Constitutive Expression of RbAp46 Induces Epithelial-mesenchymal Transition in Mammary Epithelial Cells

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Abstract. *Background: Epithelial-mesenchymal transition (EMT) is a component of several morphogenetic and organogenetic processes during embryonic development. EMT is a strictly regulated process and its aberrant regulation may contribute to cancer progression and metastasis. The retinoblastoma suppressor (Rb)-associated protein 46 (RbAp46) is a component of the histone-modifying and -remodeling complexes. Materials and Methods: Western blot and immunofluorescent assays with epithelial and mesenchymal markers and cell migration and invasion assays were employed. Results: Mammary epithelial cells, MCF10AT3B, that express recombinant RbAp46 exhibited an EMT associated with the down-regulation of epithelial markers and up-regulation of mesenchymal markers, as well as an increased migration and invasion phenotype. Conclusion: Rbap46 may be an important player in EMT during embryonic development. Dysregulated RbAp46 expression may contribute to cancer progression and metastasis.*

Epithelial to mesenchymal transition (EMT) is an important morphogenetic event occurring during embryonic development and in pathological states, such as tissue repair and tumor invasion. Several signaling pathways have been found to play roles in EMT (1-3). Signals that trigger EMT originate from outside the epithelial cells to genetically reprogram epithelial cells to mesenchymal and may activate mesenchymal-promoting genes and inactivate epithelial-related genes. Extracellular matrix (ECM) components, such as collagens, as well as soluble factors, such as EGF, scatter factor/hepatocyte growth factor (SF/HGF) and members of the FGF and TGF-β families, were shown to contribute to EMT induction (1-3).

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Recently, increasing evidence supports the view that EMT plays an important role in the progression of many carcinomas (3). Most invasive and/or metastatic cancers adopt partial or complete EMT (3). Some oncogenes, such as Src, Ras and Fos, are involved in EMT (1-3). These results led to the conclusion that the EMT process is an important step required for the invasiveness and metastasis of tumors with epithelial origin.

Fully polarized epithelial cells display keratin filaments and membrane-associated specialized junctions, such as desmosomes and adherens junctions (4, 5). In monolayers formed by epithelial cells, intercellular adhesion plays an important role in the maintenance of cell phenotype. During the EMT process, adherent junctions and desmosomes are disrupted and massive cytoskeleton reorganization takes place, involving the remodeling of the actin microfilament mesh. After EMT, epithelial cells lose the epithelial phenotypes, disassemble intercellular adhesions and increase the ability to invade and migrate through extra cellular matrix. They also acquire mesenchymal characteristics, including expression of vimentin filaments and N-cadherin and exhibition of a flattened, spindle-like phenotype.

The retinoblastoma suppressor (Rb)-associated protein 46 (RbAp46) was first identified as a protein in Hela cells that binds to an Rb affinity column (6) The cDNA encoding RbAp46 was later cloned (7, 8). The predicted amino acid sequence contains four typical WD repeats which end with WD residues, and four non-typical WD repeats which end with WN, FD or YD residues (8). RbAp46 is a component of the histone deacetylase (HDAC) complex mSin3 (9), which is involved in the transcriptional repression. RbAp46 is also known as the histone acetyltransferase (hat) type B subunit 2 in which RbAp46 binds selectively to H2A and H4 histones and greatly stimulates hat activity (10). RbAp46 is a subunit in the NuRD, a multi-subunit complex containing chromosome-remodeling activity (11). Thus, RbAp46 functions as a core-histone-binding protein that targets chromatin assembly factors, chromatin-remodeling factors, histone acetyltransferases and deacetylases to their histone substrates.

