Introduction

Progression through the cell cycle is driven by cyclin-dependent kinases (Cdk) whose catalytic activity and substrate specificity depend on their association with regulatory sub-units called cyclins. Altered expression of cyclins can drive aberrant proliferation in cancer (1). Cyclin E is of particular importance, as its expression is deregulated in many cancers, most notably breast cancer (2–4). One mode of deregulation of cyclin E expression is the generation of low-molecular-weight (LMW) isoforms following cleavage of full-length cyclin E by an elastase-like protease (5). A consequence for cells with LMW-isoforms following cleavage of full-length cyclin E is the generation of low-molecular-weight cyclin E in mammary tumorigenesis, which may be the result of the ability of LMW-cyclin E to bind and activate its Cdk partner, Cdk2. These isoforms bind more tightly to Cdk2, which leads to increased Cdk2 kinase activity and decreased sensitivity of the Cdk2 complex to inhibition by p21 and p27 (6, 11). The LMW-cyclin E expressing cells are also resistant to anti-estrogens (6) and to aromatase inhibitors (12) due to decreased sensitivity of the LMW-cyclin E/Cdk2 complexes to p21 and p27, which are induced in response to anti-estrogen treatment.

Cdk2 is a critical enzyme in the transition of cells from G1 to S-phase, and its deregulation in cancer could be causative of oncogenesis. One of the functions of Cdk2 (in complex with either cyclin E or cyclin A) is to phosphorylate substrates such as the retinoblastoma protein (pRB) that activates the genes necessary for S-phase through E2F-dependent transcription. Additional substrates of cyclin E/Cdk2 complexes include NPAT, a transcription factor that controls cell cycle–dependent histone gene transcription (13), nucleophosmin (14), CP110 (15), and Mps1 (16), proteins involved in centrosome duplication, Brca1 (17) and Ku70 (18), involved in DNA repair, and Cdk inhibitors, p21 and p27. However, a number of kinase-independent functions of cyclin E have been described including a role in replication endocycle (19, 20), in replication licensing during exit from quiescence (21), in oncogenic...
transformation by ras and dominant negative p53 (21), and in cell fate determination (22).

Cdk2-independent functions of cyclin E are also inferred from in vivo models. For example, knockout of both cyclin E1 and cyclin E2 genes in the mouse leads to embryonic lethality due to defects in the endo-reduplication of trophoblast cells (19, 20), whereas Cdk2 knockout mice do not have placental defects suggesting that E-type cyclins have Cdk2-independent roles in early development (21). A kinase-deficient cyclin E mutant can partially restore MCM loading and exit from quiescence in cyclin E-null cells (21). Cyclin E also localizes to the centrosomes in a Cdk2-independent fashion, and mutation of the centrosomal localization sequence prevents the mutated overexpressed cyclin E to increase the S-phase fraction of cells (23). In Drosophila, even when p21 is overexpressed, cyclin E is required for the initial asymmetric division of certain neuroblasts for neural segment-specific versus abdomen lineages suggesting a role in cell fate determination (22). These kinase-independent functions of the overexpressed cyclin E might facilitate the escape of tumor cells from quiescence, and might modify their cell fate, thereby contributing to cancer formation. A major, unresolved issue is the contribution of these various kinase-dependent and kinase-independent functions of LMW-cyclin E to tumorigenesis. In this study, we have addressed the question of the requirement of Cdk2 for LMW-cyclin E–induced mammary tumorigenesis function by generating LMW-cyclin E transgenic mice with either a Cdk2 wild-type or knockout background. These studies show that LMW-cyclin E–mediated tumorigenesis is completely dependent on the presence of Cdk2.

