Tumor-Specific Low Molecular Weight Forms of Cyclin E Induce Genomic Instability and Resistance to p21, p27, and Antiestrogens in Breast Cancer


ABSTRACT

The deregulated expression of cyclin E as measured by the overexpression of its low molecular weight (LMW) isoforms is a powerful predictor of poor outcome in patients with breast cancer. The mechanism by which these LMW forms give tumor cells a growth advantage is not known and is the subject of this article. In this article, we provide the pathological mechanisms of how these LMW forms are involved in disease progression. Specifically, we show that overexpression of the LMW forms of cyclin E but not the full-length form in MCF-7 results in (a) their hyperactivation because of increased affinity for cdk2 and resistance to inhibition by the cyclin-dependent kinase inhibitors p21 and p27, (b) resistance to the growth inhibiting effects of antiestrogens, and (c) chromosomal instability. Lastly, tumors from breast cancer patients overexpressing the LMW forms of cyclin E provide a molecular mechanism for the poor clinical outcome observed in breast cancer patients harboring tumors expressing high levels of the LMW forms of cyclin E. These properties of the LMW forms suggest that they are not just surrogate markers of poor outcome but bona fide mediators of aggressive disease and potential therapeutic targets for patients whose tumors overexpress these forms.

INTRODUCTION

The cell cycle is, by definition, deregulated in human cancer cells (1). In particular, the transition from G₀/S-phase is the most commonly noted cell cycle abnormality in tumors (1). In breast cancer, cyclin E, a G₁ cyclin that plays a key role in G₁-S transition, is overexpressed through gene amplification, mRNA stabilization, and a hazard ratio of 1.8 for positive nodes.

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Note: This work is dedicated to the memory of Shahrazad Soltani.

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prognostic significance of cyclin E was particularly striking in patients with stage I disease, where only the 12 patients (of 114) whose tumors overexpressed cyclin E died of disease with a median time to death of 4.1 years. Using a Cox proportional hazards model, total (i.e., LMW + full length) cyclin E levels remained the most important predictor of death from breast cancer with a hazard ratio of 13.3 compared with a hazard ratio of 2.1 for high levels of LMW cyclin E and a hazard ratio of 1.8 for positive nodes.

The mechanism by which overexpression of cyclin E and the appearance of the LMW forms are prognostic of poor clinical outcome is unclear. We do know that only tumor cells have the machinery to process cyclin E into its LMW forms (9). This tumor-specific processing is catalyzed by an elastase-like protease that cleaves the full-length cyclin, EL1 (Mₛ 50,000), at two distinct sites in the NH₂ terminus, generating five isoforms that differ by their molecular weights. EL2 (Mₛ 45,000) and EL3 (Mₛ 44,000) are generated by cleavage at N40–N45, whereas EL5 (Mₛ 35,000) and EL6 (Mₛ 33,000) are generated by cleavage at D70. Isoform EL4 (Mₛ 40,000) is generated by using an alternate start codon at methionine 46. In vitro, we have discovered that introduction of these LMW forms into immortalized, nontumorigenic breast epithelial cell lines results in alterations in the G₀-S checkpoint (10, 11).
The LMW forms of cyclin E lack varying amounts of the NH2-terminal of the native molecule, which are generated by proteolytic cleavage, mediated by elastase, a protease that itself has been implicated in breast cancer metastasis. Hence, these LMW forms of cyclin E may be merely a surrogate marker of poor prognosis and not directly related to disease progression and metastasis. In this study, we have directly addressed the biological role of the LMW forms of cyclin E using a model system overexpressing the native full-length or each of the LMW forms in the estrogen-responsive, antiestrogen-sensitive breast tumor cell line MCF-7. We show that MCF-7-overexpressing the LMW forms and not the full-length form have higher cyclin E-associated kinase activity and are resistant to inhibition by p21 and p27 in a cellular context where p53 and p21 are significantly induced. These LMW-overexpressing cells are also resistant to the growth inhibitory activity of antiestrogens mediated through the resistance of these cells to p21. We also show that among patients with ER-positive tumors that express high levels of cyclin E, disease-specific survival (DSS) rate is not improved by treatment with antiestrogens. Lastly, the LMW forms of cyclin E also induce chromosomal abnormalities, and patients with high levels of cyclin E are more prone to have polyploid tumors than those with low levels of cyclin E. Collectively, the data presented in this report provide evidence that the processing of full-length cyclin E into its LMW forms may be one of the key early events leading to a more aggressive and lethal phenotype of breast cancer.

