A Role for TAZ in Migration, Invasion, and Tumorigenesis of Breast Cancer Cells

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Abstract

TAZ (WWTR1), identified as a 14-3-3 binding protein with a PDZ binding motif, modulates mesenchymal stem cell differentiation. We now show that TAZ plays a critical role in the migration, invasion, and tumorigenesis of breast cancer cells. TAZ is conspicuously expressed in human breast cancer cell lines in which its expression levels generally correlate with the invasiveness of cancer cells. Overexpression of TAZ in low-expressing MCF10A cells causes morphologic changes characteristic of cell transformation and promotes cell migration and invasion. Conversely, RNA interference–mediated knockdown of TAZ expression in MCF7 and Hs578T cells reduces cell migration and invasion. TAZ knockdown in MCF7 cells also retards anchorage-independent growth in soft agar and tumorigenesis in nude mice. Significantly, TAZ is overexpressed in ~20% of breast cancer samples. These results indicate that TAZ plays a role in the migration, invasion, and tumorigenesis of breast cancer cells and thus presents a novel target for the detection and treatment of breast cancer. [Cancer Res 2008;68(8):2592–8]

Introduction

Breast cancer is the leading type of cancer occurring in women and it is the second leading type of death-causing cancer in women in the United States (1). Understanding the molecular mechanisms governing the initiation, progression, and metastasis of breast cancer is important for the prevention, detection, and treatment of this prevailing disease. A recent genomics study revealed that the gene for the tumor suppressor p53 is the most frequently mutated in breast cancers (2). An average of 12 genes were detected to be mutated in a given breast cancer with a high degree of heterogeneity such that more than 100 genes in total were found to be mutated in the breast cancers and breast cancer cells analyzed (2). Breast cancers can be grouped into those positive for estrogen receptor-α (ER-α) and those negative for ER-α. ER-α–positive cancers can be effectively treated by inhibiting the function of ER-α because ER-α is important for the growth of these cells, whereas the growth of ER-α–negative cells is no longer dependent on ER-α. Accordingly, ER-α–negative breast cancers are resistant to ER-α–based treatments (3). The availability of a spectrum of established cell lines derived from human breast cancers has facilitated detailed studies of breast cancer development (4–7). Recent analysis suggests that these cells exhibit many characteristics of oncogenic and genomic alterations which occur in primary breast tumors, and thus, they validate cellular systems to define the molecular mechanisms involved in the development of breast cancer (8). Similar to the cancers *in vivo*, the cancer cell lines are broadly divided into ER-α–positive and ER-α–negative cells. MCF10A represents a nontransformed but immortalized breast epithelial cell line being used widely to represent "normal" breast epithelial cells (9). The ER-α–negative Hs578T, MDA-MB-231, BT-549, and MDA-MB-435S cells have been classified as highly invasive cells, whereas ER-α–positive MCF7, T-47D, ZR-75-1, BT-474, and MDA-MB-361 cells as well as ER-α–negative MDA-MB-453, MDA-MB-468, BT-20, and SK-BR-3 are weakly invasive cells (4).

TAZ (transcriptional co-activator with PDZ-binding motif) was initially identified through its ability to interact with 14-3-3 proteins and is also called WWTR1 (WW domain–containing transcription regulator 1). Sharing amino acid sequence homology with YAP (Yes-associated protein), TAZ (400 residues) contains a conserved WW domain (around residues 125–157) capable of interacting with a PPXY motif, a coiled-coil region (around residues 223–265) implicated in protein-protein interaction, and a COOH-terminal motif capable of interacting with the PDZ domain. Phosphorylation of the Ser residue at position 89 (S89) is responsible for its interaction with 14-3-3 (10). In addition to 14-3-3, TAZ has been shown to interact with many other proteins such as NHERF-2 (10), ZO-1, TTF-1 (11), Cbfa1/Runx2 (12), polyomavirus T antigens (13), TEF-1 (14), TBX5 (15), and Pax3 (16). By coactivating Runx2-dependent gene transcription while repressing PPARγ-dependent gene transcription, TAZ is now known to modulate mesenchymal stem cell differentiation toward osteoblasts over adipocytes (17, 18). Knockout of the TAZ gene in mice suggests that only minimal developmental abnormalities were seen in surviving TAZ-deficient mice, although the number of pups born was only about half the expected Mendelian ratio in some mouse strains. In addition, the mice also were reported to have distorted lung and kidney architectures (19, 20).

We report here that TAZ is highly expressed in invasive breast cancer cell lines as well as in a significant fraction of primary breast cancers. Overexpression of TAZ in MCF10A cells induces morphologic changes characteristic of cell transformation, and enhances cell migration and invasion, whereas knockdown of TAZ in MCF7 and Hs578T cells suppresses cell migration and invasion. TAZ knockdown in MCF7 cells also compromises their growth both *in vitro* in soft agar and *in vivo* in nude mice. These results show that TAZ contributes to the tumorigenesis of breast cancer cells by promoting cell migration, invasion, and anchorage-independent growth.

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

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Materials and Methods

Cell lines and plasmids. Cell lines are described in detail in the Supplementary Materials and Methods. The cDNAs of human TAZ was from MCG clone 19891. The full-length TAZ or Flag-tagged TAZ were constructed by PCR using the MCG19891 clone and cloned into *Bam*HI/*Sal*I sites of the retroviral vector pBABEpuro. The Flag-tagged mouse TAZ (mTAZ) was constructed by PCR using mouse cDNA library from Naiyang Fu (Institute of Molecular and Cell Biology) and cloned into *Sna*BI/*Sal*I sites of the retroviral vector pBABEhygromycin from Sofie Van Huffel (Institute of Molecular and Cell Biology, Singapore). pGEX-TAZ (amino acids 160–229) and pET-TAZ (amino acids 160–229) were constructed by cloning the PCR-amplified cDNA fragment encoding the indicated amino acids into *Eco*RI/*Xho*I sites of pGEX4T-1 (Amersham Biosciences) and pET32a (Novagen), respectively. pGEX-YAP (amino acids 206–262) was constructed by cloning the PCR-amplified fragment from human cDNA library into the *Eco*RI/*Sal*I sites of pGEX4T-1. Purification of GST-tagged protein and His-tagged proteins was done as described in the Supplementary Materials and Methods.

