Hbo1 Is a Cyclin E/CDK2 Substrate That Enriches Breast Cancer Stem-like Cells

MyLinh T. Duong1,4, Said Akli1, Sira Macalou1, Anna Biernacka1, Bisrat G. Debeb2, Min Yi3, Kelly K. Hunt3, and Khandan Keyomarsi1,4

Abstract

Expression of cyclin E proteolytic cleavage products, low-molecular weight cyclin E (LMW-E), is associated with poor clinical outcome in patients with breast cancer and it enhances tumorigenicity in mouse models. Here we report that LMW-E expression in human mammary epithelial cells induces an epithelial-to-mesenchymal transition phenotype, increases the CD44hi/CD24lo population, enhances mammosphere formation, and upregulates aldehyde dehydrogenase expression and activity. We also report that breast tumors expressing LMW-E have a higher proportion of CD44hi/CD24lo tumor cells as compared with tumors expressing only full-length cyclin E. In order to explore how LMW-E enriches cancer stem cells in breast tumors, we conducted a protein microarray analysis that identified the histone acetyltransferase (HAT) Hbo1 as a novel cyclin E/CDK2 substrate. The LMW-E/CDK2 complex phosphorylated Hbo1 at T88 without affecting its HAT activity. When coexpressed with LMW-E/CDK2, wild-type Hbo1 promoted enrichment of cancer stem-like cells (CSC), whereas the T88 Hbo1 mutant reversed the CSC phenotype. Finally, doxorubicin and salinomycin (a CSC-selective cytotoxic agent) synergized to kill cells expressing LMW-E, but not full-length cyclin E. Collectively, our results suggest that the heightened oncogenicity of LMW-E relates to its ability to promote CSC properties, supporting the design of therapeutic strategies to target this unique function. Cancer Res; 73(17): 1-13. ©2013 AACR.

Introduction

Before the G1-S phase boundary, cyclin E associates with cyclin-dependent kinase 2 (CDK2) to further phosphorylate and inactivate the pRb–E2F complex and promotes the S-phase entry (1–3). Cyclin E overexpression by genomic and transcriptional amplification has been reported in multiple cancers, particularly in breast cancer (4–8). In addition, posttranslational proteolytic cleavage of cyclin E mediated by a serine protease generates two low-molecular weight cyclin E (LMW-E) isoforms in cancer cells (9, 10). Compared with full-length cyclin E (EL), the LMW-E isoforms are hyperactive by forming tighter interaction with CDK2 and are more resistant to p21 and p27 inhibition, resulting in higher rate of proliferation (11, 12, 13). Transgenic mice expressing LMW-E isoforms are themselves malignant as approximately 27% of LMW-E transgenic mice develop mammary adenocarcinomas compared with 10% of transgenic mice with EL expression (14). Furthermore, approximately 25% of the LMW-E tumors develop metastasis compared with 8% in the EL tumors (14). We recently showed preferential accumulation of LMW-E/CDK2 complexes in the cytoplasm (15). The altered subcellular localization of LMW-E renders it less susceptible to Fbw7-directed proteasomal degradation. This finding implicates many possibilities that can explain the particular oncogenic characteristics of LMW-E that are distinct from full-length cyclin E, and that these effects could be independent from cell-cycle regulation.

Compared with the EL isoform, overexpression of LMW-E induces aberrant acinar morphogenesis and mammary tumorigenesis in a CDK2-dependent manner (15, 16). In addition, examination of 395 breast cancer patient tissues identified LMW-E as an independent and powerful prognostic and predictive marker of patient outcome (17). Finally, we recently reported that the b-Raf-ERK1/2-mTOR pathway was activated in LMW-E–expressing patient samples, and activation of this pathway was associated with poor disease-specific survival (16).

Here, we provide evidence that the tumorigenicity of LMW-E is mediated through induction of the epithelial-to-mesenchymal transition (EMT) that leads to enrichment of the cancer stem-like cells (CSC) population via the phosphorylation of Hbo1 [HAT binding to ORC1 (origin recognition complex 1)].
and shows that these tumor cells can be effectively targeted by combination treatments targeting both cell proliferation (i.e., doxorubicin) and CSCs (i.e., salinomycin).

Materials and Methods

Detailed description of methods on bright field and indirect immunofluorescence analysis, quantitative real-time PCR (qRT-PCR) analysis, tissue samples, immunohistochemical staining, Western blot analysis, drug treatment, high-throughput survival assay (HTSA), histone acetyltransferase (HAT) activity colorimetric assay, ALDEFLUOR assay, lentiviral packaging, infection of short hairpin RNAs (shRNA), scratch assay, and sorting of CD44hi/CD24lo is provided in the Supplementary Methods.