MATERIALS AND METHODS

Cell Culture and Establishment of Stable Cell Lines. The cell lines derived from MCF-7 human breast cancer cells were cultured as described elsewhere (24). Empty vector pcDNA 4.0 (Invitrogen, Carlsbad, CA), cyclin EL1-FLAG, and cyclin E-FLAG constructs Trunk 1 (T1) and Trunk2 (T2), described elsewhere (5), were transfected into MCF-7 cells by using FuGENE (Invitrogen). After transfection, the cells were placed in medium containing 80 μg/ml zeocin (Invitrogen), and individual colonies were isolated and propagated. Positive transfecants were identified by their immunoreactivity on Western blots (40 μg of protein/lane) probed with monoclonal anti-FLAG (M2; Sigma, St. Louis, MO) and monoclonal anti-cyclin E (HE-12; Santa Cruz Biotechnology, Santa Cruz, CA). For experiments investigating the effect of ICI 182,780 on the estrogen- and antiestrogen-induced expression of each of the LMW forms in the estrogen-responsive, antiestrogen-sensitive breast tumor cell line MCF-7, we show that MCF-7-overexpressing the LMW forms and not the full-length form have higher cyclin E-associated kinase activity and are resistant to inhibition by p21 and p27 in a cellular context where p53 and p21 are significantly induced. These LMW-overexpressing cells are also resistant to the growth inhibitory activity of antiestrogens mediated through the resistance of these cells to p21. We also show that among patients with ER-positive tumors that express high levels of cyclin E, disease-specific survival (DSS) rate is not improved by treatment with antiestrogens. Lastly, the LMW forms of cyclin E also induce chromosomal abnormalities, and patients with high levels of cyclin E are more prone to have polyploid tumors than those with low levels of cyclin E. Collectively, the data presented in this report provide evidence that the processing of full-length cyclin E into its LMW forms may be one of the key early events leading to a more aggressive and lethal phenotype of breast cancer.

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Western Blot Analysis. Cell lysates were prepared and subjected to Western blot analysis as described previously (25). Briefly, 50 μg of protein were subjected to electrophoresis on SDS-PAGE and transferred to Immobilon P, blocked, and incubated with the indicated antibodies as already described.

Culture Conditions for S9 Cells, Virus Stocks, and Infections. Recombinant baculoviruses expressing CDK2, cyclin EL, T1, and T2 were produced from a pVL1392-expressing vector using a Baculovirus Gold kit and S9 insect cells as suggested by the manufacturers (PharMingen, San Diego, CA). In coinfection experiments, the two desired viruses were mixed together at the same titer andcoinfected with the S9 cells at a multiplicity of infection of 0.7 each. For controls, S9 cells were infected with the individual recombinant virus at multiplicity of infection of 1.4. For in vitro binding assays, purified HA-p21 (obtained from J. Wade Harper) was added to 300 μg of total cell lysate in a series of nine different concentrations (0–500 nm) that were incubated at 4°C for 30 min followed by immunoprecipitation with anti-CDK2 antibody and coupling to Sepharose protein A beads. The HA-p21 complexes were then subjected to Western blot analysis with either anti-CDK2 or anti-p21 monoclonal antibodies diluted at 1 μg/ml in BLOTTO.

Cell Cycle Analysis. Cells were pelleted and resuspended in 1.5 ml of PBS, then fixed in 3.5 ml of 95% ethanol overnight at −20°C. After being washed, the pellets were resuspended in a solution of PBS containing 10 μg/ml propidium iodide, 20 μg/ml RNase A, 0.5% Tween 20, and 0.5% BSA and incubated at 37°C for 30 min. The profiles of cells in the G1, G2, S, and G0-M phases of the cell cycle were analyzed at the M. D. Anderson Cancer Center Cytometry Core Facility on a FACScalibur machine equipped with Cellquest or ModFit software.

Cytogenetic Analysis. Exponentially growing MCF-7 control and E-EL and LMW cyclin E cells were fed 24 h before harvesting for chromosome preparation. Cytological preparations were made following the standard procedures; briefly, cells were exposed to Colcemid (0.04 μg/ml) for 1 h, subjected to hypotonic treatment (0.075 M KCl for 20–25 min at room temperature), and fixation in a mixture of methanol and acetic acid (3:1 by volume; Ref. 26). Slides were stained in Giemsa and examined blindly for structural and numerical abnormalities. These slides were decoded only after the entire scoring of aberrations was completed. From each sample, a minimum of 35 metaphase spreads were analyzed, and representative spreads were captured using a Genetiscan imaging system. One slide from each sample was also G-banded to confirm the identity of the MCF-7 cell line based on the presence of characteristic marker chromosomes (data not shown).

Patient Characteristics. The median age of the study population was 64 years (range, 29–95 years). The majority (92%) had stage I, II, or III breast cancer. Sixty-seven percent of the total study population and 50% of patients with stage I disease had received adjuvant therapy. After a median follow-up interval of 6.4 years (range, 1.5–11.0 years), 121 of the 395 patients (30.6%) had died of breast cancer. The 5- and 10-year DSS rates for the entire cohort of patients were 71% and 62%, respectively. Overall survival rates were 66% at 5 years and 47% at 10 years. The results of the univariate analysis of DSS and overall survival according to clinical factors and biological markers were
As expected, there was a significant association between clinical stage and outcome.

Statistical Methods. Results shown as mean ± SD were compared by ANOVA with a significant level of \( P < 0.05 \) and \( P < 0.01 \). The nonparametric Spearman correlation was used to quantify the relationship between protein expression of Fig. 1B. The \( P \) (two-tailed) with a 95% confidence interval was calculated with a significant level of \( P < 0.05 \). DSS was calculated from the date of surgical excision of the primary tumor to the date of death from breast cancer. Surviving patients and patients who died of causes other than breast cancer were censored at the time of last follow-up or death. DSS curves were computed using the method of Kaplan and Meier. Univariate analyses of DSS by cyclin E LMW expression and other factors (ploidy or treatment) were performed using a two-sided log-rank test.

