Low Molecular Weight Cyclin E Overexpression Shortens Mitosis, Leading to Chromosome Misseggregation and Centrosome Amplification

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Abstract

Overexpression of the low molecular weight isoforms (LMW-E) of cyclin E induces chromosome instability; however, the degree to which these tumor-specific forms cause genomic instability differs from that of full-length cyclin E (EL), and the underlying mechanism(s) has yet to be elucidated. Here, we show that EL and LMW-E overexpression impairs the G2-M transition differently and leads to different degrees of chromosome instability in a breast cancer model system. First, the most significant difference is that EL overexpression prolongs cell cycle arrest in prometaphase, whereas LMW-E overexpression reduces the length of mitosis and accelerates mitotic exit. Second, LMW-E–overexpressing cells are binucleated or multinucleated with amplified centrosomes, whereas EL-overexpressing cells have the normal complement of centrosomes. Third, LMW-E overexpression causes mitotic defects, chromosome missegregation during metaphase, and anaphase bridges during anaphase, most of which are not detected on EL induction. LMW-E induces additional mitotic defects in cooperation with p53 loss in both normal and tumor cells. Fourth, LMW-E–overexpressing cells fail to arrest in the presence of nocodazole. Collectively, the mitotic defects mediated by LMW-E induction led to failed cytokinesis and polyplody, suggesting that LMW-E expression primes cells to accrue chromosomal instability by shortening the length of mitosis. Lastly, LMW-E expression in human breast cancer tissues correlates with centrosome amplification and higher nuclear grade. These results suggest that LMW-E overexpression leads to higher centrosome numbers in breast cancer, which is a prerequisite for genomic instability. Cancer Res; 70(12): 5074–84. ©2010 AACR.

Introduction

The majority of solid tumors display abnormal chromosomal segregation at cell division (1, 2). These abnormalities play a role in tumorigenesis and possibly metastasis by increasing the rate of chromosome mutations, including deletion and amplification of genes involved in cellular proliferation and/or survival (1). Chromosome aberrations can be caused by either the loss of chromosomes at metaphase/anaphase or multipolar divisions associated with an abnormal number or structure of centrosomes (2). During the progression toward higher stages of malignancy, chromosome aberrations become more frequent. This may be explained by a process initiated by telomeric dysfunction and anaphase bridging, which may give rise to an increased frequency of multinucleated cells because of cytokinesis failure (3). These multinucleated cells contain an abnormal number of centrosomes, leading to multipolar mitotic figures at the next cell division. A key question in understanding genomic instability is to delineate the events causing the initial abnormalities in the centrosome number.

Cyclin E, a regulatory subunit of cyclin-dependent kinase 2 (CDK2), is an important regulator of entry into the S phase in mammalian cells (4–6). Using cell-free and Xenopus model systems, several independent groups have provided evidence showing that in normal proliferating cells cyclin E/CDK2 is an important regulator of the initiation of both centrosome duplication and DNA replication during the G1 phase (7–9). Constitutive overexpression of cyclin E does not significantly increase the frequency of gene amplification but does increase the frequency of polyploid cells (10). Inhibition of cyclin E degradation in cell culture models has also been associated with the formation of micronuclei, which are suggestive of the generation of aneuploid cells (11). These studies suggest that maintaining normal levels of wild-type cyclin E is critical for preserving an unperturbed genome. In many aggressive and highly metastatic cancers, full-length cyclin E is proteolytically cleaved to its low molecular weight isoforms (LMW-E; ref. 12). The tumor-specific overexpression of LMW-Es was originally discovered in breast cancer cell lines; however, these isoforms are also overexpressed in some ovarian cancers (13, 14), melanomas (15), colorectal
cancers (16), and renal cell carcinomas (17). These LMW-E isoforms are generated from the NH2-terminal elastase cleavage of the 50-kDa, full-length cyclin E1 protein (termed EL; ref. 18). LMW-Es are tumor specific, are predominantly cytoplasmic (19), and have enhanced biochemical and biological properties that differ from EL. LMW-Es are also resistant to inhibition by the CDK inhibitors p21 and p27 and the growth-inhibitory effects of antiestrogens (20, 21).

