Induction of ZEB Proteins by Inactivation of RB Protein Is Key Determinant of Mesenchymal Phenotype of Breast Cancer

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Background: Inactivation of RB is a key event for induction of EMT in cancers.

Results: ZEB proteins are markedly up-regulated through the reduction of miR-200 family of microRNAs in RB-inactive cancer cells.

Conclusion: RB/ZEB pathway plays a pivotal role in mesenchymal and aggressive phenotype in breast cancers.

Significance: Suppressing ZEB1 by cyclin-dependent kinase inhibitors provides a novel therapeutic strategy for RB-inactive breast cancers.

We previously showed that depletion of the retinoblastoma protein (RB) induces down-regulation of the adhesion molecule E-cadherin and thereby triggers the epithelial-mesenchymal transition. To further characterize the effect of RB inactivation on the phenotype of cancer cells, we have now examined RB expression in human breast cancer cell lines and clinical specimens. We found that RB-inactive cells exhibit a mesenchymal-like morphology and are highly invasive. We also found that ZEB proteins, transcriptional repressors of the E-cadherin gene, are markedly up-regulated in these cells in a manner sensitive to the miR-200 family of microRNAs. Moreover, depletion of ZEB in RB-inactive cells suppressed cell invasiveness and proliferation and induced epithelial marker expression. These results implicate ZEB in induction of the epithelial-mesenchymal transition, as well as in maintenance of the mesenchymal phenotype in RB-inactive cells. We also developed a screening program for inhibitors of ZEB1 expression and thereby identified several cyclin-dependent kinase inhibitors that blocked both ZEB1 expression and RB phosphorylation. Together, our findings suggest that RB inactivation contributes to tumor progression not only through loss of cell cycle control but also through up-regulation of ZEB expression and induction of an invasive phenotype.

Triple-negative breast cancer (TNBC) is defined as breast cancer negative for the estrogen receptor (ER), progesterone receptor, and human epidermal growth factor receptor 2 (also known as neu) and accounts for 15 to 20% of all breast cancer cases. TNBC has distinct clinical and pathological features and is clinically problematic because of its relatively poor prognosis, aggressive behavior, and lack of targeted therapies, with chemotherapy remaining the mainstay of treatment. Most TNBCs belong to the basal-like molecular subtype of breast cancer, characterized by up-regulation of epidermal growth factor receptor and basal cytokeratin (CK) 5/6.

The epithelial-mesenchymal transition (EMT) is defined by the loss of epithelial characteristics and the acquisition of a mesenchymal phenotype. A key molecular feature of the EMT is the down-regulation of E-cadherin, a cell adhesion molecule present in the plasma membrane of most normal epithelial cells. In carcinoma cells, the EMT can be associated with increased aggressiveness, as well as the development of invasive and metastatic potential. Basal-like breast cancers show up-regulation of EMT markers and mesenchymal properties, features that may be related to the aggressiveness of these tumors.

The retinoblastoma protein (RB) is a tumor suppressor that plays key roles in diverse biological processes including cell proliferation, differentiation, senescence, and apoptosis. We recently showed that RB also plays an important role in maintenance of the epithelial phenotype by up-regulating the expression of E-cadherin and that RB inactivation induces the EMT. Furthermore, microarray analysis has shown that human basal-like breast cancers frequently exhibit inactivation of RB signaling, suggesting that RB inactivation is a key event underlying the mesenchymal and aggressive phenotypes of TNBC. Exploration of the molecular links between RB inactivation and the EMT may thus reveal new targets for TNBC treatment.
We have now examined the expression of RB in TNBC specimens, as well as the expression of EMT-related transcription factors in RB-depleted cells. We found that ZEB (zinc finger E-box-binding homeobox) proteins play a pivotal role in induction of EMT-like changes, as well as in maintenance of the mesenchymal phenotype in RB-inactive cells. On the basis of these findings, we developed a screening program to identify small molecule compounds capable of suppressing ZEB1 (also known as TCF8) expression. We thereby identified several cyclin-dependent kinase (CDK) inhibitors that induced down-regulation of ZEB1 expression and suppressed RB phosphorylation.

EXPERIMENTAL PROCEDURES

Cell Lines—The human breast cancer cell lines HCC1428, MCF7, T47D, MDA-MB-436, BT549, MDA-MB-157, MDA-MB-231, and Hs578T were obtained from American Type Culture Collection. MDA-MB-231-Luc-D3H2LN cells were from Caliper Life Sciences.

