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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Estrogenic steroids have diverse physiological functions and are critically involved in the pathogenesis of breast cancer. The transcriptional regulator c-Myc has been implicated in estrogen-induced mitogenesis and we are therefore investigating the contribution of c-Myc to various downstream molecular and cellular events after estrogen stimulation. We have now developed a series of cell lines expressing c-Myc and mutants lacking one or both of the conserved 'Myc boxes' required for transcriptional regulation activity, under the control of an inducible promoter. Ongoing experiments are examining whether these mutants can mimic the effects of estrogen in activating cyclin E-Cdk2 and inducing cell cycle progression. We have recently shown that decreased expression of the CDK inhibitor p21, a c-Myc target, is critical for estrogen activation of cyclin E-Cdk2 and consequently the effect of the c-Myc mutants on p21 expression is of particular interest. We have also shown that decreased c-Myc expression can mimic the effects of antiestrogens. These data emphasise the importance of c-Myc regulation in estrogen and antiestrogen action.

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INTRODUCTION

This project focuses on the role of c-Myc in estrogen induction of proliferation in breast epithelial cells. Specifically, the aim is to determine the contribution of c-Myc to various downstream molecular and cellular events after estrogen stimulation. The rationale for this investigation lies in: the established intrinsic role of estrogens in mammary gland development and etiology of breast cancer; the proven therapeutic efficacy of antiestrogens in breast cancer; and compelling evidence that has implicated the cell cycle regulatory molecules c-Myc and cyclin D1 in estrogen-induced mitogenesis. An excellent model established in this laboratory wherein antiestrogen-arrested cells synchronously re-enter the cell cycle after treatment with estrogen has facilitated observation of various molecular events downstream from estrogen in breast cancer cells. Induction of c-Myc can also stimulate re-entry into the cell cycle in this model, and we have previously shown that this recapitulates the effects of estrogen on cyclin E-Cdk2 activation as well as S phase progression. Our experimental approach involves ectopic expression of wild-type, mutant and dominant-negative variants of c-Myc to modulate c-Myc function and hence determine the necessity for various functional domains of c-Myc in mediating estrogen stimulation of breast cancer cell proliferation.

BODY: ANNUAL SUMMARY

The aims of this project as outlined in the original application were to address several hypotheses arising from previous work highlighting the importance of c-Myc in estrogen stimulation of proliferation. These were that:

- Induction of c-Myc may precipitate a cascade of molecular events which mimic the effects of estrogen treatment i.e. c-Myc expression is *sufficient* to mimic the effects of estrogen.
- Activation of cyclin E-Cdk2 is a key element in both Myc- and estrogen-induced cell cycle progression and may occur by the same mechanism.
- c-Myc induction may be *necessary* for estrogen effects on molecular events leading to cell cycle progression, including cyclin E-Cdk2 activation.
- Given the essential requirement for estrogen in mammary gland development, c-Myc function may be also required for development of the mammary ductal tree

Work during the past year has focussed on identifying those functions of c-Myc which are necessary to mimic the effects of estrogen treatment and those which are dependent on c-Myc action. Progress is outlined below.

1. *Sufficiency of c-Myc expression for overcoming antiestrogen-induced arrest* (Task 1 in Statement of Work)

c-Myc is a bHLH/LZ (basic helix-loop-helix/leucine zipper) transcriptional regulator which forms heterodimeric complexes with another bHLH/LZ protein, Max. Myc can both activate and repress transcription and these functions require the amino terminus of c-Myc which contains two highly conserved Myc box regions. MbI (Myc box I, amino acids 45-63) is necessary for transcriptional activation; and MbII (amino acids 129-141) is necessary for transcriptional repression, c-Myc-induced cell proliferation and transformation. This raises the question of whether c-Myc-regulated transcriptional activation, repression or both are involved in estrogen stimulation of cell proliferation. Since MbII is necessary for c-Myc-induced cell proliferation in other systems we predict that c-Myc repressed genes are likely to be involved in both estrogen- and Myc-induced cyclin E-Cdk2 activation and G_1-S phase progression.

We have tested this hypothesis by examining the ability of various c-Myc mutants to promote cell cycle progression in antiestrogen-arrested MCF-7 cells, using an experimental design similar to that used to demonstrate that full-length c-Myc can mimic the effects of estrogen. The mutants are: c-MycΔMbII, which has a deleted MbII domain (1); and c-MycS, a naturally occuring translational form of c-Myc which lacks 100 amino-terminal amino acids, including the MbI domain, and cannot activate transcription but can stimulate proliferation (7, 8). We also have a construct lacking both Myc boxes, c-Zip (3). As outlined in the previous Annual Summary, a number of technical problems were encountered in attempts to constitutively express the c-Myc mutants and so given the previous success within the laboratory with zinc-inducible constructs (4, 5), it was decided to use this approach instead.

The c-Myc mutants were cloned into pΔMT, the vector used in our previous studies, downstream of the metallothionein promoter. Each mutant and wildtype c-Myc was transfected into MCF-7 cells together with a hygromycin resistance plasmid and multiple clonal cell lines were selected. Not all clonal cell lines selected in this way contain the ΔMT construct as well as the resistance plasmid, and there is significant variability in basal expression and inducibility in the ΔMT-transfected lines. In addition, there is variability in the estrogen responsiveness of different clonal MCF-7 derivatives. We therefore selected more than 20 clones for each construct and characterised those expressing the transfected c-Myc construct for estrogen/antiestrogen responsiveness, basal and zinc-induced expression as well as exponential growth rates (Table 1). This extensive characterisation (Table 2) allowed us to identify clonal cell lines with estrogen/antiestrogen responsiveness and growth rate comparable to parental cells which in addition display low basal and high inducible levels of each mutant. Interestingly, consistently higher numbers of cells continued to progress through S phase in the presence of antiestrogens in the c-Myc-expressing clones compared with parental cells or empty vector-transfected cells. This observation requires further investigation, but suggests that c-Myc expression may confer antiestrogen resistance.