Whereas the action of EL on the transitions between different phases of the cell cycle has been established, the role of the tumor-specific LMW-Es in maintaining a genomic balance and how they may differ from that of EL has not yet been investigated. In addition, no model systems have been developed to delineate how EL and LMW-E overexpression differentially alter cell cycle progression. In this report, we show that induction of LMW-E in breast cancer is much more potent than EL at inducing supernumerary centrosomes, abnormal mitotic spindles, chromosome segregation problems, and multinucleated cells. LMW-E primes the cells to accrue genomic instability by shortening the length of mitosis, whereas EL-overexpressing cells have a lengthened M phase. Human breast cancers that overexpress LMW-E harbor supernumerary centrosomes, in contrast to tumors that only express EL. These results collectively suggest that overexpression of LMW-E (but not EL) can induce chromosomal instability and supernumerary centrosomes by shortening the timing of M-phase progression.

Materials and Methods

Plasmids

pTRE-tight and pcDNA3.1-EL and pcDNA3.1-LMW-E were each digested with NheI and EcoRV. The insert encoding EL or LMW-E was ligated into the NheI and EcoRV site of pTRE-tight to generate pTRE-tight EL and pTRE-tight LMW-E.

Cell cultures and transfections

Tetracycline-inducible cell lines expressing Flag-tagged EL and LMW-E were generated by transfection of MCF-7 cells. Plasmid transfections were performed using FuGENE (Roche) according to the manufacturer’s protocols. MCF-7 cells were transfected with the doxycycline-inducible vector pTRE-tight (Clontech) harboring constructs expressing Flag-tagged EL or the EL-5/6 LMW-E isoforms. For all experiments herein, we used a construct representing the smallest LMW-E isoform, T2, as it does not contain additional elastase cleavage sites. For simplicity, we have referred to this form as LMW-E throughout the manuscript. Stable transformants were established by selection for 2 weeks with 1 μg/mL G418 (Invitrogen) and 100 μg/mL hygromycin (Merck). These cells were isolated, propagated, and analyzed by Western blotting for cyclin E expression in the presence or absence of doxycycline. Representative clones for EL and LMW-E were then selected and used for analysis.

The immortalized human mammary epithelial cell lines 76NE6 and 76NF2V were a generous gift from Dr. Vimla Band (University of Nebraska Medical Center, Omaha, NE); these cells were maintained in DCFI-1 media (D1-media; ref. 22). MCF-7 cells stably expressing a small interfering RNA vector targeting p53 were a generous gift from Dr. Xinbin Chen (University of California at Davis, Davis, CA); these cells were maintained in α-MEM supplemented with 0.5 μg/mL puromycin (Sigma).

Immunofluorescence staining

MCF-7 (induced or uninduced) or HeLa, 76NE6, 76NF2V, and MCF-7 short hairpin RNA (shRNA) p53 (infected with EL or LMW-E adenovirus) cells were grown in six-well plates with cover slides and fixed in cold 4% neutral paraformaldehyde in PBS for 20 minutes at room temperature. The cells were then washed in PBS, permeabilized in cold methanol for 5 minutes, and blocked in 5% bovine serum albumin in PBS. Incubation with a primary antibody was carried out for 1 hour at 37°C. Incubation with a secondary antibody was carried out for 1 hour at room temperature, followed by staining of DNA with 4′,6-diamidino-2-phenylindole (DAPI) for 10 minutes. Slides were mounted with Vectashield antifade medium (Vector Laboratories) after three washes with PBS and examined using a Leica DM4000B microscope equipped with ×63 Plan-Apochromat oil immersion objective.

Generation of recombinant adenoviruses

Cyclin E adenoviruses were constructed according to the manufacturer’s protocols using the AdEasy XL adenoviral vector system (Stratagene). Tumor cells were infected at a multiplicity of infection selected to ensure >70% transduction efficiency.