Antibodies. Commercial TAZ antibody was purchased from Abcam and Imgenex. YAP antibody was purchased from Cell Signaling. Actin antibody was from Sigma. Rabbit polyclonal TAZ-specific antibodies were raised by injecting rabbits thrice with GST-TAZ (amino acids 160–229) followed by two additional boost injections with His-TAZ (amino acids 160–229) before serum was collected. The antibody was affinity-purified using immobilized His-TAZ. To detect TAZ specifically, antibody was used in the presence of 100-fold excess of GST-YAP. Western blot analyses were done as described in the Supplementary Materials and Methods.

Retrovirus generation and infection. The amphotropic Phoenix packaging cells were transfected with the indicated retroviral vectors using Lipofectamine according to the instructions of the manufacturer (Invitrogen). After 48 h, the retroviral supernatants were collected, filtered (0.45 μm; Millipore), and added onto the target cells in the presence of 5 μg/mL of polybrene (Sigma-Aldrich) for 6 to 8 h. Infection was done twice. After infection, the cells were selected with puromycin (1 μg/mL) for a week before being analyzed for TAZ expression by Western blotting. For reexpression of mTAZ in MCF7-KD-715 and MCF7-KD-652 cells, the retroviral supernatants derived from tranfecting the amphotropic Phoenix cells with pBABEhygromycin-mTAZ were added to MCF7-KD-715 and MCF7-KD-652 cells, which were selected with hygromycin (500 μg/mL) and puromycin (1 μg/mL) for a week before experiments were done.

Short hairpin RNA-mediated knockdown of TAZ. Short hairpin RNAs (shRNA) against human TAZ were designed using a small interfering RNA design program from Dharmacon and subcloned into the *Bgl*II/*Xho*I sites of the pSuper.Retro.puro vector (Oligoengine). The efficacy of the constructs was tested through transduction into MCF7 and Hs578T cells and Western blot analysis of total cell lysates with the TAZ antibody. The sequences for the sense oligonucleotides for the knockdown construct were KD-1, 5′-GATGAATCCGGCCTCGGCGCC-3′; KD-650, 5′-AGAGGTACTTCCTCAATCA-3′; KD-652, 5′-AGGTACTTCCTCAATCACA-3′; KD-715, 5′-CAGCCTCTGAATCATATGA-3′; and KD-1331, 5′-AACAAACGTTGACTTAGGA-3′. The wound-healing assay, cell motility and invasion assays (Transwell assays), and anchorage-independent growth in soft agar are described in detail in the Supplementary Materials and Methods.

Tumorigenesis in nude mice. Four- to 6-week-old female nude mice were inoculated s.c. in the left and right hind flanks or into the thoracic mammary fat pad with 5×10^6 MCF7-KD-715 or MCF7-KD-652 cells suspended in 100 μL of PBS, simultaneously receiving a 60-day release pellet containing 0.72 mg of β-estradiol (Innovative Research of America). Tumor development was monitored and pictures of mice were taken when the tumor sizes were >5 mm.

Immunohistochemistry. Human breast tissue arrays (InnoGenex) were used to examine the expression of TAZ and cytokeratin (Cam 5.2, Becton Dickinson) in normal and cancer tissues. Immunohistochemistry was done using Dako Envision System K 1395 (Dako). The slides were dewaxed thrice in fresh xylene for 5 min and rehydrated sequentially with 100%, 95%, 80%, and 75% ethanol and PBS (5 min for each step), followed by antigen retrieval with 2100-Retriever (PickCell Laboratories BV, Prestige Medical, Ltd.) for 12 min in sodium citrate buffer (pH 6). After cooling for 4 h at room temperature, the slides were rinsed with water and PBS with 0.1% Tween 20 before quenching with 0.6% H_2O_2 in the dark for 20 min. After rinsing with PBS, the slides were blocked with PBS with 5% goat serum and 2% bovine serum albumin for 2 h at room temperature and incubated overnight with the primary antibody at 4°C. Subsequently, the slides were washed with PBS and 0.1% Tween 20, followed by biotinylated secondary antibody for 2 h. After washing, the slides were incubated with Vectastatin ABC reagent for 60 min. The diaminobenzidine tetrahydrochloride peroxidase substrate was applied to slides for 3 to 5 min in the dark and reactions were terminated by washing with PBS. The results were analyzed under a microscope.