Table 1: Selection of clonal MCF-7 cell lines expressing wildtype and mutant c-Myc proteins

	Number of clones selected	**Number expressing transfected construct**
Empty vector	8	-
c-Myc	25	9
c-MycS	23	5
c-MycΔMBII	22	7
c-Zip	22	9
Rep-Max	22	4

Table 2: Characterisation of clonal MCF-7 cell lines expressing wildtype and mutant c-Myc proteins

Clone	Basal expression†	Induced expression†	Antiestrogen arrest§	Estrogen rescue§
MCF-7 parent			+++	+++
Empty vector				
#101			+++	++
#102			+++	+++
#103			+++	++
#104			+++	+++
#105			+++	++
#108			+++	+
c-Myc				
#101	-	+	+++	+++
#102	-	+++	+++	++
#107	-	++	+++	+++
#108	-	+	+++	++
#112	-	+	+++	++
#114	+	+++	+++	+
#117	-	++	++	+++
#120	-	+++	+++	++
#121	++	+++	++	+++
c-MycS				
#102	++	++	+++	+++
#105	-	+++	++	+++
#106	+	+++	+++	+++
#110	-	+	++	++
#112	++	++	++	+++
c-MycΔMBII				
#106	+/-	+++	++	+++
#108	+	+++	+++	+++
#112	-	+++	+++	+++
#114	+	+++	+++	+++
#116	+	+++	+++	++
#121	+	+	+++	++
#122	-	++	+++	++

Clone	Basal expression†	Induced expression†	Antiestrogen arrest§	Estrogen rescue§
c-Zip				
#101	-	+/-	+++	+
#103	-	+	+++	+++
#105	++	++	+++	+++
#106	+	++	+++	+++
#108	-	+	+++	+
#109	-	++	+++	+++
#110	+	++	+++	+
#111	-	++	+++	+++
#112	-	+	+++	+

† -: undetectable; + - +++: low –high expression.
§ Relative S phase fractions. +++: comparable to parental cells; + - ++: reduced response

Initial experiments examined which c-Myc mutants were capable of mimicking the effects of estrogen in 'rescuing' cells from antiestrogen-mediated growth arrest. After 48 h treatment with the antiestrogen ICI 182780, cells were treated with zinc to induce c-Myc mutant expression, in the presence of nocodazole. Nocodazole blocks cell cycle progression in G_2, preventing division of cells which have re-initiated cell cycle progression and thus allowing them to be readily enumerated. Estrogen 'rescue' was also performed in parallel for each cell line. These experiments showed that both c-Myc and c-MycS recapitulated the effects of estrogen, stimulating 50-70% of cells to pass through S phase and into G_2 over 36 h. In contrast, c-MycΔMBII and c-Zip did not lead to detectable reinitiation of cell cycle progression. These data suggest that a minor subset of c-Myc functions, i.e. those mediated by the 16 amino acids comprising the MBII transrepression domain, is required to overcome antiestrogen arrest. Current experiments are aimed at dissecting the relationship between the ability of c-Myc mutants to induce cell cycle progression in this model and their ability to mimic estrogen effects on cyclin E-Cdk2 activation. Recent collaborative studies from the laboratory have argued that decreased synthesis of the CDK inhibitor p21 is essential for estrogen activation of cyclin E-Cdk2 (Prall et al, submitted). Since c-Myc represses the p21 promoter (2), investigating the effects of the various c-Myc mutants on p21 transcription will be a priority for these experiments.

2. *Necessity of c-Myc expression for estrogen-induced cell cycle progression* (Task 2 in Statement of Work)

These experiments are complementary to those described above and utilise overlapping approaches. Other colleagues within the laboratory have refined antisense oligonucleotide approaches to decreasing c-Myc abundance. These have been successful in exponentially proliferating cells and we have demonstrated that the decreased cellular proliferation of MCF-7 cells caused by decreased c-Myc abundance is a consequence of inhibition of cyclin D1 expression and subsequent redistribution of p21 from cyclin D1-Cdk4 to cyclin E-Cdk2, inhibiting cyclin E-Cdk2 activity (Carroll et al., in preparation). These molecular events mimic the effects of

antiestrogens, emphasising the importance of c-Myc regulation in estrogen/antiestrogen action. However, introduction of antisense oligonucleotides into antiestrogen-arrested cells and subsequent estrogen rescue presented technical problems despite several attempts at modifying the protocol. Consequently we decided to focus on a dominant-negative approach to identifying those aspects of estrogen induction of cell cycle progression which are dependent on c-Myc action.

Two proteins with previously-demonstrated dominant negative activity are under study: c-Zip and Rep-Max, a synthetic chimera of the mSin interaction domain of Mxi and the bHLH-LZ region of Max (6). Both these dominant-negative proteins dimerise with Max and therefore compete with endogenous c-Myc but do not regulate c-Myc-responsive genes (3, 6). As outlined above, we have selected and characterised clonal cell lines expressing c-Zip. Cell lines expressing Rep-Max have also been selected (Table 1) and will be characterized on completion of the experiments described in Task 1. Selected cell lines will then be used to determine the effect of induction of c-Zip or Rep-Max on estrogen-mediated reinitiation of cell cycle progression in breast cancer cells.

3. *Role of c-Myc in normal mammary gland development* (Task 3 in Statement of Work)

This part of the project was dependent on successful completion of the experiments described in Part 2 above and, as outlined in the previous Annual Summary, further consideration of the experimental approach in consultation with colleagues familiar with the techniques involved identified a number of potential technical problems which would likely preclude successful completion of these experiments. Therefore, during the final year of funding the focus remained on the *in vitro* approaches in Tasks 1 and 2.