SDS-PAGE electrophoresis, Western blotting, immunoprecipitation, and kinase assay

SDS-PAGE electrophoresis, Western blotting, immunoprecipitation, and kinase assays were performed as previously published (19).

Live cell microscopy

We used H2B-GFP HeLa cells, which allow high-resolution imaging of both mitotic chromosomes and interphase chromatin in live cells as described (23). Briefly, H2B-GFP HeLa cells were treated with 2 mmol/L thymidine for 18 hours at 37°C, washed, released for 8 hours, and treated again with 2 mmol/L thymidine for an additional 18 hours before release to a thymidine-free medium. Cells were then transduced with 1,000 virus particles (EL or LMW-E) per cell in complete medium for 24 hours during the second thymidine treatment. Cells were followed for 24 hours, starting 4 hours after release from the block, and photographed once every 5 minutes. We set the 0-minute time point at nuclear envelope breakdown (NEB; ref. 24) and the end point at the completion of anaphase. For these experiments, three different fields per condition were photographed, and a total of 20 cells were followed in three independent experiments. The apparatus for time-lapse experiments consisted of an Olympus IX81 spinning disc confocal microscope and a CO2-equilibrated chamber heated to 37°C and equipped with ×40 and ×60 objectives. Movies were generated from the acquired images.
Tissue samples and immunoperoxidase staining

Tumor samples were obtained from patients who underwent surgery at The University of Texas M.D. Anderson Cancer Center. Sequential sections from formalin-fixed, paraffin-embedded tissue blocks were cut 4-μm thick with a microtome, fixed to charged slides, and immunostained with a centrosome-specific antibody to pericentrin (Abcam) or cyclin E antibody (Santa Cruz Biotechnology). To measure centromeral aberrations, at least 10 different high-powered fields per case were examined by light microscopy (Leica), photographed with a SPOT digital camera, and analyzed with ImagePro image analysis software. Centrosomes were considered abnormal if they had a diameter at least twice that of centrosomes in adjacent nonneoplastic mammary gland tissue, if there were more than two per cell, or if the ratio of the greatest to the smallest diameter exceeded 2. Mean percentages of centromeral abnormalities were calculated by including all cells harboring centrosomes per sample. Cyclin E staining intensity and percent positivity were evaluated both in the nucleus and in the cytoplasm in invasive carcinoma and in nonneoplastic breast epithelial cells as previously described (25).

Patient characteristics

Patient characteristics are described in the legends of Supplementary Table S1.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software with two-way ANOVA or StatsDirect software. All statistical tests were two-sided and were considered to be statistically significant at $P < 0.05$.

Results

Induction of LMW-E expression causes centrosome amplification

We initially set out to address whether EL and LMW-E have different effects on the induction of chromosome instability by measuring the number of centrosomes in cells upon EL or LMW-E induction. For these analyses, we generated MCF-7 cells that can inducibly express Flag-tagged EL (Fig. 1A, left) or LMW-E (Fig. 1A, right) upon treatment with doxycycline. In induced cells, the CDK2 kinase activity associated with Flag-LMW-E was 1.5-fold higher than that of Flag-EL despite similar levels of EL and LMW-E (Fig. 1B). We used this inducible system to explore whether induction of EL and LMW-E differentially affects centrosome numbers. Centrosomes were stained with anti-γ-tubulin antibody. Induction of EL did not result in a significant increase in the number of cells with more than two centrosomes (Fig. 1C and D). In contrast, upon induction of LMW-E, there was a 2.5-fold increase in the number of cells with more than two centrosomes (Fig. 1C and D).

Spindle defects and chromosome missegregation in cyclin E–overexpressing cells

We next set out to examine whether there are mitotic defects associated with centrosome amplification in EL-overexpressing or LMW-E–overexpressing cells using antibodies to β-tubulin (green) to stain microtubules and γ-tubulin (red) to stain centrosomes (Fig. 2). Among the uninduced EL and LMW-E cells, 90% to 95% of the cells in mitosis showed normal chromosome condensation and congression on a bipolar spindle (Fig. 2A, −Dox). After induction of EL, only 20% of the mitotic cells had defects, whereas after induction of LMW-E, 56% of the mitotic cells had defects associated with abnormal spindles, including branched and splayed spindles (71%), chromosome alignment defects (9%), and abnormal centrosome numbers (19%; Fig. 2A and B). Furthermore, cells overexpressing LMW-E had 3-fold more mitotic defects than EL-overexpressing cells (Fig. 2B).