Materials and Methods

Generation of Cdk2-deficient mice (24), p53-deficient mice (25), and MMTV-LMW-cyclin E transgenic mice (10) were described previously. As Cdk2–/– mice were reported to be sterile, the mouse strain was maintained as Cdk2+/– and genotyped as previously described (24). Because all our transgenic lines were created and maintained on an inbred FVB/N background, Cdk2+/– mice created on a 129/CD-1 background were backcrossed 7 generations into an FVB/N background prior to crosses with p53+/– and LMW-cyclin E transgenic lines. Mating MMTV-LMW-cyclin E; Cdk2+/– mice with Cdk2+/– mice yielded the 3 experimental groups MMTV-LMW-cyclin E; Cdk2+/+, MMTV-LMW-cyclin E; Cdk2+/–, and MMTV-LMW-cyclin E; Cdk2–/–. Crossing MMTV-LMW-cyclin E; Cdk2+/– mice with p53–/–; Cdk2+/– mice yielded the 3 experimental groups MMTV-LMW-cyclin E; p53+/+; Cdk2+/+, MMTV-LMW-cyclin E; p53+/–; Cdk2+/+, and MMTV-LMW-cyclin E; p53+/–; Cdk2–/–. Mice were kept as virgins and were monitored biweekly, by palpation, for tumor occurrence. Animals showing tumors (with a mean diameter of no more than 1.5 cm) were killed, and the presence of mammary adenocarcinomas was confirmed histologically. All experiments were approved by the Animal Care and Use Committee at the University of Texas, MD Anderson Cancer Center (Houston, TX), and were performed in accordance with relevant institutional and national guidelines and regulations.

Roscovitine and meriolin 5 treatment of mice

Roscovitine and meriolin 5 were supplied by Dr. Laurent Meijer (Station Biologique de Roscof, Roscof, France). A roscovitine stock solution of 10 mg/mL (28 mmol/L) in 50 mmol/L HCl pH 2.5 was prepared and stored at −20°C. Mice were randomly separated in the control and test groups, which were injected intraperitoneally (i.p.) with 50 mmol/L HCl pH 2.5 or roscovitine (75 mg/kg) respectively, at 10 μL of stock solution/g of mouse weight i.p. 2 times daily for 7 days. A meriolin 5 stock solution of 3.333 mg/mL was prepared in dimethyl sulfoxide (DMSO). For daily injections of laboratory mice, a working solution of 0.1 mg/mL was freshly prepared with sterile PBS (30:70, v/v). Mice were randomly separated in the control and test groups, which were injected i.p. with DMSO/PBS (30:70, v/v) or meriolin 5 (1 mg/kg/d), for 2 series of 5 days with a 2-day break in between. Statistical significance was assessed using the log-rank test of Kaplan–Meier analysis.

Hormone treatment of mice

Twelve-week-old nulliparous mice were treated for 48 hours with a single interscapular s.c. injection of 17β-estradiol benzoate (1 μg) and progesterone (1 mg) in 100 μL of sesame oil (all from Sigma). Two hours before killing, animals were injected i.p. with 5-bromo-2-deoxyuridine (BrdUrd; 0.03 mg/g of body weight; Sigma). Mammary glands were removed and fixed in 10% formalin overnight. After embedding in paraffin, tissues were sectioned (5–7 μm) onto Probe-On Plus charged slides (Fisher Scientific).

Whole-mount mammary gland staining and histology

Mammary glands from selected mice were dissected out and fixed on glass slides with Carnoy’s solution (glacial acetic : chloroform : ethanol, 1:3:6) overnight at room temperature. The glands were dehydrated prior to overnight staining in aluminum carmine (1 g carmine, 2.5 g aluminum potassium sulfate boiled for 20 minutes in distilled H2O, filtered, and brought to a final volume of 500 mL). The glands were then dehydrated, cleared with xylene, and mounted. Photographs were taken under 4× power objective using a digital camera mounted on a Leica MZ125 microscope. For histologic analysis, 6-mm sections were cut and stained with hematoxylin and eosin.

BrdUrd incorporation assay

To assess cell proliferation, mice were injected i.p. with 0.25 mg BrdUrd/g of body weight 2 hours before killing. BrdUrd incorporation was detected on sections by immunohistochemistry using a cell proliferation kit (Amersham) following the manufacturer’s instructions. The numbers of BrdUrd-positive cells in wild-type and MMTV-cyclin E mammary glands were counted in 10 fields under a 40× objective lens.

