lines transfected with the vector alone (7). Zinc treatment resulted in a rapid induction of cyclin D1 mRNA and protein beginning 1 h after treatment and reaching typically 5-fold levels of protein between 3–12 h. This was accompanied by a profound increase in the proportion of cells reaching S-phase between 12 and 24 h after treatment (Fig. 4). Subsequent experiments demonstrated that this was due to both an increase in the rate of G_1 transit and an increase in the proportion of the cell population engaged in cell cycle transit (7). Thus, cyclin D1 induction mimicked the effects of mitogenic stimulation by growth factors and steroids.

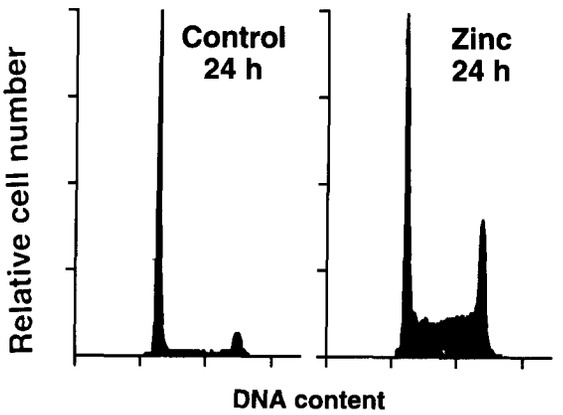

Fig. 4. Effect of cyclin D1 on cell cycle progression in cycling cells. DNA histograms of T-47D cells transfected with cyclin D1 under control of the zinc-inducible metallothionein promoter after treatment for 24 h with $ZnSO_4$ or vehicle (control).

To test whether cyclin D1 was sufficient for cell cycle progression T-47D cells growth arrested by serum depriv-

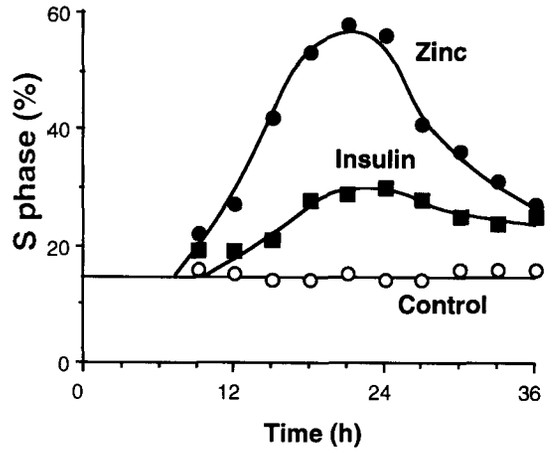

Fig. 5. Effect of cyclin D1 induction in growth-arrested cells. T-47D cells transfected with cyclin D1 under control of the zinc-inducible metallothionein promoter were growth-arrested in unsupplemented serum-free medium and then treated with either vehicle (Control, ○), 50 μM $ZnSO_4$ (Zinc, ●) or 10 μg/ml insulin (■).

ation were treated with zinc or insulin. Interestingly zinc treatment was significantly more potent than insulin in stimulating cell cycle progression (Fig. 5). Arrested cells traversed G_1 phase and completed the cell cycle following either treatment indicating that induction of cyclin D1 alone was sufficient to take growth-arrested cells from G_1 to mitosis.

The proportion of breast cancer cells initiating progress through the cell cycle after induction of ectopic cyclin D1 expression depended on the cyclin D1 level (7). These observations are consistent with data showing a relationship between endogenous cyclin D1 levels and the proportion of cells subsequently entering S-phase after mitogen stimulation (6, 20, 21). Furthermore, since cyclin D1 is sufficient for completion of G_1 in breast cancer cells, cyclin D1 overexpression might reduce the effects of physiological growth restraints, i.e. the requirement for steroids or growth factor stimulation, particularly in tumour cells which are already transformed. This is supported by the observation that both rodent fibroblasts and human breast cancer cells overexpressing cyclin D1 maintain a higher S-phase fraction upon serum deprivation than control cells (7, 22).

Cyclin amplification and overexpression in breast cancer

Their central role in cell cycle control has led to the suggestion that cyclins are proto-oncogenes (9). The cyclin D1 gene (PRAD1) is located at 11q13, one of the most frequently amplified regions in human carcinomas (23) and is the favoured candidate oncogene associated with translocations involving the BCL1 locus in a subset of B-cell lymphomas (9). More recently studies in transgenic mice have demonstrated the oncogenic potential of the human cyclin D1 gene in both the mammary gland (4) and lymphoid system (3). In addition, other cyclin genes including cyclins A and E, have been implicated in oncogenesis (9).

To search for aberrant expression of cyclin genes in breast cancer a series of human breast cancer cell lines was first screened for the expression of cyclin A, B1, C, D1 and E mRNAs (5). These cell lines encompass a spectrum of phenotypes, ranging from oestrogen receptor-positive, hormone-responsive, more differentiated cell lines to oestrogen receptor-negative, hormone-independent cell lines, representative of more aggressive, poorly differentiated tumours. Although the cyclins were uniformly expressed in the majority of the cell lines, increased expression of one or more of the cyclin A, B1, D1 or E genes was found in 7/19 cell lines (5).