Cyclin E expression cooperates with p53 loss in causing mitotic defects and chromosome missegregation

The presence of the tumor suppressor p53 is a crucial component of a checkpoint that limits the accumulation of cells with supernumerary centrosomes (24). To examine whether p53 loss cooperates with cyclin E overexpression (EL or LMW-E) to induce mitotic defects, we introduced EL and LMW-E by adenoviral infection into human mammary epithelial 76NF2V and 76NE6 cells (Fig. 3A). The 76NE6 cell line was transfected with the E6 gene of human papillomavirus; this immortal phenotype lacks p53 due to E6-directed proteasomal degradation (26). The 76NF2V cell line was transfected with a mutant E6 gene (F2V) incapable of degrading p53 but still able to immortalize cells (27). Mitotic defects were recorded by staining the cells with antibodies to β-tubulin and γ-tubulin (Fig. 3C). Although, in response to LMW-E, both 76NE6 and 76NF2V cells underwent mitotic defects, there was a significantly higher number of mitotic defects in 76NE6 cells overexpressing LMW-E compared with 76NF2V cells (Fig. 3C and D, left). Specifically, 48% of 76NE6 cells overexpressing LMW-E contained mitotic defects compared with 34% of 76NF2V cells overexpressing LMW-E ($P < 0.01$). The mitotic defects included monopolar, tripolar, and multipolar cells as well as those with centrosomes clustering, lagging chromosomes, and spindle polarity defects (Fig. 3C). Similarly, EL-overexpressing 76NE6 cells incurred more mitotic defects (28%) compared with EL-overexpressing 76NF2V cells (17%; $P < 0.05$; Fig. 3C and D, left). The same experiment was repeated in another model system in which p53 was stably downregulated with p53 shRNA in MCF-7 cells (Fig. 3B and C). We found that both EL and LMW-E cooperate with the loss of p53 to induce mitotic defects, but the percentage of
mitotic defects was higher in the LMW-E-overexpressing cells (66%; \(P < 0.001\); Fig. 3D, right) than the EL-overexpressing cells (40%; \(P < 0.01\), Fig. 3D, right). These results show that the loss of p53 cooperates with LMW-E and, to a lesser extent, with EL to induce mitotic defects in both immortalized and breast cancer cell lines.

**LMW-E overexpression causes multinucleation, polyploidy, and compromised spindle checkpoint activation**

We next examined the effect of EL and LMW-E on genomic instability by measuring the number of multinucleated and polyploid cells on induction of either EL or LMW-E in two different systems (Fig. 4, right). Using the MCF-7–inducible system, we found a significant difference in the number of multinucleated cells between the EL-induced and LMW-E–induced cells. Specifically, 12% of all LMW-E–overexpressing cells and 95% of those with multiple centrosomes were binucleated or multinucleated versus only 4% of EL-overexpressing cells (40%; \(P < 0.01\), Fig. 3D, right). These results show that the loss of p53 cooperates with LMW-E and, to a lesser extent, with EL to induce mitotic defects in both immortalized and breast cancer cell lines.