Cell Culture—HCC1428 cells were cultured in RPMI 1640 medium supplemented with 10% FBS. T47D cells were cultured in RPMI 1640 supplemented with 10% FBS and bovine insulin (0.01 mg/ml; Sigma-Aldrich). BT549 cells were cultured in RPMI 1640 supplemented with 10% FBS and human insulin (0.023 IU/ml; Sigma-Aldrich). MCF7 cells were cultured in DMEM supplemented with 10% FBS. Hs578T cells were cultured in DMEM supplemented with 10% FBS and bovine insulin (0.01 mg/ml). MDA-MB-231-Luc-D3H2LN cells were cultured in minimum essential medium/Earle's Balanced Salt Solutions (Hyclone) supplemented with 10% FBS, nonessential amino acids, l-glutamine, sodium pyruvate (Hyclone), and minimum essential medium vitamin solution (Invitrogen). HCC1428, T47D, BT549, MCF7, Hs578T, and MDA-MB-231-Luc-D3H2LN cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂. MDA-MB-231 and MDA-MB-157 cells were cultured in Leibovitz's L-15 medium supplemented with 10% FBS. MDA-MB-436 cells were cultured in Leibovitz’s L-15 medium supplemented with 10% FBS, human insulin (0.01 mg/ml), and glutathione (0.01 mg/ml; Sigma-Aldrich). MDA-MB-231, MDA-MB-157, and MDA-MB-436 cells were maintained at 37 °C in a humidified atmosphere without CO₂. All of the cells were propagated for a short time after receipt and then frozen in batches as stock. A new batch was resuscitated every 2 months (corresponding to 15–20 passages).

Antibodies—The following antibodies were obtained from the indicated sources: mouse monoclonal antibodies to RB (Cell Signaling), E-cadherin, N-cadherin, fibronectin (BD Transduction Laboratories), vimentin, Ki67 (Dako), α-tubulin, β-actin (Sigma-Aldrich), CK18 (Abcam), ERα, p53, and cyclin B (Santa Cruz Biotechnology); rabbit polyclonal antibodies to Ser587/589-phosphorylated RB (Cell Signaling), RB2/p130 (Rockland), p107, and p16 (Santa Cruz Biotechnology); and goat polyclonal antibodies to ZEB1 and SLUG (Santa Cruz Biotechnology). In addition, immunohistochemical analysis was performed with mouse monoclonal antibodies to RB and to p16 obtained from BD Pharmingen and with those to E-cadherin obtained from Dako.

PCR Primers—PCR primer sequences (forward and reverse, respectively) were as follows: 5'-TTCCAAACTTTTCTCC- TGAACC-3' and 5'-TCAAGGTCATCGTGATGCTGAG-3' for vimentin; 5'-CCAGTCCTACAACAGTATTCTC-3' and 5'-CTTCTCTGTACGCTTGACATGC-3' for fibronectin; 5'-CCTCAAGATGCAAGGCAGAAG-3' and 5'-ACATGGCTTGTAGCAGCGCA-3' for SNAI1; 5'-GGCACAACTGCGAGAGAGAAG-3' and 5'-GGCACAACTGCGAGAGAGAAG-3' for TWIST1; 5'-CCCCACATTACCTTGTGTTGCAA-3' and 5'-CAAATGCTCTTGTCATGAGG-3' for SNAI2; 5'-GGCAAGAATGAGGGAGAAG-3' and 5'-CTTCTGAGCCTACTACTC-3' for ZEB1; 5'-TGTGACAGAGCATCCAGAGAAG-3' and 5'-AAAATGAGGGAGAAG-3'.

siRNAs, shRNAs, and miRNA Adenoviruses—RB siRNA (5'-GAAAGGATGAACTTATTATT-3'), a negative control siRNA and ZEB1, ZEB2, and E2F1 siRNA SMART pools were obtained from Dharmacon. MDA-MB-231 and BT549 cells were transfected with siRNAs with the use of the Oligofectamine reagent (Invitrogen), whereas T47D, MCF7, and HCC1428 cells were transfected with the use of the Lipofectamine RNAiMAX reagent (Invitrogen). A plasmid encoding a ZEB1 shRNA (Sure Silencing shRNA plasmid for human ZEB1) and a negative control plasmid were obtained from SA Biosciences and introduced into cells by transfection with the use of Lipofectamine 2000 (Invitrogen). MDA-MB-231 cells stably expressing ZEB1 or control shRNAs were selected by exposure to puromycin (2 μg/ml). miRDIAN microRNA Mimic Negative Control, miRDIAN Mimic Human has-miR-200a, and miRDIAN Mimic Human has-miR-200b were obtained from Dharmacon. The cells were transfected with the mimic negative control miRNA or a mixture of both mimic miR-200a and mimic miR-200b.