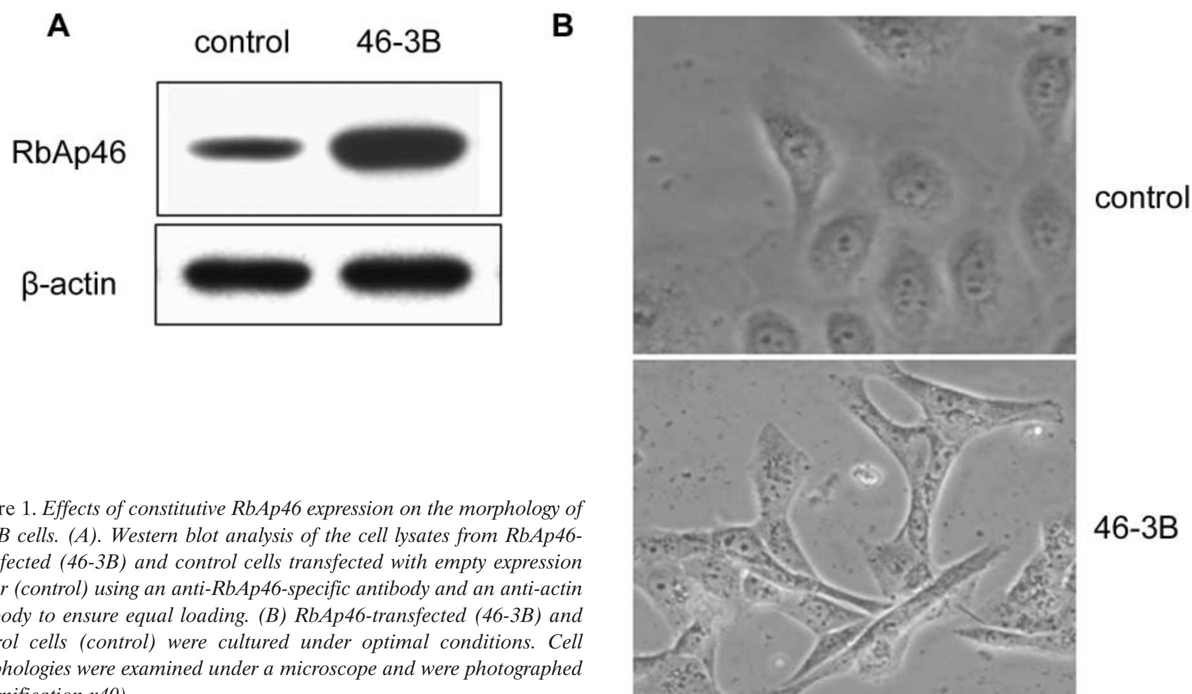

Figure 1. *Effects of constitutive RbAp46 expression on the morphology of TG3B cells. (A). Western blot analysis of the cell lysates from RbAp46-transfected (46-3B) and control cells transfected with empty expression vector (control) using an anti-RbAp46-specific antibody and an anti-actin antibody to ensure equal loading. (B) RbAp46-transfected (46-3B) and control cells (control) were cultured under optimal conditions. Cell morphologies were examined under a microscope and were photographed (magnification x40).*

Previously, RbAp46 was isolated as a downstream gene of the Wilms' tumor suppressor gene, WT1 (12) and was found to function as a potent growth inhibitor to suppress transformed phenotypes of different cancer cells (12-14). Here, the constitutive RbAp46 expression in mammary epithelial cells is reported to result in an EMT.

Materials and Methods

Cell culture and stable cell lines. MCF10AT3B epithelial cells were obtained from Karmanos Cancer Institute (Detroit, MI, USA). MCF10AT3B cells and its derivatives were maintained at 37˚C in a 5% CO_2 atmosphere in DMEM/F12 supplemented with 5% horse serum, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), hydrocortisone (0.5 μg/ml), insulin (10 μg/ml), EGF (2 ng/ml) and cholera toxin (0.1 μg/ml).

The MCF10AT3B cells stably transfected with RbAp46 expression vector (46-3B) and control cells transfected with empty expression vector were established as described previously (15).

Western blot analysis and antibodies. Cells were washed with PBS and lysed with lysis buffer (50 mM Tris-HCl pH8.0, 150 mM NaCl, 0.25 mM EDTA pH8.0, 0.1% SDS, 1% Triton X-100, 50 mM NaF, and protease inhibitor cocktail from Sigma, St. Louis, MO, USA). Total cell lysates adjusted to the same total protein content were analyzed by Western blot analysis. The lysates were boiled for 5 min in SDS gel loading buffer and separated on a 10% or 6% SDS-PAGE gel. After electrophoresis, the proteins were transferred to a PVDF membrane (BioRad Laboratories, Hercules, CA, USA). The membranes were probed with different first antibodies, appropriate HRP-conjugated secondary antibodies and were visualized with enhanced chemiluminescence (ECL) detection reagents (Amersham Bioscience, Little Chalfont, Buckinghamshire, UK). The same membranes were stripped and reprobed with an antibody against β-actin as loading controls.