CD24/CD44 FACS analysis

Half a million cells were washed three times with PBS containing 1% horse serum and resuspended with 10 μL phycoerythrin (PE) anti-mouse CD24, 10 μL allophycocyanin (APC) anti-mouse CD44 (BD Pharmingen), and 30 μL of the 1% serum PBS buffer. Samples were incubated for 20 minutes on ice, washed with 1% serum PBS buffer, and analyzed with the FACS Calibur. The data were analyzed by the Flowjo software (version 8.8.6; TreeStar, Inc.). Cells with only a single antibody label were used to set up the gates for analysis.

Mammosphere formation efficiency

To generate primary mammospheres, cells were grown in serum-free, growth factor–enriched media in low attachment plates as previously described by Dontu and colleagues (18). Briefly, cells were trypsinized, and single cells seeded in 6-well ultralow attachment plates (40,000 cells/mL) in serum-free Minimum Essential Medium supplemented with 20 ng/mL basic fibroblast growth factor (bFGF), 20 ng/mL EGF and B27 (Invitrogen), and incubated for 5 to 7 days. For secondary mammosphere assay, cells from primary mammospheres were dispensed with 0.05% trypsin, seeded in 6-well ultralow attachment plates (20,000 cells/mL) in mammosphere media, and incubated for 5 to 7 days. Mammospheres with at least a size of 100 μm were counted with an automated colony counter (Oxford Optronix) following MTT staining (Sigma-Aldrich) to increase the contrast and allow automatic detection of mammospheres.

ProtoArray human protein microarray analysis

The ProtoArray Human Protein Microarrays (Invitrogen) contain more than 6,100 kinase substrates expressed as N-terminus glutathione S-transferase (GST) fusions (www.invitrogen.com/protoarray). Purified FLAG-EL/CDK2 and FLAG-LMW-E/CDK2 complexes were produced in s9 insect cells, purified by immunoprecipitation (IP) with FLAG-tagged antibody and eluted with 3× FLAG peptide (Sigma). Arrays were incubated either with recombiant active EL/CDK2 or LMW-E/CDK2 at a concentration of 50 nmol/L in the presence of 33P-γ-ATP for 1 hour at 30°C. After washing and drying, arrays were exposed to X-ray film overnight. Imaging and data analysis were conducted as recommended by the manufacturer.

Statistical analysis

Each cell culture experiment was carried out at least three times. Continuous outcomes were summarized with mean and SDs. Comparisons among groups were conducted using SPSS software, version 12.0 through either unpaired Student t test or Pearson χ² test as indicated in the figure legends.

Results

LMW-E activates gene expression associated with the EMT and enhances cell migration

The cell line system used in this report was previously derived and described (16). Briefly, to investigate the functional tumorigenic difference between the EL and LMW-E isoforms, we used the 76NE6 cell line [which is a human mammary epithelial cell (hMEC) derived from reduction mammoplasty] and exogenously introduced empty vector, EL, and LMW-E cyclin E isoforms to generate stable cell lines (herein referred to as 76NE6-V, 76NE6-EL, and 76NE6-LMW-E). These cells were subjected to xenograft transplantation and in vivo pasaging. Only the 76NE6-LMW-E (but not the 76NE6-V or 76NE6-EL) cells were tumorigenic. The tumors were removed, prepared for culture in vitro, and then injected back into the mice for four successive generations. As a result, the cell lines derived from in vivo pasaging of the 76NE6-LMW-E cells and used in this article are T1G2.2, T1G3.1, and T1G4.2, in which T denotes tumor and G generation and are herein referred to as tumor-derived cells (TDC). We found that continued pasaging of the 76NE6-LMW-E cells in vivo generated more aggressive tumors with a selection for an increase in the level of LMW-E protein and a decrease in EL protein level (16).

We initially observed that the TDCs displayed reduced cell-cell contact. Immunostaining shows dramatically reduced E-cadherin expression in LMW-E–expressing cells (Fig. 1A and B), particularly at the membrane junction, whereas the parental 76NE6 cells display strong membranous E-cadherin staining (Fig. 1A, white arrows).

76NE6-LMW-E and the TDCs showed high expression of cyclin E mRNA levels, and reduced expression of E-cadherin mRNA levels (Fig. 1C and D). Furthermore, the cells that show high cyclin E expression also upregulate N-cadherin, twist, slug, and vimentin mRNA levels, which are the genes associated with the EMT (Fig. 1E–H). Taken together, exogenous LMW-E expression in the 76NE6 cells resulted in reduced cell-cell adhesion and induced transcriptional changes that are features of the EMT.