KEY RESEARCH ACCOMPLISHMENTS

- Selection and characterisation of multiple clonal MCF-7 cell lines expressing c-Myc, c-MycS, c-Myc ΔMBII, c-Zip and RepMax. Selection of multiple clonal MCF-7 cell lines expressing RepMax.

- Demonstration that c-Myc ΔMBII cannot mimic the effects of estrogen while c-MycS, lacking MBI, is as effective as wildtype c-Myc.

- In collaborative studies, elucidation of the role of decreased c-Myc expression in antiestrogen-mediated inhibition of proliferation and identification of a key role for the c-Myc target p21 in estrogen activation of cyclin E-Cdk2 .

REPORTABLE OUTCOMES

Manuscript

Carroll JS, Prall OWJ, Sergio CM, Rogan EM, Watts CKW, Musgrove EA, Sutherland RL.
Estrogen/antiestrogen antagonist regulation of the cell cycle in breast cancer cells. Steroid hormones and cell cycle regulation. In press.
Bernstein, KL, ed. Kluwer Academic Publishers, Boston.

Invited Presentations

Sutherland RL. SERMS: Mechanism of Action as Growth Inhibitory Agents. ANZ Breast Cancer Trials Group Meeting, Queenstown, New Zealand, 5-8 July 2000.

Sutherland RL. ER signalling into the cyclin/CDK/Rb pathway. 23rd Annual San Antonio Breast Cancer Symposium, San Antonio, TX, 6-9 December 2000.

Sutherland RL, Carroll JS, Lee CSL, Prall OWJ, Rogan EM, Swarbrick A, Watts CKW, Musgrove EA. Cell cycle regulation by oestrogens and progestins in breast cancer cells. Breast Cancer 2001, Melbourne Vic, Australia, 18-20 March, 2001.

Sutherland RL, Carroll JS, Lee CSL, Prall OWJ, Rogan EM, Swarbrick A, Watts CKW, Musgrove EA. Cell cycle regulation by oestrogens and progestins in breast cancer cells. Endocrine Society 83rd Annual Meeting, Denver CO, 20-23 June, 2001.

Cell lines

Clonal MCF-7 derivatives transfected with c-Myc or c-Myc mutants, as listed in Table 1.

CONCLUSIONS

We have demonstrated that c-Myc is sufficient to initiate re-entry of antiestrogen-arrested cells into the cell cycle, thus mimicking the effects of estrogen, and that this occurs via a pathway that is initially distinct from cyclin D1-activated events. However, both pathways converge on activation of cyclin E-Cdk2. Further, we have shown that the region of c-Myc encompassing MBII is necessary for this response. This implies that previously undefined transrepressed genes are critical for estrogen-induced mitogenesis in addition to those estrogen-induced genes that have been the major focus of research in this area to date. A prime candidate is the CDK inhibitor p21 which is downregulated by c-Myc in other systems and which we have shown is critical for estrogen activation of cyclin E-Cdk2. In experiments that are currently in progress, we are examining the relationship between the ability of c-Myc mutants to induce cell cycle progression in this model and their ability to mimic estrogen effects on p21 expression and cyclin E-Cdk2 activation, and will go on to identify which of these effects of estrogen are dependent on c-Myc. These should be completed in the near future. The cell lines developed in the course of these studies will provide an invaluable resource for further experimentation, for example identifying and characterising other c-Myc targets which are potentially involved in estrogen-induced mitogenesis.

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5

ESTROGEN/ESTROGEN ANTAGONIST REGULATION OF THE CELL CYCLE IN BREAST CANCER CELLS

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INTRODUCTION

The role of estrogen in the growth of breast cancer was recognised over a century ago when it was shown that ovariectomy in premenopausal women with breast cancer resulted in tumor regression (Beatson 1896). Subsequent research showed that estrogen exerted its proliferative effects through a specific receptor (estrogen receptor-ER) and was essential for the initiation and progression of mammary cancer in experimental animals. This and other observations, such as the correlation between ER status of the tumor and a positive response to endocrine therapy, led to the development of estrogen antagonists (antiestrogens) for the treatment of breast cancer (Lerner and Jordan 1990). Tamoxifen, the antiestrogen most commonly employed in the treatment of hormone sensitive breast cancer, significantly decreases the rates of both disease recurrence and death (Early Breast Cancer Trialists' Collaborative Group 1992; Early Breast Cancer Trialists' Collaborative Group 1998; Fisher et al. 2001). However, tamoxifen therapy is limited by the frequent development of cellular resistance. In addition, synthetic non-steroidal antiestrogens like tamoxifen possess both estrogen agonist and antagonist activity and as such have the potential to induce proliferative side effects in other reproductive organs such as the endometrium (MacGregor and Jordan 1998). Due to these clinical limitations, more potent antiestrogens have been developed which do not have estrogen agonist properties, have

prolonged effectiveness and are potentially efficacious in cancers that have developed resistance to nonsteroidal antiestrogens such as tamoxifen (Wakeling and Bowler 1987; Howell et al. 1995). This structurally distinct class of antiestrogens includes ICI 182780 (Faslodex) which is currently in clinical trials both as a primary treatment and for the treatment of tamoxifen resistant cancers (DeFriend et al. 1994; Howell et al. 1995). The mechanistic basis for the anti-tumour effects of antiestrogens is inhibition of estrogen mediated mitogenesis, but the molecular events in antiestrogen induced growth arrest are not fully understood. Similarly, there is an incomplete understanding of the molecular events that mediate estrogen-induced mitogenesis in breast cancer cells. This chapter summarizes recent data from this and other laboratories on antiestrogen action in breast cancer cells and provides insight into the role of estrogen in mitogenic stimulation of target cells.

