PTEN Loss Induces Epithelial–Mesenchymal Transition in Human Colon Cancer Cells

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Abstract. *Background: The epithelial–mesenchymal transition is a critical early event in the invasion and metastasis of many types of cancer, including colorectal cancer (CRC). Chronic inflammation is an inducer of several cancer types and inflammatory cytokines have been implicated in tumor invasion. Materials and Methods: Human colon cancer cell lines HCT116 and SW480 were transfected with phosphatase and tensin homolog deleted on chromosome 10 (PTEN) siRNA or non-targeting control (NTC). Invasiveness was measured using a modified Boyden chamber assay and migration was assessed using a scratch assay. Results: PTEN knockdown increased the invasion and migration of CRC cells and the addition of medium containing tumor necrosis factor-α (TNF-α) further enhanced the migration and invasion. PTEN knockdown resulted in nuclear β-catenin accumulation and increased expression of downstream proteins c-Myc and cyclin D1. Conclusion: Our study supports the findings of clinical studies identifying an association of PTEN loss with late stage cancer. Cellular factors secreted from the surrounding tumor milieu likely act in concert with genetic changes in the tumor cells and contribute to enhanced tumor invasion.*

The epithelial–mesenchymal transition (EMT) is a process that occurs normally during critical phases of embryonic development in mammals, including embryonic neural crest migration and multi-organ development (1). The migration and subsequent reorganization of embryonic organelles in EMT has led to a comparison with the process of tumor metastasis. Progression from premalignant disease to a malignant phenotype is accomplished by a number of acquired genetic alterations and reversible changes attributable to the tumor–host microenvironment which are exceedingly important in tumor invasion and metastasis (2, 3). During EMT, the expression of E-cadherin, referred to as the 'caretaker' of the epithelial phenotype, decreases, resulting in loss of cell–cell adhesion and increased migration (4). The nuclear accumulation of β-catenin, a key component of the Wnt signaling pathway, is also noted at the invasive front, a high density area of inflammatory cells that surrounds the primary cancer mass (5).

Tumor necrosis factor-α (TNF-α), an inflammatory cytokine involved in the acute phase reaction, is implicated in a variety of human diseases, including cancer (6). TNF-α is mainly produced by macrophages, but it is also produced by a variety of other cell types including mast cells, lymphoid cells and fibroblasts (7). The increased density of inflammatory cells, including macrophages that are located at the invasive front of primary tumors, has led to the supposition that TNF-α plays a key role in tumor–host crosstalk. Recent evidence supports the notion that the innate immune system plays a key role in tumor invasion by the release of cytokines mainly from macrophages that are found in high density at the invasive front (8). The effect of TNF-α is well established; however, its involvement as a mediator at the invasive front of primary tumors has yet to be determined.

Activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway is important for normal cellular metabolism and growth and progression of certain types of cancer (9). PI3K/Akt pathway mutations are found in several human malignancies including breast, ovarian, pancreatic, and colorectal cancer (CRC) (10, 11). Evidence that the PI3K pathway plays a key role in CRC is supported by studies showing that the absence of phosphatase and tensin homolog deleted on chromosome ten (*PTEN*), the natural PI3K inhibitor, is associated with increased intestinal mucosal tumors (12-14). Recent studies suggest that loss of *PTEN* expression is more prevalent in CRCs than originally thought, particularly in later stage cases. Rychahou *et al*. (15) from our laboratory demonstrated loss of *PTEN* expression

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in approximately 83% of metastatic CRCs. In agreement with these findings, *PTEN* was noted to be inactivated (expression was reduced or lost in 66-70% of CRCs) and was identified more frequently in association with microsatellite instability (16, 17). Additionally, TNF-α results in the down-regulation of *PTEN* (18). Together, the silencing of *PTEN*, and TNF-α stimulation may provide an environment for the migration, invasion and subsequent metastasis of CRCs.

The tumor microenvironment is increasingly recognized as a source of the soluble factors and inflammatory cytokines important in the progression of various cancer types (19, 20). The purpose of our current study was to evaluate the importance of reduced *PTEN* expression, coupled with the response of TNF-α, on CRC cell phenotype and invasion.

