

Rb Depletion Results in Deregulation of E-Cadherin and Induction of Cellular Phenotypic Changes that Are Characteristic of the Epithelial-to-Mesenchymal Transition

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Abstract

The retinoblastoma tumor suppressor protein (Rb) is mutated or expressed at very low levels in several tumor types, including retinoblastoma and osteosarcoma, as well as small cell lung, colon, prostate, bladder, and breast carcinomas. Loss or reduction of Rb expression is seen most commonly in high-grade breast adenocarcinomas, suggesting that a relationship may exist between loss of Rb function and a less-differentiated state, increased proliferation, and high metastatic potential. In this study, we found that knockdown of Rb by small interfering RNA in MCF7 breast cancer cells disrupts cell-cell adhesion and induces a mesenchymal-like phenotype. The epithelial-to-mesenchymal transition (EMT), a key event in embryonic morphogenesis, is implicated in the metastasis of primary tumors. Additionally, Rb is decreased during growth factor- and cytokine-induced EMT and overexpression of Rb inhibits the EMT in MCF10A human mammary epithelial cells. Ectopic expression and knockdown of Rb resulted in increased or reduced expression of E-cadherin, which is specifically involved in epithelial cell-cell adhesion. Other EMT-related transcriptional factors, including Slug and Zeb-1, are also induced by Rb depletion. Furthermore, we confirmed that Rb binds to an E-cadherin promoter sequence in association with the transcription factor activator protein-2 α . Finally, in breast cancer specimens, we observed a concurrent down-regulation of Rb and E-cadherin expression in mesenchymal-like invasive cancers. These findings suggest that Rb inactivation contributes to tumor progression due to not only loss of cell proliferation control but also conversion to an invasive phenotype and that the inhibition of EMT is a novel tumor suppressor function of Rb. [Cancer Res 2008;68(13):5104–12]

Introduction

The breakdown of epithelial cell homeostasis leading to aggressive cancer progression is correlated with a loss of epithelial characteristics and the acquisition of a migratory phenotype. This phenomenon, called the epithelial-to-mesenchymal transition (EMT), is considered to be a crucial event in malignancy. The

EMT is also an essential component of embryonic development, tissue remodeling, and wound repair (reviewed in refs. 1, 2). During this transition, the epithelial phenotype, characterized by strong cell-cell junctions and polarity, is replaced by a mesenchymal phenotype, with reduced cell-cell interactions, a fibroblastic morphology, and increased motility. Given the importance of EMT in carcinoma progression, there is considerable interest in understanding the mechanisms that contribute to this complex process.

A hallmark of EMT is the loss of E-cadherin expression. E-cadherin is a key component of adherens junctions, structures that play crucial roles in the maintenance of epithelial integrity (3). E-cadherin is a calcium-dependent cell adhesion molecule that mediates homophilic interactions and controls the formation of catenin-containing complexes that link E-cadherin to the actin and microtubule cytoskeleton (3, 4). In cancer, loss of E-cadherin function through genetic or epigenetic mechanisms has been implicated in the progression and metastasis of numerous malignancies (5, 6). Therefore, deregulation of E-cadherin expression may contribute to tumorigenesis. Several proteins that down-regulate E-cadherin expression, including Snail (7, 8), Twist (9), SIP1 (10), Slug (11), and ZEB1/ δ EF1 (12), have been identified. In contrast, activator protein-2 α (AP-2 α) is a transcription factor that positively regulates expression of E-cadherin (13–15). Gain of negative regulators and loss of positive regulators of E-cadherin transcription have been reported for some cancers (15, 16).

The retinoblastoma (Rb) gene product is a tumor suppressor that regulates multiple cellular processes, such as growth, differentiation, and apoptosis, and is inactivated by mutations in many types of human cancers (17–19). The tumor suppressor activity of Rb derives from its ability to inhibit cell cycle transition by repressing the transcription of genes required for the G₁-S phase transition, such as E2F/DPI-dependent transcription (reviewed in ref. 20). The loss of Rb is critical for tumorigenesis. Somatic inactivation of both Rb gene alleles has been found in a number of common mesenchymal and epithelial malignancies (21). Furthermore, functional loss of the Rb gene has been implicated in advanced stages of various cancers, including carcinoma of the breast, bladder, liver, esophagus, and colon (22–26). Rb expression levels have been inversely correlated with tumor invasiveness in gastric carcinoma (27). A recent report indicates that the basal cluster subtype of invasive breast tumors tends to express relatively low levels of Rb and high levels of p16 (28). Given that low levels of Rb expression correlate with clinical outcome, Rb inactivation may contribute to tumor initiation, progression, and metastasis. However, the molecular mechanisms of this contribution have not been elucidated.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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In this study, we show that depletion of Rb in breast cancer cells results in the specific down-regulation of E-cadherin expression and induction of an EMT-like phenotype with high production of extracellular matrix and increased cell motility. In addition, we found that Rb is degraded during the EMT. Furthermore, reexpression of Rb inhibited EMT and induced E-cadherin expression in Rb-negative cells. Rb binds to AP-2 α and promotes E-cadherin expression (13), and, indeed, we confirmed that Rb binds to the E-cadherin promoter sequence with AP-2 α . Our observations support the hypothesis that Rb plays a crucial role in maintenance of the epithelial phenotype by up-regulating the expression of E-cadherin. Thus, these results indicate that Rb is a key player in EMT inhibition and that Rb inactivation is associated with invasion and metastasis of cancer cells.

Materials and Methods

Cell lines and cell cultures. MCF10A cells were cultured in mammary epithelial growth medium containing BPE, hydrocortisone, human epidermal growth factor, insulin, and GA-1000 (MEGM BulletKit, Takara) at 37°C in a humidified atmosphere of 5% CO₂. MCF-7, MDA-MB-468, and DLD-1 cells were cultured in DMEM supplemented with 10% fetal bovine serum. MCF10A cells were incubated with transforming growth factor- β 1 (TGF β 1; Sigma), tumor necrosis factor- α (TNF α ; Calbiochem), MG132 (Calbiochem), Z-Val-Ala-Asp(OMe)-fmk (Enzyme Systems Products), or TGF β type I receptor (T β RI) kinase inhibitor II (Calbiochem), as indicated.

Small interfering RNA transfection and adenoviral infections. Rb small interfering RNA (siRNA; #1, 5'-GAAAGACATGTGAACCTATT-3' and #2, 5'-CGAAATCAGTGTCCATAAATT-3') and negative control siRNA were purchased from Dharmacon. Stealth p53 siRNA (5'-CCAGTGGTAATC-TACTGGGACGGAA-3') and negative control siRNA were purchased from Invitrogen. Cells were transfected using the Lipofectamine 2000 reagent (Invitrogen). Ad-CMV-Rb was purchased from Vector Biolabs. Ad5CMV-Luc encoding the *Luciferase* gene was used as a control. Cells were cultured with adenovirus at a multiplicity of infection of 100.