EFFECTS OF ESTROGENS AND ANTIESTROGENS ON CELL CYCLE PROGRESSION

Early information on the growth-inhibitory actions of antiestrogens originated from *in vitro* studies on breast cancer cell lines. These experiments suggested that growth rates (measured as changes in both cell number and tritiated thymidine incorporation into DNA) were significantly reduced by antiestrogen treatment (Lippman and Bolan 1975; Lippman et al. 1976). In the MCF-7 breast cancer cell model which has been the most widely studied experimental paradigm (Levenson and Jordan 1997), the typical growth inhibitory response to antiestrogens (both non-steroidal and steroidal antiestrogens) is a decrease in the proportion of cells synthesising DNA (S phase) after approximately 8 hours of antiestrogen treatment. This decrease in S phase coincides with an increase in the proportion of cells in G_0/G_1. It is clear that only cycling cells in early to mid G_1 are sensitive to antiestrogens (Sutherland et al. 1983; Taylor et al. 1983; Reddel et al. 1984; Musgrove et al. 1989; Wakeling et al. 1989) which coincides with the period of the cell cycle when cells are sensitive to mitogenic stimulation.

The growth arrest following antiestrogen treatment of breast cancer cells has been used to study the effects of estrogen on cell cycle progression, since subsequent estrogen 'rescue' from antiestrogen-mediated growth arrest results in semi-synchronous progression of MCF-7 cells from G_0/G_1 through to S phase. The profile of such changes in cell cycle parameters is shown in Figure 1. This model has provided a robust experimental system

to develop greater insight into the molecular events involved in mitogenic stimulation by estrogen in breast cancers.

CELL CYCLE PROGRESSION

G0/G1 to S phase progression is mediated by the action of a family of serine/threonine kinases, the cyclin-dependent kinases (CDKs), which in conjunction with their regulatory partners, cyclins, phosphorylate pRb and other members of the pocket protein family, p107 and p130 (Dyson 1998). The phosphorylation of pRb during G1 phase is mediated via two temporally distinct stages, in which initial phosphorylation by cyclin D1-Cdk4/6 is followed by cyclin E-Cdk2 phosphorylation to complete inactivation of pRb. A major level of cyclin-CDK regulation is from two families of specific inhibitors. The INK4 inhibitors, $p16^{INK4a}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$, have as primary targets Cdk4 and Cdk6 (Sherr and Roberts 1999). Members of the second family, which include $p21^{WAF1/Cip1}$ and $p27^{Kip1}$, have a dual function: inhibition of CDKs including cyclin E-Cdk2 as well as facilitation of the assembly of cyclin D-Cdk4/6 complexes at low stoichiometries (Sherr and Roberts 1999) and inhibition of cyclin D-Cdk4/6 complexes at higher concentrations.

The association of the pocket proteins with members of the E2F family of transcription regulators is responsible for a major part of their growth inhibitory actions. Phosphorylation and hence, inactivation, of the pocket proteins results in dissociation of E2Fs, allowing subsequent transcription of genes required for progression through late G1 and S phase (Dyson 1998). While the availability of 'free' E2Fs is an important determinant of transcriptional activity, it is also apparent that pocket protein-E2F complexes can actively repress transcription of target genes, in addition to simply blocking the action of E2F. Specific complexes between different E2F family members and pocket proteins are characteristic of particular stages of the cell cycle. During G1 phase, pRb preferentially complexes with E2Fs 1-3, although pRb-E2F4 complexes are present in some cells. p107 is not abundant or active until late G1, where it negatively regulates E2F4 and E2F5 at the G1 to S phase boundary. p130 is the predominant pocket protein in quiescent (G0) and differentiated cells and interacts with E2F4 in these cells (Dyson 1998).

Figure 1: Changes in cell cycle profile after estrogen 'rescue' of antiestrogen arrested breast cancer cells

Another important component of the cell cycle machinery is the c-Myc oncoprotein, the product of the immediate early gene myc. This was one of the first candidate genes investigated as a potential target of estrogen induced mitogenesis, primarily due to the rapid induction of the gene after

estrogen stimulation. We and others have confirmed the important role that c-Myc plays in estrogen-induced progression through the cell cycle in *in vitro* models, where accumulation of c-Myc follows stimulation of quiescent cells with estrogen (Prall et al. 1997) and where induction of c-Myc on its own is sufficient to induce cell cycle progression (Henriksson and Luscher 1996). Activation of conditional alleles of c-Myc is followed by the activation of G1 specific cyclin-Cdk complexes (Steiner et al. 1995; Rudolph et al. 1996; Perez-Roger et al. 1997), highlighting the importance of c-Myc in cell cycle control. The c-Myc protein is estrogen stimulated in estrogen receptor positive cell lines via an estrogen response element (ERE) in the c-myc promoter and is constitutively overexpressed in hormone-independent breast cancer cells via undefined mechanisms (Dubik et al. 1987; Dubik and Shiu 1992).

MOLECULAR MECHANISM OF GROWTH REGULATION BY ANTIESTROGENS

The estrogen receptors are members of the nuclear hormone receptor superfamily and as such recruit various subsets of coactivators and corepressors (Shang et al. 2000), which regulate transcription from estrogen response elements, AP-1 sites and other potential DNA motifs. Various classes of antiestrogens induce specific conformational changes in the structure of the estrogen receptor (Brzozowski et al. 1997; Shiau et al. 1998). These distinct conformations facilitate association with different groups of coactivators or corepressors, which likely explain the various responses to different structural classes of antiestrogens (McDonnell et al. 1995; MacGregor and Jordan 1998). However, to date, the cell cycle effects of the various classes of antiestrogens appear similar.