An adenovirus encoding a constitutively active mutant of human RB (Ad-RB Am) that is refractory to CDK-cyclin-mediated phosphorylation was kindly provided by E. Knudsen. Ad-RB obtained from VectorBiolabs. Ad-Luc encoding the Luciferase gene was used as a control. The cells were cultured with adenovirus at a multiplicity of infection of 100 as described previously (9).

Inhibitors—U0126, CDK4 inhibitor, CDK2/9 inhibitor, and MG132 were obtained from Calbiochem. CDK1/2 inhibitor III, puruvanolan A, and alsterpaullone were obtained from Alexis Biochemicals. PD0332991 was obtained from Pfizer.

RT and Real Time PCR Analysis—Total RNA was isolated from cells with the use of an RNeasy mini kit (Qiagen), and portions (1 μg) of the RNA were subjected to RT with an Advantage RT-for-PCR kit (BD Biosciences). The resulting cDNA was subjected to real time PCR analysis with SYBR Pre-
mix ExTaq (TaKaRa) and a Thermal Cycler Dice RealTime System (TB800, TaKaRa) for 40 cycles of 95 °C for 30 s and 60 °C for 30 s. Product specificity was verified by agarose gel electrophoresis and melting curve analysis. Primer sequences are listed in “PCR Primers.” The amount of each target mRNA was normalized by that of hypoxanthine phosphoribosyltransferase 1 (HPRT1) mRNA. For RT and real time PCR analysis of miRNAs, total RNA (10 ng) was subjected to RT with the use of a TaqMan MicroRNA reverse transcription kit and with the RNA-specific RT primer from the TaqMan MicroRNA assay (Applied Biosystems). The resulting cDNA was then subjected to real time PCR analysis with the use of miRNA-specific TaqMan probes provided in the microRNA assay. The amount of each target miRNA was normalized by that of U6 RNA with the use of the provided software (Applied Biosystems).

**Immunofluorescence Analysis**—The cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, washed with PBS, and permeabilized for 5 min at room temperature with 0.2% Triton X-100 in PBS containing 3% bovine serum albumin. They were then stained with primary antibodies for 1 h at room temperature, washed with PBS, incubated with Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies. They were then stained with primary antibodies. They were then washed with PBS, incubated with biotinylated secondary antibodies (Vector Laboratories) and 3,3′-diaminobenzidine (Vector Laboratories). The sections were counterstained with Mayer’s hematoxylin, dehydrated in a graded series of alcohol solutions, and mounted in Malinol (Muto Pure Chemicals).

**Immunoblot Analysis**—The cells were washed with PBS, lysed with SDS sample buffer (2% SDS, 10% glycerol, 50 mM Tris-HCl, pH 6.8), subjected to ultrasonic treatment, mixed with bromphenol blue and dithiothreitol (final concentration, 100 mM), and incubated at 95 °C for 5 min. Lysates (30 µg of protein) were then fractionated by SDS-polyacrylamide gel electrophoresis on a 5–20% gradient gel (Pagel, Atto), and the separated proteins were transferred to a nitrocellulose membrane (Hybond; GE Healthcare). The membrane was exposed to primary antibodies, and immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare and Dako) and enhanced chemiluminescence reagents (PerkinElmer Life Sciences).

**Immunohistochemical Analysis**—Sections of formalin-fixed, paraffin-embedded tissue were de-paraffinized, hydrated, and exposed to microwave radiation in 10 mM sodium citrate buffer (pH 6.0) for 10 min. The sections were incubated with 3% H2O2 for 5 min to inhibit endogenous peroxidase activity, washed with PBS, exposed to 3% bovine serum albumin for 20 min at room temperature, and incubated overnight at 4 °C with primary antibodies. They were then washed with PBS, incubated with biotinylated secondary antibodies (Vector Laboratories) for 30 min at room temperature, and washed again, after which immune complexes were detected with the use of an avidin-peroxidase complex (Vector Laboratories) and 3,3′-diaminobenzidine (Vector Laboratories). The sections were counterstained with Mayer’s hematoxylin, dehydrated in a graded series of alcohol solutions, and mounted in Malinol (Muto Pure Chemicals).

**Invasion Assays**—Cell invasiveness was measured with a Boyden chamber (Falcon BD). The cells (5 × 10^4 in 0.5 ml of serum-free medium) were seeded on a filter membrane in the upper chamber, and serum-containing medium was placed in the lower chamber. After incubation for 24 h at 37 °C, the cells were scraped from the upper chamber with a cotton swab, and those on the underside of the membrane were fixed with 100% methanol, stained with Giemsa solution, and counted. The cells were transfected with siRNAs or infected with adenoviruses for 48 or 24 h, respectively, before performance of the invasion assay.