LMW-E–inducible MCF-7 cells in the presence or absence of doxycycline for 14 days and subjected these cells to metaphase spread, which we then enumerated to measure the number of diploid cells (Fig. 4C). These analyses revealed that there was no difference in cell number between the induced and uninduced EL and LMW-E cells at the end of 14 days (data not shown). However, LMW-E–overexpressing cells had 20% fewer diploid cells, whereas the percentage of diploid cells was unchanged in the EL-overexpressing cells (Fig. 4C). We next assessed the integrity of the mitotic spindle checkpoint in EL-induced and LMW-E–induced MCF-7 cells by measuring the mitotic index in cells treated with nocodazole for 16 hours. The control cells showed a robust mitotic arrest, with 28% of the cells arrested by 16 hours following nocodazole treatment (Fig. 4D). However, the induction of EL versus LMW-E resulted in different mitotic indices. For example, whereas the induction of EL slightly increased the mitotic index, the induction of LMW-E resulted in a 60% drop in the mitotic index (to 8%) compared with that of control or EL cells (Fig. 4D). Thus, induction of LMW-E compromised the spindle checkpoint activation, as shown by a strongly reduced mitotic index following 16 hours of nocodazole treatment. These results collectively show that genomic instability, as measured by the extent of multinucleated and polyploid cells and compromised mitotic index, was significantly altered on induction of LMW-E but not EL.
LMW-E overexpression causes cells to enter and exit mitosis prematurely

The studies on spindle checkpoint activation showed that induction of LMW-E induces more mitotic defects than EL. We next asked if the mitotic defects observed were due to the alteration of the transition of the LMW-E–induced cells through mitosis using real-time microscopy. HeLa cells expressing green fluorescent protein (GFP)–tagged histone H2B were transduced with EL, LMW-E, or control adenovirus and imaged as they progressed through mitosis. We detected EL and LMW-E expression within 24 hours of adenoviral infection by Western blot analysis (Fig. 5A). After synchronization in G1-S, the cells expressing EL and LMW-E entered mitosis earlier than control cells, with mitotic entry starting 4 to 16 hours (EL), 4 to 12 hours (LMW-E), and 10 to 16 hours (control) after release from G1-S arrest, respectively (Fig. 5B). In addition, the LMW-E–expressing cells exited mitosis 4 hours earlier than control cells (Fig. 5B), whereas the EL-expressing cells exited mitosis 2 hours later than control cells (Fig. 5B). To dissect the timing of mitotic exit in EL-expressing and LMW-E–expressing cells, we measured the time from NEB until anaphase onset in these cells. We found that in EL-expressing cells the time from NEB to the anaphase transition ranged from 305 to 645 minutes, with a

Figure 2. LMW-E overexpression leads to mitotic defects. A, cells were treated with doxycycline (1 μg/mL) for 24 h; fixed; stained for β-tubulin (green), γ-tubulin (red), or DNA (blue); and analyzed by immunofluorescence microscopy. B, quantification of spindle defects in 200 mitotic cells per experiment from 10 independent experiments. Columns, percentage of cells with mitotic defects; bars, SD. *, P < 0.001. C, pictorial presentation of various mitotic errors (micronuclei, chromosomes missegregation, anaphase bridges, and cytokinesis failure) in LMW-E–overexpressing cells. D, quantification of mitotic errors in control, EL-expressing, and LMW-E–expressing cells. Graphs indicate the percentage of cells with micronuclei, metaphase chromosome missegregation, anaphase bridges, and cytokinesis failure. Columns, mean (n = 3); bars, SD. *, P < 0.01; **, P < 0.001.
median of 415 minutes (n = 20) compared with a median of 130 minutes in control cells (Fig. 5C and D). In contrast, in LMW-E-expressing cells, the time from NEB until anaphase onset ranged from 60 to 135 minutes, with a median of 80 minutes (Fig. 5C and D). The differential mitotic exit times mediated by LMW-E versus EL are also depicted in several movies taken from normal cells (Movie 1), EL-overexpressing cells showing premature mitotic entry and prolonged mitotic exit (Movie 2), LMW-E-overexpressing cells showing a faster mitotic transition (Movie 3) and mitotic defects (Movie 5), and binucleated cells (Movie 6). Because EL is not modified by proteolytic cleavage in HeLa cells, we asked whether expression of LMW-E could rescue the mitotic arrest induced by EL. To this end, we overexpressed both EL and LMW-E in HeLa H2B-GFP cells (Fig. 5A, lane 4). The median time from NEB to anaphase was ~120 minutes (Fig. 5C and D; Movie 4), suggesting that the presence of LMW-E overrides the mitotic block induced by EL. Lastly, we observed that some mitotic cells expressing LMW-E (7%, n = 40) had multipolar spindles, resulting in a longer time from NEB to anaphase onset