Antiestrogens increase hypophosphorylation of the pocket proteins

The stereotypic pocket protein, pRb has been implicated in growth arrest and is a central regulator of cell cycle progression in late G1 phase. There is a time-dependent change in pRb phosphorylation after ICI 182780 treatment of MCF-7 cells, such that by 4-6 hours, hypophosphorylated pRb begins to accumulate and by 48 hours pRb is unphosphorylated and reduced in abundance by approximately 80% (Watts et al. 1995). These changes occur in both MCF-7 and T-47D breast cancer cells following treatment with hydroxytamoxifen (the active metabolite of tamoxifen) or ICI 182780, confirming that the changes in pRb are universal to growth arrest by different classes of antiestrogens (Musgrove et al. 1993; Wilcken et al. 1996).

More recent work investigated changes in the other two pocket proteins, p107 and p130. Hypophosphorylated forms of p130 (forms 1 and 2) are detected between 4 and 10 hours and predominate by 24 hours. Interestingly, the total protein levels of p130 increase substantially, such that after 24 hours of antiestrogen treatment they increase 3 to 4 fold (Carroll et al. 2000). In contrast to p130, a decrease in p107 phosphorylation status is not clear until 24 hours, coinciding with a loss of total protein levels of p107. It is thought that p130 and p107 are reciprocally regulated as dependent transcriptional events (Smith et al. 1998).

Analysis of complex formation by the pocket proteins reveals substantial changes in their association with the E2F transcription factors (Carroll et al. 2000). Levels of pRb-E2F1 complexes decrease after 16 hours of antiestrogen treatment and fall to approximately 20% of control levels by 48 hours, which correlates with the loss of total pRb protein. p130-E2F4 complexes become clearly detectable by 24 hours and increase by more than 20-fold within 48 hours. The formation of this complex and the presence of hyperphosphorylated forms of E2F4 are specific markers of quiescence (G_0) (Thomas et al. 1998) and have therefore been used as signatures of a G_0 growth arrest state. Our analysis of E2F4 phosphorylation from MCF-7 cells treated with the pure antiestrogen ICI 182780 shows hyperphosphorylation of E2F4 at 24 hours, which increases substantially at 48 hours (Carroll et al. 2000). This and the presence of p130-E2F4 complexes suggest that this pure antiestrogen arrested cells in a G_0 as opposed to a G_1 state. The generality of this phenomenon to other classes of antiestrogens/SERMs awaits further investigation.

Antiestrogen regulation of cyclin-Cdk activity

The most likely explanation for the changes in pocket protein phosphorylation described above is the loss of cyclin-Cdk activity. The activity of the G_1 specific cyclin-Cdk complexes, cyclin D1-Cdk4/6 and cyclin E-Cdk2 was assessed after treating MCF-7 cells with ICI 182780. The kinase activity of cyclin D1-Cdk4 declines to 40% of control levels by 12 hours and by 80% at 24 hours, preceding the changes in S phase (Watts et al. 1995). The activity of cyclin E-Cdk2 also decreases in the presence of ICI 182780 and it too, decreases prior to changes in S phase, supporting a causative relationship (Carroll et al. 2000). The activity of cyclin E-Cdk2 falls to less than 20% of control levels by 24 hours. Thus, both cyclin D1-Cdk4 and cyclin E-Cdk2 enzymatic activity is lost before cells exist the cell cycle. This is consistent with the hypothesis that a loss of

cyclin-Cdk activity in early G1 contributes to the cell cycle arrest. Recent work by another laboratory suggested that similar events occur during tamoxifen-mediated growth arrest of MCF-7 cells (Cariou et al. 2000).

The loss of cyclin D1-Cdk4 and cyclin E-Cdk2 activity does not result from changes in the protein levels of Cdk2, Cdk4 or cyclin E (Watts et al. 1995; Carroll et al. 2000). However, mRNA and protein levels of cyclin D1 decrease rapidly (within the first 6 hours) to approximately 50% of control levels. The mechanism of cyclin D1 gene regulation by ICI 182780 is unknown but recent work has identified a putative cAMP response element in the proximal promoter of cyclin D1 which is activated by estrogen and inhibited by antiestrogen (Sabbah et al. 1999). ER appears to interact with ATF-2/c-Jun heterodimers to activate cyclin D1 gene transcription and this effect may be mediated in part by estrogen-induced c-Jun expression (Sabbah et al. 1999). Thus a potential mechanism of antiestrogen inhibition of ER-mediated cyclin D1 transcription may be via disruption of ER-ATF-2/c-Jun complexes. Furthermore, recent studies on estrogen induced transcription suggest that coactivators, such as AIB1 are required for cyclin D1 transcription by estrogens (Planas-Silva et al. 2001). Whether antiestrogens can directly regulate the levels of coactivators, or can physically inhibit association of these molecules with the estrogen receptors and therefore inhibit transcription of the cyclin D1 gene is unknown. Although the mechanisms that result in decreased cyclin D1 gene expression remain to be fully defined, the decline in cyclin D1 protein is an important early event in growth arrest, since a forced decrease in cyclin D1 levels with antisense oligonucleotides mimics the growth arrest seen after antiestrogen treatment (Carroll et al. 2000).

Antiestrogen regulation of Cdk-inhibitors

Another major level of regulation of cyclin-Cdk activity is by association with the two classes of inhibitors, the KIP and INK4 proteins. Our work suggests that ICI 182780 does not change the mRNA levels of p15, p18 and p19 INK4 molecules during the first 12-16 hours of treatment (Carroll et al, unpublished). The gene encoding p16 is deleted in MCF-7 cells, also suggesting that the INK4 proteins do not play a critical role in ICI 182780-mediated inhibition of cyclin-Cdk complexes and consequent growth arrest.