Reverse transcription-PCR assays. Total RNA was isolated using an RNeasy Mini kit (QIAGEN). The cDNA was synthesized from 1 μ g of total RNA with the Advantage RT-for-PCR kit (BD Biosciences). Semiquantitative PCR was performed using Taq DNA polymerase (Roche) on a Peletier Thermal Cycler (MJ Research) for 25 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The PCR products were separated by electrophoresis on 1.5% agarose gels. Real-time PCR was performed using SYBR Premix ExTaq (TaKaRa) on 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 the supplementary information.

Immunoblot analysis. Cells were rinsed in PBS and lysed with NuPAGE LDS sample buffer (Invitrogen). Whole-cell extracts were heated at 70°C for 10 min after the addition of DTT. Samples were subjected to 5% to 20% (REAL GEL PLATE, Bio Craft) or 3% to 8% (NuPAGE Tris-Acetate Gel, Invitrogen) gradient SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes (Millipore). Immunodetection was performed using the enhanced chemiluminescence system (Amersham).

Immunofluorescence analysis. Cells were fixed with 4% paraformaldehyde-PBS for 15 min at room temperature, washed with PBS, and then permeabilized with 0.2% TritonX-3% bovine serum albumin-PBS for 5 min at room temperature. Cells were stained with primary antibodies for 1 h at room temperature. After being washed with PBS, the cells were incubated with AlexaFluor-conjugated secondary antibodies (Molecular Probe, Inc.), counterstained with 4',6-diamidino-2-phenylindole (DAPI), and observed with a fluorescence microscope (ECLIPSE E1000, Nikon).

Antibodies. The following antibodies were used: mouse monoclonal antibodies for Rb (4H1, Cell Signaling), E-cadherin (BD Transduction Laboratories), p53 (DO-1, Oncogene Science), cytokeratin 18 (Abcam), and fibronectin (P1H11, Santa Cruz); rabbit polyclonal antibodies for phosphorylated Smad3(ser^{423/425})/Smad1(ser^{463/465}, Cell Signaling), β -catenin (H-102,

Santa Cruz), AP-2 α (Santa Cruz); goat polyclonal antibodies for β -actin and Samd2/3 (Santa Cruz). Horseradish peroxidase-conjugated secondary antibodies were obtained from Amersham Biosciences. AlexaFluor-conjugated secondary antibodies were obtained from Molecular Probes, Inc. For immunohistochemistry, anti-E-cadherin, anti-vimentin (Dako), and anti-Rb (BD PharMingen) mouse monoclonal antibodies were used.

Cdc42 activation assay. Cell lysates were incubated with GST-Pak1-p21 binding domain agarose beads (EZ-Detect Cdc42 activation kit, Pierce). GTP-bound Cdc42 that precipitated with the beads was detected by SDS-PAGE and immunoblotting with an anti-Cdc42 antibody.

Matrigel assay. For the bottom layer, 300 μ L of Matrigel (BD Biosciences) was added to 24-well tissue culture plates and incubated at 37°C for 30 min to allow the Matrigel to solidify. Cells were transfected with siRNAs 24 h before the assay, and 2 \times 10⁴ cells (0.5 mL) were then mixed with Matrigel (4%; 0.5 mL) at 4°C and added over the solidified bottom layer. After incubation at 37°C for 3 d, cell growth was analyzed.

Invasion assays. Cell motility was measured using a Boyden Chamber (Falcon BD). Cells were transfected with siRNAs 24 h before the assay, and 5 \times 10⁴ cells (0.5 mL) were then seeded in serum-free medium in the upper chamber with serum-containing medium in the lower chamber. After 24 h at 37°C, cells were scraped from the upper chamber with a cotton swab, and the undersides of the membranes were fixed in 100% methanol, stained with Giemza, and counted.

Biotinylated dsDNA pull-down assay. Cells were lysed in TNTE buffer [20 mmol/L Tris-HCl (pH 7.5), 120 mmol/L NaCl, 1 mmol/L EDTA, and 0.5% Triton-X 100] containing protease and phosphatase inhibitors. Cell extracts were incubated with biotinylated double-stranded E-cadherin nucleotides comprising the core promoter region (-308/+21) and poly(deoxyinosinic-deoxycytidylic acid) for 18 h. DNA-bound proteins were collected with streptavidin-agarose beads for 4 h, washed with TNTE buffer, separated by SDS-PAGE, and identified by immunoblotting.

Results

Inactivation of Rb leads to EMT-like changes. To characterize the effect of the inactivation of Rb on epithelial morphology, we transfected siRNAs targeting two different regions of Rb into human breast cancer MCF7 cells. Depletion of Rb induced a morphologic change of MCF7 cells from a paving stone sheet-like structure to fibroblastic-spindle shape, which represents one characteristic of EMT (Fig. 1A). When epithelial cells are undergoing EMT, their morphology changes from well-organized cell-cell adhesion to loss of cell-cell contacts and cell scattering, which is often associated with loss of epithelial markers and gain of mesenchymal markers. Thus, we examined the localization and expression of several critical molecules, such as E-cadherin, β -catenin, and fibronectin, which are altered during the conversion of epithelial-like cells into mesenchymal-like cells. Whereas both E-cadherin and β -catenin were mainly localized along cell-cell contact sites in control MCF7 cells, they were detected mainly in the cytoplasm in Rb-depleted cells (Fig. 1B). Furthermore, fibronectin expression, which is a mesenchymal marker, was significantly increased in Rb-depleted cells (Fig. 1C). Moreover, expression of other EMT markers, including vimentin and N-cadherin, was also detected, as described below. The induction of an EMT-like phenotype by the depletion of Rb was also observed in human colon cancer DLD-1 cells (Supplementary Fig. S1). Expression of adenoviral Rb restored the morphologic changes and E-cadherin localization in Rb-depleted cells (Supplementary Fig. S2). These results indicate that inactivation of Rb in these cells is associated with morphological and molecular changes of EMT.

Another important change induced by depletion of Rb was the suppression of cell growth (Supplementary Fig. S3). The trypan blue exclusion test revealed that >40% of cells had died within