Materials and Methods

Antibodies. Antibodies against E-cadherin and α-catenin were purchased from BD Transduction Laboratories (San Jose, CA, USA). Antibodies against PTEN, phosphorylated-Akt, Akt, phosphorylated-β-catenin, β-catenin, phosphorylated-GSK-3β, and GSK-3β were purchased from Cell Signaling (Danvers, MA, USA). Antibodies against Lamin B and IκBα, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody for β-actin was from Sigma-Aldrich (St. Louis, MO, USA)

Cell culture. Human colon cancer cell lines, HCT116 and SW480, rat intestinal epithelial cell lines (RIE-1 and IEC-6), the murine macrophage cell line RAW, and the human intestinal epithelial cell line (HIE) were obtained from American Type Culture Collection (Manassas, VA, USA). HCT116 cells were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS). RAW cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. RIE-1 cells were maintained in DMEM-H supplemented with 5% FBS. IEC-6 cells were maintained in DMEM with 5% FBS, and HIE cells were maintained in Opti-MEM medium supplemented with 4% FBS. Tissue culture media and reagents were purchased from Life Technologies, Inc. (Grand Island, NY, USA). SW480 cells were maintained in Liebovitz L 15:DMEM (1:1 ratio) with 10% FBS.

To generate the *PTEN* shRNA metastatic (m)SW480 cell line, *PTEN* shRNA green fluorescent protein-tagged SW480 cells were injected into the spleen of athymic nude mice. After 4 weeks a liver metastasis was isolated, dissected into small fragments, and transferred to a flask. Hank's balanced salt solution was added to the flask and cells were incubated for 3 h. Cells were then passed through a nylon mesh filter, spun down, and plated. Cells were maintained in SW480 media supplemented with ampicillin and streptomycin for 2 weeks and then selected with puromycin media with for 2 weeks. *PTEN* shRNA mSW480 cells maintained their green fluorescent protein-tag, and puromycin selection ensured that all other cells were eliminated.

Macrophage conditioned media. For isolation and culture of human macrophages, buffy coats were collected from the blood of healthy donors at The University of Texas Medical Branch Blood Bank. Primary blood mononuclear cells were isolated by density-gradient centrifugation through Ficoll/Hypaque (Amersham Bioscience, Piscataway, NJ, USA), suspended (8×10^6 cell/ml) in RPMI-1640 medium with 15% heat inactivated human serum (Sigma), and seeded into flasks. After incubation for 2 h at 37˚C, adherent cells were detached with trypsin and resuspended at 1×10^6 cells/ml in medium supplemented with 40 ng/ml macrophage colony-stimulating factor (Pepro Tech Inc, Rocky Hill, NJ, USA). Cells were allowed to differentiate for seven days in macrophage colony-stimulating factor. On day seven, cell media were replaced with fresh media without macrophage colony-stimulating factor and cells were cultured for an additional 24 h. Human macrophages were finally exposed to lipopolysaccharide (100 ng/ml, Sigma) for another 24 h. The culture media were collected, centrifuged, stored in aliquots at –80˚C, and defined as macrophage–conditioned media (MCM).

Cell transfections. For migration and invasion experiments, HCT116 and SW480 cells were transfected with *PTEN* siRNA (Dharmacon, Lafayette, CO, USA). HCT116 (2.5×10^6), SW480 (4×10^6), RIE-1 (8×10^6), IEC-6 (8×10^6), and HIE (8×10^6) cells underwent electroporation in pre-sterilized 4 mm gap electroporation cuvettes (Molecular BioProducts, San Diego, CA, USA). A *PTEN* non-inducible vector system (Dharmacon) was used to create SW480 and HCT116 polyclonal stable shRNA populations with puromycin antibiotic selection. Stable clones were established for at least 2 weeks before they were sorted twice by FACS Aria (Becton Dickinson, Franklin Lakes, NJ, USA). Effective knockdown was confirmed by Western blot and real-time reverse transcriptase–polymerase chain reaction (RT–PCR) as we have previously described (18).

Migration assay. A monolayer scratch assay was used to compare the migratory ability of HCT116, SW480, and mSW480 with *PTEN* shRNA. *PTEN* and non-targeting control (NTC) siRNA-transfected cell lines or shRNA cell lines were cultured to confluency, scratched and photographed using phase-contrast microscopy at 0, 24 and 48 h. In some migration experiments, different concentrations of MCM was added at time 0 h. The minimum distance in millimeters between the wound edges of the scratch area was analyzed using Adobe Photoshop 7.0. All experiments were performed in triplicate.