The levels of KIP proteins, p21 and p27, increase following ICI 182780 treatment, but not until 12 hours after the addition of the antiestrogen (Watts et al. 1995; Carroll et al. 2000) and are preceded by changes in S

phase and the loss of cyclin D1-Cdk4 and cyclin E-Cdk2 activity. The increase in p21 protein levels at late timepoints occurs as a result of increased transcription, while elevated levels of p27 occur due to increased protein stability ((Carroll et al. 2000) and unpublished data). Therefore, although elevated levels of p21 and p27 at late timepoints may contribute to long term growth arrest there is no early increase in protein levels that can account for the initial loss of cyclin-Cdk activity.

Several investigations have provided evidence that a redistribution of inhibitors, rather than an increase in their levels, can result in inhibition of cyclin-Cdk complexes (Reynisdottir et al. 1995; Foster and Wimalasena 1996; Planas-Silva and Weinberg 1997; Prall et al. 1997; Liu et al. 2000; Swarbrick et al. 2000). We have recently shown that the loss of cyclin D1 observed after ICI 182780 treatment results in an increase in unbound, 'free' p21 which can subsequently associate with and inhibit cyclin E-Cdk2 leading to an increase in cyclin E-Cdk2-p21 complexes within 6 hours of ICI 182780 treatment (Carroll et al. 2000). p27 is also released from cyclin D1-Cdk4 complexes as a result of decreases in cyclin D1 levels, but p27 is phosphorylated by active cyclin E-Cdk2 and targeted for proteosome mediated degradation (Vlach et al. 1997). Therefore the current hypothesis is that p21 binds and inhibits cyclin E-Cdk2 sufficiently to cause a shift in the ratio of phosphorylated to unphosphorylated p27. Thereafter, p27 accumulates and cooperates with p21 to inhibit the remaining cyclin-Cdk complexes and maintain cell cycle arrest. The forced loss of p21 through inhibition of gene expression with p21 antisense oligonucleotides can attenuate the growth inhibitory effects of ICI 182780 implicating this molecule as an essential component of growth arrest (Carroll et al. 2000). This is not unique to ICI 182780 arrest, since a loss of either p21 or p27 could reverse the growth inhibitory effects of hydroxytamoxifen (Cariou et al. 2000), therefore confirming the universal importance of p21 and p27 in growth arrest of breast cancer cells by diverse antiestrogens.

c-Myc and its role in antiestrogen-induced growth arrest

Antiestrogen regulation of c-Myc at both the mRNA and protein levels has been observed in *in vitro* and *in vivo* models (Santos et al. 1988; Wong and Murphy 1991; Musgrove et al. 1993), with a loss of c-Myc mRNA observed within 2 hours in T-47D breast cancer cells treated with the pure antiestrogen ICI 164384 (Musgrove et al. 1993). More recent work has confirmed that ICI 182780 also has a rapid and profound effect on c-Myc protein levels in MCF-7 cells, with levels declining to 25% of control within 2 hours, suggesting that the loss of c-Myc is a common feature of

antiestrogen action. To further elucidate the importance of c-Myc in antiestrogen-mediated growth arrest we attempted to mimic the downstream events of ICI 182780 treatment in MCF-7 cells by specifically repressing the production of the c-Myc protein. Treatment with antisense oligonucleotides to c-myc induce the expected loss of c-Myc protein levels and subsequent growth arrest. Growth arrest following loss of c-Myc is accompanied by similar molecular changes to ICI 182780. A loss of cyclin D1 is observed and p21 is redistributed from cyclin D1-Cdk4 to cyclin E-Cdk2 complexes, resulting in a decrease in cyclin E-Cdk2 activity. This inhibition in cyclin E-Cdk2 activity is clearly mediated through p21 since inhibition of p21 gene expression with antisense oligonucleotides attenuates the inhibition of cyclin E-Cdk2 activity and rescues cell proliferation (Carroll et al. 2000). These data emphasise the important role of c-Myc in antiestrogen mediated growth arrest, since a loss of c-Myc alone is sufficient to induce the initial downstream events that culminate in growth arrest after ICI 182780 treatment.

Figure 2. Current model of antiestrogen action in breast cancer cells

ESTROGEN-INDUCED CELL CYCLE PROGRESSION IN BREAST CANCER CELLS

The role of c-Myc in estrogen-induced proliferation

The role that c-Myc plays in estrogen-induced mitogenesis has become clearer in recent years, but it still far from fully defined. Increases in c-Myc transcription and protein accumulation are early responses to estrogen and are essential for G1 to S phase progression. To help understand the complex role that c-Myc plays in regulating components of the cell cycle, we constructed MCF-7 cells that expressed c-Myc under the control of an inducible promoter (Prall et al. 1998). Expression of c-Myc is sufficient to rescue the MCF-7 cells from antiestrogen arrest providing further support for the oncogenic properties of c-Myc. Entry into S phase following induction of c-Myc does not influence levels of cyclin D1 protein but is sufficient to activate cyclin E-Cdk2 complexes and induce hyperphosphorylation of the pocket proteins. The cellular proliferation that results from induction of c-Myc is dependent on the activation of cyclin E-Cdk2, since a specific chemical inhibitor of Cdk2 can attenuate progression through the cell cycle. Interestingly, this re-activation of cyclin E-Cdk2 results from complex changes that include a loss of cyclin E-Cdk2 associated p21 and a subsequent increase in p130 associated cyclin E-Cdk2 complexes (Prall et al. 1998). The peptide motifs required for cyclin E-Cdk2 binding are shared by p21 and p130 ensuring that their association is mutually exclusive.

An important transcriptional target of c-Myc is the phosphatase Cdc25A (Galaktionov et al. 1996), which is positively regulated by estrogen and c-Myc and is required for activation of cyclin-Cdk complexes. Cdc25A and cyclin E have been implicated in growth promotion via a pRb independent pathway (Santoni-Rugiu et al. 2000) and microinjection of antibodies to Cdc25A is sufficient to block cell cycle progression (Jinno et al. 1994; Foster et al. 2001) implicating this c-Myc downstream target as an essential component of the G1-S transition. In MCF-7 cells which have been growth arrested by overexpression of the INK4 protein, p16, the consequent inhibition of cyclin E-Cdk2 activity can be reversed by the addition of Cdc25A, further implicating Cdc25A in cell cycle progression (Foster et al. 2001).