Western blot analysis, immunoprecipitation, kinase assays, and immunohistochemistry

Cell lysates were prepared and subjected to Western blot analysis as previously described (26). Briefly, 50 μg of protein...
was subjected to electrophoresis on SDS-PAGE and transferred to Immobilon P overnight at 4°C at 35 mV constant voltage. The blots were blocked overnight at 4°C in BLOTTO (5% nonfat dried milk in 20 mmol/L Tris, 137 mmol/L NaCl, 0.05% Tween, pH 7.6). After being washed, the blots were incubated in primary antibodies for 3 hours. Primary antibodies used were cyclin E (HE-12; Santa Cruz Biotechnology), Cdk2 (Transduction Laboratories), and actin (Chemicon International Inc.). Blots were incubated with goat anti-mouse immunoglobulin-gorseradish peroxidase conjugate at a dilution of 1:5,000 in BLOTTO for 1 hour and finally washed and developed by using the Renaissance chemiluminescence system as directed by the manufacturer (Perkin-Elmer Life Sciences, Inc.). For immunoprecipitation, 250 μg of tissue extracts were used per immunoprecipitation with polyclonal antibody to cyclin E coupled to protein A beads. After being washed, the immunoprecipitates were incubated with kinase assay buffer containing 60 mmol/L cold ATP, 5 mCi of [32P]ATP, and 5 μg of histone H1 (Roche Diagnostics Corporation) in a final volume of 30 μL at 37°C for 30 minutes. The products of the reaction were analyzed on 13% SDS-PAGE gels, and the gels were stained, destained, dried, and exposed to X-ray film. For quantitation, the protein bands corresponding to histone H1 were excised, and the radioactivity of each band was measured by Cerenkov counting. For immunohistochemistry, the sections were incubated in 1% H2O2 to block endogenous peroxidase activity. To retrieve nuclear antigens on paraffin embedded sections, slides were incubated for 20 minutes in 10 mmol/L sodium citrate buffer (pH 6.0) at 90°C. The sections were then incubated for 60 minutes in 5% fetal calf serum (FCS) overnight with primary antibodies, followed by 2-hour incubation at room temperature with appropriate secondary antibodies. Nuclei were counterstained with hematoxylin. Rabbit polyclonal anti–cyclin E (Santa Cruz Biotechnology, sc-198 [C19]) that specifically recognized the human cyclin E protein was used for the transgenic cyclin E. For detection, the Vectastain ABC Elite kit (Vector Labs) was used.

Compounds, cell lines, and culture conditions

CVT-313, a selective and potent inhibitor of Cdk2, was obtained from Enzo Life Sciences International, while roscovitine and meriolin 5 were provided by Dr. Laurent Meijer. Serum was purchased from Hyclone Laboratories and cell culture medium from Life Technologies, Inc. The culture conditions for HCC1806, HCC1569, ZR75-1, UACC812, and MCF-7 breast cancer cell lines were described previously (27, 28). All cell lines were purchased from American Type Culture Collection and used within 6 months.

RNA interference

The synthetic small interfering RNA (siRNA) oligonucleotides were synthetized by Sigma. siRNAs were targeting Cdk2 on nucleotides 274–292 in NM_001798 for Cdk2 siRNA#1 (ID# SASI_Hs01_00060175) and on nucleotides 308–326 in NM_001798 for Cdk2 siRNA#2 (ID# SASI_Hs01_00060174). A MISSION siRNA Universal Negative Control #1 was used as negative control (Neg). Breast cancer cell lines were cultured for 24 hours in 6-well plates and transfected with 2 μg siRNA using X-tremeGENE siRNA Transfection Reagent (Roche) according to manufacturer’s protocol. For MCF-7 LMW-cyclin E (T2) cells, doxycycline (1 μg/mL) was used to induce expression of LMW (T2) cyclin E for 24 hours before siRNA transfection.

MTT metabolism assay

Cell proliferation assays were carried out using MTT assays, where MTT is reduced to purple formazan in the mitochondria of living cells. Increase in cell number is detected by increased MTT metabolism, and decrease in cell number is reflected by decrease in MTT metabolism. MCF-7 cells that can inductively express Flag-tagged LMW-cyclin E (T2) upon treatment with doxycycline [described in (7)] were plated at a density of 2,500 cells per well in 96-well plates and cultured overnight in MEM supplemented with 10% FBS. Cells were incubated with or without doxycycline (1 μg/mL) for 24 hours, and cells were then treated with either diluent (DMSO) or CVT-313 (29), or meriolin 5 (30), or roscovitine (31) at 8 different concentrations for 48 hours. Stock solution for each drug was made in DMSO at a concentration of 25 mmol/L for CVT-313, 10 mmol/L for roscovitine, and 1 mmol/L for meriolin 5 followed by 2-fold serial dilution in the medium. Each well was replaced with 200 μL of fresh medium containing MTT (0.5 μg/μL) and incubated for 4 hours. The medium was then removed and 100 μL of solubilization buffer (0.04M HCl, 1% SDS in isopropyl alcohol) was added to each well. The plate was agitated in the dark for 5 minutes to dissolve the MTT-formazan crystals. The absorbance of the samples was recorded at 590 nm in a multiwell plate reader (Perkin-Elmer Victor 1420). Results were plotted as the mean [95% confidence interval (CI)] values of quadruplicates from a representative experiment that was repeated at least 2 independent times.