Invasion assay. A modified Boyden chamber invasion assay with Matrigel-coated Transwell chambers was performed with HCT116 and SW480 cell lines. Cell lines transfected with *PTEN* siRNA were compared to those transfected with NTC to determine invasiveness. MCM was used as the chemoattractant and the cell line is complete medium was used as the control. After 72 h, the cells were fixed with 3% glutaraldehyde and stained with 4',6-diamidino-2-phenylindole (DAPI) fluorescent staining. DAPI-stained cells were counted in 4 different fields with an inverted fluorescence microscope. For co-culture experiments using *PTEN* shRNA SW480 with RAW macrophages, TNF-α antibody (R&D, Minneapolis, MN, USA) and/or isotype control IgG was added and renewed every 24 h. All experiments were performed in triplicate.

Soft agar assay. A soft agar assay was used to assess anchorage-independent growth of *PTEN* shRNA HCT116 and SW480 stable transfectants compared with control cells. Cells were grown in 0.35% Seaplaque agarose gel (Lonza, Switzerland) using 24–well plates. The cells were plated at a density of 1.6×10^4 cells/ml and plated onto the gel. After 2 weeks the cells were stained with crystal violet, counted, and photographed. All experiments were performed in triplicate.

Western blot. Western blotting was performed as previously described (21). Briefly, total protein (60 μg) was resolved on a 10% Nu-PAGE Bis-Tris gel and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were then incubated overnight at 4˚C in blotting solution, followed by 1 h incubation with primary antibodies. The membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. After three additional washes, the immune complexes were visualized by enhanced chemiluminescense detection.

Enzyme-linked immunosorbent assay (ELISA). A monoclonal antibody specific for human TNF α coated on a 96-well plate was obtained from Bio Scientific (Austin, TX, USA) and the assay was performed according to the manufacturer's instructions. Briefly, standards and samples were added to the wells, the wells were washed, and biotinylated monoclonal anti-human TNF-α antibody was added. After a second wash, avidin-horseradish peroxidase was added, producing an antibody-antigen-antibody conjugate. The wells were again washed and a substrate solution was added, which produces a blue color in direct proportion to the amount of human TNF-α present in the initial sample. The stop solution changes the color from blue to yellow and the wells were read at 450 nm. All experiments were performed in triplicate.

Immunostaining. Experiments were performed as described previously (9). For immunofluorescent staining, cells were grown on chamber slides, fixed with 4% paraformaldehyde, and incubated with primary antibodies. Secondary antibodies were fluorescein-labeled (FITC)-conjugated goat anti-mouse from Invitrogen (Carlsbad, CA, USA).

Statistical analysis. For invasion assay, cell counts were analyzed using analysis of variance for a three-factor experiment. The factors were siRNA (NTC, *PTEN* siRNA), media (control, MCM) and experiment (conducted at 3 different times). For wound assay, distances were measured at 6 different locations and averaged for each scratch at each time point. The averaged data were analyzed using analysis of variance for a two-factor experiment with repeated measures on time. The two factors were siRNA (NTC, *PTEN* siRNA) and time (0, 24, 48 h). Main effects and interactions were assessed at the 0.05 level of significance. Multiple comparisons were conducted using a t–statistic with the standard error computed from a linear combination of appropriate mean squares in the analysis of variance, with an approximated degrees of freedom using Satterthwaite method, and with Bonferroni adjustment for the number of comparisons. Statistical computations were carried out using PROC GLM and PROC MIXED in SAS®, Release9.1.

Results

PTEN knockdown increases CRC cell migration. Migration and invasion are both key components of EMT (22). Since the HCT116 CRC cell line is a metastatic cell line that invades through the basement membrane (23), we tested the hypothesis that the knockdown of *PTEN* would increase migration and invasion. HCT116 cells were transfected with NTC siRNA or *PTEN*-targeted siRNA and scratch assays were performed. There was near complete closure of the scratch in *PTEN* siRNA HCT116 cells when compared to control (Figure 1A left). These results were quantified by measuring the distance between the scratch edges and found to be statistically significant at 48 h after the scratch (Figure 1A middle). Western blot analysis confirmed *PTEN* knockdown in HCT116 cells (Figure 1A right). Next, a non-metastatic cell line, SW480, was transfected with NTC or *PTEN* siRNA and scratch assays were performed. *PTEN* siRNA SW480 cells demonstrated complete closure of the scratch after 48 h when compared to the control (Figure 1B). Together, these findings demonstrate increased CRC migration associated with reduction of *PTEN* expression.