Results

TAZ expression in breast cancer cell lines. The expression of TAZ in breast cancer cell lines was examined by immunoblot analysis using total cell lysates. Testing several commercially available as well as in-house–generated antibodies showed that all antibodies reacted with both TAZ and the homologous YAP. Due to efficient detection of both TAZ and YAP by a commercial antibody (Imgenex), we have used this antibody throughout the entire study except for the experiment specified later. The results derived from a representative experiment, shown in Fig. 1*A*, indicate that TAZ was expressed at varying levels in all breast cancer cell lines examined. The expression levels of both TAZ and YAP were normalized against those of actin and the quantitative results derived from three independent experiments are presented in arbitrary units shown in Fig. 1*B*. Among the breast cancer cell lines, high levels (approximately four arbitrary units) of TAZ were detected in Hs578T, BT-549, and MDA-MB-435S cells, whereas moderate levels (approximately two arbitrary units) were observed in MDA-MB-231, BT-20, and T-47D cells. MCF10A, MCF7, MDA-MB-453, ZR-75-1, and BT-474 cells express low levels (approximately one arbitrary unit) of TAZ. Significantly, three (Hs578T, BT-549, and MDA-MB-435S) of the four highly invasive cancer cell lines exhibited high levels of TAZ expression, with MDA-MB-231 cells expressing moderate levels. Most (five out of seven) of the weakly invasive cells expressed low levels of TAZ with two cell lines (BT-20 and T-47D) expressing moderate levels. These results suggest that the majority of highly invasive breast cells express high levels of TAZ, whereas the majority of weakly invasive cells express low levels of TAZ. No such correlation of YAP expression levels with invasiveness of breast cancer cells was noticed (Fig. 1*B*). The correlation of TAZ expression level with the invasiveness of breast cancer cells suggests that TAZ may be part of the mechanism governing the invasiveness of breast cancer cells.

Overexpression of TAZ in MCF10A cells induces fibroblast-like morphology and promotes cell migration and invasion. To examine the functional consequence of TAZ expression in breast cancer cells, we overexpressed TAZ or Flag-tagged TAZ in MCF10A cells (which exhibit low endogenous levels of TAZ) to levels that are ~2- to 3-fold of those found in high-expressing invasive cells such as Hs578T and BT-549 (Fig. 2*A*). This was achieved by retrovirus-mediated transduction, as MCF10A cells are not satisfactorily transfected to express exogenous proteins. Pools of MCF10A cells infected with the appropriate retrovirus were analyzed to avoid clonal variations. Enhanced green fluorescent protein (EGFP)–expressing cells (Fig. 2*B, left*) retained the epithelial appearance seen in parental MCF10A cells. However, cells overexpressing TAZ (Fig. 2*B, right*) or Flag-TAZ (data not shown) developed a more

spindle-shaped fibroblast-like morphology characteristic of cell transformation.

Because TAZ expression levels correlate with the invasiveness of breast cancer cells, we examined whether TAZ overexpression could promote cell migration and invasion. Using the wound-healing assay, we compared the cell mobility of MCF10A cells expressing TAZ relative to the cells expressing EGFP (Fig. 2*B*). The mobility of TAZ-expressing cells was dramatically enhanced. Within 14 hours, the area of the wound was significantly recovered by the migrating TAZ-overexpressing cells, and by 24 hours (*right*), the wound area had been completely recovered by migrating cells. In marked contrast, the wound closure of MCF10A cells expressing EGFP was not significant within 14 hours and was only partial within 24 hours (*left*). The motility and invasiveness of these cells were independently assessed using the Transwell assay (Fig. 2*C*). The migration and invasiveness of TAZ-expressing cells increased ~5-fold and 2- to 3-fold, respectively, compared with control cells expressing EGFP. These results suggest that TAZ promotes cell migration and invasion, and these properties may contribute to the altered morphology of cells overexpressing TAZ.

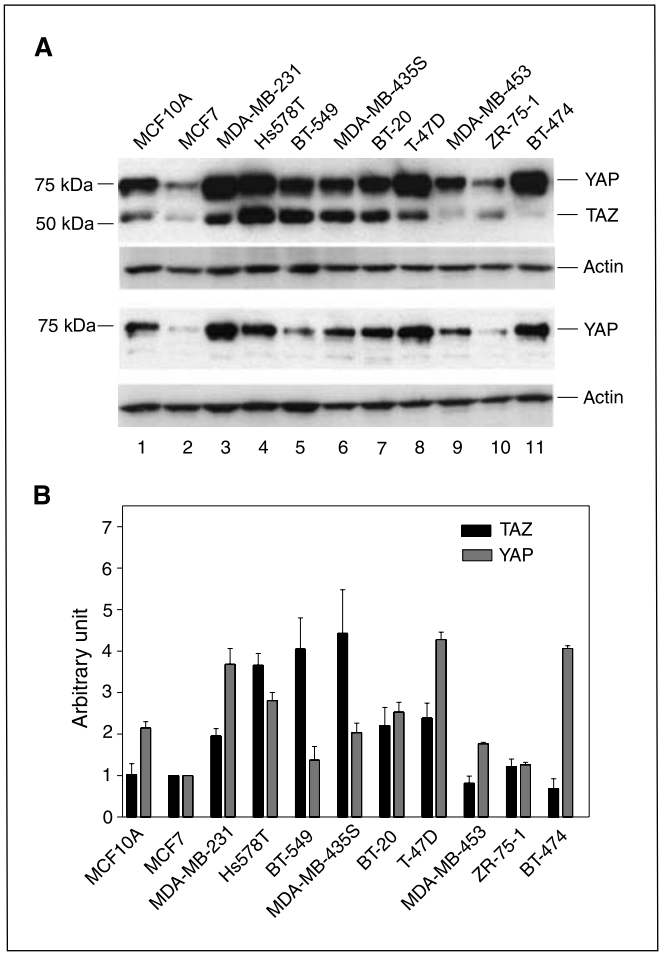

Figure 1. TAZ is preferentially overexpressed in highly invasive breast cancer cells. *A,* lysates derived from 11 breast cancer cell lines were analyzed by Western blot using anti-TAZ antibodies which also reacted well with YAP (*top*) or anti-YAP antibodies (*bottom*). The levels of actin as detected by anti-actin antibodies were used as loading controls. *B,* the expression levels of TAZ and YAP were quantified in three independent experiments and the averages were presented as arbitrary units relative to actin.