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Figure 3. Cyclin E overexpression cooperates with p53 loss to induce mitotic defects. A and B, 76NF2V (p53 positive) and 76NE6 (p53 deficient), as well as MCF-7 breast cancer cells, parental and stably transfected with either control or p53 shRNA, were infected with the adenoviruses indicated. p53, cyclin E, and vinculin expression were detected by Western blotting. C, cells treated as in A and B were fixed and then stained for γ-tubulin (red) or β-tubulin (green), counterstained with DAPI (blue), and analyzed by immunofluorescence microscopy. Representative images of mitotic abnormalities observed in 78NE6 and MCF-7 p53 shRNA cells infected with LMW-E-expressing adenovirus are shown. D, quantification of mitotic defects in LacZ control, EL-expressing, and LMW-E-expressing 76NF2V/76NE6 cells (right) and control shRNA/p53 shRNA MCF-7 cells (left). Two hundred and fifty mitotic figures were counted for each condition in two independent experiments. *, P < 0.01; **, P < 0.001.
(195 min), but were able to still divide (Fig. 5D). These results show that although overexpression of both EL and LMW-E results in faster mitotic entry, LMW-E-expressing but not EL-expressing cells exit mitosis much faster than control cells, suggesting that LMW-E expression alters mitotic exit differently than does EL expression.

**LMW-E expression is positively correlated with centrosome amplification and nuclear grade in breast cancer specimens**

Both chromosomal instability and centrosome defects are common features of epithelial cancers (28–30). Because LMW-E overexpression causes centrosome amplification in cell lines, we examined whether a correlation exists between centrosome abnormalities in human breast tumors and expression of LMW-E isoforms. To this end, we used Western blotting to analyze cyclin E expression in 30 primary invasive breast carcinomas and adjacent nonneoplastic breast tissue samples as well as in normal breast tissues from reduction mammoplasty specimens. The tissue samples were also analyzed by immunohistochemistry for pericentrin, which detects centrosome abnormalities (Fig. 6A and B; Supplementary Fig. S2). The clinicopathologic characteristics of all patients are summarized in Supplementary Table S1. We quantitated EL and LMW-E levels by densitometry, normalized those levels to the EL level in adjacent normal breast tissue, and analyzed the correlation between EL and LMW-E levels and the degree of centrosome abnormalities. These analyses revealed that the invasive tumor tissues had 20% more centrosome abnormalities, including supernumerary centrosomes, abnormally shaped centrosomes, and centrosomes of larger diameter, than tissue from normal adjacent epithelium (Fig. 6A). Multiple linear regression analysis revealed that LMW-E expression, but not EL expression, is an independent predictor of centrosome alteration (P > 0.0005; Fig. 6C). In addition, centrosome abnormalities (r² = 0.137779, P = 0.0434) and LMW-E expression (r² = 0.18627, P = 0.0172) were positively correlated with a higher modified Black's nuclear grade (Supplementary Fig. S1B and C). The data from the breast cancer patients suggest that overexpression of LMW-E expression is positively correlated with centrosome amplification and nuclear grade in breast cancer specimens.

**Figure 4.** LMW-E overexpression leads to polyploidy. A, pTRE-LMW-E-expressing MCF-7 cells were treated with or without doxycycline (1 μg/mL) for 24 h; stained with β-tubulin (red), phalloidin (green), or DAPI (blue); and analyzed by immunofluorescence microscopy. Right, the percentages of binucleated and multinucleated cells are plotted. Columns, mean from three independent experiments; bars, SD. B, HeLa cells infected with EL or LMW-E adenovirus for 24 h and stained with β-tubulin (red), phalloidin (green), or DAPI (blue). Right, the percentages of binucleated and multinucleated cells are plotted. One thousand cells were analyzed for each group for binucleated or multinucleated cells. Columns, mean from three independent experiments; bars, SD. C, pTRE-EL and pTRE-LMW-E cells were treated with doxycycline (1 μg/mL) for 14 d and subjected to metaphase spread. The percentage of diploid cells was counted. Columns, mean from three independent experiments; bars, SD. D, MCF-7 EL and MCF-7 LMW-E cells were treated with or without doxycycline for 24 h and nocodazole for 16 h, and the number of mitotic cells was counted microscopically. *, P < 0.01.
LMW-E, but not EL, is associated with centrosome abnormalities and higher grade of tumors.