**Cell Cycle Analysis**—The cells were collected by exposure to trypsin, washed with PBS, fixed in ice-cold 70% ethanol, and stored at −20 °C until analysis. They were subsequently washed twice with PBS, incubated for 30 min at room temperature with RNase A (100 µg/ml), and stained with propidium iodide (25 µg/ml) for 30 min. Flow cytometry was performed with a FACS Calibur instrument, and data were analyzed with CellQuest software (BD Biosciences).

**Patients and Tumor Samples**—Eighteen patients with primary, invasive, TNBC who were surgically treated at the National Cancer Center Hospital were retrospectively evaluated in this study. For the pathological examination, the surgically resected specimens were fixed in 10% formalin. For diagnosis of TNBC, ER and progesterone receptor status were determined by immunohistochemistry. Tumors with >10% positive-stained cells were defined as positive. Human epidermal growth factor receptor 2 overexpression in the primary tumor was defined according to the criteria recommended in the American Society of Clinical Oncology 2006 guidelines. The protocol for this study was reviewed by the institutional review board of the National Cancer Center.

**Cell Transplantation**—MDA-MB-231-Luc-D3H2LN cells (2 × 10^6 in 30 µl of PBS) were injected as a single-cell suspension into the no. 4 mammary fat pad of 4-week-old female Balb/c nu/nu immune-deficient mice (Charles River) that had been anesthetized by exposure to 1 to 3% isoflurane. PD0332991 (75 mg/kg) or vehicle (saline) was then administered orally once per day on days 1–3, 6–10, 13, and 14. The physical condition of the mice was monitored daily, and body weight and tumor volume were measured every 2 or 3 days. Tumor volume was calculated as length × width^2/2.

**Bioluminescence Imaging**—Mice anesthetized as above were injected intraperitoneally with D-luciferin (20 mg/kg) 10 min before whole body imaging with an in vivo fluorescence imager (ClairvivoOPT; Shimadzu).

**Statistical Analysis**—The data are presented as the means ± S.D. and were analyzed with Student’s paired t test. A p value of <0.05 was considered statistically significant.

**RESULTS**

**Depletion of RB Induces EMT-like Morphological Shift and RB Controls Invasive Potential in Breast Cancer Cells**—We have previously shown that depletion of RB triggers the down-regulation of E-cadherin and thereby induces the EMT in MCF7 breast cancer cells (7). The induction of vimentin mRNA (supplemental Fig. S1A) and an EMT-like morphological shift (Fig. 1A) by siRNA-mediated depletion of RB were also observed in T47D and HCC1428 breast cancer cells. Boyden chamber Transwell® assays revealed that depletion of RB in these cells also increased cell invasiveness (Fig. 1B). To further assess the contribution of RB to the epithelial phenotype, we infected RB-negative BT549 breast cancer cells with an adenovirus encoding human RB (Ad-RB) (supplemental Fig. S1B). RT and real
time PCR analysis revealed a marked increase in the expression of both E-cadherin (CDH1) and CK18 (KRT18) genes in the Ad-RB-infected cells compared with cells infected with a control adenovirus encoding luciferase (Ad-Luc) (Fig. 1C). Moreover, Boyden chamber Transwell® assays revealed that infection with Ad-RB resulted in a marked decrease in the invasiveness of BT549 cells (Fig. 1D). A similar attenuation of invasiveness was also observed on infection of RB-inactivated MDA-MB-231 and MDA-MB-157 breast cancer cells with Ad-RB Am, an adenovirus encoding a constitutively active mutant of RB that is refractory to CDK-cyclin-mediated phosphorylation (Fig. 1D). These results indicated that inactivation of RB in epithelial cancer cells is associated with an EMT-like morphological shift.