Increased expression of cyclin D1 was the most common alteration in cyclin gene expression noted in these cell lines. This gene was highly expressed in MDA-MB-134, -175, -330 and -453 cells and one of two MCF-7 variants, compared with the level of mRNA observed in the majority of the breast cancer cell lines and in two strains of normal, non-transformed breast epithelial cells (5). Cyclin D1 gene amplification was detected in six cell lines but amplification was not a prerequisite for, and did not always lead to, increased cyclin D1 expression. Amplification of the cyclin E gene was present in the MDA-MB-157 cell line, accompanying increased expression of cyclin E mRNA.

Table

Overexpression of cyclin D1 in breast cancer

	Number of tumours	Greater than normal expression	Staining intensity		
			Weak	Moderate	Strong
Cyclin D mRNA					
Buckley et al. (5)	124	56 (45%)			
Immunohistochemistry					
Bartkova et al. (18)	147		61	42 (29%)	44 (30%)
Gillett et al. (24)	63		36		27 (43%)

The frequency of 11q13 amplification in breast cancers has been summarized by Fantl et al. (23) where studies of over 2 000 tumour samples indicated a mean frequency of amplification of about 13%. In view of the dissociation between cyclin D1 amplification and mRNA expression in breast cancer cell lines, we examined cyclin D1 expression in a series of 124 well-characterised breast tumours and 16 samples of histologically normal breast tissue (5). All tumour samples had detectable levels of cyclin D1 mRNA although large variations in the level of expression were observed. Fifty-six of the 124 tumours analysed (45%) expressed levels of cyclin D1 mRNA that exceeded the maximum level observed in 16 specimens of histologically normal breast tissue (5). Thus, the increased expression of cyclin D1 displayed by some breast cancer cell lines was also observed in vivo. Support for a higher proportion of cyclin D1 overexpression than predicted from studies of 11q13 amplification is evident from two recently published studies employing immunohistochemical detection of cyclin D1 protein (18, 24). These data demonstrating 30–43% overexpression are in good agreement with our estimate based on mRNA expression (Table).

The relationship between cyclin D1 amplification and selected clinical and pathological parameters has been reported but to date there are limited data on the relationship of cyclin D1 overexpression with such parameters. Several studies have suggested a weak association between 11q13 amplification and tumour size and lymph node status, a strong association with oestrogen receptor status and claims that amplification predicts for poor survival (23). We have confirmed a strong association between cyclin D1 mRNA expression and oestrogen receptor mRNA levels. Whether or not cyclin D1 overexpression identifies a subunit of poor prognosis patients within the good prognosis, oestrogen receptor-positive group is a major unresolved question. It is clear, however, that cyclin D1 overexpression is apparent in early lesions (18) and may therefore have some role in tumourigenesis.

CDKs and CDK inhibitors

Together these observations provide evidence for a central role for cyclin D1 in breast cancer cell proliferation and suggest that molecules which regulate cyclin D1 expression or the function of associated kinases, particularly CDK4, might also play a critical role in the control of cell cycle progression in these cells. Recently a number of endogenous inhibitors of CDK activity have been identified. These include: $p16^{INK4}$, which specifically inhibits the catalytic activity of cyclin D/CDK4 complexes; $p27^{KIP1}$, which inhibits both cyclin D/CDK4 and cyclin E/CDK2 complexes and appears to provide a link between the functions of these kinases; and $p21^{WAF1/CIP1}$, a p53-induced protein involved in growth arrest after DNA damage (9). The latter is of particular importance given the established role of the gene encoding p53 as a tumour suppressor gene in breast cancer (25).

Since the activity of the cyclin D/CDK4 complex is modulated by the CDK4 inhibitor, $p16^{INK4}$, deletion or inactivation of INK4 alleles with resultant loss of $p16^{INK4}$ activity may have effects similar to overexpression of cyclin D1, i.e. INK4 may be a tumor suppressor gene in breast cancer. Consistent with this possibility 5 of 19 breast cancer cell lines (BT-20, Hs-578T, MCF-7, MDA-MB-231 and -330) did not express detectable levels of INK4 mRNA due to homozygous deletion of the INK4 gene. Furthermore, loss of heterozygosity at this locus was detected in another 2 cell lines (BT-474 and MDA-MB-361). These data, demonstrating frequent deletions of INK4 (36%) in breast cancer cell lines, suggest that abnormalities in CDK4 function or activation, including loss of $p16^{INK4}$ inhibitor function, may be involved in human breast cancers. Testing of this hypothesis requires the analysis of significant numbers of breast carcinomas but to date no data have been published. Even if no major INK4 abnormalities are found in breast tumours, the data presented here indicate that molecules involved in the regulation of cyclin D1 expression and function are likely to have an important role in determining rates of breast cancer cell cycle progression and in turn the phenotype of a particular tumour.

Conclusions

The data summarized above demonstrate that abnormalities of cyclin gene expression are relatively common events in breast cancer. Amplification and overexpression of the G_1 cyclin, cyclin D1, is the most common abnormality of cyclin expression; this gene is amplified in about 15% of breast cancers and overexpressed in up to 45% of cases. Since cyclin D1 is an early response gene following mitogenic stimulation and is both rate-limiting and sufficient for G_1 progression in breast cancer cells, overexpression is likely to have a significant influence on phenotype. Although the properties of such tumours have yet to be defined in detail, likely consequences of cyclin D1 overexpression include the development of autonomous growth and loss of sensitivity to some endocrine therapies, including growth inhibitory antioestrogens. These hypotheses require urgent testing both with cell cultures and in large numbers of breast carcinomas from well-defined patient populations.

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