The passage of cells through an EMT is associated with increased migratory and invasive properties. Previously (16), we showed that hMECs with LMW-E expression invaded significantly more than cells with EL expression. Therefore, to test if LMW-E may also contribute to the migratory phenotype of these cells, we subjected the TDCs and the parental cells to in vitro scratch assay (Supplementary Fig. S1). The average area closure for the 76NE6-EL cells was 28.8% ± 7.9% at 16 hours compared with 43.8% ± 6.9% (P < 0.05) for the 76NE6-LMW-E and 61.7% ± 12.3%, 66.0% ± 8.7%, and 57.8% ± 9.0% (P < 0.01) for the T1G2.2, T1G3.1, and T1G4.2 cells, respectively. This result shows that LMW-E expression enhances the migratory property of mammary cells significantly more than EL
expression and is consistent with the invasive (16) and EMT properties associated with LMW-E expression.

**LMW-E expression enriches the mammary CSC population**

The connection between induction of the EMT and generation of cells with CSC properties (19) led us to suspect that the tumorigenicity of LMW-E could be due to its ability to induce the EMT and enrich CSCs. To investigate whether ectopic expression of EL or LMW-E can enrich this population, cells were stained using antibodies to CD24 and CD44, and their relative expression levels were measured by fluorescence-activated cell sorting (FACS) analysis (Fig. 2).

We found that expression of LMW-E increased the number of cells with CD44hi/CD24lo phenotype compared with the EL expressing 76NE6 cells and to vector control cells (P < 0.05; Fig. 2A). The difference was more remarkable in the TDCs, in which most of the clones contained approximately 60% of this CSC population. Furthermore, Western blot analysis using CD24 and CD44 antibodies confirmed that the LMW-E–expressing cells downregulated CD24 and upregulated CD44 protein levels (Fig. 2E). Because in vivo passaging led to the enhancement of LMW-E expression as well as enrichment for the CD44hi/CD24lo population, we propose that cyclin E is involved in selecting for this CSC population. Cyclin E expression levels also positively correlated with the CD44hi/CD24lo population with $R^2 = 0.853$ (P < 0.0001; Fig. 2B).

CSCs also have the increased ability to form mammospheres (18, 20). As expected, the 76NE6-V control cells underwent apoptosis as apparent by extensive membrane blebbing (Fig. 2C). In contrast, the 76NE6-LMW-E cells and the TDCs were able to form more and larger mammospheres compared with the 76NE6-EL cells (P < 0.05; Fig. 2C and D). The breast cancer cell line Sum159, which contains a high percentage of CD44hi/CD24lo cells and forms robust mammospheres, served as a positive control (21). Similar to primary mammospheres, all the LMW-E–overexpressing 76NE6 cells formed significantly more secondary mammospheres than EL-expressing 76NE6 cells (Fig. 2D) confirming their self-renewal abilities.
To determine if the CSCs (CD44<sup>hi</sup>/CD24<sup>lo</sup>) are able to differentiate into non-CSCs, we sorted the CSC fraction (CD44<sup>hi</sup>/CD24<sup>lo</sup>) of each of the 76NE6 cell lines (EL, LMW-E, T1G2.2, T1G3.1, and T1G4.2) and examined the distribution of cell types after growth of isolated CSCs from 76NE6 cell lines for 1 week (Supplementary Fig. S2). The results revealed that sorted CSCs rapidly differentiate into non-SCCs after 7 days (53% for EL, 30% for LMW-E, and 13%–43% for the TDCs). This
result shows that LMW-E–generated CSCs are also capable of generating non-CSCs.

High aldehyde dehydrogenase (ALDH) protein and enzymatic levels are also associated with CSC phenotypes (22). The 76NE6-LMW-E cells and the TDCs express high LMW-E and ALDH protein levels (Fig. 2E) along with statistically higher ALDH activity compared with the 76NE6-EL and vector control cells (P < 0.05; Fig. 2F). Collectively, the evidence we provide in these series of experiments suggest that LMW-E enriches cells with CSC properties by upregulating the ALDH activity level and enabling hMECs to survive and undergo self-renewal in the absence of basement membrane attachment.

**Hbo1 is a novel substrate of the cyclin E/CDK2 complex**

To elucidate the signaling pathway downstream of LMW-E, we used the ProtoArray Human Protein Microarray from Invitrogen. Recombinant EL/CDK2 and LMW-E/CDK2 complexes were expressed and purified from insect cell lysates and a kinase assay was conducted using GST-Rb as a substrate to confirm that these complexes have robust kinase activity (Supplementary Fig. S3A). Next, the Protein Microarray, which contains more than 9,000 unique human proteins (Supplementary Fig. S3C), was subjected to kinase assay with purified EL/CDK2 and LMW-E/CDK2 complexes. A list of proteins that were most differentially phosphorylated by EL/CDK2 and LMW-E/CDK2 complexes was generated (Supplementary Table S1). Our screen identified 146 substrates common to both EL/CDK2 and LMW-E/CDK2 complexes. Interestingly, we only identified four proteins that were phosphorylated by EL/CDK2 significantly more than by LMW-E/CDK2 as compared with the 14 potential substrates that were preferentially phosphorylated by LMW-E/CDK2 (Supplementary Fig. S3D). This suggests that by losing the N-terminal portion of cyclin E, the LMW-E/CDK2 kinase complex is able to specifically interact and phosphorylate additional proteins.