MCM further increases CRC migration after PTEN knockdown. MCM is known to contain a variety of growth factors and cytokines, most importantly TNF-α (24, 25). To simulate the tumor microenvironment, MCM (containing 992 pg/ml TNF-α as measured by enzyme-linked immunosorbent assay) was added to HCT116 and SW480 cells after *PTEN* siRNA or NTC transfection and scratch assays were performed. The addition of MCM to HCT116 (Figure 2A) and SW480 (Figure 2B) *PTEN* siRNA cells resulted in near complete closure of both cell lines by only 24 h after the initial scratch compared with our results with *PTEN* siRNA alone, which resulted in near complete closure of the wound by 48 h. The synergistic increase of migration that was observed after *PTEN* knockdown and the addition of MCM containing TNF-α suggests that factors produced by the tumor microenvironment act in concert to enhance CRC migration.

Stimulation by TNF-α increases CRC invasion after PTEN knockdown. Invasion is a critical step in EMT and is characterized by the loss of epithelial adhesion molecules, cell dissociation, and penetration through the basement membrane (26). To determine if an inflammatory microenvironment is a hallmark of increased invasion of SW480 and HCT116 cell lines after *PTEN* knockdown, a modified Boyden chamber invasion assay with thin-layer Matrigel-coated Transwell chambers was performed. Cells were transfected with *PTEN* siRNA or NTC, plated onto Matrigel-coated chambers and, after 72 h, fixed and stained with DAPI. There was a significant increase in the invasion of HCT116 and SW480 cells in chambers with MCM compared to control media (Figure 3A). There was also a significant increase when compared to serum free–media (data not shown). MCM further enhanced the invasion of metastatic HCT116 cells and increased the invasiveness of the non-metastatic SW480 cell line.

To further assess the role of TNF-α in the enhanced invasion noted above, a co-culture assay was performed with *PTEN* shRNA SW480 cells. Isotype control antibody or TNF-α blocking antibody was added to the bottom chamber: a significant decrease in invasion was observed when TNF-α blocking antibody was used (Figure 3B left).Western blot