The polyclonal anti-RbAp46 antibody was produced as a customer service by Alpha Diagnostic International (San Antonio, TX, USA) by immunizing rabbits with a synthetic peptide corresponding to the last 15 amino acids of RbAp46 and the antibody was purified by affinity purification with immunogen peptide. For Western analysis, the antibody was used at a concentration of 1:5000. Antibodies against E-cadherin, N-cadherin, α-catenin, β-catenin and γ-catenin were purchased from BD Science Transduction Laboratories (Lexington, KY, USA). Antibodies against Desmoplakin I&II, vimentin (AB-2), Epithelial Specific Antigen (Ab-2) and Epithelial Membrane Antigen (Ab-2) were obtained from LabVision Corporation (Fremont, CA, USA).

Immunofluorescence staining and confocal microscopy. The control cells and RbAp46-transfected 46-3B cells grown on 8-well BIOCOAT chamber slides (BD Science Discovery Labware, Bedford, MA, USA), washed twice with PBS and fixed with 4% paraformaldehyde in PBS (pH 7.4) for 30 min at room temperature. After washing with PBS, cells were permeabilized with 0.5% (v/v) Triton X-100 for 10 min, washed with PBS and blocked with 3% serum in PBS at room temperature for 1 h. The slides were incubated with different primary antibodies at room temperature for 1 h and washed three times with PBS containing 0.5% Triton X-100 (PBST), then incubated with different fluorescein isothiocyanate (FITC)-conjugated secondary antibodies, respectively. The specificity of staining was verified by omitting the primary antibodies. Finally, the slides were washed