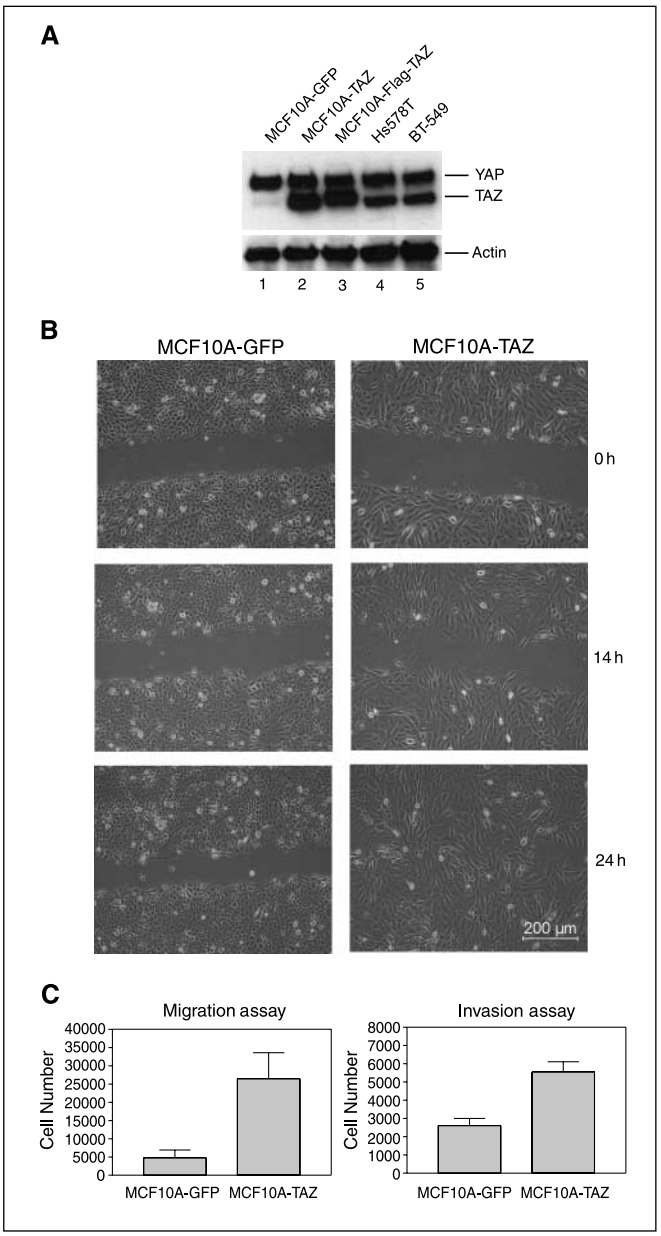

Figure 2. TAZ promotes the migration and invasion of MCF10A cells. *A,* the levels of TAZ in MCF10A cells transduced with retrovirus expressing EGFP (*lane 1*), TAZ (*lane 2*), or Flag-TAZ (*lane 3*) were assessed by Western blot. The levels of TAZ in Hs578T (*lane 4*) and BT-549 (*lane 5*) cells were assessed as comparisons. *B,* wound-healing migration assay for MCF10A cells expressing EGFP (*left*) and MCF10A cells expressing TAZ (*right*). The healing of wounds by migrated cells at time 0, 14, and 24 h was imaged. MCF10A cells expressing TAZ have a more motile and spindle-shaped fibroblast-like appearance and migrate faster than MCF10A cells expressing EGFP. *C,* the migration (*left*) and invasion (*right*) of MCF10A cells expressing EGFP and TAZ were assessed by transwell assays. *Columns,* mean of three independent experiments; *bars,* SEM.

shRNA-mediated knockdown of TAZ in MCF7 and Hs578T cells suppresses cell migration and invasion. To verify the role of TAZ in cell migration and invasion, a complimentary but independent approach was used that is based on RNA interference (RNAi)–mediated knockdown of gene expression. For sustained knockdown while avoiding clonal variations, we analyzed pools of cells stably infected with a retrovirus-based vector expressing