RESULTS

Expression of the Full-Length (EL1) and LMW Forms of Cyclin E in Breast Cancer Cells. As a first step to understanding the biological properties of the LMW forms of cyclin E in breast cancer, we generated a model system using stably transfected human mammary epithelial MCF-7 cells. The parental cells are derived from a human breast adenocarcinoma. They are ER positive, express the tumor-specific LMW forms at low levels (4), and are a model for estrogen-responsive, antiestrogen-sensitive breast tumors. MCF-7 cells were transfected with either the vector backbone (4.0) alone or one of three FLAG-tagged cyclin E constructs termed EL1, T1, or T2.

Fig. 1. Expression of the EL1 and low molecular weight of cyclin E in breast cancer cells. A, schematic representation of FLAG-tagged cyclin E constructs. The three cyclin E constructs were engineered with a 3'-FLAG sequence represented by \( \text{FLAG} \). B, empty vector (4.0), cyclin EL1-FLAG, and cyclin E-FLAG constructs T1 and T2 were transfected into MCF-7 cells by using FuGENE. The transfected cells were selected using 80 \( \mu \)g/ml zeocin. Positive transfectants were identified by their immunoreactivity on Western blots (50 \( \mu \)g of protein/lane) probed with monoclonal anti-FLAG (M2; Sigma) and monoclonal anti-cyclin E (HE-12; Santa Cruz Biotechnology). C, MCF-7 stable transfectants were subjected to histone H1 kinase analysis. Equal amounts of protein (250 \( \mu \)g) from cell lysates were prepared, immunoprecipitated with anti-FLAG or anti-cyclin E antibody coupled to protein A beads, and incubated in the kinase buffer using histone H1 as substrate. Histone H1-associated kinase activities were quantitated by Cerenkov counting (number of independent clones \( n \) for 4.0, \( n = 2 \) for EL1, \( n = 7 \) for T1, and \( n = 7 \) for T2 for cyclin E kinase activity; \( n = 2 \) for EL1, \( n = 3 \) for T1, and \( n = 3 \) for T2 for FLAG kinase activity; **, \( P < 0.01 \) and *, \( P < 0.05 \) versus empty vector-only cells for cyclin E kinase activity and versus EL1 cells for FLAG kinase activity).
The EL1 construct codes for the full-length M, 50,000 form of cyclin E (termed EL1; Fig. 1A), the T1 construct codes for the M, 45,000 and M, 44,000 forms (termed EL2 and EL3), and the T2 construct codes for the M, 35,000 and M, 33,000 forms (termed EL5 and EL6). Hence, the forms generated by the T1 and T2 constructs correspond to the endogenous LMW forms that we termed EL2,3 and EL5,6, respectively (5). EL2,3 (i.e., T1) and EL5,6 (i.e., T2) are expressed in ER-negative breast cancer cells generated after cleavage by an elastase-like protease at the amino acid 40–45 and A69-D70 sites, respectively (5). Several clones representing each of the transfected vectors were selected, and their expression of cyclin E was assessed by Western blot analysis using anti-FLAG and anti-cyclin E antibodies. These experiments revealed different levels of expression of each of the cyclin E isoforms (Fig. 1B), and the level of cyclin E expression in each clone corresponded to its kinase activity: T1 and T2 clones showed 3–5-fold higher FLAG-associated kinase activity and 2-fold higher cyclin E-associated kinase activity than EL1 clones (Fig. 1C). Moreover, overexpression of cyclin E resulted in increases in p53 (p = 0.0039) and p21 (p = 0.0446) expression levels, whereas p27 (p = 0.3423) and Cdk2 (p = 0.2584) levels remained relatively unchanged when compared with those in empty vector-only cells.

LMW-Associated Kinase Activity Is Resistant to p21 and p27 Inhibition. We next examined the biochemical interactions of the LMW forms of cyclin E with its kinase partner CDK2 and the CDK2 inhibitors (CKIs) p21 and p27 in three clones overexpressing cyclin EL1 or one of the LMW forms (Fig. 2). These clones were chosen as they overexpressed cyclin E, T1, and T2 at similar levels, (i.e., 4–6-, and 5-fold higher levels, respectively, than the endogenous cyclin E protein), and this overexpression coincided with p53 activation and parallel p21 induction. In the EL1-overexpressing cells, both p53 and p21 were induced by 2-fold over the parental or vector-only controls, whereas in the T1 and T2 clones, p53 and p21 were induced by 2.5 and 5 times, respectively, over the controls (Fig. 2A). Despite the high levels of p21 in cyclin E-transfected cells, T1- and T2-overexpressing cells manifested 4–5-fold greater FLAG-associated kinase activity than the EL1 form, and ~3-fold greater cyclin E-associated kinase activity and CDK2 kinase activity compared with the empty vector-only cells (Fig. 2B). Hence, although the levels of cyclin E overexpression between EL1 and LMW forms were similar (Fig. 2A; i.e., all ~5-fold higher than endogenous cyclin E), the LMW-overexpressing clones had higher FLAG, cyclin E, and Cdk2 kinase activity than the EL1 clones (Fig. 2B), even in the presence of high levels of p21.