Results obtained from 2 independent ProtoArray microarray experiments identified Hbo1 as a novel substrate for LMW-E/CDK2 with the phosphorylation signal being 3-fold higher than that noted by EL/CDK2 (Fig. 3A). Hbo1 is highly conserved from yeast to humans and has been implicated in regulating gene expression, DNA replication, and DNA repair (23–26). Ectopic overexpression of Hbo1 in MCF7 and SKBR3 cells enhanced soft agar colony formation, whereas knockdown of Hbo1 with siRNA blocked S-phase progression and reduced cell proliferation (27, 28). Furthermore, Hbo1 is highly expressed in breast cancer tissues and correlates positively with histologic grade in estrogen receptor–positive tumors (29). Therefore, we decided to further investigate its relationship with cyclin E/CDK2 in mediating mammary tumorigenesis.

To examine if Hbo1 is in the same complex as LMW-E/CDK2, we coexpressed FLAG-EL or FLAG-LMW-E, HA-CDK2, with Myc-Hbo1 into HEK293T cells. We observed binding of EL and LMW-E as well as CDK2 with Hbo1 both using Myc and Flag antibodies (Fig. 3B).

To confirm whether Hbo1 is a substrate of the cyclin E/CDK2 kinase complex, purified EL/CDK2 and LMW-E/CDK2 kinase complexes were used in kinase assays (Fig. 3C). Both kinase complexes phosphorylate Hbo1 at similar levels, and addition of roscovitine (a CDK1/2 inhibitor) efficiently inhibited the Hbo1 phosphorylation signal (Fig. 3D). There are six potential CDK2 phosphorylation sites on the Hbo1 gene sequence (Fig. 3E) that were mutated to alanine to identify which site is being phosphorylated by the LMW-E/CDK2 complex. The mutant proteins were transfected into HEK293T cells, purified by IP, and subjected to kinase assay (Fig. 3F). Only the T88A-mutant variant of Hbo1 showed abolished radioactive signal, suggesting that of the six potential phosphorylation sites, the LMW-E/CDK2 complex is phosphorylating Hbo1 only at the T88 residue (Fig. 3G). Collectively, these results identify Hbo1 as a novel substrate of the LMW-E/CDK2 complex that may mediate critical downstream signaling to contribute to the ability of LMW-E/CDK2 to enrich a CSC phenotype.

**Cyclin E/CDK2 phosphorylation of Hbo1 does not affect the HAT activity of Hbo1**

CDK1 phosphorylates Hbo1 at T85/T88 to create a docking site for polo-like kinase 1 (Plk1; ref. 30), which subsequently activates the HAT enzymatic activity of Hbo1. We speculated that the interaction and phosphorylation of Hbo1 by the cyclin E/CDK2 complex may also affect the HAT activity of Hbo1. To test this hypothesis, we purified the following proteins from HEK293T cells overexpressing Myc-Hbo1 (wt, T88A, and T88D), streptavidin-flag-S protein (SFB-CDK2 by IP with Myc or FLAG antibodies and subjecting to an in vitro HAT activity colorimetric assay (Supplementary Fig. S4A and S4B). As shown in Supplementary Fig. S4C, phosphorylation of Hbo1 at T88 did not alter the HAT activity of Hbo1 as the T88A and T88D mutants exhibited similar HAT activity levels to wild-type (wt) Hbo1. Furthermore, inhibition of the kinase activity of EL/CDK2 and LMW-E/CDK2 by addition of roscovitine did not affect the HAT activity level. These results suggest that the HAT activity of Hbo1 is unlikely to modulate the LMW-E–mediated CSC phenotype.

**Hbo1 is overexpressed in breast cancer cell lines and when coexpressed with LMW-E/CDK2, enhances self-renewal capability of hMECs**