Statistical analysis

Tumor onset data were analyzed for statistical significance by using survival analysis methods. Within each genotype, tumor-free survival curves were estimated by the Kaplan–Meier method and compared by using the log-rank test using Prism (GraphPad software, Inc.). Results are shown as mean ± SD. Differences were considered significant when the 2-tailed Student’s t test showed differences at P < 0.05.

Results

Decreased branching and proliferation in Cdk2−/− mammary glands can be rescued by ovarian hormone treatment

We compared the appearance of mammary glands of adult, virgin Cdk2−/− females with that of Cdk2+/+ and Cdk2+/− by whole mounts stained with carmine red (Fig. 1A). The results reveal that although the development of the whole mammary glands proceeds relatively normally in all 3 genotypes, the branching morphogenesis is decreased in the Cdk2−/− animals (Fig. 1A). This raised the question of whether there is a defect in the ability of the mammary cells to proliferate in the Cdk2−/− mice, or if the decreased branching is linked to an indirect effect of Cdk2 loss on the
synthesis of ovarian hormones due to the reduced size of the ovaries. To address this question, we examined the ability of mammary epithelial cells in each of the 3 genotypes to proliferate in response to pregnancy hormones, estrogen and progesterone. To mimic the burst of proliferation observed during early pregnancy, Cdk2\(^+\)/+ mice, Cdk2\(^+\)/− mice, and Cdk2\(−/−\) mice were treated acutely with 1 mg of estrogen and 1 mg of progesterone either once for 48 hours or 3 times, 48 hours apart (E+P, 3x 48 hours). Two hours before killing, the mice were injected i.p. with BrdUrd (0.03 mg/g of body weight). Following treatment, the contralateral inguinal gland was removed (Cdk2\(^+\)/+ mice, n = 3; Cdk2\(^+\)/− mice, n = 3; Cdk2\(−/−\) mice, n = 3, 11–32 weeks of age at day 0 of experiment) and whole-mounts were prepared. B and C, proliferation is quantified by BrdUrd staining. NS, not significant.

Figure 1. Hormone-induced proliferation in Cdk2\(−/−\) mammary glands. A, a cohort of mice were left untreated (no hormones) or treated acutely (48–50 hours) with estradiol benzoate (E, 1 \(\mu\)g, Sigma) and P (1 mg, Sigma) in 100 \(\mu\)L sesame oil via a single interscapular s.c. injection behind the neck once (E+P, 48 hours) or 3 times, 48 hours apart (E+P, 3x 48 hours). Two hours before killing, the mice were injected i.p. with BrdUrd (0.03 mg/g of body weight). Following treatment, the contralateral inguinal gland was removed (Cdk2\(^+\)/+ mice, n = 3; Cdk2\(^+\)/− mice, n = 3; Cdk2\(−/−\) mice, n = 3, 11–32 weeks of age at day 0 of experiment) and whole-mounts were prepared. B and C, proliferation is quantified by BrdUrd staining. NS, not significant.
not altered the ability of mammary epithelial cells to proliferate in response to stimuli, in this case pregnancy hormones.

**Resistance of Cdk2–/– mice to LMW-cyclin E–induced breast cancer**

The critical role of LMW-cyclin E in mammary oncogenesis and tumorigenesis (6, 32) may be the result of the ability of LMW-cyclin E to bind and activate Cdk2. To specifically test the importance of LMW-cyclin E–associated Cdk2 kinase activity in mammary oncogenesis, we generated double and triple transgenic mice with the genotypes LMW-cyclin E/ Cdk2–/– and LMW-cyclin E/p53+/–/Cdk2–/–. The transgene in all these animals is under the control of the MMTV promoter. We followed the MMTV-LMW-cyclin E mice in the different genotypes for mammary tumor incidence and found that the loss of Cdk2 protected Cdk2–/– mice from breast cancer induced by LMW-cyclin E in the p53+/+ (Fig. 2A) or p53+/– (Fig. 2B) background. Whereas LMW-cyclin E mice in Cdk2+/– or Cdk2+/+ background succumb to mammary tumors with mean latencies of 16 and 19.5 months respectively, their LMW-cyclin E; Cdk2–/– littersates do not form mammary tumors (Fig. 2A).