To explore the mechanism for the differences in kinase activities between the EL1 and the LMW forms, we analyzed the composition of the cyclin E immune complexes in each of the clones (Fig. 2C). For these experiments, cyclin E was immunoprecipitated using a polyclonal antibody as described elsewhere (5, 7) followed by Western blot analysis with the indicated monoclonal antibodies (Fig. 2C). Our results revealed that the 5-fold increase in cyclin E level was accompanied by increased binding of p21 and p27 to cyclin E complexes. Specifically, the EL1 complexes contained 7 times more p21 than the controls, whereas the T1 and T2 complexes contained 15 times more p21 than the controls. Similarly, the extent of binding of p27 to the T1 and T2 complexes was much higher than that to EL1 cells or controls (Fig. 2C), despite there being no increase in the basal levels of p27 (Fig. 2A). This binding of p21 and p27 resulted in decreased activity of the cyclin EL1 complexes. In T1 and T2 complexes, however, even with high levels of p21 and p27 binding, the cyclin E kinase was 2-fold greater than that of the EL1-associated kinase, suggesting that the cyclin E kinase activities associated with the LMW form complexes are more resistant to p21 and p27 inhibition than the cyclin E kinase activity associated with the full-length cyclin E.

Next, we examined the composition of the CDK2 complexes in the cyclin E clones and found that 4 times more p21 and p27 was bound to CDK2 in T1 and T2 clones than in EL1 clones (Fig. 2D). This increased binding was due in part to higher CDK2 levels in the T1 and T2 clones than in the EL1 clones (Fig. 2A). We also found greater CDK2 kinase activity in the T1 and T2 clones than in the EL1 clones, despite increased binding of p21 and p27 to T1- and T2-Cdk2 complexes (Fig. 2, B and D). These results suggest that the resistance of the LMW cyclin E forms to inhibition by p21 and p27 is not because of lack of interaction between the LMW forms and these inhibitors.

Because the levels of CDK2 were 2-fold higher in the T1 and T2 clones than in the EL1 clones (Fig. 2A), the greater kinase activity associated with both CDK2 and cyclin E in the T1 and T2 clones could reflect the higher CDK2 levels and/or the greater affinity of T1 and T2 than of EL1 for CDK2 (Fig. 2, C and D). Hence, the increased cyclin E kinase activity in LMW-overexpressing cells may have been due in part to increased affinity of T1 and T2 for Cdk2 and in part to their decreased sensitivity to p21 and p27 inhibition.

To further address this question, we examined the kinase activity associated with p21 and p27 as well as the immune complexes associated with each CKI (Fig. 2, E and F). For this analysis, the CKIs were immunoprecipitated with polyclonal antibodies to p21 or p27 and then subjected to both kinase assays and Western blot analysis with the indicated antibodies (Fig. 2, E–H). The results reveal that both p21- and p27-associated kinase activities were significantly greater in the T1 and T2 clones than in the EL1 clones. Specifically, the kinase activity associated with p21 in T1 and T2 clones was 8-fold greater than the kinase activity in EL1 clones, parental or vector-only controls (Fig. 2, E and F). Similarly, the p27-associated kinase activity was 3-fold greater in T1 and T2 clones than in EL1 clones or controls (Fig. 2, G and H). Although the levels of p21 were induced in T1 and T2 clones, the levels of p27 remained unchanged in these clones as compared with EL1 clones or controls (Fig. 2A), suggesting that the increased kinase activity associated with the CKIs was not due purely to increased levels of these inhibitors in T1 and T2 clones. These results reveal that the LMW forms of cyclin E are resistant to p21 and p27 inhibition, whereas EL1 remains sensitive, suggesting that p21 and p27 could be assembly factors for T1 and T2 forms of cyclin E, similar to their role in this capacity when complexed with CDK4 (27, 28).

LMW Forms of Cyclin E Are Resistant to Inhibition by Purified p21. To confirm the increased resistance of LMW forms of cyclin E to CKI inhibitors, we used the baculovirus expression system, which allows precise control of the expression (i.e., amount) of the different proteins implicated in the activity of the cyclin E/CDK2 complexes. In these experiments (Fig. 3), the insects cells were initially coinfected with the three different cyclin E vectors and CDK2. The homogenates were incubated in presence of increasing concentration of purified HA-p21 (0–500 nM), and the CDK2 complexes were then affinity purified and subjected to kinase assays using GST-Rb as substrate. Additionally, the binding of p21 to cyclin E/CDK2 complexes was also assessed. These analyses revealed that the concentration of HA-p21 required to inhibit the cyclin E/CDK2 activity by 50% (i.e., IC50) was 50 nM. However the IC50 of p21 required to inhibit the LMW forms of cyclin E was 3–5-fold higher than the one inhibiting the full length. Furthermore, whether the relative or the normalized CDK2-associated GST-Rb kinase activity values were used, the IC50s were identical, suggesting that the resistance of the LMW forms of cyclin E to p21 is not because of higher basal activity of these forms as compared with the full length. When we examined if the binding of the p21 to the LMW forms was in anyway compromised to account for the resistance of these forms to inhibition, we found that the LMW forms of cyclin E/CDK2 complexes bind to p21 at concentrations where these inhibitors do not