**Cyclin E localizes to the cytoplasm in cells with centrosome amplification**

We recently tested the hypothesis that LMW-E isoforms have an altered subcellular localization because they lack a portion of the EL NH₂ terminus containing a nuclear localization sequence. Here, we asked whether patient tumor samples overexpressing LMW-E have cytoplasmic expression of LMW-E and if the cytoplasmic staining of cyclin E is predominant in patients with high pericentrin levels. Immunostaining of tumor tissues showed high cytoplasmic localization of cyclin E in samples with high degree of centrosome abnormalities detected by immunohistochemistry for pericentrin (Supplementary Fig. S1A). We also analyzed 10 reduction mammoplasty samples from nontumor-bearing patients by immunohistochemistry for pericentrin and by Western blot and immunohistochemistry for cyclin E (Supplementary Fig. S2). We found an absence of LMW-E on Western blot of normal breast tissues, nuclear localization of cyclin E immunostaining, and pericentrin in centrosomes.

Figure 5. Expression of EL and LMW-E induces premature mitotic entry and differential mitotic exit times. A, Western blot analysis of HeLa cells infected with adenovirus expressing LacZ control, EL, LMW-E, or a combination of EL and LMW-E using a cyclin E antibody. B, HeLa H2B-GFP cells were synchronized by double thymidine block; infected with adenovirus expressing LacZ, EL, or LMW-E during the second thymidine block; and analyzed by time-lapse microscopy 4 h after release for 24 h. Cells were photographed every 5 min from three different fields. Mitotic cells were counted and plotted as cumulative numbers. Data are percentage of mitotic cells (mitotic index) at different time points after thymidine release. C, the time from NEB to anaphase onset was measured by time-lapse microscopy in control cells and in cells infected with EL, LMW-E, or both EL and LMW-E adenoviruses. Each symbol in the scatter plot represents a single cell. D, representative fluorescence videomicroscopy series from cells described in C; numbers denoted are times (min).

LMW-E Overexpression Shortens Mitosis

Cancer Res; 70(12) June 15, 2010 www.aacrjournals.org
normal in number and structure. These data suggest that cytoplasmic cyclin E is tumor specific and linked to aberrant centrosomes.

**Discussion**

In this report, we have shown that expression of LMW-E induces genomic instability by causing cells to progress faster through mitosis without proper chromosome segregation and with failure of cytokinesis and the generation of multinucleated cells with supernumerary centrosomes. As previously suggested, failure to execute a crucial step in the cell division process, such as late anaphase and cytokinesis, leads to the formation of cells with two nuclei; these 4N cells can then undergo either bipolar or multipolar mitosis with amplified centrosomes (31). We observed that most cells with more than two centrosomes were binucleated or multinucleated, and DNA content analysis showed polyploid cells,

![Figure 6](image)