**Basal-like Breast Cancer Cell Lines Show Mesenchymal Phenotype Accompanied by RB Inactivation**—To further characterize the effect of RB inactivation on the phenotype of breast cancer cells, we analyzed RB expression in seven breast cancer cell lines. Immunofluorescence analysis revealed that HCC1428, MCF7, and T47D cells were E-cadherin-positive epithelial-like cells, whereas MDA-MB-436, BT549, MDA-MB-157, and MDA-MB-231 cells were vimentin-positive mesenchymal-like cells (Fig. 2A). Immunoblot analysis showed that the epithelial-like cell lines expressed CK18, as well as E-cadherin, whereas the mesenchymal-like cell lines expressed fibronectin, vimentin, or N-cadherin (Fig. 2B). Such analysis also detected RB in the epithelial-like cell lines. In contrast, with regard to the mesenchymal-like cell lines, MDA-MB-436 and BT549 cells were negative for RB expression, MDA-MB-157 cells exhibited a low level of RB expression, and MDA-MB-231 cells expressed the hyperphosphorylated (inactive) form of RB (Fig. 2B). MDA-MB-436, BT549, and MDA-MB-157 cells exhibited a high level of p16 expression, suggesting that this endogenous CDK inhibitor was up-regulated through a negative feedback mechanism triggered by the loss of RB. Boyden chamber Transwell® assays revealed that cell invasiveness was markedly increased in the RB-inactivated (MDA-MB-436, BT549, MDA-MB-157, and MDA-MB-231) cells compared with that in the epithelial-like (HCC1428, MCF7, and T47D) cells (supplemental Fig. S1C). These data thus indicated that RB function is commonly suppressed in mesenchymal breast cancer cells and that inactivation of RB contributes to mesenchymal-like morphology with an invasive phenotype. The expression of p53 or of other RB family proteins, including RB2 (p130) and p107, did not show any association with epithelial or mesenchymal phenotypes.

We next examined whether RB is inactivated in clinical specimens of TNBC. The expression of p16, RB, and phosphorylated (Ser627/628) RB was assessed by immunohistochemical analysis of formalin-fixed specimens. Of the 18 TNBC samples...
examined, four samples were normally positive for RB, five samples expressed hyperphosphorylated RB, and nine samples were negative for RB expression, suggesting that RB tends to be inactivated in TNBCs (Fig. 2C). The expression level of p16 was high in TNBCs expressing phosphorylated RB (P1 sample) or in those considered negative for RB (P2 to P4).

Inhibition of EMT in RB-depleted T47D Cells by Knockdown of ZEB—We next investigated the mechanism by which RB inactivation induces the EMT. We first examined the effects of RB depletion in MCF7, T47D, and HCC1428 cells on expression of the genes for various transcription factors, including SNAI1, SNAI2, TWIST1, ZEB1, and ZEB2, that are known to induce the EMT through suppression of E-cadherin expression (10–15). RT and real time PCR analysis revealed that RB depletion increased the amounts of ZEB1 and ZEB2 mRNAs in all three cell lines, whereas it substantially increased those of SNAI1, SNAI2, and TWIST1 mRNAs in subsets of these cell lines but had a minimal effect on that of the mRNA for E2F1, a major downstream target of RB (Fig. 3A). In addition, SNAI2, ZEB1, and ZEB2 were expressed at a higher level in breast cancer cell lines with RB inactivation than in those without it (supplemental Fig. S1D), and ZEB1 protein was detected in the former but not the latter cells (Fig. 2B). Furthermore, depletion of both ZEB1 and ZEB2 with specific siRNAs inhibited induction of the EMT-like change by RB knockdown in T47D cells (Fig. 3, B and C). In contrast, the depletion of E2F1 did not inhibit either the mesenchymal-like phenotype or the up-regulation of ZEB1, ZEB2, or SNAI2 expressions induced by RB knockdown in these cells (Fig. 3, B–D). Collectively, these data indicated that ZEB1 and ZEB2 are key mediators of EMT induction by RB inactivation.

Overexpression of miR-200 Inhibits ZEB Expression Induced by Depletion of RB—We next investigated the mechanism by which RB inactivation results in up-regulation of ZEB1 and ZEB2 expression. miRNAs are small noncoding RNAs that are able to silence cognate target genes by binding specifically to mRNAs and inhibiting their translation. ZEB proteins and miRNAs of the miR-200 family have been shown to be reciprocally linked in a feedback loop, with each strictly controlling the expression of the other (16–19). Indeed, we found that depletion of RB reduced the abundance of miR-200 family members (supplemental Fig. S2A). We therefore tested whether forced expression of miR-200a and miR-200b mimics prevented the increase in ZEB1 and ZEB2 expression induced by RB depletion in T47D (Fig. 4A) and MCF7 (Fig. 4B) cells. These results thus suggested that down-regulation of miR-200 family members contributes to the EMT induced by depletion of RB.