We next set out to directly decipher if Hbo1 has a role in inducing the CSC properties of the LMW-E–expressing cells. We first determined that Hbo1 is highly expressed in 13 of 18 breast cancer cell lines tested with no breast cancer subtype specificity compared with the relatively low levels in the three hMEC lines (Fig. 4A). The 76NE6-LMW-E-T1G2.2, T1G3.1, and T1G4.2 cells also express high levels of Hbo1 protein compared with the 76NE6-V and 76NE6-EL cells (Fig. 4B). To determine whether coexpression of cyclin E (EL or LMW-E), CDK2, and Hbo1 in the 76NE6 cells affect the CSC population, these gene constructs were introduced into the 76NE6 cells via lentiviral infection and stable cell lines were generated through antibiotic selection (Fig. 4C). The 76NE6 cells coexpressing wt Hbo1 with the LMW-E/CDK2 complex (Fig. 4C, lane 8) formed significantly more mammospheres than those with the EL/CDK2 complex (Fig. 4C, lane 7; P = 0.0027).
The data in Fig. 4C further strengthen our observation in Fig. 4A and B, which is that breast cancer cell lines express high levels of Hbo1 proteins and that the 76NE6-LMW-E, and the TDCs T1G2.2, T1G3.1, and T1G4.2 cells also express high level of Hbo1 protein compared with the 76NE6-V and 76NE6-EL cells. The parental 76NE6 cell line expresses very low level of Hbo1 and thus explains why retroviral induction of EL/CDK2 or LMW-E/CDK2 does not result in a difference in the ability of these cells to form mammospheres (Fig. 4C, lanes 5 and 6). However, when Hbo1 is concomitantly overexpressed, we observed a significant difference in the number of mammospheres formed between EL- and LMW-E-expressing cells, indicating that LMW-E/CDK2 coexpression with Hbo1 enhances self-renewal capability of hMECs to a higher extent that with EL/CDK2 (Fig. 4C, compare lane 7 with lane 8). Perhaps during tumor evolution, Hbo1 upregulation is selected for tumor growth advantage as we observe that most of the breast cancer cell lines that we examined express very high level of Hbo1 protein in Fig. 4A.

The phosphorylation signals indicate relative radioactive signal detected in the microarray spots. B, FLAG-EL or LMW-E, HA-CDK2, and Myc-Hbo1 constructs were transfected into HEK293T cells and IP with either Myc- or FLAG-tagged antibodies and probed with the indicated antibodies. IB, immunoblot. C, SFB-EL or SFB-LMW-E was cotransfected with SFB-CDK2 into HEK293T cells, purified using FLAG-tagged antibody, eluted with 3X FLAG peptide, and visualized by Western blot analysis. Myc-Hbo1 was transfected into HEK293T cells and purified using Myc-tagged antibody. D, the EL/CDK2 or LMW-E/CDK2 kinase complex was incubated with purified Hbo1 in the presence of [γ-32P]-ATP, with or without roscovitine. The samples were separated by SDS-PAGE and exposed to X-ray films. E, schematic of the Hbo1 gene construct with the potential phosphorylation sites predicted on the basis of the CDK2 consensus phosphorylation motif (S/T-P-X-R/K/H). F and G, the six potential phosphorylation sites were mutated to alanine, expressed, purified, and subjected to similar kinase assay as in D. IB, immunoblot.

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In addition, coexpression of the T88A Hbo1 mutant in the 76NE6 cells (Fig. 4C, lanes 9 and 10) reduced the number of mammospheres to similar levels as in the cells without Hbo1 overexpression (Fig. 4C, lanes 5 and 6), suggesting that the phosphorylation of Hbo1 by the cyclin E/CDK2 complex is critical for the enhanced self-renewal capability of these cells. Furthermore, the cells with the T88D Hbo1 phosphorylation mimetic expression (Fig. 4C, lanes 11 and 12) showed increase in mammosphere formation compared with T88A Hbo1 (Fig. 4C, lanes 9 and 10) underscoring the importance of the phosphorylation of Hbo1 at T88 by the cyclin E/CDK2 complex.

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Figure 4. Hbo1 is overexpressed in breast cancer cell lines and coexpression with LMW-E/CDK2 enhances self-renewal capability of hMECs. A, cell lysates from three hMECs lines and 18 breast cancer cell lines were subjected to Western blot analysis with antibodies to Hbo1 and β-actin. B, the 76NE6 stable cell panel and the TDCs were subjected to similar analysis as in A. C, lentivirus generated in HEK293T cells and carrying the EL, LMW-E, CDK2, or Hbo1 shRNA were used to transduce the 76NE6 cell line, and the knockdown efficiency was assessed by Western blot analysis. D, the 76NE6 stable cell panel and the TDCs were subjected to similar analysis as in A.
to generate a docking site for protein recruitment. Collectively, these results indicate that Hbo1 along with LMW-E/CDK2 function to alter the physiology of the cell to generate cell properties associated with CSCs.