Similarly, although MMTV-LMW-cyclin E/p53+/–– transgenic mice in Cdk2+/– or Cdk2+/+ background succumb to mammary gland tumors between 7 and 13 months with 100% penetrance, their MMTV-LMW-cyclin E; p53+/+/–; Cdk2–/– littersates are resistant to tumor formation (Fig. 2B). These results clearly show that Cdk2 is critically required for LMW-cyclin E–induced mammary tumorigenesis and consequently, the loss of Cdk2 renders Cdk2–/– mice resistant to breast cancer induced by LMW-cyclin E expression.

**Cdk2 absence does not prevent LMW-cyclin E expression in the mammary gland**

Next, we examined if in the absence of Cdk2 the cyclin E transgene is expressed equally in each genotype. To this end we examined cyclin E levels by immunohistochemical analysis in the mammary glands of LMW-cyclin E transgenic mice in a Cdk2+/+ or Cdk2–/– background (Fig. 3A). The results revealed that similar levels of the transgene expression in the mammary glands of Cdk2+/+; and Cdk2–/– females were observed. In addition, the ducts in each genotype had similarly normal morphology (Fig. 3A). These results rule out the possibility that the differences in breast cancer free

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**Figure 2. Resistance of Cdk2–/– mice to LMW cyclin E–induced breast cancer.** Percentage of mammary tumor free mice among MMTV-LMW cyclin E-T1; Cdk2+/+; MMTV-LMW cyclin E-T1; Cdk2+/-; MMTV-LMW cyclin E-T1; Cdk2–/– females in A and MMTV-LMW cyclin E-T1; p53+/–; Cdk2+/+; MMTV-LMW cyclin E-T1; p53+/–; Cdk2+/-; MMTV-LMW cyclin E-T1; p53+/–; Cdk2–/– females in B. Whereas LMW-cyclin E-T1 mice in Cdk2+/- or Cdk2+/+ background succumb to mammary tumors with mean latencies of 16 and 19.5 months respectively, their LMW cyclin E-T1; Cdk2–/– littersates do not form mammary tumors (P = 0.05, Cdk2+/- vs. Cdk2–/–; P = 0.0252, Cdk2+/+ vs. Cdk2–/–; P = 0.1457, Cdk2+/+ vs. CDk2+/–, log-rank test). Similarly, MMTV-LMW-cyclin E T1/p53+/– transgenic mice in Cdk2+/- or Cdk2+/+ background succumb to mammary gland tumors between 7 and 13 months with 100% penetrance, their MMTV–LMW-cyclin E-T1/p53+/–; Cdk2–/– littersates are resistant to tumor formation (P < 0.01, Cdk2+/+ vs. Cdk2–/–; P = 0.011, Cdk2+/- vs. Cdk2–/–; P = 0.5072 Cdk2+/+ vs. Cdk2–/–, log-rank test).
survival could be caused by an inadequate expression of LMW-cyclin E transgene in the mammary glands of Cdk2−/− animals and that Cdk2 absence does not prevent LMW-cyclin E expression in the mammary glands.

We then asked if the cyclin E–associated kinase activity in the Cdk2+/− mammary glands was completely dependent on Cdk2. We measured the cyclin E–associated kinase activity using histone H1 and found a total absence of cyclin E–associated kinase activity in the Cdk2+/− mammary glands (Fig. 3B). Hence, even though the LMW-cyclin E is being expressed in the mammary gland (Fig. 3A), the protein does not retain any cyclin E–associated kinase activity in a Cdk2+/− background.