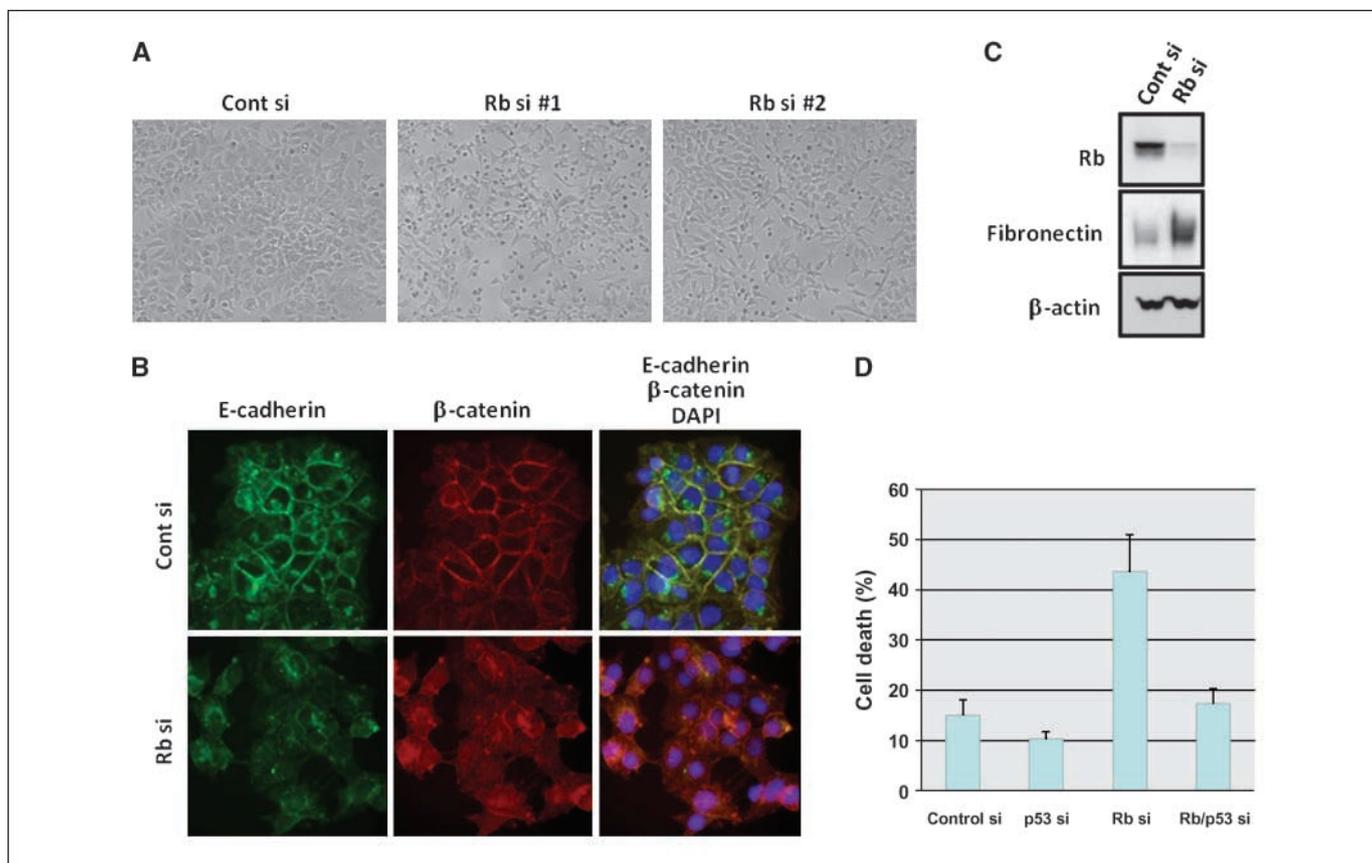


Figure 1. Depletion of Rb induces EMT-like changes in MCF7 cells. *A*, cells were transfected with two separate siRNAs targeting the human Rb sequence or with a control siRNA. After 48 h of incubation, cells were examined by phase-contrast microscopy. *B*, immunolabeling localization of E-cadherin and β -catenin. MCF7 cells were transfected with siRNAs for 48 h and then stained with antibodies to E-cadherin (green) and β -catenin (red) and with DAPI (blue). *C*, whole-cell lysates were subjected to an immunoblot analysis with antibodies to Rb, fibronectin, or β -actin. *D*, cells were transfected with the indicated siRNAs for 48 h. Cell viability was measured by the trypan blue exclusion method.

48 h after transfection (Fig. 1D), suggesting that the suppression of cell growth in Rb-depleted cells is due to cell death. Given that the cell death induced by the depletion of Rb was completely rescued by p53 siRNA (Fig. 1D), the Rb depletion activates p53-dependent apoptotic pathways.

Depletion of Rb induces Rho family GTPase-mediated actin reorganization and cell invasion. To further show that the depletion of Rb induces EMT, we examined whether the depletion was able to mediate migratory and invasive phenotypes, which are characteristics of the EMT. Local polymerization of actin, making lamellipodia-like and filopodia-like structures an important signature of cell migration, was observed in Rb-depleted cells (Fig. 2A). The Rho family GTPases mediate modulation of the actin cytoskeleton: filopodia are induced to form by Cdc42, lamellipodia by Rac1, and stress fibers by RhoA. To test the involvement of Rac1/Cdc42 activation in the Rb depletion-induced EMT, we used GST pull-down assays to analyze the active forms of Cdc42. As expected, Cdc42 was activated in Rb-depleted cells (Fig. 2B). Therefore, depletion of Rb induces the activation of Cdc42, leading to reorganization of the actin cytoskeleton at the cell membrane, which may explain the motility of the cells. Notably, the depletion of p53 also induced reorganization of the actin cytoskeleton, formation of filopodia (Fig. 2A), and activation of Cdc42 (Fig. 2B), as previously reported (29). However, in contrast to the depletion of Rb, the down-regulation of E-cadherin expression (Supplementary

Fig. S4) and the expression of fibronectin were not observed in p53-depleted cells, suggesting that the EMT is not induced by the inactivation of p53 alone. One of the hallmarks of the invasive mesenchymal phenotype is the formation of branching colonies in three-dimensional Matrigel cultures. Control MCF7 cells form alveolar spherical colonies in Matrigel. In contrast, Rb depletion impairs the formation of the spherical structures (Fig. 2C), although depletion was insufficient to form the branching colonies. Furthermore, Boyden chamber transwell assays revealed that siRNA-mediated depletion of Rb in T47D or MCF7 cells led to an increase in cell invasiveness (Fig. 2D). However, this increase in invasiveness was significantly inhibited when MCF7 cells were treated with Toxin B, a Rho family small GTPase inhibitor. In addition, we investigated the effect of expression of a Cdc42 dominant-negative form on the EMT phenotype induced by Rb depletion. The expression of Cdc42 dominant-negative restored stress fiber formation in Rb-depleted cells (Supplementary Fig. S5). This result indicates that activation of Cdc42 by Rb depletion is an important mechanism in induction of filopodia and reduction of stress fibers, which are related to increased motility of Rb-depleted cells. All these findings suggest that depletion of Rb induces invasiveness and that Rho family GTPases play a role in the Rb siRNA-mediated proinvasive phenotype.

Down-regulation of Rb expression during EMT in mammary epithelial cells. In the course of the siRNA analysis, we found that

Rb expression is required for the maintenance of an epithelial phenotype in MCF7 breast cancer cells. We next attempted to determine whether Rb expression is down-regulated during the induction of EMT. Previously, treatment with a combination of TGF β and TNF α was shown to induce EMT in some cell types (30, 31). When MCF10A normal mammary epithelial cells were treated with TGF β /TNF α , morphologic change from an epithelial-like to mesenchymal-like appearance was induced (Fig. 3A). In TGF β /TNF α -treated MCF10A cells, the localization of both E-cadherin and β -catenin was altered from cell-cell contacts to the cytoplasm, and the expression of another epithelial marker, cytokeratin 18, was reduced (Fig. 3A and C). Moreover, expression of a mesenchymal marker, fibronectin, increased (Fig. 3B). The effect of TGF β /TNF α treatment on Rb levels in MCF10A cells was determined by immunoblot analysis. Both Rb and p53 levels were significantly reduced during EMT (Fig. 3B). TGF β stimulates Smad-mediated signaling by binding to and activating the type I and type II TGF β receptors. Smad2 and Smad3 are activated by phosphor-

ylation of the COOH terminal SXS motif by the T β RI kinase, and Smad3 is an essential mediator of the TGF β -mediated EMT (reviewed in ref. 32). The TGF β /TNF α -induced reduction in Rb and p53 expression, as well as fibronectin expression, was totally blocked by treatment with a T β RI kinase inhibitor, suggesting that the reduction was dependent on the T β RI-Smad3 signaling pathway (Fig. 3C). We also found that transfection with a combination of Rb siRNA and p53 siRNA induced down-regulation of E-cadherin expression and reorganization of actin in MCF10A cells (Supplementary Fig. S6).