Relation between RB and ZEB1 Expression in Primary Breast Tumor Specimens—Given that a reduction in RB expression was associated with up-regulation of ZEB expression and the mesenchymal phenotype in breast cancer cell lines, we exam-
ined whether such a relation was apparent in tumor specimens from breast cancer patients. Whereas antibodies to ZEB1 readily detected endogenous ZEB1 in RB-inactivated cell lines (Fig. 2B), we were not able to obtain antibodies capable of detecting endogenous ZEB2. We therefore performed immunohistochemical analysis to detect the expression of ZEB1, RB, E-cadherin, and vimentin in formalin-fixed TNBC specimens. RB was expressed in normal mammary gland cells positive for membrane-associated E-cadherin (Fig. 5A). In contrast, down-regulation of RB and E-cadherin expression was observed in invasive ductal carcinoma cells positive for vimentin, and ZEB1 was highly expressed in the invasive lesions (Fig. 5B). Of 14 TNBC samples showing RB inactivation, six samples contained ZEB1-positive tumor cells. None of the four samples positive

FIGURE 3. Inhibition of the EMT in RB-depleted cells by knockdown of ZEB. A, MCF7, T47D, and HCC1428 cells were transfected with control or RB siRNAs for 48 h and were then subjected to RT and real time PCR analysis of SNAI1, SNAI2, TWIST1, ZEB1, ZEB2, and E2F1 mRNAs. The data were normalized by the amount of HPRT1 mRNA and expressed relative to the corresponding value for cells transfected with the control siRNA. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus corresponding control value (Student’s paired t test). B, T47D cells were transfected with RB (RB si), ZEB1 (ZEB1 si), ZEB2 (ZEB2 si), E2F1 (E2F1 si), or control siRNAs (Cont si), as indicated, for 72 h and were then examined by phase contrast microscopy. Scale bar, 100 μm. C, Boyden chamber Transwell® assay of the invasiveness of T47D cells transfected with the indicated siRNAs for 48 h. D, RT and real time PCR analysis of E2F1, ZEB1, ZEB2, and SNAI2 mRNAs in T47D cells transfected with control, RB, or both RB and E2F1 siRNAs for 48 h. The data were normalized by the amount of HPRT1 mRNA and expressed relative to the corresponding value for cells transfected with the control siRNA. The data in A, C, and D are the means ± S.D.

FIGURE 4. Depletion of RB induces ZEB expression in a manner sensitive to the miR-200 family. T47D (A) and MCF7 (B) cells were transfected for 72 h with control (Cont si) or RB siRNAs (RB si), as well as with a control miRNA mimic (miR-cont) or a mixture of miR-200a and miR-200b mimics. The amounts of RB1, ZEB1, and ZEB2 mRNAs were then determined by RT and real time PCR analysis. The data were normalized by the amount of HPRT1 mRNA, are expressed relative to the corresponding value for cells transfected with the control siRNA and control miRNA mimic, and are the means ± S.D. ***, p < 0.001 (Student’s paired t test). NS, not significant.
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Depletion of ZEB Induces Epithelial Cell Marker Expression and Inhibits Both Cell Invasiveness and Growth—We next examined the role of ZEBs in maintenance of the mesenchymal-like phenotype. Depletion of both ZEB1 and ZEB2 in MDA-MB-231 and BT549 cells induced a morphological change typical of the mesenchymal-epithelial transition (Fig. 6A). The morphology of cells undergoing a mesenchymal-epithelial transition changes as a result of the formation of organized cell-cell adhesion complexes, and this change is associated with the loss of mesenchymal markers and the gain of epithelial markers. Expression of the mammary epithelial marker genes CDH1, KRT18, and KRT19 was markedly increased in ZEB-depleted MDA-MB-231 cells (Fig. 6B) and BT549 cells (supplemental Fig. S2B). Immunoblot analysis also showed that the abundance of E-cadherin and CK18 was increased in ZEB-depleted MDA-MB-231 cells (Fig. 6C). The mesenchymal-like MDA-MB-231 and BT549 cells thus reverted to an epithelial-like phenotype as a result of ZEB knockdown. We also generated MDA-MB-231 cells stably depleted of ZEB. Given that MDA-MB-231 cells express ZEB1 at a much higher level than ZEB2 (supplemental Fig. S1D), we suppressed ZEB1 expression in these cells by transfection with a plasmid encoding a shRNA specific for ZEB1 mRNA. Such stable depletion of ZEB1 resulted in marked suppression not only of cell invasiveness (Fig. 6D) but also of cell proliferation (Fig. 6E).