Roscovitine and meriolin treatment delay LMW-cyclin E–induced breast cancer

Next, we set out to investigate if targeting Cdk using roscovitine and meriolin, two Cdk2 inhibitors, would suppress mammary tumorigenesis in MMTV-LMW-cyclin E transgenic mice in a Cdk2+/− background. Roscovitine (31) is a potent and selective inhibitor of Cdk1, Cdk2, and Cdk5, with strong antiproliferative and apoptotic effects in preclinical models and antitumor activity in several xenograft models. Roscovitine is well tolerated when administered by i.p. injection, and displays high bioavailability (33). To assess if treatment of the LMW-cyclin E transgenic mice with roscovitine would delay tumor formation, we i.p. injected a group of 7 mice and a group of 7 mice with roscovitine starting at 7 months, twice a day for 7 days. We used the LMW-cyclin E; p53+/−/+− mice in a Cdk2+/+, Cdk2+/−−, or Cdk2−−/−− background was carried out with a cyclin E antibody (HE12) using histone H1 as substrate. The histogram shows the relative activities after densitometric scanning (arbitrary units).

![Figure 3. Cdk2 absence does not prevent LMW-cyclin E expression in the mammary gland. A, immunohistochemistry for human cyclin E protein in mammary glands of MMTV-LMW-cyclin E-T1; p53+/−−; Cdk2+/+/++ and MMTV-LMW-cyclin E-T1; p53+/−−; Cdk2+/−−− B, cyclin E-associated kinase assays of mammary gland protein lysates from age-matched wild-type (Non-Tg), MMTV-LMW-cyclin E; p53+/−− mice in Cdk2+/+, Cdk2+/−−, Cdk2−−/−− background.](image-url)
staining followed by FACS demonstrated that Cdk2 knockdown in LMW-cyclin E-expressing line HCC1569 and 1806 led to a strong induction of apoptosis with a 3- to 4-fold increase in the percentage of cells in sub-G1, compared with cells transfected with the negative control siRNA. In contrast, in the low LMW-cyclin E lines, there is less than a 2-fold increase in the percentage of cells in sub-G1. These results suggest that Cdk2 knockdown causes apoptosis/cell death in LMW-cyclin E-expressing breast cancer cell lines (Fig. 5C).

Next, we asked if cell death observed following Cdk2 inhibition in breast cancer cells is dependent on LMW-cyclin E expression. For these experiments we used MCF-7 cells that can inducibly express Flag-tagged LMW-cyclin E (T2) upon treatment with doxycycline (7). LMW-cyclin E (T2) was induced for 24 hours followed by silencing of Cdk2 expression by transient siRNA transfection (Fig. 5D). Cdk2 downregulation resulted in selective inhibition of cell growth at 48 hours when LMW-cyclin E is induced. Cell proliferation is reduced by 11.5% for Cdk2 siRNA#1 and by 18.5% for siRNA#2, \( P = 0.05 \) and 0.002, compared with control siRNA transfected cells only when LMW-cyclin E is induced. Downregulation of Cdk2 had no affect on cell proliferation when LMW-cyclin E was not induced (Fig. 5D). To extend these findings to clinically applicable compounds, we used 3 different Cdk inhibitors (CVT-313, meriolin 5, and roscovitine) at different concentrations and compared the sensitivity of MCF-7 cells in the LMW-cyclin E on (with doxycycline) and off (without doxycycline) settings. CVT-313 is a potent Cdk2 inhibitor, which was identified from a purine analog library and was shown to arrest human cells in G1 (29). For each of the 3 drugs, the IC\(_{50}\) was reduced by at least 2-fold when LMW-cyclin E was expressed (Fig. 5E). These findings show that cell growth inhibition following Cdk inhibition is dependent on LMW-cyclin E overexpression.

**Discussion**

Previously we showed that expression of LMW-cyclin E under the control of a mouse mammary tumor virus (MMTV) promoter results in the development of mammary tumors in 27% of the transgenic mice with a latency of 17 to 19 months and a third of these mice develop lung metastasis. When MMTV-LMW-cyclin E mice are crossed with \( p53^{+/−} \) mice, 100% of double transgenic mice develop mammary tumors with a latency of 11 months (10). In our cohort of an FVB strain of mice, the \( p53^{+/−} \) allele does not predispose to p53-mediated mammary tumor formation. Expression of LMW-cyclin E in breast cancer cells induces genomic instability (6–8), and resistance of Cdk2 complexes to inhibition by p21 and p27 (32). Our results, presented in this article, show that Cdk2 is essential for oncogenic LMW-cyclin E–induced breast tumorigenesis. Consistent with this finding, inhibition of Cdk activities using roscovitine or meriolin delays LMW-cyclin E–induced breast cancer in this mouse model. Furthermore, targeted silencing of Cdk2-induced cell death in LMW-overexpressing breast cancer cell lines, but not in cell lines with no LMW expression. Consequently, mammary epithelial cells expressing LMW-cyclin E are unable to initiate transformation.