The role of cyclin D1 and p21 in estrogen-induced mitogenesis

As previously identified, modulation of cyclin D1 protein levels is an important early event in breast cancer cell cycle progression, such that a loss of cyclin D1 by treatment with antiestrogens, antisense oligomers or microinjection of antibodies to cyclin D1 (Baldin et al. 1993) is sufficient to induce cell cycle arrest. Increases in cyclin D1 levels are a rapid event following rescue of antiestrogen arrested cells with estrogen and the downstream events that follow include activation of cyclin D1-Cdk4 and phosphorylation of pRb (Foster and Wimalasena 1996; Planas-Silva and Weinberg 1997; Prall et al. 1997). We have developed inducible cyclin D1 expressing MCF-7 cells to provide further insight into the potential role of cyclin D1 in estrogen action (Prall et al. 1998). Activation of cyclin D1 in these cells results in cell cycle re-entry from an antiestrogen arrested state, without any effects on c-Myc protein levels. The mechanism of cell cycle progression is otherwise similar to that seen following induction of c-Myc, including activation of cyclin E-Cdk2 via a loss of p21 association and subsequent phosphorylation of pRb. However, the use of a specific Cdk2 inhibitor does not attenuate the growth promoting effects of cyclin D1 expression to the same degree as it does on c-Myc induced cell cycle entry. This suggests that cyclin D1 mediated cell cycle re-entry is less dependent on cyclin E-Cdk2 activation (despite a significant increase in cyclin E-Cdk2 activation following induction of cyclin D1) but can induce pocket protein phosphorylation via changes in other mechanisms, likely an increase in cyclin D1-Cdk4 activity.

The activation of cyclin E-Cdk2 complexes following either estrogen exposure or induction of c-Myc or cyclin D1 requires a decreased association of cyclin E-Cdk2 with p21. The mechanisms involved in these changes in p21 association and inhibitory potential have recently been clarified (Prall et al. 2001). It is now clear that the total cellular protein pool of p21 is not the major factor determining the inhibitory activity of p21, but rather, the decrease in p21 available to bind cyclin E-Cdk2 following estrogen stimulation of MCF-7 cells occurs as a result of a decrease in newly synthesised p21 (Prall et al. 2001). Newly synthesised p21 appears to have a greater inhibitory activity then pre-existing p21, which has been incorporated into multimeric complexes (Prall et al. 2001). Coupled with the loss of newly synthesised p21 due to estrogen-mediated transcriptional inhibition, increases in the synthesis of cyclin D1 are an important component of activation of cyclin E-Cdk2 complexes. However, an increase in cyclin D1 alone is not sufficient to restore full activation of cyclin E-Cdk2 following growth arrest, unless cyclin D1 levels are elevated to approximately 2-fold the level induced by maximally stimulatory concentrations of estrogen. Therefore, in breast cancer cells the estrogen-induced increases in cyclin E-Cdk2 that contribute to cell

cycle re-entry occur via two distinct mechanisms. These are an increase in cyclin D1 synthesis and a decrease in the production of newly synthesised, 'active' p21 (Prall et al. 2001). The mechanism involved in the repression of p21 following estrogen induced proliferation may include transcriptional repression by c-Myc (Coller et al. 2000; Gartel et al. 2001).

Figure 3: A model of estrogen effects on the cell cycle machinery that result in cell cycle progression.

CONCLUSIONS

Recent work by this and other laboratories has provided a greater depth of understanding of the molecular mechanisms that mediate the anti-proliferative action of antiestrogens and the mitogenic properties of estrogen in breast cancer cells. We now know that the pure estrogen antagonist, ICI 182780 can elicit an anti-proliferative effect on cycling

cells that are in early to mid G1 phase of the cell cycle, as a sequence of events that include an acute decrease in c-Myc, a subsequent decline in cyclin D1 and consequent loss of cyclin D1-Cdk4 complexes. This results in re-distribution of p21 from cyclin D1-Cdk4/6 complexes to cyclin E-Cdk2 complexes, inhibition of cyclin E-Cdk2, hypophosphorylation of the pocket proteins and ultimately growth arrest in a state with characteristics of quiescence (Carroll et al. 2000). Figure 2 presents our current model of antiestrogen-induced growth arrest in breast cancer cells. Whether the growth arrest of cells in G0 is common to all antiestrogens or is specific to pure antiestrogens in the focus of ongoing studies. Also, the question of whether the state of growth arrest of a population of cells following antiestrogen treatment influences their subsequent sensitivity to mitogenic re-stimulation is also being investigated.

Estrogen induced mitogenesis from this arrested state not surprisingly involves a similar subset of cell cycle components including c-Myc which is rapidly up-regulated and on its own is sufficient to induce cell cycle re-entry (Prall et al. 1998). The subsequent up-regulation of cyclin D1 is also a relatively early event. This increase in cyclin D1 is also sufficient to induce cell cycle re-entry. However, an ectopic increase in c-Myc does not influence cyclin D1 levels in our experimental paradigm and inducible cyclin D1 expression does not directly affect c-Myc levels. This suggests that the increase in both c-Myc and cyclin D1 following estrogen stimulation occur via independent pathways (see Figure 3 for our current model of estrogen stimulation of breast cancer cell proliferation). These pathways converge on cyclin E-Cdk2 activation, which is now known to result primarily from an increase in cyclin D1 synthesis and a decrease in newly synthesised p21.