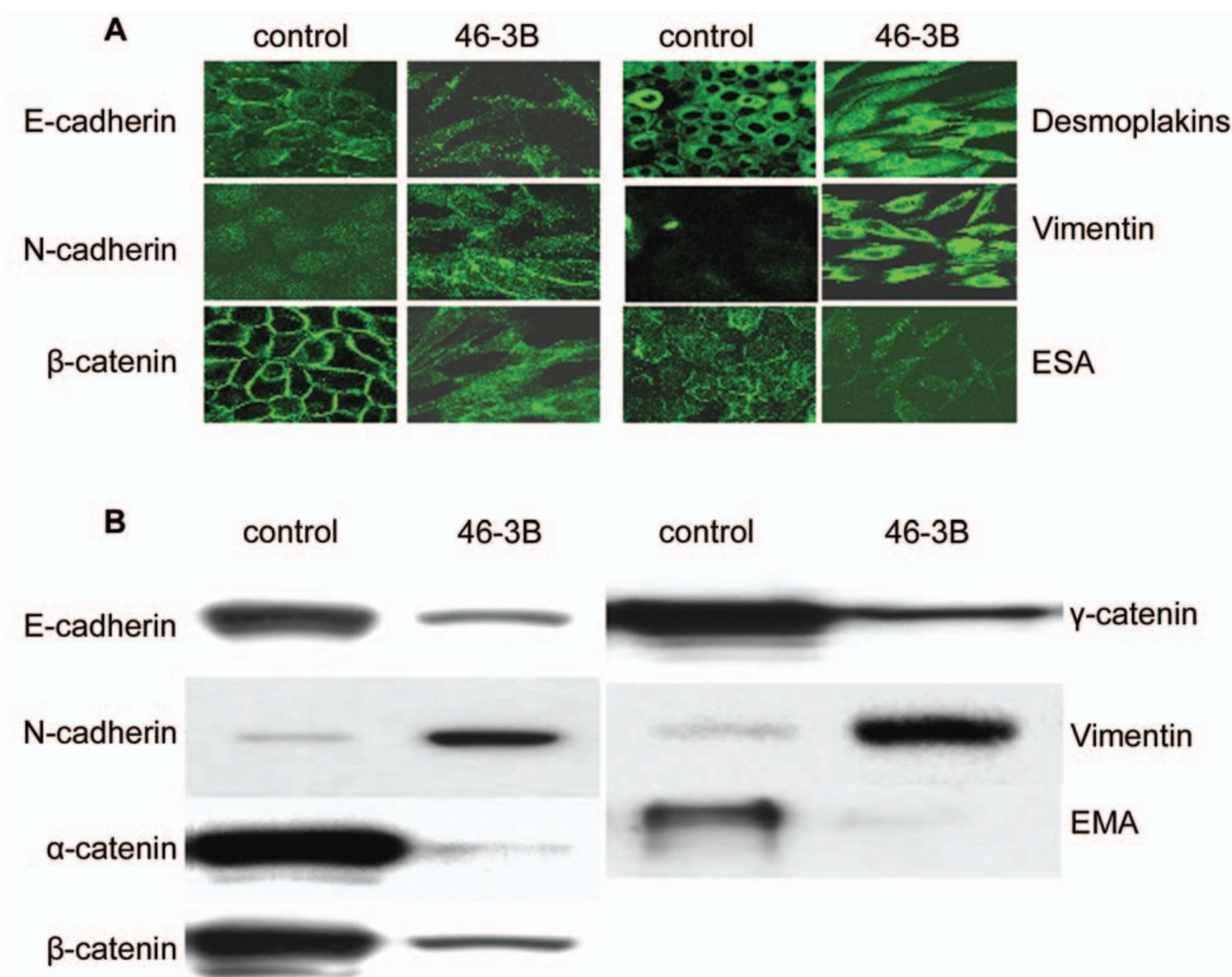

Figure 2. *Constitutive RbAp46 expression induced an epithelial-mesenchymal transition associated with the loss of epithelial markers and the gain of mesenchymal markers. (A). Cells were fixed and immunofluorescent staining was performed using antibodies specific to E- and N-cadherin, β-catenin, desmoplakin I&II, vimentin and epithelial specific antigen (ESA) and FITC-conjugated secondary antibodies. The images were photographed under a Bio-Rad MRC1024 confocal scanning laser microscope (magnification X 60). (B). Western blot analysis of expression levels of epithelial and mesenchymal markers in the 46-3B and control cells using antibodies specific to E- and N-cadherin, α-, β- and γ-catenin, vimentin, and epithelial membrane antigen (EMA).*

three times with PBST, one time with PBS, then mounted with anti-fade medium (Molecular Probes, Eugene, OR, USA) and examined under a Nikon Microscope at 60x magnification using appropriate filters. Images were captured by the MRC-1024 confocal imaging system (Bio-Rad).

In vitro cell migration and matrigel invasion assays. To test cell motility, the cells were plated at a density of $1x10^6$ per 100-mm dish, incubated for 24 h and a line was drawn with a sterilized pipette tip on the dish bottom to take off the cells and cells were replayed on fresh medium. After incubating for another 24 h, cell migration was checked with an inverted microscope and was photographed.

To measure the invasion ability of RbAp46-expressing cells, a 24-well BioCoat Matrigel Invasion Chamber (BD Science Discovery Labware) was used. An equal number of cells ($2x10^5$/ml) in 0.5 ml of medium was loaded onto the top chamber of each well. After incubating the chamber for 8 h, non-migrating cells were scraped from upper surface of the filter. The cells on lower surface of the chambers were fixed, stained with Diff-Quik Stain Set (Baxter Healthcare, Deerfield, IL, USA) and photographed under a light microscope. The cells present in the lower compartment and those adhering to the lower surface of the filter were collected and counted.