Next, we interrogated the role of Hbo1 in enriching the CSC population. For these studies, shRNAs targeting the Hbo1 mRNA were packaged into lentivirus with lentivirus carrying the shHbo1–5R construct followed by the same analyses. The FACS and mammosphere formation results were averaged from at least 2 independent experiments and the statistical analysis used was unpaired Student t test; Fig. 5D). In addition, high LMW-E expression levels as measured by Western blot analysis significantly associated with cytoplasmic cyclin E staining (P = 0.0194, Pearson χ² test; Fig. 5G), whereas EL (Fig. 5F) and total cyclin E (Fig. 5E) expression levels did not. In other words, the cytoplasmic cyclin E staining observed from IHC analysis is likely to represent LMW-E cyclin E and not of full-length cyclin E consistent with our previous report showing that LMW-E preferentially accumulates in the cytoplasm in complex with CDK2 and associated kinase activity (32). Interestingly, the altered subcellular localization of the LMW-E isoform renders it less susceptible to proteasomal degradation by Fbw7. As a result, we believe that the cytoplasmic localization of LMW-E further enhances the stability of the LMW-E/CDK2 complex compared with the EL/CDK2 complex. The results found here further support this observation (32). In conclusion, similar to our cell line data, tumor tissue from patients with breast cancer who have high CD44hi/CD24lo population significantly correlate with cytoplasmic cyclin E, and we have shown that cytoplasmic cyclin E protein represents the LMW isoforms.

**Doxorubicin synergizes with salinomycin to kill LMW-E–expressing tumor cells**

Doxorubicin was recently identified from a large screen of small-molecule inhibitors to specifically kill CSCs (33). Breast cancer cells treated with salinomycin showed a decrease in the CD44hi/CD24lo population and reduction in their ability to form mammospheres, and the 4T1 xenograft tumors formed fewer metastases compared with parallel paclitaxel treatment (33). Indeed, we showed that salinomycin treatment reduced the CD44hi/CD24lo population of the LMW-E–expressing cells in a dose-dependent manner (Fig. 6A and Supplementary Fig. S6). The TDCs contain approximately 80% of the CD44hi/CD24lo population, in which only 10% of these cells remained

that correspond to increasing percentage of CD44hi/CD24lo population (Fig. 5A). In addition, duplicate slides from the same patient cohort were subjected to IHC staining using a cyclin E antibody and a scoring system as defined in Fig. 5B. Finally, lysates prepared from these patient samples were also analyzed for the protein levels of EL and LMW-E by Western blot analysis followed by densitometric analysis (Fig. 5C). Statistical analysis was applied to data obtained from (i) CD24/CD44 double IHC staining, (ii) cyclin E IHC staining, and (iii) EL versus LMW-E protein levels from Western blot analysis.

Statistical analysis revealed that more than 60% of the patient tissues contain less than 10% of the CD44hi/CD24lo population (i.e., tumors that received a score of 0 or 1; n = 72), and this patient distribution was similar to that previously reported (Table 1; ref. 31). In Fig. 5D, we show that cytoplasmic staining of cyclin E significantly correlated with high percentage of the CD44hi/CD24lo population (P = 0.0188, Student t test; Fig. 5D). In addition, high LMW-E expression levels associated with cytoplasmic cyclin E staining (P = 0.0194, Pearson χ² test; Fig. 5G), whereas EL (Fig. 5F) and total cyclin E (Fig. 5E) expression levels did not. In other words, the cytoplasmic cyclin E staining observed from IHC analysis is likely to represent LMW-E cyclin E and not of full-length cyclin E consistent with our previous report showing that LMW-E preferentially accumulates in the cytoplasm in complex with CDK2 and associated kinase activity (32). Interestingly, the altered subcellular localization of the LMW-E isoform renders it less susceptible to proteasomal degradation by Fbw7. As a result, we believe that the cytoplasmic localization of LMW-E further enhances the stability of the LMW-E/CDK2 complex compared with the EL/CDK2 complex. The results found here further support this observation (32). In conclusion, similar to our cell line data, tumor tissue from patients with breast cancer who have high CD44hi/CD24lo population significantly correlate with cytoplasmic cyclin E, and we have shown that cytoplasmic cyclin E protein represents the LMW isoforms.

**Cytoplasmic cyclin E expression correlates with high CD44hi/CD24lo population in breast cancer patient tissue**

Given that cyclin E expression positively associates with the CD44hi/CD24lo population in hMECs, we aimed to determine whether this correlation also exists in tumor tissue samples from patients with breast cancer. Breast cancer patient tissues (n = 118) were analyzed for CD24 and CD44 expression by double IHC staining and visualized by 3,3'-diaminobenzidine (DAB) and permanent red, respectively (Fig. 5A). The clinico-pathologic characteristics of the patient cohort are presented in Supplementary Table S2. The slides were given a score of 0 to 5
after treatment with 8 μmol/L salinomycin. We show that salinomycin treatment results in the enrichment of the cell population with non-CSC (CD44hi/CD24lo) cells that are not killed by salinomycin. In addition, salinomycin treatment effectively disrupted their ability to form mammospheres (Fig. 6B). This observation led us to hypothesize that cotreatment with doxorubicin, which is expected to target the non-CSC population with non-CSC (CD44hi/CD24hi) cells that are not killed by salinomycin, and then followed by salinomycin to target the CSC population. Most highly proliferative cancer cells are sensitive to conventional chemotherapy drugs, whereas CSCs are believed to be more resistant to these agents, resulting in high frequency of tumor recurrence (34). As anticipated, doxorubicin treatment with increasing drug concentration did not affect the distribution of the CD44hi/CD24lo population of any of the cell lines examined (Fig. 6C). Therefore, a combination treatment regimen was used in which the LMW-E tumor cells were first treated with doxorubicin to eliminate the non-CSC population and then followed by salinomycin to target the CSC population. An advantage of combining drug treatment is that it often allows for administration of the drugs at lower concentrations and therefore lessens the toxic side effects if the drugs are synergistic. We used the HTSA to examine the toxicity of combining doxorubicin and salinomycin (Supplementary Fig. S3).