It is clear from the data summarised in this review that there is much overlap in the pathways that drive estrogen mitogenesis and mediate growth arrest by antiestrogens. Positive or negative fluctuations in c-Myc protein levels (by estrogens and antiestrogens, respectively) is sufficient to induce downstream events that propel a breast cancer cell through one round of the cell cycle or initiate the events that culminate in growth arrest. This fluctuation in c-Myc converges on changes in cyclin E-Cdk2 activity, which is influenced by changes in p21 association. The appropriate modulation of cyclin E-Cdk2 activity converges on pocket protein phosphorylation and dictates the state of cell cycle progression. This significant overlap between pathways has allowed clarification of the important protagonists in the complex functioning of the cell cycle in breast cancer cells.

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Figure 1

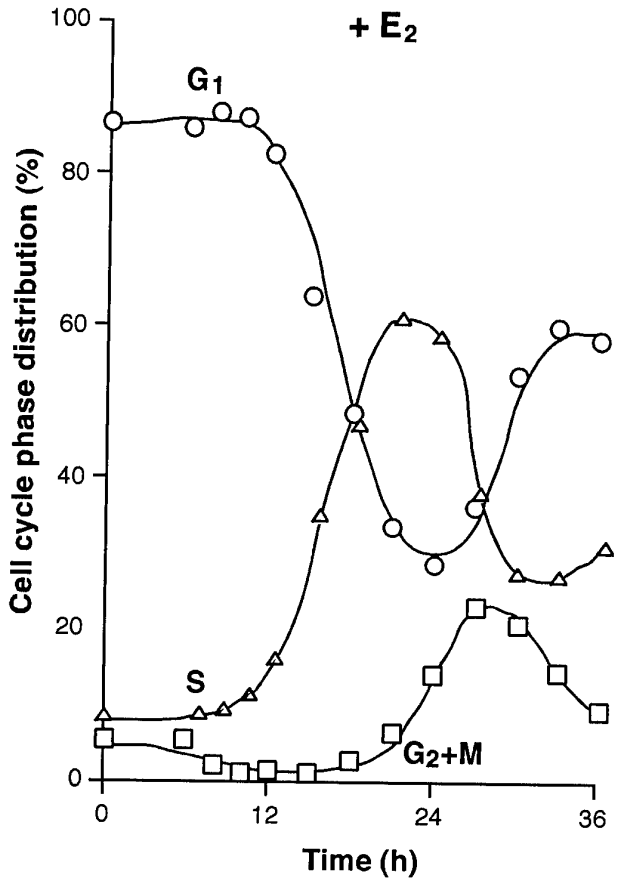
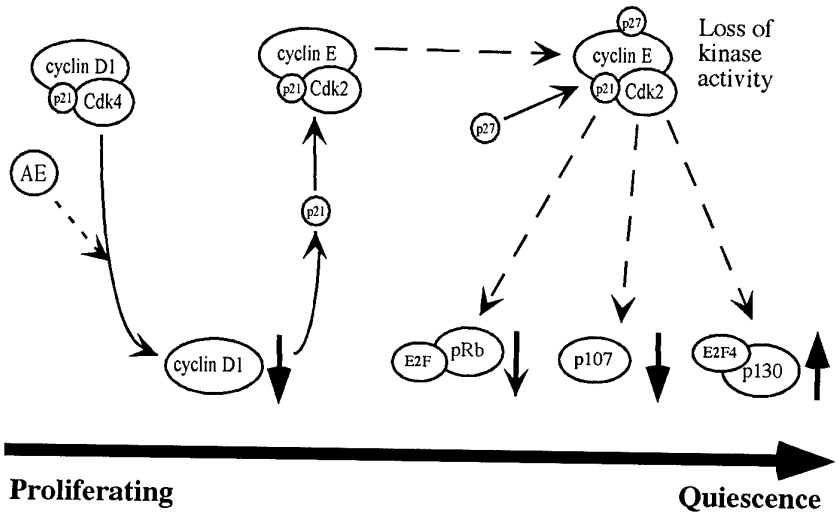
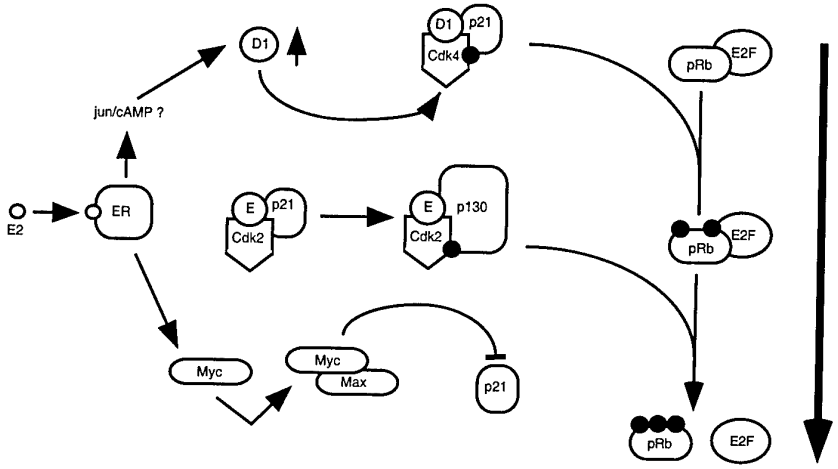

+ E2
G1
S
G2+M
Cell cycle phase distribution (%)
100
80
60
40
20
0
0
12
24
36
Time (h)

Figure 2


cyclin D1
p21
Cdk4
AE
cyclin D1
cyclin E
p21
Cdk2
p21
p27
cyclin E
p21
Cdk2
p27
Loss of
kinase
activity
E2F
pRb
p107
E2F4
p130
Proliferating
Quiescence


D1
D1
p21
Cdk4
pRb
E2F
jun/cAMP ?
E2
ER
E
p21
Cdk2
E
p130
Cdk2
pRb
E2F
Myc
Myc
Max
p21
pRb
E2F