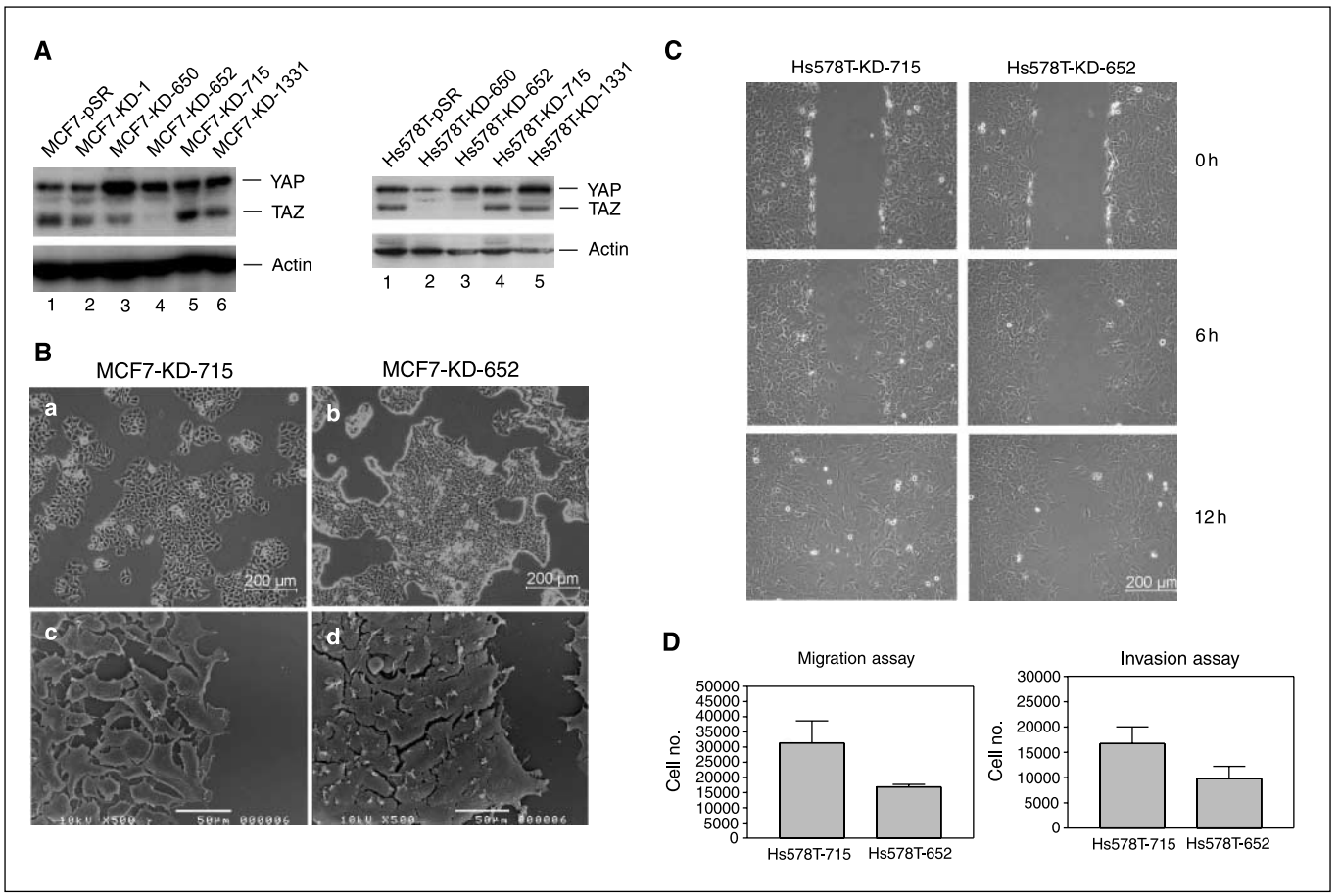

Figure 3. TAZ knockdown in MCF7 cells enhances epithelial morphology and in Hs578T cells suppresses cell migration and invasion. *A,* the expression levels of TAZ in MCF7 (*left*) and Hs578T (*right*) cells transduced with the vector (*lane1*) and various shRNAs (*lanes 2–6*) targeting different sites of TAZ mRNA were assessed by Western blot. shRNA-652 is most potent in suppressing TAZ expression in both MCF7 and Hs578T cells. *B,* TAZ knockdown in MCF7 cells results in clusters of more densely packed and compact sheets of cells. When plated at low to medium densities, both MCF7-KD-715 cells and MCF7-KD-652 cells grew as clusters of cells. However, the cell density of the clusters is obviously enhanced in the MCF7-KD-652 cells (*b*) as compared with MCF7-KD-715 cells (*a*). Scanning electron microscopy revealed that the space between cells was reduced in MCF7-KD-652 cells (*d*) as compared with MCF7-KD-715 cells (*c*). This resulted in the appearance of more tightly aligned/packed and compact epithelia when TAZ expression was knocked down. *C,* TAZ knockdown in Hs578T cells suppresses cell migration. Wound-healing migration assay for Hs578T-KD-715 (*left*) and Hs578T-KD-652 (*right*) cells was performed. The healing of wounds by migrated cells at time 0, 6, and 12 h was imaged. Hs578T-KD-652 cells with TAZ knockdown have a much reduced motility as compared with Hs578T-KD-715 cells which have a similar migration to parental and vector-transduced Hs578T cells. *D,* the migration (*left*) and invasion (*right*) of Hs578T-KD-715 and Hs578T-KD-652 cells were assessed by transwell assays. *Columns,* mean of three independent experiments; *bars,* SEM.

shRNA targeting various regions of TAZ mRNA. One reported RNAi target site (KD-1) plus four other sites of TAZ mRNA were tested for their susceptibility to knocking down the expression of TAZ protein in MCF7 cells as assessed by immunoblot analysis. As shown in Fig. 3*A* (*left*), the shRNA based on the reported target noticeably reduced the level of TAZ protein. Among the shRNAs based on the four new targets, two (715 and 1331) had no significant effect on TAZ protein levels, one (650) had an RNAi effect comparable to the reported one, whereas another (652) had the most efficient effect of suppressing TAZ expression. The four shRNA-expressing retroviruses were also used to infect Hs578T cells (*right*) and pools of stably transduced cells were analyzed for TAZ expression. Again, shRNA 715 and 1331 had no significant effect, whereas both shRNA 650 and 652 reduced the protein level of TAZ significantly. We therefore used cells knocked down with shRNA 652 for subsequent analysis in comparison with cells transduced with shRNA715, as cells transduced with shRNA715 behaved like parental and vector-transduced cells in all analyses.