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Fig. 2. Cyclin E/Cdk2-LMW-associated kinase activities are more resistant to p21 and p27 inhibition than the cyclin E/Cdk2-EL1-associated kinase activity in MCF-7 cells. A, three stable clones overexpressing cyclin EL1, T1, or T2 at similar levels were subjected to Western blot analysis (50 μg of cell lysate) with the indicated antibodies. B, FLAG, cyclin E, and Cdk2 kinase assays were performed on the same cell extracts by immunoprecipitating equal amounts of cell lysate (250 μg) with monoclonal antibodies to FLAG and cyclin E or polyclonal antibody to Cdk2 coupled to protein A or G beads, using histone H1 as substrate. C and D, immune complex formation with cyclin E (C) or Cdk2 (D) was assessed for the same samples by subjecting the anti-cyclin E and anti-Cdk2 immunoprecipitates to Western blot analysis using the same antibodies as for the kinase assays. Immune complex formation with p21 (E) or with p27 (G) was assessed for the same samples by subjecting the anti-p21 and anti-p27 immunoprecipitates to Western blot analysis using the same antibodies as for the kinase assays. p21- and p27-kinase assays were performed on the same cell extracts by immunoprecipitating equal amounts of cell lysate (250 μg) with polyclonal antibodies to p21 and p27 coupled to protein A beads, using histone H1 as substrate. Histone H1-associated kinase activities were quantitated by Cerenkov counting (mean ± SD for two independent experiments).
mediate any inhibition (i.e., Fig. 3, Lanes 4–6). In fact, there was no significant difference in the binding of p21 to the full-length versus LMW/CDK2 complexes, although the full-length cyclin E is much more sensitive to the inhibition by p21 than the LMW forms. Similar results were obtained using HA-p27 (data not shown) confirming our in vivo results.

**The LMW Forms of Cyclin E Are More Resistant to Antiestrogen-Induced G₁ Arrest Than the Full-Length Form of Cyclin E.** One of the biological consequences of cells overexpressing the LMW forms is that they may become resistant to the growth inhibitory effects of drugs whose mechanism of growth inhibition is through induction of p21 and/or p27 resulting in CDK2 inhibition and subsequent G₁ arrest. One such class of agents is the antiestrogens that are commonly used for the treatment of estrogen receptor positive breast cancer. Because antiestrogen-induced G₁ arrest in MCF-7 cells is mediated through the CKIs p21 and p27 and through a decrease in cyclin E/CDK2 activity (29, 30), we questioned what effect, if any, overexpression of cyclin E would have on antiestrogen sensitivity. Furthermore, because p21 is induced between 2- and 5-fold in cyclin E-overexpressing MCF-7 cells (Fig. 2A), the role of p21 in mediating antiestrogen-induced G₁ arrest in these cells can be readily examined. The pure antiestrogen ICI 182.780 was used to discern the antiestrogenic responses of parental and of EL1- and LMW-overexpressing cells (Fig. 4). For these studies, MCF-7 clones were plated at low density and incubated in the presence of 17β-estradiol for 48 h to generate an asynchronous, proliferating population. The 17β-estradiol-containing medium was then removed, and fresh medium containing 10 nM ICI 182.780 was added to block ER signaling, and cells were collected at the indicated posttreatment time intervals and analyzed for cell cycle distribution (Fig. 4A). The results reveal that ICI 182.780 caused a gradual decrease in the percentage in S phase of cells transfected with empty vector beginning after 12 h of treatment and reaching a minimum of 10% S-phase cells at 48 h. In contrast, cells transfected with cyclin EL1 or its LMW isoforms were much more resistant to the growth inhibitory effects of ICI 182.780 such that at the end of 48 h of treatment, 31, 40, and 48%, respectively, of EL1-, T1-, and T2-transfected MCF-7 cells were still in S phase. These results suggest that cyclin E overexpression can mediate partial resistance to ICI 182.780-induced G₁ arrest. Moreover, cells transfected with T1 or T2 appeared more refractory to ICI 182.780-induced G₁ arrest than cells transfected with the full-length form. In the ER-negative breast cancer cell line MDA-MB-436, ICI 182.780 had no effect on cell cycle phase distribution over 48 h. In empty vector-only cells, the drop in the S-phase cell fraction was preceded by peak induction of p21 at 12 h and a slight increase in p27 protein level, whereas in cyclin E-overexpressing cells, p21 and p27 levels were not modulated by ICI 182.780 treatment (Fig. 4B).

To better quantify the difference in sensitivity to ICI 182.780-induced G₁ arrest in EL1 and LMW clones, we analyzed the effects of increasing concentrations of ICI 182.780 over several time intervals (Fig. 4C). In these experiments, the 17β-estradiol-containing medium was removed, fresh medium containing ICI 182.780 at various concentrations was added, and cells were collected at 24, 48, and 72 h after treatment and subjected to flow cytometric analysis. As shown in Fig. 4C, the EL1-overexpressing cells revealed partial resistance at 24 h, becoming less pronounced at 48 h, and revealed no resistance by 72 h. In contrast, T1- and T2-overexpressing cells were significantly more resistant to ICI 182.780-induced G₁ arrest than EL1 and empty vector-only cells at all three time points examined.