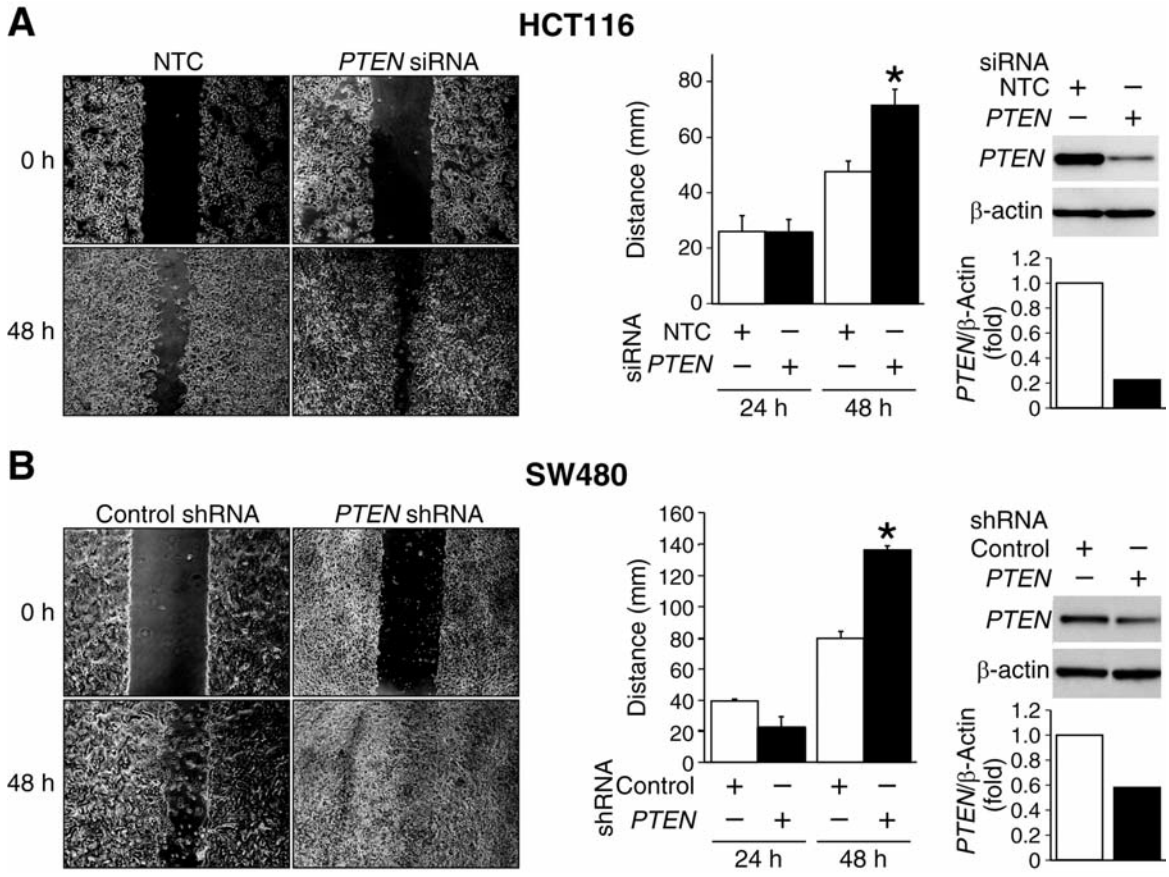

Figure 1. *PTEN knockdown increases migration of HCT116 and SW480 cells. A, PTEN siRNA and NTC transfected cell lines were grown to confluency, scratched, and photographed 0 and 48 h. A significant increase in migration was observed after PTEN knockdown in HCT116 cells (*p<0.05). Western blot analysis of protein expression demonstrated knockdown of PTEN in HCT116 cells. B, A significant increase in migration was observed after PTEN knockdown in SW480 cells (*p<0.05). Western blot analysis of protein expression demonstrated knockdown of PTEN in SW480 cells.*

analysis showed knockdown of *PTEN* in the SW480 stable cell line (Figure 3B right). The increased invasion of CRC cells toward TNF-α containing MCM further supports the importance of the tumor microenvironment in tumor invasion. These results, coupled with the significant decrease in invasion of CRC cells with TNF-α blocking antibody, suggest that TNF-α is a critical cytokine regulating the increased invasion noted with the addition of MCM.

Nuclear β-catenin is up-regulated after PTEN knockdown. Activation of the Wnt/β-catenin pathway and the subsequent nuclear accumulation of β-catenin is a common feature of CRC (27). The nuclear accumulation of β-catenin at the invasive front has also been associated with EMT (28, 29). To determine whether an increase in total β-catenin and nuclear β-catenin occurs after the targeted knockdown of *PTEN*, intestinal epithelial cell lines RIE-1, IEC-6, and HIE and the CRC cell line SW480 were analyzed. Cells were transfected with *PTEN* siRNA or NTC and cell lysates were analyzed by Western blot for nuclear and cytoplasmic β-catenin expression. *PTEN* knockdown increased β-catenin expression levels in IEC-6 and HIE cells with a slight increase noted in SW480 cells, suggesting a general regulation of β-catenin expression by *PTEN* (Figure 4A).

To detect whether β-catenin levels in the nucleus were increased, SW480 and RIE cells were transfected with *PTEN* or NTC siRNA and nuclear and cytosolic protein was isolated after 48 h. The knockdown of *PTEN* increased cytosolic as well as nuclear β-catenin expression levels as noted by Western blot analysis (Figure 4B). Lamin B, an integral protein of the nuclear envelope, and IκBα, located in the cytoplasm, were used as controls for the nuclear and cytosolic fractions, respectively. To assess whether the increased β-catenin altered targeted protein expression, cyclin D1 and c-Myc expression was determined. Inhibition of PI3K using wortmannin or LY294002 reduced c-Myc and cyclin D1 expression, and knockdown of *PTEN* increased the expression of these proteins in RIE-