We next attempted to determine whether Rb and p53 are affected at the transcriptional or protein levels in cells undergoing EMT. Semiquantitative reverse transcription-PCR (RT-PCR) analysis revealed that mRNA levels of p53 were markedly suppressed in TGF β /TNF α -treated MCF10A cells compared with untreated cells, whereas those of Rb were unchanged (Fig. 3D, left). The decline of both Rb and p53 levels in TGF β /TNF α -treated cells was effectively prevented by the presence of MG132,

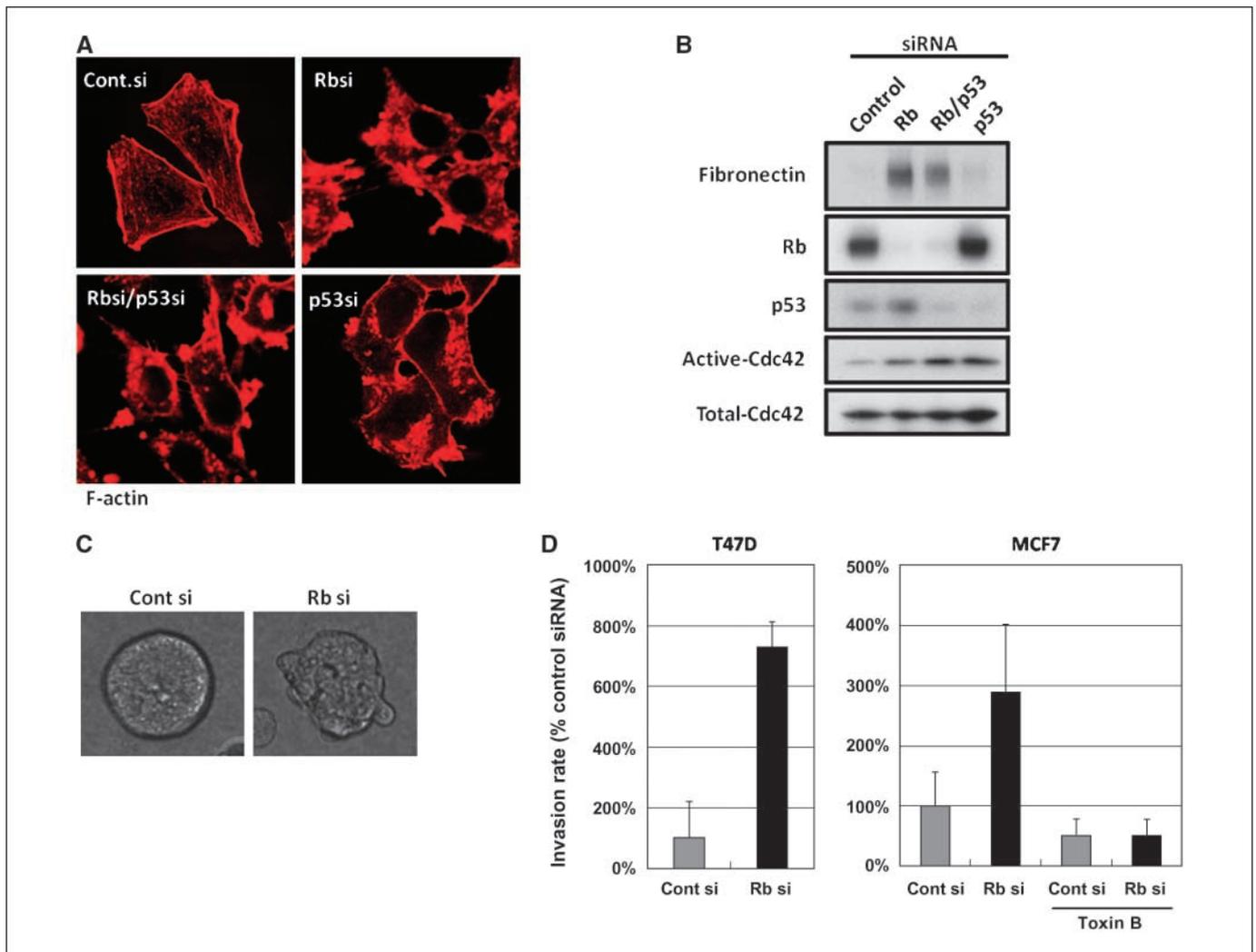


Figure 2. Depletion of Rb alters reorganization of the actin cytoskeleton and invasive potential in MCF7 cells. *A*, cells were transfected with the indicated siRNAs. After 48 h, cells were stained for filamentous actin (*F-actin*) using rhodamine-conjugated phalloidin and observed with confocal laser scanning microscopy. *B*, cells were transfected with siRNAs for 48 h and then serum starved for 4 h, after which cell lysates were subjected to immunoblotting with antibodies to Rb, p53, cdc42, and the mesenchymal marker fibronectin. A pull-down assay was performed to capture the active form of cdc42. *C*, MCF7 cells were subjected to a Matrigel analysis. After 3 d, the colonies were photographed. *D*, Boyden chamber transwell assay. Depletion of Rb significantly promoted the invasiveness of T47D and MCF7 cells. Cells were treated with Toxin B (10 ng/mL) to inhibit Rho family small GTPases.