**Resistance of LMW-Overexpressing Cells to ICI 182.780-Induced G₁ Arrest Is Caused by Decreased Sensitivity to p21- and p27-Mediated G₁ Arrest.** The increased resistance of T1 and T2 to ICI 182.780-induced G₁ arrest could be because of the increased resistance of these LMW-overexpressing cells to p21 and p27 inhibition. To test this hypothesis, we examined the expression and composition of the immune complexes of cyclin E after ICI 182.780...
Low molecular weight-overexpressing MCF-7 cells are more resistant to ICI 182,780-induced G1 arrest than EL1-overexpressing MCF-7 cells. A, exponentially proliferating MCF-7 cells were treated with ICI 182,780 (10 nM) or ethanol vehicle, and cells were harvested at the time points indicated for flow cytometric analysis of propidium iodide-stained nuclei. The numbers given are mean values ± SD (number of independent clones n = 3 for 4.0, n = 2 for EL1, n = 6 for T1, n = 3 for T2; **, P < 0.01 versus empty vector-only cells). B, the three stable clones overexpressing EL1, T1, or T2 at similar levels, previously selected, were subjected to the same experiment as in A. Cell extracts were collected at the indicated time points and were subjected to Western blot analysis (50 μg of cell lysates) with the indicated antibodies. Similar results were obtained with at least one additional clone for each vector.

Consequences of EL1 and LMW Overexpression on Proliferation and Ploidy of Breast Cancer Cells. Next, we addressed whether cyclin E overexpression in MCF-7 cells would have an impact on proliferation or DNA index of the cells (Fig. 6). Initially, we measured the proportion of cells in the different cell cycle phases by flow cytometry and found that the cyclin E-positive clones had lower G1 and higher S-phase cell populations than cyclin E-negative clones. In control MCF-7 cells (empty vector-only clones), the percentage of cells in S phase was 22.5 ± 8.2%, whereas in cyclin E-overexpressing cells, the percentages of cells in S phase were 30.7 ± 1.1% for EL1, 31.8 ± 6.5% for T1, and 26.2 ± 3.6% for T2 (Fig. 6A). The flow cytometry analysis also showed that cyclin E overexpressing cells had a 4–6-fold increase in the percentage of cells with a polyploid DNA contents when compared with empty vector cells (Table 2, P < 0.01). These changes in cell cycle distribution and ploidy correlated with the level of cyclin E overexpression. Despite these increases in the S-phase population, no decrease in doubling time or increase in plating efficiency was observed in cyclin E-overexpressing clones (data not shown). Microscopic examination of cyclin E-overexpressing colonies in the colony-forming assay, however, showed greater frequency of giant polyploid cells than in empty vector-only cells (Fig. 6B), suggesting that nuclear DNA content is disrupted in the T1 and T2 clones, consistent with the ploidy data from these cells (Table 1).

Overexpression of Cyclin E in MCF7 Cells Induces Genomic Instability. A relevant feature of the pathobiology of the overexpression of the LMW forms of cyclin E could be their ability to induce chromosomal instability in human breast epithelial cells. Because chromosomal abnormalities are a feature of all solid tumors and increase with progression of the disease process, we questioned if the overexpression of the LMW forms of cyclin E could result in genomic instability. To this end, we undertook a detailed chromosome analysis of the EL-, T1-, and T2-overexpressing MCF-7 clones. Compared with vector transfected and untransfected parental cell lines, transfection of T1 and T2 constructs induced a dramatic 7- and 4-fold increase in the number of chromosomal aberrations/cell, respectively (Fig. 7). These aberrations included multiple chromosomal fragments, breaks, and subtelomeric chromatid breaks, resulting in telomeric associations (Fig. 7B–E). In addition, a 3–4-fold increase in the number of polyploid and tetraploid cells were observed in T1 and T2 transfectants, most likely a result of chromosomal endoreduplication (Fig. 7B–D). Although chromosome aberrations were also present in cells transfected with EL1-cyclin E, the total number of structural aberrations was significantly reduced compared with those observed in T1- and T2-transfected cell lines (Table 2). These results suggest that overexpression of the LMW forms of cyclin E induces genomic instability by promoting aneuploidy and the formation of structural chromosomal lesions.
Presence of LMW Forms of Cyclin E in Tumors from Breast Cancer Patients Correlates with Polyploidy and Lack of Response to Antiestrogen Therapy. The ability of the LMW forms of cyclin E to give tumor cells a growth advantage as assessed by increased genomic instability and resistance to CKIs and antiestrogens have been shown with MCF-7 as a model system (Figs. 1–7). The question still remains, however, if the overexpression of the LMW forms of cyclin E in tumors of breast cancer patients correlates with polyploidy and/or resistance to antiestrogens—two parameters, which we assessed in our model system. For this purpose, we examined the relationship between ploidy and cyclin E protein levels among 331 stage I–III breast cancer patients and found a significant correlation, \( P = 0.0003 \).