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**Silencing of Cdk2 is lethal to LMW-expressing breast cancer cell lines**

To evaluate Cdk2 as a potential drug target, we silenced Cdk2 expression by siRNA in 2 breast cancer cell lines expressing LMW-cyclin E, HCC1806, and HCC1569, and in 2 breast cancer cell lines with no LMW-cyclin E expression, ZR75-1 and UACC812 (Fig. 5A). Two days following Cdk2 siRNA transfection, all the cell lines transfected with the scrambled siRNA increased their cell number by 1.44, 1.16, 2.93, and 1.55-fold for HCC1806, HCC1569, ZR-75-1, and UACC812, respectively (Fig. 5B). Cdk2 siRNA transfection did not affect the growth of the 2 cell lines with no LMW expression, whereas Cdk2 knockdown in LMW-expressing line HCC1806 and 1569 led to a strong diminution in cell number so that at 2 days following transfection only 50% of plated cells for 1806 and 10% of plated cells for 1569 remained on each plate (Fig. 5B). We next asked if the decrease in cell number following Cdk2 knockdown is due to cell-cycle arrest or due to cell death. Propidium iodide staining followed by FACS demonstrated that Cdk2 knockdown in LMW-cyclin E-expressing line HCC1569 and 1806 led to a strong induction of apoptosis with a 3- to 4-fold increase in the percentage of cells in sub-G1, compared with cells transfected with the negative control siRNA. In contrast, in the low LMW-cyclin E lines, there is less than a 2-fold increase in the percentage of cells in sub-G1. These results suggest that Cdk2 knockdown causes apoptosis/cell death in LMW-cyclin E-expressing breast cancer cell lines (Fig. 5C).

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Figure 5. Cdk2 inhibition causes apoptosis and inhibition of cell growth in LMW-cyclin E overexpressing breast cancer cell lines. A, 2 breast cancer cell lines expressing LMW-cyclin E, HCC1806 and HCC1569, and 2 breast cancer cell lines with no LMW-cyclin E expression, ZR75-1 and UACC812, were harvested at 48 hours and immunoblotted for cyclin E. B, 100,000 cells for each cell line were plated at day 0, transfected with scrambled siRNA (Neg) or siRNA against Cdk2 (#1 and #2). Samples were harvested at 48 hours and counted using a Coulter counter. C, FACS analysis of the same cells as in B (left) and Western blot analysis for Cdk2 and actin (right). The levels of proteins were measured by densitometric scanning of the corresponding bands and normalized using actin values. The values indicated at the bottom (in %) were compared with the values obtained with scrambled siRNA transfected cells set at 100%. D, pTRE-LMW-cyclin E (T2) stably transfected MCF-7 cells were incubated with or without doxycycline (Dox, 1 µg/mL) for 24 hours then transfected with 21-bp double-strand siRNA against Cdk2 or with a negative control siRNA. Cells were collected 48 hours later and used for counting or Western blot analysis with cyclin E (T2), Cdk2, and actin antibodies. E, effect of the expression of LMW-cyclin E (T2) induced by doxycycline in MCF-7 cells on cyclin-dependent kinase inhibitor response. pTRE-LMW-cyclin E–transfected MCF-7 cells (2,500 cells per well) were incubated in 96-well plates for 24 hours, incubated with or without doxycycline (1 µg/mL) for 24 hours, and then treated with either diluent (DMSO) or CVT-313, or meriolin 5, or roscovitine at 8 different concentrations for 48 hours and MTT metabolism was measured. Absorbance was measured at 590 nm. The mean absorbance values of diluent-treated samples were taken as 100%. Absorbance values of the cells treated with different drugs are plotted as percentages with respect to the mean value of the diluent-treated samples. A least squares fit was obtained to estimate the IC50. Means and 95% CIs of an experiment representative of 3 independent experiments performed in quadruplicate are shown.
in the absence of Cdk2, whereas the oncogenic LMW-cyclin E/Cdk2 kinase provides breast cancer cells with the capabilities to resist cell death and as such is required to sustain tumor cell proliferation.