Using the CalcuSyn software, the isobolograms of the 76NE6-V and 76NE6-EL cell lines indicated that the combined drug treatment resulted in additive to antagonistic effects (Fig. 6D and E). In contrast, the drug treatment showed a synergistic effect in the LMW-E–expressing cells (76NE6-LMW-E, T1G2.2, T1G3.1, and T1G4.2) and the MDA-MB-231 breast cancer cells, which express endogenous LMW-E isoforms. An average of the

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combination indices confirmed that the doxorubicin and salinomycin combination resulted in additive/antagonistic effects in the 76NE6-V and 76NE6-EL cells but synergistic effects in the 76NE6-LMW-E and the TDCs (Fig. 6F). These results suggest a promising therapeutic strategy of combining doxorubicin and salinomycin to selectively treat patients with breast cancer who have high levels of LMW-E expression and enriched CSC population.

**Discussion**

The role of LMW-E in breast cancer was strongly established by its association with poor clinical outcome and was subsequently reinforced by the demonstration that transgenic mice with ectopic LMW-E expression develop mammary carcinoma and metastasis at significantly higher frequency compared with transgenic mice with EL expression (15, 17). We recently described similar findings in a xenograft mouse model in which hMECs expressing LMW-E/CDK2 yielded higher tumor incidence compared with cells with EL/CDK2 expression (16).

By expressing LMW-E, the 76NE6 mammary epithelial cells underwent a morphologic switch by adopting mesenchymal characteristics as well as CSC properties (16). Here, we elucidate the mechanism that provides LMW-E-expressing cells increased tumorigenic potential. LMW-E–expressing cells contain a high percentage of cells in the CD44\textsuperscript{hi}/CD24\textsuperscript{lo} population, were able to form mammospheres in nonadherent culture, and had upregulated expression and enzymatic activity of ALDH. More importantly, the phenotypes observed were more robust with exogenous LMW-E overexpression than with EL overexpression.

Furthermore, we observed a strong positive association between LMW-E expression in breast cancer tissues from patients with tumors that contain high CD44\textsuperscript{hi}/CD24\textsuperscript{lo} population, suggesting that breast cancer cells with LMW-E expression have CSC-like characteristics. More importantly, statistical analysis also revealed a significant association between LMW-E expression and cytoplasmic localization from IHC staining of the tumor tissues, which is a more efficient way to identify LMW-E expression in tumor tissues by IHC rather than by Western blot analysis.

We have previously reported that overexpression of the LMW-E protein in mammary epithelial cells results in an increase in the expression of full-length cyclin E (EL; ref. 16). This phenomenon was observed in both cell line and transgenic mouse model systems with inducible LMW-E expression. Perhaps during tumor progression, LMW-E induces an unchecked G\textsubscript{1}–S phase progression, thus resulting in a positive feedback loop that drives increased cyclin E expression. As a result, EL overexpression further contributes to the tumorigenic phenotypes. In all of our functional assays, we observed that overexpression of EL resulted in a mild induction in the phenotypes compared with those from overexpression of LMW-E (∼50%). However, LMW-E/CDK2 is a stronger and more stable kinase complex compared with the EL/CDK2 complex (32) and given that approximately 30% of patients with breast cancer express high levels of LMW-E, their tumor induction and progression may be due to an enhanced CSC population.

Our screen for novel substrates of the cyclin E/CDK2 complex identified Hbo1, which is a HAT that can alter the expression pattern of multiple genes. The consensus phosphorylation motif for CDK2 is S/T-P-X-R/K/H, and we found that cyclin E/CDK2 phosphorylated Hbo1 at T\textsuperscript{88}KK conferring that the phosphorylation is specific. T88 is located within the N-terminal serine-rich region of the Hbo1 gene, which is the regulatory domain, whereas the C-terminal region contains the Zn-finger and a HAT domain that carries out the enzymatic function (26, 35). The results from our *in vitro* kinase assay showed similar phosphorylation levels of Hbo1 by both the EL/CDK2 and LMW-E/CDK2 kinase complexes. The reason no difference in the extent of Hbo1 phosphorylation between the EL/CDK2 and LMW-E/CDK2 complexes is detected is due to the saturated concentrations of the kinase as well as the substrate in this *in vitro* kinase assay condition compared with the condition conducted for the ProtoArray. The kinase concentration was 50 nmol/L in the microarray experiment and was approximately 500 nmol/L in this *in vitro* kinase assay. In addition, as the LMW-E isoforms are overexpressed in breast cancer, we speculate that it is these truncated forms of cyclin E in complex with CDK2 that phosphorylate Hbo1 and not the full-length cyclin E form.