Twenty-one of 105 patients with diploid tumors had high levels of total cyclin E (i.e., full-length + LMW forms), compared with 69 of 226 patients with polyploidy tumors (\( P = 0.045 \), \( \chi^2 \) test). Further-

Fig. 5. Low molecular weight-overexpressing cells are resistant to ICI 182,780-induced G\(_1\) arrest. A, levels of p21, p27, Cdk2, and cyclin E in empty vector-only cells and the three cyclin E-overexpressing clones previously selected. Total lysates (250 \( \mu \)g) of cells treated with or without antiestrogen [10 nM ICI 182,780 (+) or ethanol vehicle (−)] and harvested at 48 h were separated by SDS-PAGE and analyzed by Western blot with the indicated antibodies. B, analysis of the stoichiometry of the cyclin E complexes by immunoprecipitation followed by Western blot with the indicated antibodies. The same cell lysates as in A (250 \( \mu \)g) were immunoprecipitated by using a monoclonal cyclin E antibody coupled to protein G beads, and the immune complexes were resolved by SDS-PAGE; this was followed by immunoblotting using the indicated antibodies or by kinase assay, using histone H1 as substrate. C, histogram showing the arbitrary values of the cyclin E kinase activity by the immune complex kinase assay 48 h after addition of ICI 182,780. Histone H1-associated kinase activities were quantitated by Cerenkov counting. Similar results were obtained with at least one additional clone for each vector.

Table 1 Percentage of polyploidy in vector and cyclin E-overexpressing MCF7 cells as determined by flow cytometry analysis

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<tr>
<th>MCF-7 clones</th>
<th>% polyploidy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector</td>
<td>2.6 ± 3.2</td>
</tr>
<tr>
<td>Cyclin EL</td>
<td>10.9 ± 3.0( ^a )</td>
</tr>
<tr>
<td>Cyclin E-T1</td>
<td>15.2 ± 4.9( ^a )</td>
</tr>
<tr>
<td>Cyclin E-T2</td>
<td>13.5 ± 2.8( ^a )</td>
</tr>
</tbody>
</table>

\( ^a P < 0.01 \) compared with empty vector cells.
more, of the 241 patients whose tumors expressed low levels of cyclin E, the DNA content of the tumors did not impact DSS rate; the outcomes of this subgroup were uniformly favorable (Fig. 8A). Among the remaining 90 patients whose tumors expressed high levels of cyclin E, on the other hand, the 5-year DSS rate was significantly lower among those with polyploid tumors ($P = 0.02$, log-rank test). At a longer follow-up interval, however, this difference in survival was lost ($P = 0.083$, log-rank test) because of late disease-related deaths among patients with diploid tumors (Fig. 8B). The survival data, therefore, suggest that diploid tumors expressing high levels of cyclin E have a more indolent course than polyploid tumors yet remain fully malignant, with survival approaching 0% at 8 years.

To examine if overexpression of the total cyclin E is associated with resistance to antiestrogen treatment, we investigated the impact of antiestrogens among 150 stage I–III ER+ patients who had high cyclin E. We found no difference in DSS among patients receiving antiestrogen treatment compared with those receiving adjuvant chemotherapy but no antiestrogens (Fig. 8C), suggesting that patients with high cyclin E-overexpressing tumors may be resistant to the effects of antiestrogen therapy. The data combined from these clinical studies provide additional support for the relevance of the LMW forms of cyclin E to human disease.

**DISCUSSION**

In this article, we describe the development of a breast cancer cell model system stably overexpressing full-length (EL1) or LMW (T1 and T2) forms of cyclin E in the estrogen-responsive, antiestrogen-sensitive breast tumor cell line MCF-7. Our results reveal that cyclin E overexpression induces p53 and p21 expression, resulting in increased binding of p21 and p27 to cyclin E, inactivating cyclin E and CDK2 activities in cells overexpressing the full-length form. Cells overexpressing the LMW forms of cyclin E, on the other hand, are resistant to these inhibitors and consequently are functionally hyperactive. The increased cyclin E kinase activity in T1- and T2-overexpressing cells results from increased affinity for CDK2 and resistance to inhibition by the CDK inhibitors p21 and p27. Furthermore, when both the full-length and LMW forms of cyclin E are coexpressed in cells in the insect expression system, p27 preferentially binds to the LMW forms and is unable to inhibit Cdk2 activity.

The resistance of the LMW forms of cyclin E to p21 and p27 inhibition is not because of lack of interaction between the LMW forms and these inhibitors. Our *in vitro* data revealed that despite p21 and p27 binding to LMW forms of cyclin E, CDK2 kinase activity is not inhibited. Sequence analysis of cyclin EL1 has shown that the NH$_2$ terminus region forms two tandem hairpin loop-type structures that may expose the critical residues cleaved by the elastase-like protease (5). The structure of cyclin A/Cdk2 bound to the NH$_2$ terminus of p27 has been previously described (32), and the region of cyclin A interacting with p27 is conserved and present in LMW forms of cyclin E (residues 139–145). Because activation of the kinase by cyclin binding induces conformational changes (33), the absence of the NH$_2$-terminal region in the LMW forms of cyclin E could remove negative interactions for CDK2 binding so that the LMW forms bind CDK2 more effectively than EL1. The absence of this region does not affect the binding of p21 or p27 inhibitors to the LMW forms but may affect interactions of the inhibitors with the kinase domain of the complex, preventing inhibition of the catalytic cleft of CDK2.