**Figure 6.** Centrosome abnormalities correlate with LMW-E expression. A, human breast cancers and adjacent nonneoplastic breast tissues were immunostained with a pericentrin antibody to visualize centrosomes. Nuclei were stained light blue with hematoxylin. Numbers above the panels denote different surgical cases. Magnification, ×1,000. Insets are digitally magnified views of centrosomes. In normal epithelia, centrosomes are uniform and small, whereas in tumors they are larger, multiple, or structurally abnormal. Top row images, normal epithelium (left) and tumors with low LMW-E expression; bottom row images, normal epithelium (left) and tumors with high expression of LMW-E. B, Western blot analysis of EL and LMW-E expression in human breast cancer cell lines, human breast cancer tissue samples, and adjacent normal breast tissue samples. Vinculin was used as loading control. C, positive correlation between LMW-E expression and centrosome abnormalities. To examine centrosomes, at least 10 different high-power fields per case were analyzed. Centrosomes were considered abnormal if they had a diameter at least twice that of centrosomes in adjacent nonneoplastic mammary gland tissue, if they were present in numbers >2, or if the ratio of their greatest and smallest diameters exceeded 2. Mean percentages of centrosomal abnormalities were calculated by including all cells harboring centrosomes per sample. EL and LMW-E protein levels were measured by densitometry of bands on Western blot. Quantification of centrosome abnormalities was correlated with ratio of tumor EL to normal EL or tumor LMW-E to normal EL. Multiple linear regression analysis revealed that LMW-E expression, but not EL expression, is an independent predictor of centrosome alteration.
suggesting that LMW-E triggers accumulation of cells with centrosome amplification by abortive mitosis rather than by affecting centrosome fragmentation or duplication. Time-lapse microscopy data suggest that overexpression of LMW-E forces cells to escape mitotic arrest, reenter G1, with a 4N DNA content, survive, and increase the population of polyploid cells. However, overexpression of EL results in a much longer/delayed mitosis, suggesting that these cells do not have a growth advantage. The results we present with EL agree with previous studies showing that overexpression of EL alone does not cause centrosome amplification and leads to prolonged mitotic arrest due to inhibition of the anaphase-promoting complex (APC<sup>cdh1</sup>) and accumulation of cyclin B1 and securin (32). Prolonged mitosis in EL-overexpressing cells leads to complete alignment of chromosomes at the metaphase plate. Only cells that fail to divide their chromosomes will become polyploid, an event that is rare in EL-overexpressing cells but one that is significantly enhanced in LMW-E–overexpressing cells. What is novel and clinically relevant in the present study is that, in contrast to EL, LMW-E–overexpressing cells exit mitosis faster than the EL–overexpressing cells, leading to a failure of cytokinesis and higher levels of polyploidy. Thus, we propose that the generation of LMW-E isoforms drives chromosomal instability by perturbing the G2–M transition and the G1–S checkpoint.

LMW-E–overexpressing cells that prematurely exit mitosis must bypass the subsequent G1–S checkpoint before the next DNA replication cycle to become polyploid (33, 34). It has been suggested that this G1–S checkpoint is dependent on p53 function (33) and that loss of p53 function induces phenotypes similar to those observed with LMW-E overexpression: centrosome amplification and aneuploidy (35, 36). We have shown that cyclin E overexpression cooperates with loss of p53 in both an immortalized and a breast cancer cell line to increase mitotic defects and chromosome missegregation. These results suggest that the LMW-E–induced mitotic defects concomitant with downregulation of p53 allow cells to bypass the G1–S checkpoint and enter the next round of DNA replication, thus becoming more likely to generate polyploid cells.

We also translated our cell line observation to the clinic. Using breast cancer specimens, we showed a positive correlation between the level of centrosome amplification, the presence of LMW-E, and the nuclear grade in human breast cancer. Centrosome defects are present in low-grade tumors but increase in more aggressive carcinomas (37). In addition, LMW-E was absent in normal adjacent tissues of breast cancer patients or normal tissues obtained from reduction mammoplasty samples and was not a function of the levels of EL. LMW-E is preferentially accumulated in the cytoplasm in tumors with centrosome amplification. This is consistent with our recent finding that the subcellular localization of LMW-E is altered in cell lines in vitro (19). This tumor-specific cytoplasmic LMW-E expression suggests that cyclin E may be deregulated during tumorigenesis through altered cellular localization and suggests that centrosome abnormalities associated with LMW-E expression can predict the development of high-grade cancer and may provide a much-needed surrogate marker for aggressive disease.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Low Molecular Weight Cyclin E Overexpression Shortens Mitosis, Leading to Chromosome Misseggregation and Centrosome Amplification

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