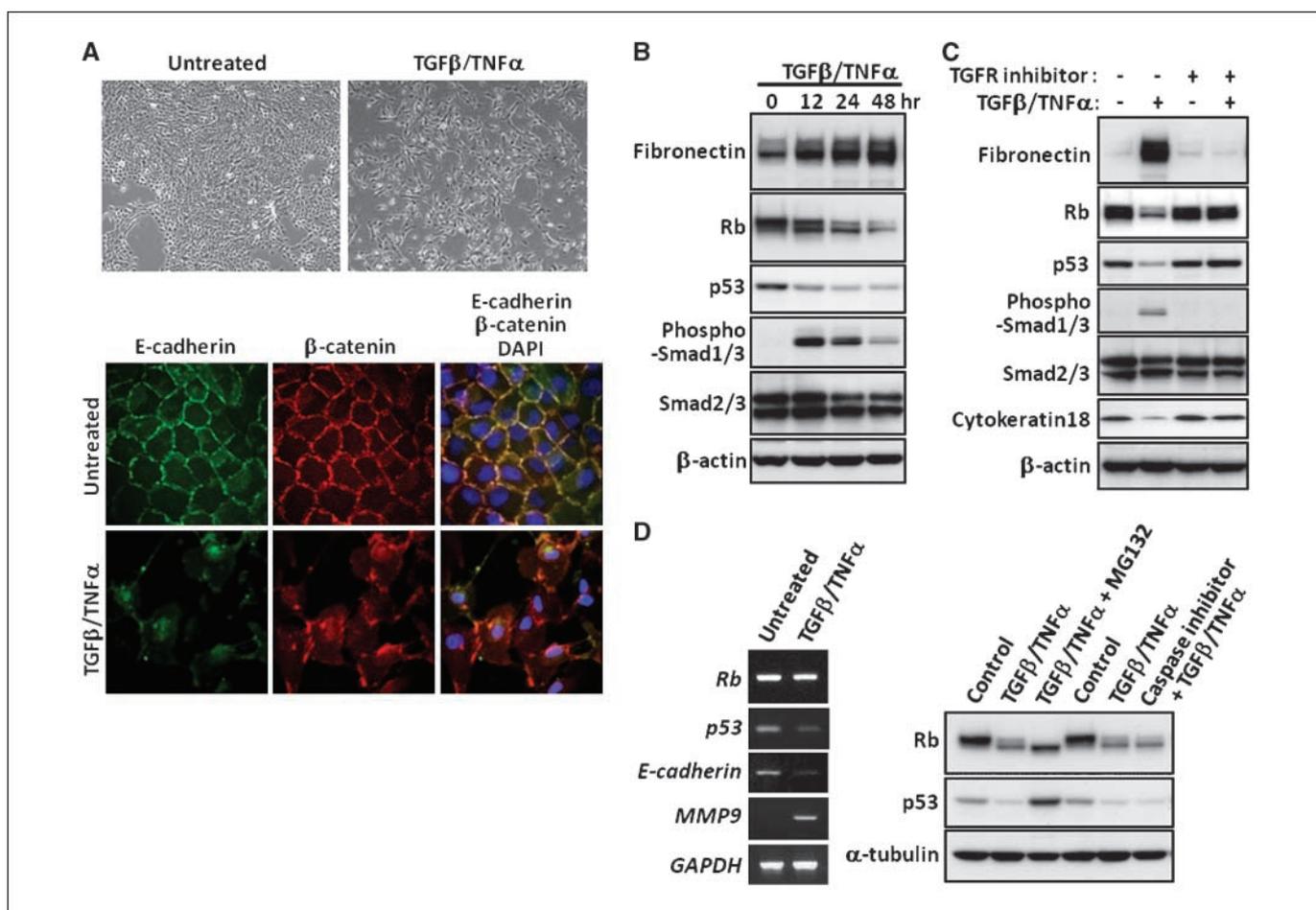


Figure 3. Rb down-regulation occurs during EMT in MCF10A normal mammary epithelial cells. **A**, cells were treated with a combination of TGF β (10 ng/mL) and TNF α (10 ng/mL) for 48 h. Cells were examined by phase-contrast microscopy and then stained with antibodies to E-cadherin (green) and β -catenin (red) and with DAPI (blue). **B**, MCF10A cells were treated with TGF β and TNF α for the indicated times, after which whole-cell lysates were subjected to an immunoblot analysis with the indicated antibodies. **C**, cells were treated with TGF β /TNF α for 48 h in the presence or absence of 1 μ mol/L TGF β receptor I kinase inhibitor. Samples were subjected to immunoblotting with the indicated antibodies. **D**, MCF10A cells were incubated for 48 h with or without TGF β /TNF α , after which the abundance of mRNAs for *Rb*, *p53*, *E-cadherin*, *MMP9*, and *GAPDH* was determined by semiquantitative RT-PCR (left). Cells were incubated with TGF β /TNF α for 24 h and then in the additional absence or presence of 10 μ mol/L MG132 for 12 h. For caspase inhibitor treatment, cells were treated with TGF β /TNF α in the presence of 20 μ mol/L Z-Val-Ala-Asp(OMe)-fmk for 48 h. Whole-cell lysates were subjected to an immunoblot analysis with antibodies to Rb, p53, or α -tubulin (right).

an inhibitor of various intracellular proteases, as well as proteasomes (Fig. 3D, right). These results indicate that the down-regulation of Rb expression is caused by ubiquitin/proteasome-mediated protein degradation via the EMT signaling pathway. Because Rb also regulates apoptosis and is cleaved by caspases in apoptotic cells (33), we determined the amount of Rb in the presence of the caspase inhibitor Z-Val-Ala-Asp(OMe)-fmk. The TGF β /TNF α -induced reduction of Rb was not blocked by the caspase inhibitor, suggesting that the reduction is not a consequence of apoptosis.

Inhibition of EMT in TGF β /TNF α -treated MCF10A cells by overexpression of Rb. We tested the possibility that constitutive Rb expression is able to block the TGF β /TNF α -induced EMT in MCF10A cells. Adenovirus-mediated overexpression of Rb, but not p53, restored E-cadherin expression and localization to cell-cell junctions and prevented the morphologic transition from an epithelial-like to mesenchymal-like appearance caused by TGF β /TNF α treatment (Fig. 4). These results suggest that Rb is required for maintenance of the epithelial phenotype and that a loss of Rb is involved in the induction of EMT.

Rb regulates E-cadherin expression. The process of EMT is associated with the functional loss of E-cadherin due largely to the repression of its transcription (34). E-cadherin plays a central role in maintaining epithelial cell-cell adhesion and polarity. Down-regulation of E-cadherin transcription is thought to be a primary contributor to the onset of EMT (34, 35). Accordingly, to investigate the involvement of Rb in E-cadherin transcription, we assessed changes in E-cadherin mRNA levels using Rb-specific siRNA in Rb-positive MCF7, MCF10A, and DLD-1 cells. RT-PCR analyses showed that depletion of Rb in these epithelial cells resulted in a decrease in E-cadherin expression (Fig. 5A). This result shows that constitutive Rb expression is at least partly responsible for maintaining high levels of E-cadherin in epithelial cells. To further assess the contribution of Rb to the expression of E-cadherin, we reexpressed Rb in Rb-negative MDA-MB-468 cells. RT-PCR analysis revealed a significant enhancement of E-cadherin mRNA expression in Ad-Rb-infected cells compared with Ad-Luc-infected control cells (Fig. 5A). To determine whether Rb directly or indirectly regulates the level of E-cadherin, we first investigated the effects of Rb depletion on the expression of various molecules that