Results

Constitutive expression of RbAp46 induced a morphological change. Previously, RbAp46 was isolated as a downstream gene of the Wilms' tumor suppressor gene product, WT1, and was found to function as a potent growth inhibitor (2, 13). Recently, stable cell lines were established from MCF10AT3B cells, a mammary epithelial cell line derived

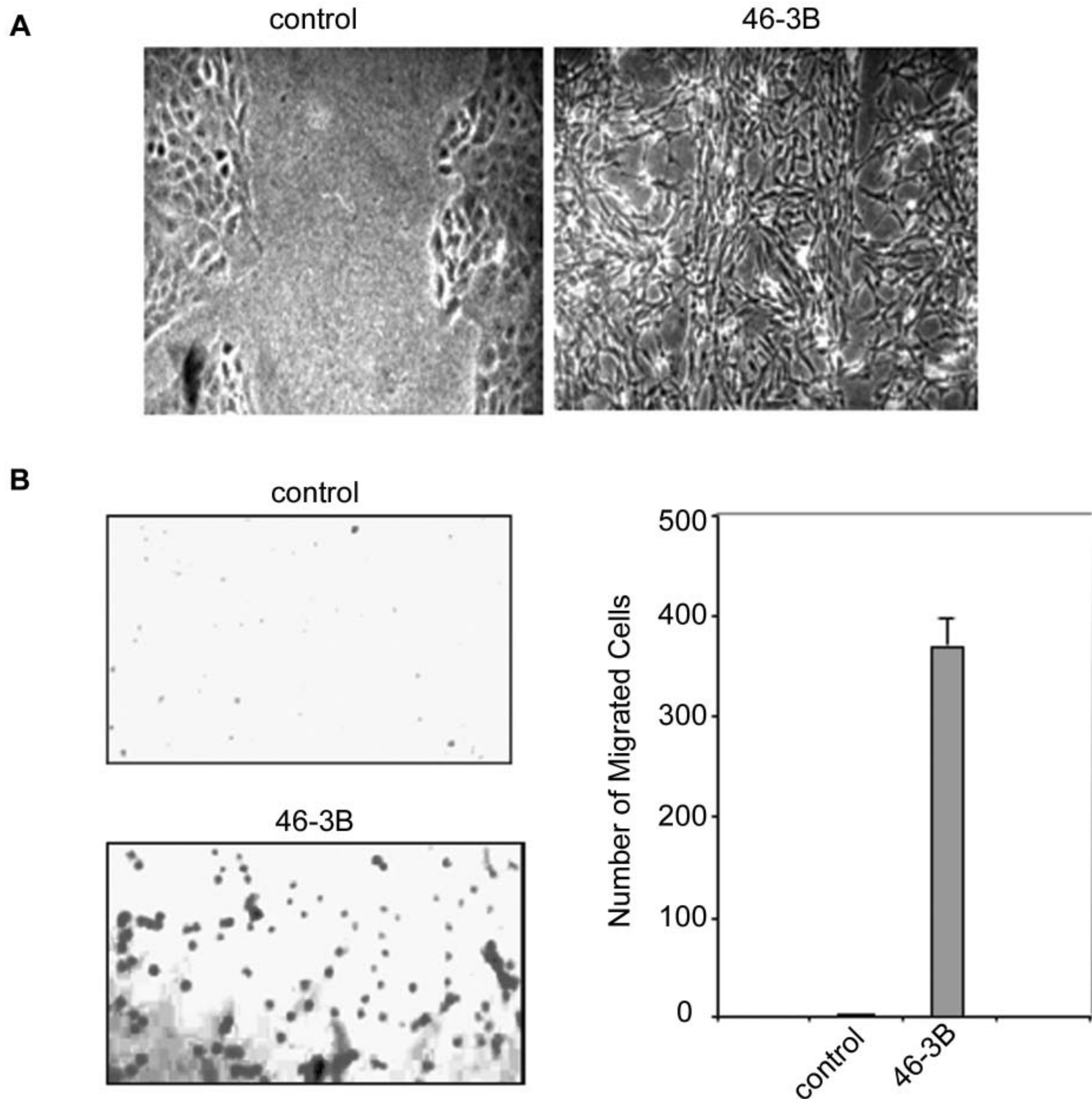

Figure 3. *Constitutive RbAp46 expression induced a migratory and invasive phenotype. (A). The motility behavior of the RbAp46-transfected (46-3B) and control cells (control) was analyzed in an in vitro wound-healing assay. Subconfluent cultures of cells were gently scratched with a pipette tip to produce a wound. Photographs of the culture were taken after 24 h in culture. (B&C). The invasive features of the control and 46-3B cells were analyzed in an invasion assay on Matrigel in a BioCoat Matrigel Invasion Chamber. The cells adhered to the lower surface of the filters were fixed and stained with a Diff-Quik Stain Set, photographed under a light microscope. The cells present in the lower compartment and those adhered to the lower surface of the filter were collected and counted. Experiments were repeated three times.*