We also show that Cdk2 development of the mammary glands and ducts proceeds relatively normally except for a slight decrease in branching in the Cdk2−/− animals with a concomitant 2-fold decrease in the proliferation of the TEBs. When stimulated by ovarian hormones, the absence of Cdk2 delays proliferation of the mammary cells but this delay can be rescued by sustained treatment with estrogen and progesterone. This rescue experiment shows that Cdk2 is not required for developmental proliferation but the delayed proliferative response of Cdk2−/− mammary cells may be linked to the reduced size of the ovaries of these mice. Other studies showed that Cdk2 is not required for proliferation and differentiation of hematopoietic cells in vivo (35), nor is it required for neural progenitor cell proliferation, differentiation, and survival of hippocampal granule neurons in vivo (36). In the adult subventricular zone, it was shown that Cdk2 is required for proliferation and self-renewal of neural progenitor cells when Cdk4 expression is too low to compensate for loss of Cdk2 (37). Consequently, functional redundancies by Cdk1 or Cdk4/6 may compensate for the lack of Cdk2 in those cell types. For example, Cdk1 was shown to bind cyclin E in various tissues and also to regulate G1/S-phase transition in mouse embryonic fibroblasts (38). However, we show here that although Cdk2 is dispensable for mammary gland development, it is required for LMW-cyclin E tumorigenesis. This reflects the requirement for the phosphorylation of a specific set of substrates that mediate the oncogenic function of LMW-cyclin E/Cdk2 kinase. This result suggests that targeting LMW-cyclin E/Cdk2 kinase activity may be a viable and specific strategy for clinical treatment of certain breast tumors.

Our experiments show that short-term exposure to roscovitine or meriolein results in a significant delay of LMW-cyclin E–induced mammary tumor development. This delay may be due to decreased proliferation and partial induction of cell death following treatment. These results also suggest that the LMW-cyclin E transgenic mouse model should be a valuable tool to specifically evaluate drugs against the oncogenic LMW-cyclin E/Cdk2 kinase frequently expressed in triple-negative breast cancers. Future studies will aim at identifying the targets of the oncogenic LMW-cyclin E/Cdk2 kinase that provide breast cancer cells with the capabilities to resist cell death.

We also propose that breast cancer patients whose tumors express the LMW-cyclin E will be most responsive to Cdk2 inhibitors such as roscovitine or its analogues. Several CDK inhibitors have entered clinical trials including flavopiridol and seliciclib (roscovitine). Phase I studies have demonstrated that these drugs can generally be administered safely (39, 40). Phase II studies with CDK inhibitors have shown little single-agent activity in solid tumors including metastatic melanoma (41) and endometrial adenocarcinoma (42) but combination with chemotherapy seems more efficient (43–45). Overall, the results from clinical trials of CDK inhibitors in breast cancer have been disappointing.

When introducing novel treatment strategies in the clinic, special attention must be given toward identifying the patient population who will respond most effectively to such treatment. In addition, particular attention must be given to the mechanism of action of the agent to decipher the best treatment modality (i.e., single agent or in combination). Specifically, for roscovitine, only those patients whose tumors express LMW-cyclin E will be most responsive to this treatment.

Our study presented here shows that CDK2 kinase inhibitor is preferentially effective in LMW-cyclin E–overexpressing breast cancer and reinforces the need for a more targeted approach for the evaluation of this class of drugs to subgroups of breast cancer patients with high LMW-cyclin E expression. Our in vitro data show that the 2 CDK inhibitors used are cytotoxic for breast cancer cells expressing LMW-cyclin E so that we could expect to reach the same level of clinical efficacy as observed for hematologic malignancies that are more sensitive to induction of apoptosis (46, 47). It should be noted that none of the patients treated with roscovitine in either phase I or phase II studies were preselected based on altered expression of cyclin E or Cdk2. As a result, the clinical experience with roscovitine has been unimpressive. If patients are preselected based on their cyclin E expression their responses will likely improve.

Disclosure of Potential Conflicts of Interest

All authors declare no conflict of interest.

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Cdk2 is Required for Breast Cancer Mediated by the Low-Molecular-Weight Isoform of Cyclin E

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