are known to regulate E-cadherin expression and to play a key role in EMT induction by real-time PCR analysis. Rb depletion significantly induced the expression of EMT-related transcription factors, including Slug and Zeb-1, both of which suppress the expression of E-cadherin (Fig. 5B). In addition, SIM2, a repressor of Slug (36), was reduced in Rb-depleted cells. Given that the transcription factor AP-2 α is associated with a GC-rich region of the E-cadherin promoter and positively regulates the expression of E-cadherin (13–15), we next examined whether Rb binds to the E-cadherin promoter sequence together with AP-2 α by conducting a biotinylated double-stranded DNA pull-down assay. In MCF7 cells, we confirmed that both Rb and AP-2 α associate with the promoter region (Fig. 5C), indicating that Rb can assist AP-2 α in up-regulating transcriptional activity at the E-cadherin promoter. To elucidate the relationship between the up-regulation of E-cadherin expression and the cell cycle, we examined the phosphorylation status of Rb bound to the E-cadherin promoter. Cells were treated with actinomycin D or aphidicolin to induce G₁ arrest or early S-phase arrest, respectively. The actinomycin D-treated cell extract contained mainly hypophosphorylated Rb, whereas the aphidicolin-treated cell extract contained hyperphosphorylated Rb. Hypophosphorylated Rb exhibited a greater association with the E-cadherin promoter than did hyperphosphorylated Rb (Fig. 5D). Interestingly, the amount of precipitated AP-2 α increased in the presence of hypophosphorylated Rb, suggesting that the hypophosphorylated Rb is an activator of AP-2 α . Collectively, our data indicate that E-cadherin expression in epithelial-derived cancer cells may be regulated not only indirectly by Slug and Zeb-1 expression as a result of Rb depletion, but also directly by Rb binding to the promoter region together with AP-2 α .

Correlation between Rb and E-cadherin expression in primary breast tumor samples. Because the reduction in Rb expression correlated with the down-regulation of E-cadherin expression in the cell line-derived EMT model, we examined whether such a correlation exists in tumor specimens from breast cancer patients. The expression levels of Rb, E-cadherin, and vimentin were determined in formalin-fixed breast cancer samples by immunohistochemical analysis. Representative immunohistochemical staining is illustrated in Fig. 6 and Supplementary Fig. S7. Rb was highly expressed in intraductal carcinoma cells that expressed membrane-associated E-cadherin (Fig. 6A). In contrast, concurrent down-regulation of Rb and E-cadherin expression was observed in invasive ductal carcinoma cells expressing vimentin, which is the major intermediate filament protein expressed in mesenchymal cells (Fig. 6B). Of the 14 breast cancer samples examined, three invasive ductal carcinomas showed down-regulation of E-cadherin expression, cancer cells in those samples acquired the mesenchymal phenotype, and all the samples expressed very low levels of Rb. In the other 11 samples, which exhibited higher expression of both Rb and E-cadherin, cancer cells maintained the epithelial phenotype. Similar findings were made in tumor specimens obtained from colorectal cancer patients (Supplementary Fig. S8). Indeed, the down-regulation of Rb expression was associated with down-regulation of E-cadherin and the mesenchymal conversion of cancer cells.

Discussion

We have investigated the effect of Rb depletion in human epithelial cells. Our data reveal that Rb is responsible for maintenance of the epithelial phenotype by preventing EMT and

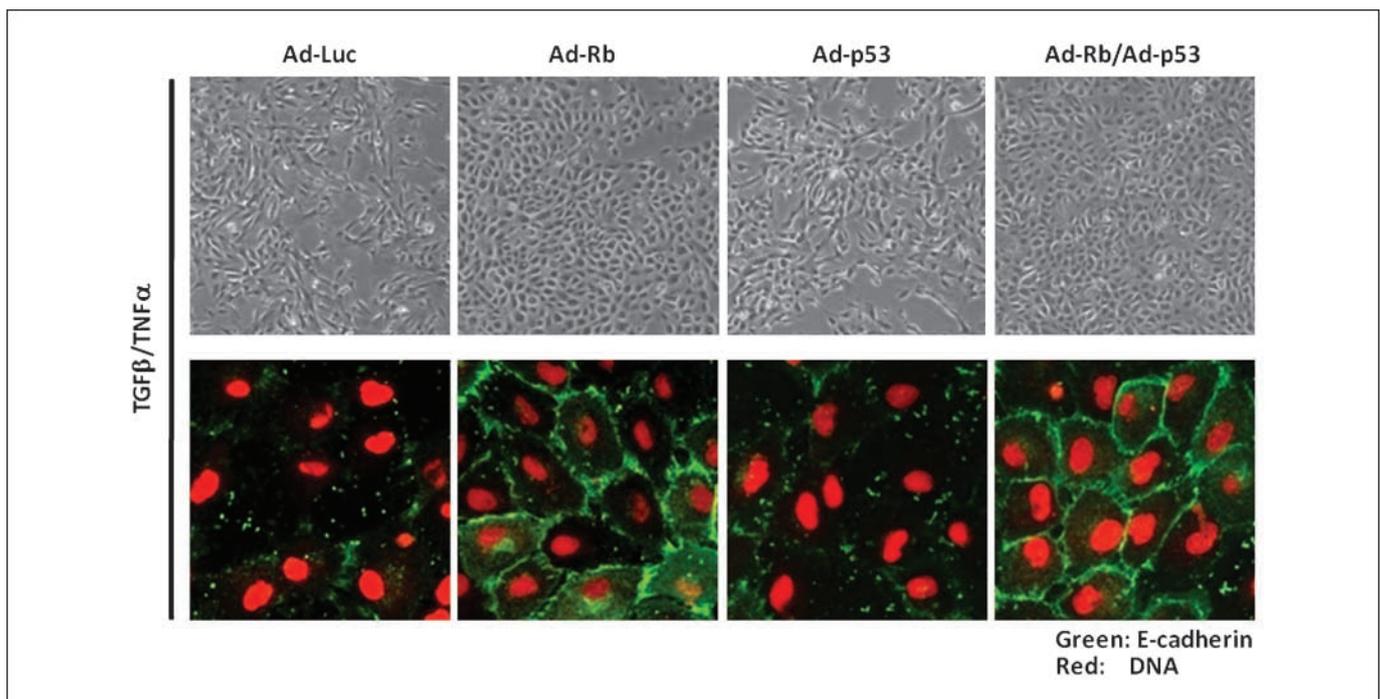


Figure 4. Inhibition of EMT in TGF β /TNF α -treated MCF10A cells by overexpression of Rb. MCF10A cells were infected with recombinant adenoviruses encoding luciferase (*Ad-Luc*), Rb (*Ad-Rb*), or p53 (*Ad-p53*) and incubated in the presence of TGF β (10 ng/mL) and TNF α (10 ng/mL) for 24 h. Treated cells were examined by phase-contrast microscopy and then stained with antibodies to E-cadherin (green) and with propidium iodide (red) and observed with confocal laser scanning microscopy.