ZEB1 Expression Is Dependent on CDK Activity—To dissect the mechanism of intracellular signaling that controls ZEB1 expression in breast cancer cells, we developed a screening program for ZEB1 inhibitors. With the use of a SCADS (Screening Committee of Anticancer Drugs) (20) inhibitor kit, we identified several chemical inhibitors of ZEB1 protein expression as a positive control, the cells were treated with U0126, an inhibitor of the MEK-ERK signaling pathway, given that this agent was previously shown to reduce ZEB1 expression in prostate cancer cells (21). The identified inhibitors of ZEB1 expression in MDA-MB-231 cells included CDK inhibitors, which also attenuated the phosphorylation of RB on Ser^807/811 (Fig. 7A). In particular, a CDK4 inhibitor induced cell cycle arrest and a mesenchymal-epithelial transition-like morphological change in MDA-MB-231 cells (Fig. 7B). In addition, the expression of the mammary epithelial marker KRT19 was increased in MDA-MB-231 cells by treatment with the CDK4 inhibitor (supplemental Fig. S3A). We also examined whether active RB was able to block the expression of ZEB1 in RB-inactivated MDA-MB-231 cells. Immunoblot and RT real time PCR analysis revealed that infection of cells with Ad-RB Am resulted in a marked decrease in the abundance of both ZEB1 protein levels (Fig. 7C) and in ZEB1 transcriptional levels (Fig. 7D). Furthermore, the CDK4 inhibitor down-regulated the expression of both ZEB1 and the EMT-related transcription factor SLUG in MDA-MB-231 and Hs578T breast cancer cells (Fig. 7E).

We next examined the mechanism underlying down-regulation of ZEB1 expression by CDK inhibitors. We found that the abundance of miR-200a and miR-200b, but not that of miR-200c, was increased in MDA-MB-231 cells by treatment with the CDK4 inhibitor (Fig. 7F). The decline in ZEB1 protein levels in CDK4 inhibitor-treated cells was prevented by MG132, an inhibitor of the proteasome and other intracellular proteases (supplemental Fig. S3B). These results thus indicated that the down-regulation of ZEB1 induced by CDK inhibition in breast cancer cells is mediated by induction of miR-200 family members and proteasome-mediated ZEB1 degradation.

The CDK4/6 Inhibitor PD0332991 Suppresses Breast Cancer Cell Growth and Cell Invasiveness—The CDK4/6 inhibitor PD0332991 has been recently tested in phase I clinical study (22). We also found that PD0332991 both inhibited the expression of ZEB1 (Fig. 8A) and reduced the proportion of Ki67-positive (proliferating) cells (Fig. 8B) in MDA-MB-231 cells. Boyden chamber Transwell® assays revealed that cell invasiveness was decreased markedly in the PD0332991-treated cells (Fig. 8C). Finally, we examined the effect of this CDK inhibitor in an MDA-MB-231 orthotopic xenograft tumor model in nude mice. Treatment with PD0332991 significantly inhibited the growth of MDA-MB-231-Luc cells (MDA-MB-231 cells that stably express luciferase) in vivo compared with a vehicle control (Fig. 8, D and E). PD0332991 treatment resulted in a marked suppression of not only cell proliferation but also of cell invasiveness. These results suggested the possibility that the effect of the CDK4/6 inhibitor PD0332991 on tumor growth was attributable, at least in part, to down-regulation of ZEB1.

DISCUSSION

We have investigated the role of RB inactivation in the EMT phenotype of TNBC. Our data have revealed that ZEB proteins are major mediators of the induction and maintenance of the mesenchymal phenotype associated with RB
The inactivation of RB and consequent up-regulation of ZEB may thus provide novel targets for the treatment of TNBC.

We previously showed that RB controls cell-cell adhesion in epithelial cells by up-regulating E-cadherin gene (CDH1) transcription through interaction with the transcription factor AP-2α (7). We have now shown that depletion of RB induced the expression of the genes for the EMT-related transcription factors ZEB1 and ZEB2. These results thus indicate that the inactivation of RB gives rise to the EMT through multiple molecular events. We also found that RB inactivation induces down-regulation of the miR200 family of miRNAs, which leads to up-regulation of ZEB. Members of the miR-200 family are key regulators of the EMT and act to maintain the epithelial phenotype by targeting expression of the E-cadherin transcriptional repressors ZEB1 and ZEB2 (16, 23, 24). However, the precise molecular mechanism by which RB regulates expression of miR-200 family members remains unknown. Expression of the miR-200a/141 cluster was recently shown to result in G1 arrest caused by increased p27Kip1 and decreased CDK6 expression and that expression of the miR-200bc/429 cluster promotes G2 arrest through up-regulation of the inhibitory phosphorylation of Cdc25C in breast cancer cell lines (25). The down-regulation of CDK6 and cell cycle arrest induced by the miR-200 family may be related to our present observation that CDK inhibitors down-regulated ZEB1 expression in breast cancer cells, given the reciprocal suppression mediated by ZEB and miR-200.