Cells overexpressing LMW forms of cyclin E accumulated in S phase without an accompanying increase in the doubling time, suggesting a defect in S-phase progression. A significant fraction of these cells became polyploid, and cytogenetic analysis revealed that numerous structural chromosome aberrations, including chromosome breaks, fragments, and fused chromosomes, were higher in MCF-7 cells expressing T1 and T2 than in cells expressing the EL1 construct. In addition, the frequency of poly/tetraploid metaphases also increased in T1 and T2 cells. The presence of endoreduplication in T1 and T2 expressing cells suggest that two rounds of chromosome

---

**Table 2** Chromosomal aberrations in control cell lines and cell lines overexpressing different forms of cyclin E

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of metaphases analyzed</th>
<th>Total % of metaphases with aberrations</th>
<th>No. of total aberrations per metaphase</th>
<th>No. of chromatid breaks/cell</th>
<th>No. of TA$^*$s/cell</th>
<th>% polyploid/tetraploid cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>85</td>
<td>11.0</td>
<td>1.09</td>
<td>1.01</td>
<td>0.08</td>
<td>9.4</td>
</tr>
<tr>
<td>Vector</td>
<td>87</td>
<td>20.7</td>
<td>0.72</td>
<td>0.47</td>
<td>0.25</td>
<td>6.9</td>
</tr>
<tr>
<td>T1 form cyclin E</td>
<td>88</td>
<td>35.2</td>
<td>6.7</td>
<td>6.07</td>
<td>0.6</td>
<td>28.4</td>
</tr>
<tr>
<td>T2 form cyclin E</td>
<td>90</td>
<td>44.4</td>
<td>3.9</td>
<td>3.4</td>
<td>0.51</td>
<td>22.2</td>
</tr>
<tr>
<td>EL1</td>
<td>40</td>
<td>30.8</td>
<td>3.0</td>
<td>2.55</td>
<td>0.45</td>
<td>17.5</td>
</tr>
</tbody>
</table>

$^*$TA, telomeric association.
LOW MOLECULAR WEIGHT CYCLIN E AND GENOMIC INSTABILITY

Fig. 8. Presence of low molecular weight forms of cyclin E in breast tumors correlates with polyploidy and lack of response to antiestrogen therapy. A. relationship between ploidy and cyclin E protein levels among 331 stage I–III breast cancer patients (P = 0.0003). When the difference between the proportion of patients with high cyclin E and diploid tumors (21 of 105; 20%) and the proportion of patients with high cyclin E and polyploid tumors (69 of 226; 30%) is compared by the χ² test the P is significant at 0.045. B. Kaplan-Meier estimates of disease-specific survival (DSS) rates in patients with stage I–III breast cancers with high total levels of cyclin E expression as a function of ploidy (n = 241). C. Kaplan-Meier estimates of DSS rates in patients with stage I–III breast cancers with high total levels of cyclin E expression as a function of ploidy (n = 90; 5-year DSS; P = 0.02, log-rank test and overall DSS, P = 0.083, log-rank test). D. Kaplan-Meier estimates of DSS rates in patients with stage I–III breast cancer stratified per level of cyclin E expression and antiestrogen treatment (n = 150).

We recently reported that high levels of either total cyclin E or of the LMW forms of cyclin E were significantly correlated to poor outcome in breast cancer patients (7). The data presented here provides a potential mechanism for how the LMW forms of cyclin E increase the aggressiveness of the disease. First, increased kinase activity in the presence of p53 activation and high p21 levels may interfere with the normal functions of cyclin E, leading to aberrant licensing of replication origins and promoting genetic instability. This genomic instability accelerates acquisition of genetic defects, driving tumor cells toward a more advanced stage of disease. Second, because LMW-overexpressing cells can bypass p21 and p27 inhibition, their overexpression confers to the tumor a growth advantage and a decreased sensitivity to antiestrogen treatment. Third, overexpression of the LMW forms of cyclin E in tumors from patients with breast cancer may confer resistance to antiestrogens.

Cyclin E is overexpressed in 25% of breast cancers (7). Two-thirds of the breast tumors examined in this and our previous study (7) were ER positive, and ER is used as a molecular target for endocrine therapy. Only 50% of patients with ER-positive tumors and 75% of patients with tumors exhibiting both ER and progesterone receptor positivity respond to endocrine therapy (38). Acquired resistance limits the effectiveness of the treatment for a finite period of time. ICI 182,780 was developed as an alternative for patients who develop resistance to tamoxifen. LMW forms, by bypassing the inhibitory effects of p21 and p27 induced by antiestrogen treatment, provide a mechanism for acquisition of de novo or acquired resistance and raise the possibility that antiestrogen treatment is ineffective in cyclin E-overexpressing breast tumor cells. We see that breast cancer patients whose tumors express high levels of cyclin E appear to derive no survival benefit from adjuvant tamoxifen therapy, providing clinical support for the hypothesis that cyclin E is an important mediator of antiestrogen resistance.
In this article, we highlight important biochemical and functional differences between EL1 and LMW isoforms of cyclin E. The functional hyperactivity of the LMW isoforms when compared with EL1 is because of (a) more effective binding to CDK2 and (b) resistance to inhibition by p21 and p27 despite these inhibitors’ binding to the LMW/CDK2 complexes. These altered biological properties of the LMW cyclin E forms provide a molecular mechanism for understanding the poor outcome of breast cancer patients whose tumors express high levels of cyclin E. Generation of LMW forms of cyclin E, therefore, provides a new mechanism for deregulating cell cycle progression and points to their potentially essential role in tumorigenesis.

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Tumor-Specific Low Molecular Weight Forms of Cyclin E Induce Genomic Instability and Resistance to p21, p27, and Antiestrogens in Breast Cancer


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