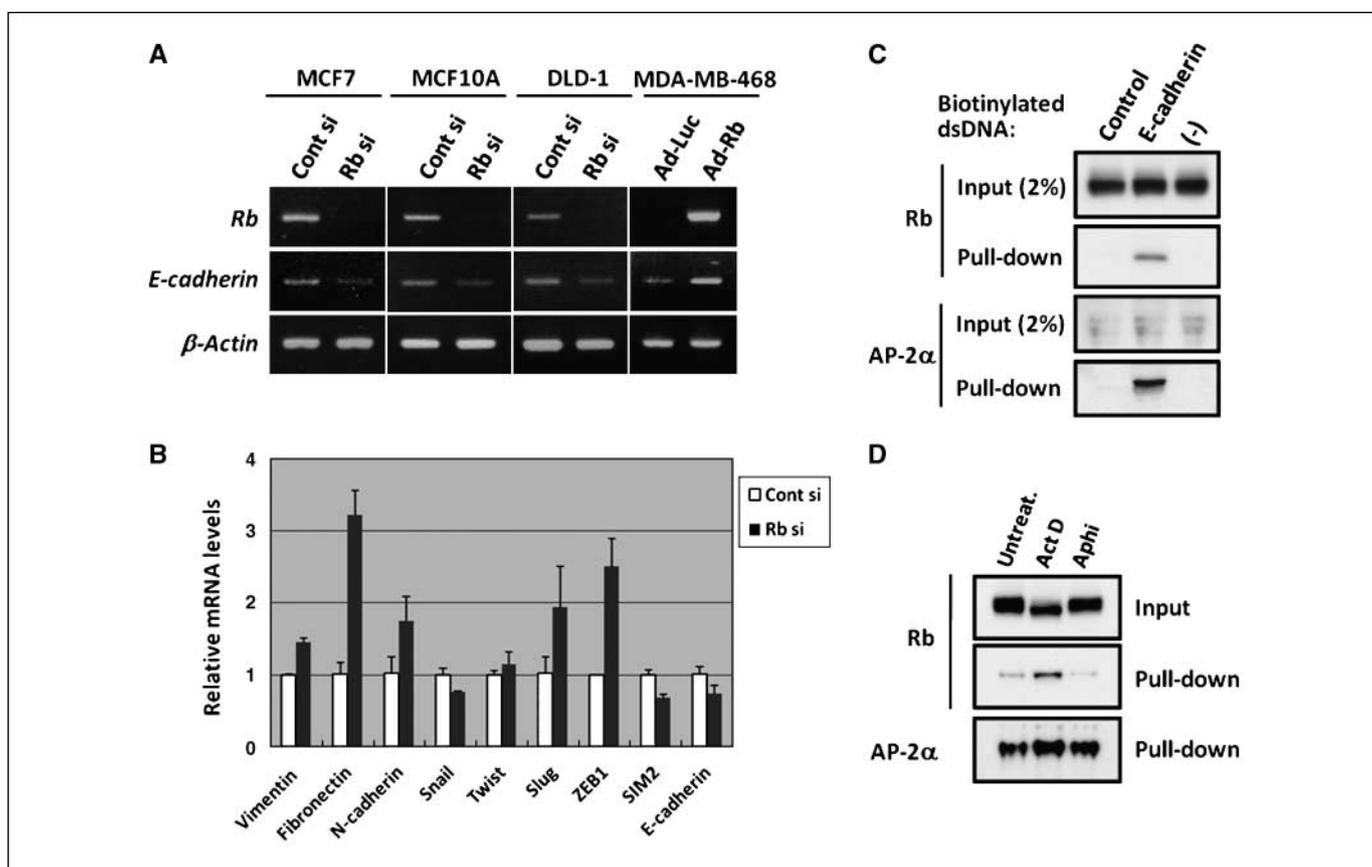


Figure 5. Rb regulates E-cadherin expression and endogenous Rb binds to the E-cadherin promoter sequence with the transcription factor AP-2 α . **A**, MCF7, MCF10A, or DLD-1 cells were transfected siRNAs and MDA-MB-468 cells were infected Ad-Luc or Ad-Rb. The mRNA levels of Rb and E-cadherin were then determined by semiquantitative RT-PCR. **B**, real-time PCR analysis of the abundance of mRNAs for *Vimentin*, *Fibronectin*, *N-cadherin*, *Snail*, *Twist*, *Slug*, *Zeb-1*, *SIM2*, and *E-cadherin* in control siRNA-transfected or Rb-siRNA-transfected MCF7 cells. **C**, association of endogenous Rb with AP-2 α on the human E-cadherin promoter sequence determined by DNA-mediated pull-down assays. MCF7 cell extracts were incubated with streptavidin-agarose beads and biotinylated dsDNA. Whole extracts (*Input*) and precipitated complexes were subjected to immunoblotting with antibodies to Rb or AP-2 α . **D**, HCT116 cells were treated with actinomycin D or aphidicolin, and the extracts included mainly hypophosphorylated or hyperphosphorylated Rb, respectively. The cell extracts were mixed with AP-2 α derived from MDA-MB-468 cells and then incubated with streptavidin-agarose beads and biotinylated E-cadherin promoter sequence dsDNA.

suppression of tumor progression and metastasis. Here, we propose that inactivation of Rb contributes not only to tumorigenesis but also to tumor progression to an invasive phenotype and that the inhibition of EMT is a novel tumor suppressor function of Rb.

Rb acts not only as a negative, but also as a positive regulator of transcription (37). The negative regulatory activity of Rb is largely viewed as an effect on cell cycle progression that influences tumorigenesis; hypophosphorylated Rb binds E2F and inhibits E2F transcriptional activity at the promoters of many genes involved in DNA replication (20, 38). In addition to its role in regulating cell proliferation through the E2F pathway, Rb plays a role in differentiation (e.g., myogenesis, adipogenesis, and osteogenesis) by positively affecting the activity of several differentiation-promoting transcription factors, such as MyoD, CCAAT/enhancer binding protein β , Runx2, and the glucocorticoid receptor (39–42). Although Rb acts as a positive regulator of transcription, whether this activity contributes to the tumor suppressor function of Rb is unknown. Here, we showed that Rb controls cell-cell adhesion in epithelial cells through positive regulation of E-cadherin transcription by interacting with AP-2 α . It is particularly important to understand whether this regulation of transcription is related to cell cycle progression. We found that hypophosphorylated Rb preferentially associates with the E-cadherin promoter compared

with hyperphosphorylated Rb. Furthermore, a previous study has shown the importance of E-cadherin in contact-dependent growth inhibition of normal epithelial cells, and E-cadherin expression also leads to dephosphorylation of Rb, resulting in an increase in the level of the cyclin-dependent kinase inhibitor p27^{kip1} and a decrease in the level of cyclin D1 (43). We hypothesize that a positive feedback loop involving E-cadherin and Rb acts to maintain epithelial cell characteristics. However, the precise molecular mechanisms by which Rb regulates the activity of AP-2 α are still unknown. Recently, it has been reported that the association of Rb with p204, which has two LXCXE Rb-binding motifs, is required for stimulation of Runx2-dependent transcription and osteogenesis (44). It is therefore possible that certain LXCXE-containing proteins cooperate with the Rb/AP-2 α complex to regulate the transcription of E-cadherin.