Concomitant with reduced TAZ expression, the clusters of cells became more densely packed and compact sheets of cells with the cell density of the clusters being enhanced in the MCF7-KD-652 cells as compared with MCF7-KD-715 cells (Fig. 3*B, top*). Scanning electron microscopy revealed that the space between cells was reduced in MCF7-KD-652 cells as compared with MCF7-KD-715 cells (Fig. 3*B, bottom*), resulting in the appearance of more tightly aligned/packed and compact epithelia. This observation is obvious when the cells are plated at either low density or high density and cultured under standard conditions. As MCF7 cells do not migrate and invade significantly using most assays, we have analyzed migration and invasion using Hs578T cells. Relative to Hs578T-KD-715 cells, with no detectable RNAi effect, which migrated robustly in the wound-healing assay (Fig. 3*C, left*), knockdown of TAZ significantly reduced the migration of Hs578T-KD-652 cells (Fig. 3*C, right*). The reduction of migratory and invasive abilities was also revealed using the Transwell assay (Fig. 3*D*). These RNAi experiments further bolster the conclusion derived from the

overexpression experiments that TAZ is an important regulator of cell migration and invasion. Its expression levels correlate negatively with the epithelial appearance of breast cells and positively with migratory and invasive properties.

TAZ is important for anchorage-independent growth and tumorigenesis. To examine the importance of TAZ in the tumorigenesis of breast cancer cells, we first assessed the anchorage-independent growth capability of TAZ–knocked-down MCF7-KD-652 cells in comparison with MCF7-KD-715 cells. MCF7-KD-715 cells grew well in soft agar (Fig. 4*A, top*), whereas the number of colonies grown in soft agar was dramatically reduced for MCF7-KD-652 cells. Compared with the well-developed spheres of cell colonies of MCF7-KD-715 cells (Fig. 4*B, left*), MCF7-KD-652 cells failed to grow up and only small aggregations of cell debris were observed (Fig. 4*B, right*). These results suggest that TAZ is essential for anchorage-independent growth of MCF7. We subsequently examined whether TAZ contributes to tumorigenesis in nude mice. MCF7-KD-715 and MCF7-KD-652 cells were separately injected into the thigh and fat pad of nude mice and the growth of the tumors was monitored (Fig. 4*C*). Compared with MCF7-KD-715 cells (right side), MCF7-KD-652 cells (left side) were compromised in forming tumors at both the thigh (left) and fat pad (right) injection sites. The quantitative analysis of the resulting tumors was shown in Supplementary Fig. S1. These results suggest that TAZ is also important for *in vivo* tumorigenesis of MCF7 cells.

Overexpression of TAZ in breast cancers. To assess whether our findings obtained from analyzing the breast cancer cells have physiologic and clinical relevance, we examined TAZ expression in tissue sections derived from primary breast cancers. To overcome the cross-reactivity of anti-TAZ antibodies with YAP, we generated rabbit antibodies to a region of TAZ that is most divergent from YAP. The affinity-purified antibodies preferentially recognized TAZ but also reacted with YAP at lower efficiency (Fig. 5*A, left*). However, the antibodies specifically recognized TAZ in the presence of 100-fold excess (over the antibody) of a recombinant YAP fragment corresponding to the TAZ region used as the antigen (Fig. 5*A, right*). Using this approach to specifically detect TAZ, immunohistochemistry was used to examine the primary breast cancer samples. Among 126 breast cancer samples analyzed, 27 (21.4%) overexpressed TAZ and most (21 samples) of the TAZ-expressing cancers were of invasive (infiltrating) ductal carcinomas (IDC), suggesting that TAZ is overexpressed in a significant fraction of IDCs. A representative positive labeling of TAZ in IDC is shown (Fig. 5*B, right*). The summary of our immunohistologic analysis of the cancer samples is shown in Table S1. These results suggest that TAZ overexpression is likely to be important for the progression of breast cancer into IDC and establish the physiologic and clinical relevance of our findings with breast cancer cell lines.

Discussion

In our ongoing proteomics analysis of proteins in human cancer cell lines, we have noticed the higher expression levels of TAZ in more invasive breast cancer cells. This observation prompted us to investigate the physiologic/clinical relevance and the role of TAZ in the tumorigenesis of breast cancer. TAZ is widely expressed in breast cancer cells. An important observation is that most highly invasive breast cancer cell lines express TAZ at levels that are approximately four times of those expressed by the majority of weakly invasive breast cancer cells, implying a role for TAZ in the invasiveness of breast cancer cells. The clinical relevance of this observation is supported by the finding that TAZ is overexpressed in a significant fraction of breast cancers (~21.4% of 126