We performed RT real time PCR analysis so as to determine whether or not the expression of ZEB1 is transcriptionally suppressed by RB. Adenovirus-mediated overexpression of RB Am in MDA-MB-231 cells resulted in a marked reduction of ZEB1 mRNA. Furthermore, adenovirus-mediated overexpression of RB in BT549 cells decreased ZEB2 mRNA (supplemental Fig. 3C). These results indicate that RB suppresses the expression of ZEBs in transcriptional levels. Our observations also indicate
that the reduction in ZEB1 expression induced by CDK inhibition is mediated by the ubiquitin-proteasome pathway. ZEB1 possesses an LXCXE RB-binding motif, suggesting that an E3 ubiquitin ligase may target the RB-ZEB1 complex to regulate ZEB1 abundance.

By screening a chemical library, we found that CDK inhibitors suppressed ZEB1 expression. We also found that a CDK4/6 inhibitor, PD0332991, attenuated phosphorylation of RB on Ser807/811 as well as induced cell cycle arrest and inhibited tumor growth. It was possible that the down-regulation of ZEB1 by CDK inhibitors was due to cell cycle-dependent expression of ZEB1. To rule out this possibility, we examined the expression of ZEB1 in HeLa cells released from a double thymidine block. We found that ZEB1 expression did not fluctuate substantially during cell cycle progression (Supplemental Fig. S3D). We also found that RB depletion did not affect the expression of E2F1 mRNA. Furthermore, the depletion of E2F1 did not inhibit either the EMT-like phenotype or the up-regulation of \( ZEB1 \) and \( ZEB2 \) mRNA induced by RB knockdown. We concluded that the E2F1-mediated signals did not contribute to mesenchymal-like properties induced by RB inactivation.

The observed suppression of tumor cell proliferation and tumor growth by CDK inhibitors was likely attributable not only to direct arrest of the cell cycle but also to down-regulation of ZEB, given that we found that shRNA-mediated depletion of ZEB1 resulted in marked inhibition of cell proliferation. Furthermore, treatment with a Cdk inhibitor (PD0332991) also suppressed the cell invasion activity, which we believe is due to the inhibition of ZEB-mediated mesenchymal phenotypes of cancer cells.

Together, our results show that RB regulates ZEB1 and that ZEB1 plays a role not only in induction of the EMT, contributing to tumor cell invasion and metastasis, but also in cell proliferation in RB-inactivated cancer cells. Indeed, the level of \( ZEB1 \) expression was previously shown to corre-
late with tumor metastasis and differentiation grade, as well as with clinical survival in lymph node negative breast adenocarcinomas (26). These observations indicate that ZEB1 expression is a potential marker of poor prognosis in breast cancer. Furthermore, our results suggest that treatment with CDK inhibitors may be effective in some patients with TNBC and that ZEB1 expression may serve as a predictive marker for the efficacy of such treatment. Further studies are necessary to determine whether the expression of ZEB1 influences prognosis of TNBC patients and which of the effects of ZEB1 (such as those on cell proliferation, survival, metastasis, and differentiation) contribute to tumor aggressiveness.

FIGURE 8. The CDK4/6 inhibitor PD0332991 suppresses ZEB1 expression and tumor growth. A, MDA-MB-231 cells treated with 10 μM PD0332991 (or dimethyl sulfoxide (DMSO)) for 48 or 96 h were subjected to immunoblot analysis of ZEB1 and phosphorylated RB. B, MDA-MB-231-Luc cells were incubated with PD0332991 or dimethyl sulfoxide for 4 days and then subjected to phase contrast microscopy, as well as to immunofluorescence staining with antibodies to Ki67 (red) and staining of nuclei with 4′,6-diamidino-2-phenylindole (DAPI, blue). Scale bars, 100 μm. C, MDA-MB-231 cells were treated with PD0332991 or dimethyl sulfoxide for 24 h and then assessed for invasiveness with a Boyden chamber Transwell® assay. The data are expressed as percentages of the corresponding value for cells treated with dimethyl sulfoxide and are the means ± S.D. from triplicate data. ***, p < 0.001 (Student’s paired t test). D, nude mice were injected with MDA-MB-231-Luc cells and treated intermittently with PD0332991 (75 mg/kg) or vehicle as described under “Experimental Procedures.” The volume of the resulting orthotopic tumors was determined at the indicated times after cell injection. The data are the means ± S.D. for four (PD0332991) or six (vehicle) mice. *, p < 0.05; **, p < 0.01; ***, p < 0.001 (Student’s paired t test) versus corresponding control value. E, in vivo detection of luciferase reporter expression by bioluminescence imaging at 3 and 17 days after cell injection in mice treated as in D.
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