A striking finding here is that interference with Rb function contributed to the induction of EMT in cancer cell lines. The Rb pathway is inactivated in the majority of human cancers. Down-regulation of Rb expression has been implicated in high-grade human breast cancers (28, 45), and Rb expression is inversely correlated with tumor invasion in gastric carcinoma (27). Additionally, loss of E-cadherin has been strongly implicated in the progression and metastasis of human cancers: aberrant

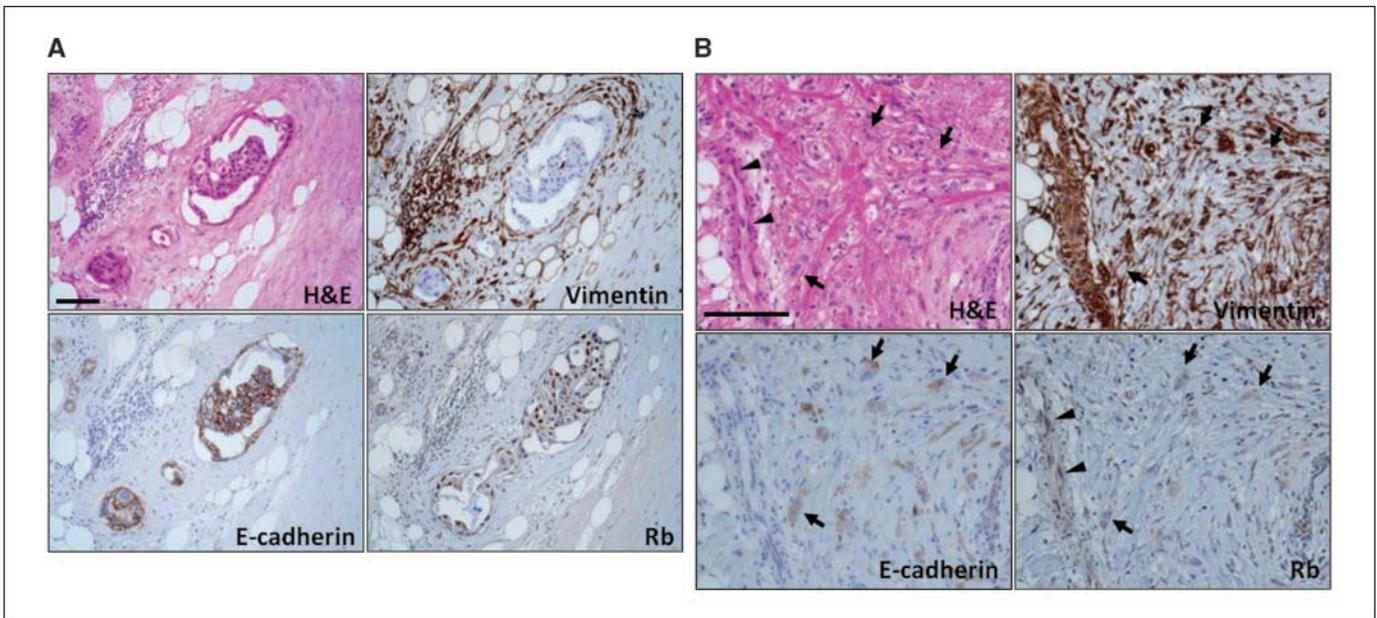


Figure 6. Correlation between Rb and E-cadherin expression in primary breast tumor samples. The expression of Rb, E-cadherin, and vimentin were immunohistochemically examined in formalin-fixed, paraffin-embedded breast cancer tissues. Rb and E-cadherin are expressed in intraductal carcinoma cells (A), whereas down-regulation of Rb and E-cadherin expression was observed in invasive ductal carcinoma cells (B). Arrows indicate cancer cells having the mesenchymal phenotype defined by both morphologic changes and vimentin expression. Arrowheads indicate vascular endothelial cells expressing Rb as a positive control. Bars, 100 μ m.

expression of E-cadherin has been correlated with metastasis of breast cancer (46, 47). Moreover, abnormal expression of E-cadherin and β -catenin is reported to be a useful prognostic factor in patients with gastric carcinoma (48). In addition to analyses using cell lines, our immunohistochemical analysis of human breast cancer samples revealed a down-regulation of E-cadherin and Rb in the invasive front where vimentin-positive cancer cells are located, but not in normal mammary gland tissues (Fig. 6 and Supplementary Fig. S7). These *in vivo* observations further suggest a role for Rb in the regulation of E-cadherin expression in primary human cancers and strengthen the biochemical and functional links between inactivation of Rb and cancer progression.

Expression of adenoviral E-cadherin partially restored the morphologic changes and cell-cell dissociation observed in Rb-depleted cells (Supplementary Fig. S9). This result suggests that inactivation of Rb induces EMT partly due to loss of E-cadherin expression. We also examined whether the depletion of E-cadherin can induce EMT in MCF7 cells (Supplementary Fig. S10). E-cadherin expression was significantly reduced in cells transfected with E-cadherin siRNA, and the E-cadherin/ β -catenin adhesion complex disappeared from cell membrane. However, the fibroblastic morphologic change characteristic of the EMT was not observed in E-cadherin-depleted cells, indicating that loss of E-cadherin alone is not sufficient for EMT induction. Several known EMT-related genes, including vimentin, N-cadherin, fibronectin, and Slug, were induced by depletion of Rb (Fig. 5B). Zeb-1 can be regulated by Rb (49), and, indeed, we confirmed that depletion of Rb induces Zeb-1 expression. Additionally, SIM2, which binds and represses the Slug promoter (36), was reduced in Rb-depleted cells. Given that Slug and Zeb-1 are known to suppress E-cadherin expression, E-cadherin expression may be regulated not only by the direct effects of Rb-binding to the promoter but also by the indirect effects of

SIM2, Slug and Zeb-1 expressions, as a result of Rb depletion. All these findings suggest that Rb is an upstream regulator of E-cadherin and other EMT-related molecules, playing a key role in the EMT/MET switch.

Loss of p53 function is also assumed to be a crucial event in the development of many types of cancers, leading to deregulation of cell cycle checkpoints and apoptosis. Our data show that combined inactivation of Rb and p53 raised the cell survival rate, although there was disruption of cell-cell interactions. These findings imply that loss of p53 enables cells to counteract p53-dependent apoptosis induced upon loss of cell-cell interactions. In addition, recent studies suggest a potential role for p53 in cell motility (29). The effects of p53 on cell motility are largely mediated through the regulation of Rho signaling, thereby controlling organization of the actin cytoskeleton. In the present study, both Rb and p53 expression levels were significantly reduced during EMT, and the combined inactivation of Rb and p53 activated Cdc42, leading to a much greater reorganization of the actin cytoskeleton than the inactivation of Rb alone. Moreover, the loss of both E-cadherin and p53 resulted in the accelerated development of invasive and metastatic mammary carcinomas in mice (50). Thus, it is very probable that the loss of p53 raises cell survival in cells with disrupted cell-cell interactions and facilitates cell motility, whereas the loss of Rb down-regulates the transcription of E-cadherin leading to EMT.

Together, the results of this study show that Rb acts as a regulator of E-cadherin and that its inactivation plays a role not only in loss of control of cell proliferation but also in induction of EMT, contributing to tumor progression, including invasion and metastasis. Further studies are necessary to understand whether the role of Rb in the EMT influences metastatic development in human cancers. Research addressing this aspect will also ascertain the relative contributions of each of the functional properties of Rb, i.e., control of the cell cycle, apoptosis, and cell-cell adhesion, in tumor aggressiveness.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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