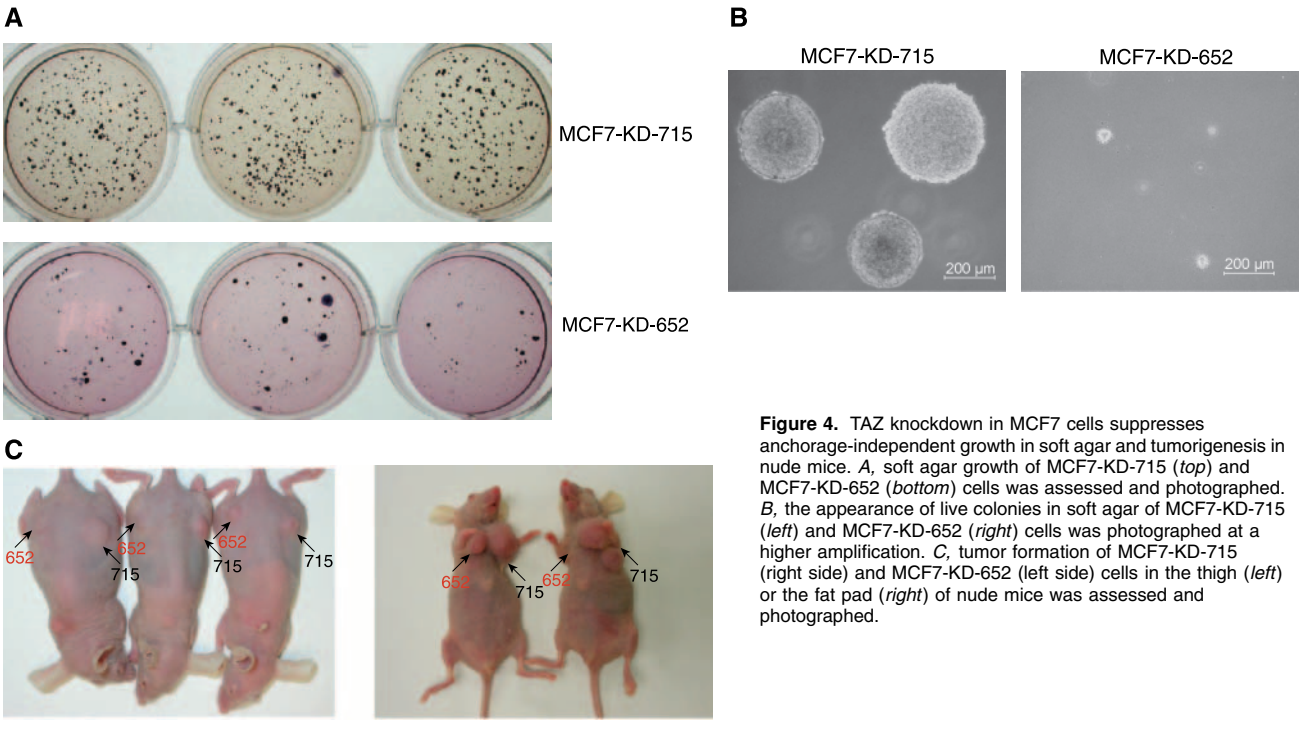

Figure 4. TAZ knockdown in MCF7 cells suppresses anchorage-independent growth in soft agar and tumorigenesis in nude mice. *A,* soft agar growth of MCF7-KD-715 (*top*) and MCF7-KD-652 (*bottom*) cells was assessed and photographed. *B,* the appearance of live colonies in soft agar of MCF7-KD-715 (*left*) and MCF7-KD-652 (*right*) cells was photographed at a higher amplification. *C,* tumor formation of MCF7-KD-715 (right side) and MCF7-KD-652 (left side) cells in the thigh (*left*) or the fat pad (*right*) of nude mice was assessed and photographed.

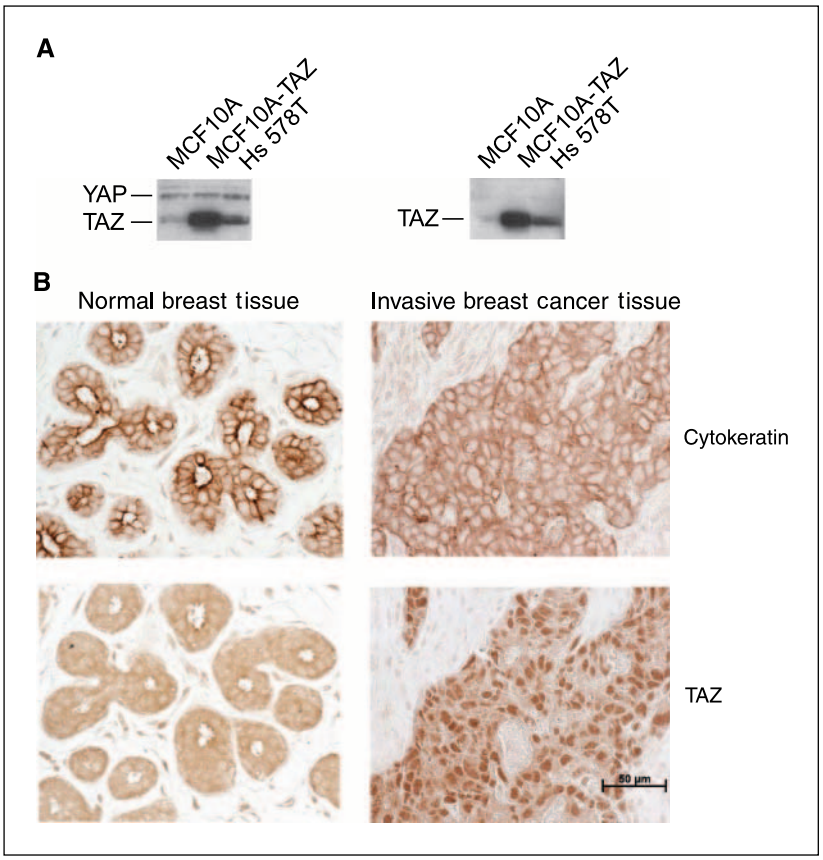

Figure 5. TAZ is overexpressed in IDCs. *A,* characterization of rabbit anti-TAZ antibodies. Lysate derived from the indicated cells were analyzed by Western blot using affinity-purified rabbit antibodies raised against fragment (amino acids 160–229) of TAZ in the absence (*left*) or the presence of 100× of recombinant YAP fragment (amino acids 206–262) corresponding to the TAZ antigen region. Although the antibodies cross-reacted with YAP (*left*), they specifically recognized TAZ in the presence of excess amounts of recombinant YAP fragment. *B,* TAZ is overexpressed in IDC. Normal breast tissues (*left*) or breast cancer tissues (IDC; *right*) were either stained with TAZ antibody (preincubated with 100-fold excess of recombinant YAP fragment) or cytokeratin antibody as a control. TAZ is overexpressed in IDC but not in normal breast tissue.