from a xenografts model of human proliferative breast disease (15) to express recombinant RbAp46. The constitutive expression of RbAp46 was observed to inhibit the malignant transformation of preneoplastic MCF10AT3B cells (14). The RbAp46-expressing MCF10AT3B cells (46-3B) are heterogeneous cell mixtures that express relatively high levels of RbAp46 compared to the control cell mixtures that were transfected with empty expression vector (control) (Figure 1A). Microscopic examination of the 46-3B cells surprisingly revealed a dramatic change of cell morphology. Whereas the control MCF10AT3B cells displayed the cubical appearance characteristic of epithelial cells, the 46-3B cells exhibited a highly elongated and spindle-shaped phenotype and failed to form extensive cell-cell contacts, *i.e.*, characteristic of

fibroblast (Figure 1B). This result suggested that constitutive RbAp46 expression induced a change in morphology.

Constitutive expression of RbAp46 induced an EMT. Since 46-3B cells acquired a fibroblast-like morphology, whether the change in morphology correlated with the disappearance of epithelial markers and the appearance of mesenchymal markers was examined. As epithelial markers, the expression and localization of desmoplakins I&II, β-catenin, E-cadherin and Epithelial Specific Antigen (ESA) were investigated by immunofluorescence staining and confocal microscopy. To characterize the mesenchymal features, N-cadherin and vimentin were employed as markers.

In the control cells, E-cadherin was located at the cell surface and appeared as a continuous line at the boundaries between neighboring cells, as typically seen in epithelial cells. However, in the 46-3B cells, this pattern disappeared and E-cadherin immunostaining signals were diffused throughout the cytoplasm of the cells and then dramatically decreased (Figure 2A). In addition, the staining patterns for β-catenin were characteristic of epithelial cells and resembled the localization of E-cadherin at the cell boundaries in control cells. Like E-cadherin, the β-catenin staining changed from a peripheral pattern to a diffuse pattern in the 46-3B cells (Figure 2A). The immunoreactive patterns for desmoplakins I & II were dot-like structures on the plasma membrane of the control cells, but disappeared from the plasma membrane and scattered throughout the cytoplasm in the these cells (Figure 2A), indicating that the desmosome structures were disrupted in 46-3B cells. Furthermore, the immunostaining signal for epithelial-specific antigen (ESA) was significantly weak and redistributed in the 46-3B cells, compared to control cells, indicating their epithelial origin (Figure 2A).

Confocal immunofluorescence microscopy also revealed a membrane as well as cytoplasmic distribution of N-cadherin, a mesenchymal marker, in the 46-3B cells whereas N-cadherin was barely detected in the control cells (Figure 2A). In addition, a dramatic change was observed in the distribution and intensity of the immunostaining signals for vimentin, a mesenchymal marker protein (Figure 2A).

Western blot analysis further demonstrated that the expression levels of E-cadherin, and the epithelial membrane antigen (EMA) were dramatically down-regulated in 46-3B cells (Figure 2B). In addition, the expression levels of α-, β- and γ-catenin were all significantly decreased in the 46-3B cells (Figure 2B). In contrast, expression levels of both N-cadherin and vimentin were highly increased in the 46-3B cells (Figure 2B). Taken together, these data demonstrate that constitutive expression of RbAp46 in mammary epithelial cells induced a typical EMT.