The fact that coexpression of Hbo1 with LMW-E/CDK2 further increased the CSC properties of hMECs, whereas knockdown of Hbo1 expression reduced the CD44\textsuperscript{hi}/CD24\textsuperscript{lo} population and mammosphere formation ability suggests that Hbo1 plays a role downstream of LMW-E in enriching the CSC population. We speculate that in the cellular context, phosphorylation of Hbo1 by the LMW-E/CDK2 complex at T88 may also create a docking site for specific kinases/proteins to be recruited to regulate Hbo1’s HAT activity.

Given that the understanding of breast CSCs has only recently evolved, clinical therapy aimed at targeting these cells is currently under active investigation. In a large screen for

**Table 1. CD44\textsuperscript{hi}/CD24\textsuperscript{lo} patient distribution**

<table>
<thead>
<tr>
<th>Score (%CD44\textsuperscript{hi}/CD24\textsuperscript{lo})</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (0%)</td>
<td>37 (31)</td>
</tr>
<tr>
<td>1 (1%–10%)</td>
<td>35 (30)</td>
</tr>
<tr>
<td>2 (11%–25%)</td>
<td>20 (17)</td>
</tr>
<tr>
<td>3 (25%–50%)</td>
<td>14 (12)</td>
</tr>
<tr>
<td>4 (51%–75%)</td>
<td>10 (8)</td>
</tr>
<tr>
<td>5 (76%–100%)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>Total</td>
<td>118 (100)</td>
</tr>
</tbody>
</table>

*NOTE: The CD24 and CD44 double staining was given a score from 0 to 5 for the respective percentage of CD44\textsuperscript{hi}/CD24\textsuperscript{lo} population in the tissue slide. At least three images from different regions of each patient slide were scored and averaged. N represents number of patients and % is the percentage of total patient population with that particular score.*
small-molecule inhibitors that can specifically kill CSCs, Gupta and colleagues identified salinomycin as a potent agent that can abrogate the phenotypes associated with CSCs and thereby reduce tumor aggressiveness and regrowth (33). Recently, salinomycin was reported to sensitize cancer cells to doxorubicin and etoposide, leading to increased DNA damage and apoptosis (36). In our study, we also showed that the combination of doxorubicin and salinomycin effectively targeted both the tumor bulk (non-CSCs) and the CSCs (37, 38). Such synergistic effect on cancer cell toxicity further encourages investigation into other drug combinations that target both the CSC and non-CSC populations, which could be used in the clinic. Furthermore, the identification of Hbo1 as a downstream substrate of the LMW-E/CDK2 complex provides a novel mechanism of how LMW-E mediates mammary tumorigenesis.

The level of Hbo1 phosphorylation can also be analyzed for association with the percentage of the CD44hi/CD24lo population as well as survival outcome in patients. For instance, a combination between a CDK inhibitor (i.e., roscovitine) and salinomycin might be effective at inhibiting the CSC population in patients with high LMW-E expression. Although CDK inhibitors such as flavopiridol and roscovitine have shown unimpressive response in clinical trials as a single agent, it must be noted that the patients in these trials were not selected on the basis of LMW-E levels (20, 39). By inhibiting the CSC population in the high LMW-E–expressing tumors, we may be able to achieve favorable outcome...
and avoid tumor relapse due to the specific targeting of the CSCs.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: M.T. Duong, S. Akli, A. Biernacka, K.K. Hunt, K. Keyomarsi
Development of methodology: M.T. Duong, S. Akli, A. Biernacka, B.G. Debeb, K.K. Hunt, K. Keyomarsi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Akli, A. Biernacka, K. Keyomarsi
Analysis and interpretation of data (e.g., statistical analysis, biosistics, computational analysis): M.T. Duong, S. Akli, A. Biernacka, M. Yi, K.K. Hunt, K. Keyomarsi
Writing, review, and/or revision of the manuscript: M.T. Duong, K.K. Hunt, K. Keyomarsi

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.T. Duong, K.K. Keyomarsi

Study supervision: M.T. Duong, K.K. Hunt, K. Keyomarsi

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MyLinh T. Duong, Said Akli, Sira Macalou, et al.

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