commercially available breast cancer samples examined). As the invasiveness of cancer cells is dependent on increased migratory and invasive properties, we have tested the hypothesis that the mechanism of action of TAZ overexpression in breast cancers and cell lines is to promote the migration and invasiveness of breast cancer cells. Both gain of function (by overexpression) and loss of function (by shRNA-mediated knockdown) approaches were used to establish the critical role of TAZ in the migration and invasion of breast cancer cells. Overexpression of TAZ in MCF10A cells to a level ~2- to 3-fold of those detected in highly invasive cells caused a morphologic change from an epithelial to a fibroblast-like appearance and dramatically increased the migratory and invasive properties of the cells. Furthermore, shRNA-mediated knockdown of TAZ expression in MCF7 and Hs578T cells reduced cell migration and invasion. The epithelial clusters of MCF7 cells became more densely packed with cells when TAZ expression was knocked down. These results indicate that TAZ is a negative regulator of epithelial morphology/architecture, as well as a positive regulator for invasive and migratory behavior. It is conceivable that TAZ overexpression in breast cancer may trigger the loss of epithelial property to promote the migratory property, an important event for ductal carcinoma *in situ* to progress into IDC. Finally, when TAZ expression is knocked down in MCF7 cells, their anchorage-independent growth in soft agar and tumorigenesis in nude mice is retarded, suggesting that TAZ overexpression is an important part of the process involved in breast cancer development and progression. We believe that our experiments have addressed the role of TAZ directly rather than as an off-target of shRNA. First, we employed several different shRNAs and observed a correlation between the extent of knockdown and the observed consequence on cellular behaviors. Secondly, results derived from overexpression in MCF10A cells lead to similar conclusions. Finally, reintroduction of RNAi-resistant mouse cDNA encoding Flag-tagged mTAZ in MCF7-KD-652 (TAZ knocked-down) significantly restored the ability of the cells to form colonies in soft agar and the results are shown in Supplementary Fig. S2.

Although the molecular mechanism governing the function of TAZ is not fully clear, one of the mechanisms for TAZ action is to trigger a loss of epithelial morphology, to promote cell migration and invasion, and to support anchorage-independent growth, all of which are important for cancer initiation, progression, and invasion. Although TAZ overexpression is not sufficient to enable MCF10A cells to grow into sizeable colonies in soft agar,[1] it is important for anchorage-independent growth of MCF7 cells. TAZ may thus play a critical role but might not be solely sufficient for the anchorage-independent growth of breast cancer cells. The morphologic change of MCF10A cells due to TAZ overexpression is similar to epithelial-mesenchymal transition (EMT; ref. 21) characterized by loss of cell adhesion and increased cell mobility, whereas the altered morphology of MCF7 cells due to TAZ knockdown might be related to the mesenchymal-epithelial transition (MET). TAZ might also be part of the regulatory machinery governing the EMT/MET events in breast epithelial cells and its deregulated expression will enhance EMT to facilitate the

[1] S.W. Chan, C.J. Lim, and W. Hong, unpublished observation.

development of breast cancer and invasive property. Preliminary studies suggests that the expression level of E-cadherin in MCF10A and MCF7 cells is not significantly altered by either overexpression or knockdown of TAZ, indicating that TAZ may regulate EMT/MET via mechanisms different from those used by Twist, snail, and slug, which are known to promote EMT by down-regulating E-cadherin (22). Based on our current knowledge of TAZ as a coactivator of gene transcription, one possible mechanism for the action of TAZ is to interact with other transcriptional activators to enhance the transcription of genes that are involved in cell migration. Our preliminary microarray analysis seems to support this possibility as many genes that are potentially involved in cell migration and other cellular processes are up-regulated by TAZ. We are in the process of verifying these results to identify the genuine downstream targets of TAZ that are involved in cell migration. At the same time, TAZ may directly interact with proteins that are involved in cell migration via its PDZ domain, WW domain, or coiled-coiled domain. Our preliminary analysis of cells expressing EGFP-tagged TAZ indicates that some EGFP-TAZ can be detected in the membrane ruffles. Further studies will be necessary to explore these possibilities.

YAP, a protein highly homologous to TAZ, was recently identified as a candidate oncogene on the chromosome 11q22 amplicon. Overexpression of human YAP in nontransformed mammary epithelial cells induces EMT, suppresses apoptosis, and promotes growth factor–independent proliferation and anchorage-independent growth in soft agar (23). Hence, YAP and TAZ may share similar or overlapping functions. However, we did not observe any obvious correlation between YAP expression levels and the invasiveness of breast cancer cells, whereas the expression of TAZ is much increased in more invasive breast cancer cell lines (Fig. 1). In conjunction with the observation that the expression of YAP was not affected by shRNA-mediated knockdown of TAZ, it seems that TAZ and YAP are independently regulated. A few other studies suggest that YAP may have tumor-suppressing properties by interacting with and stabilizing tumor suppressor p73 in the nucleus for proper execution of the cell death pathway (24). More future studies are needed to gain a full understanding about these issues.

The finding that TAZ is overexpressed in breast cancers and cancer cell lines, and its critical role in cell migration, invasion, and tumorigenesis is of significance. First, it might serve as a novel biomarker for breast cancers (especially IDCs) and our findings suggest that a comprehensive examination of TAZ expression in a large number of breast cancers in terms of the prevalence, clinical outcome, and response to various treatments is warranted. Secondly, our findings have laid down a novel and solid foundation for future studies aiming to reveal additional insights into the molecular mechanisms governing its role in breast cancer cell migration, invasion, and tumorigenesis and its interplay with other proteins involved in the development, progression, and metastasis of breast cancers. Furthermore, TAZ might offer a novel target to treat breast cancers as its expression is preferentially increased in invasive breast cancer cells and the levels correlate with invasiveness. Because TAZ plays an important role in the tumorigenesis of breast cancer cells, yet is clearly not essential for mouse development and fertility, it might be an effective yet selective target for breast cancer therapy.

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