Constitutive expression of RbAp46 enhances cell invasion and migration. Since a typical EMT is usually associated with an enhanced ability of invasion and migration, whether the 46-3B cells displayed enhanced motility was examined using an *in vitro* wound-healing assay. The 46-3B cells showed a strong increase in mobility to heal wound in culture 24 h after an incision was made, by which time the controls had barely started to move towards the wound (Figure 3A). The invasive ability of the 46-3B cells was further analyzed in an invasion assay using matrigel-coated filters. RbAp46-transfected 46-3B cells invaded and migrated through matrigel efficiently and exhibited a dramatic increase of invading cells, whereas the control cells were not invasive in this assay (Figure 3B,C). These data further demonstrate that RbAp46-transfected 46-3B cells exhibit a typical fibroblastoid phenotype.

Discussion

In this study, we demonstrated that constitutive RbAp46 expression has a profound morphological effect on the MCF10AT3B mammary epithelial cells. First, RbAp46 transfected 46-3B cells displayed elongated and spindle-shaped morphologies, a typical fibroblastoid phenotype. Second, these morphological changes are associated with the loss of epithelial markers such as E-cadherin and the gain of mesenchymal markers such as N-cadherin and vimentin. Third, the adherens junctions among cells are completely diminished and all catenins and E-cadherin are downregulated and redistributed. Fourth, the RbAp46 transfected 46-3B cells acquired enhanced motile and invasive activities. All of these changes resemble a typical EMT process, which is characterized by the loss of epithelial properties and the gain of mesenchymal features (1-3).

At the cellular level, the predominant differentiation process occurring in the developing kidney is the mesenchymal-epithelial transition (MET), a process that turns mesenchyme into functionally specialized epithelial nephrons in the mature kidney. In a previous study (12), we observed that RbAp46 was highly expressed in mesenchymal cells of the developing kidney, the precursor cells of the epithelial nephrons, but is diminished in the podocytes of the S-shaped bodies, a population of highly specialized glomerular epithelial cells, suggesting that the downregulation of RbAp46 expression is a prerequisite for the MET process in the developing kidney. Considering that RbAp46 is a subunit of the complexes involved in global gene regulation and chromosome remodeling, RbAp46 may be one of the master switches of EMT during embryogenesis.

The analysis of cellular marker protein expression indicated that the effect of constitutive RbAp46 expression on cells was not limited to cell morphology. RbAp46 affects expression of genes that determines cellular behaviors, such as the loss of the epithelial cellular marker E-cadherin and the gain of mesenchymal N-cadherin. The shift in expression from E- to N-cadherin in RbAp46 transfected cells again indicated that

constitutive RbAp46 expression induced a typical EMT. Previously, it was reported that forced expression of N-cadherin in MCF-7 breast cancer cells induced cell migration, invasion and metastasis without changes of the endogenous expression or adhesive function of E-cadherin and cell epithelial phenotypes (16), suggesting that cell migration and invasion are cellular processed independent of the EMT. Thus, it is likely that the induced N-cadherin expression in the RbAp46-transfected 46-3B cells was responsible for the enhanced cell motile and invasive activities observed, whereas E-cadherin down-regulation contributed to the disassembly of the adherens junctions during RbAp46-trigged EMT.

The importance of E-cadherin in maintaining the epithelial phenotype is well documented (17). The down-regulation of E-cadherin expression and function is an important step during EMT and in the malignant progression of most epithelial tumors (3). We observed that E-cadherin was down-regulated and redistributed in RbAp46-transfected 46-3B cells. Currently, it is unclear how constitutive RbAp46 expression results in the loss of the E-cadherin expression. It has been recently reported that the transcription repressors such as Snail (18), SIP1 (ZEB-2) (19) and Twist (20) suppress the transcription of E-cadherin gene and induce EMT. Since RbAp46 is a component of the histone deacetylase complexes that are involved in transcriptional repression mediated by many transcriptional repressors, it is possible that RbAp46 may regulate transcriptional repression activities of transcription factors such as Snail and SIP1. In addition, it was also reported that Snail expression is repressed by MTA3 (metastasis-associated gene 3), a Mi-2/NuRd histone deacetylase subunit (21). It is also possible that RbAp46 may be involved in transcriptional regulation of the transcription factors involved in regulation of E-cadherin such as Snail. Thus, it would be interesting to determine the effect of RbAp46 on these transcription repressors with regard to E-cadherin expression in future studies.

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