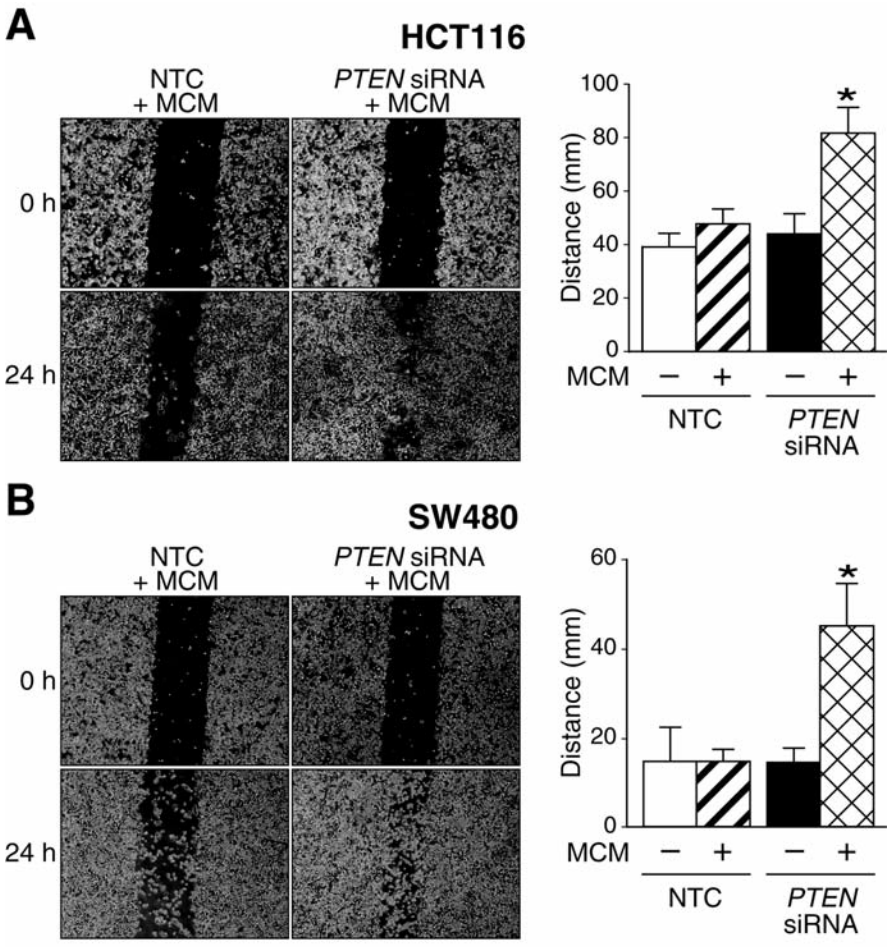

Figure 2. *MCM with TNF-α increases the migration of HCT116 and SW480 cells. A, PTEN siRNA and NTC transfected cell lines were grown to confluency, scratched and photographed at 0 and 24 h. At 0 h, MCM (125 μl) was added to PTEN siRNA HCT116 cells and NTC. MCM significantly increased the migration of PTEN siRNA HCT116 at 24 h (*p<0.05). B, At 0 h, MCM (200 μl) was added to cells PTEN siRNA SW480 cells and NTC. MCM significantly increased the rate of closure at 24 h (*p<0.05).*

1 cells; the silencing of *PTEN* also increased c Myc and cyclin D1 protein expression in IEC-6 and HIE cells (data not shown).

Epithelial markers are a consistent hallmark of EMT. For example, loss of E-cadherin is highly indicative of EMT (30). Because *PTEN* knockdown increased migration and invasion of two CRC cell lines, we hypothesized that these cells would demonstrate a decrease in epithelial characteristics. Previous reports have shown that transient knockdown with siRNA does not correlate with a change in epithelial phenotype, thus a *PTEN* non-inducible vector system was used to create SW480 and HCT116 stable transfectants. As shown in Figure 4C, *PTEN* shRNA SW480 cells lacked the epithelial characteristics of E-cadherin when compared to control cells; Western blot confirmed the down-regulation of E-cadherin and α-catenin and the up-regulation of phosphorylated-Akt(Ser 473), which confirmed a decrease in *PTEN* activity (Figure 4D). The down-regulation of E-cadherin and α-catenin was also noted in HCT 116 cells transfected with *PTEN* shRNA (Figure 4E). α-Catenin, an actin-binding protein, forms an important physical connection at the adherens junction that is present at adhesional complexes and attaches the microfilaments and associated proteins to cadherins *via* β-catenin (31). These findings are consistent with the cells undergoing a change from an epithelial to a mesenchymal phenotype after *PTEN* knockdown and further establishes a connection between EMT and the PI3K pathway in CRC.

Increased invasive phenotype noted in metastatic cells. The ability of cells to invade and disseminate is yet another characteristic of EMT (32). Anchorage-independent growth in soft agar is typically used to determine if cells will colonize *in vivo* (33). HCT116 and SW480 cells with *PTEN*

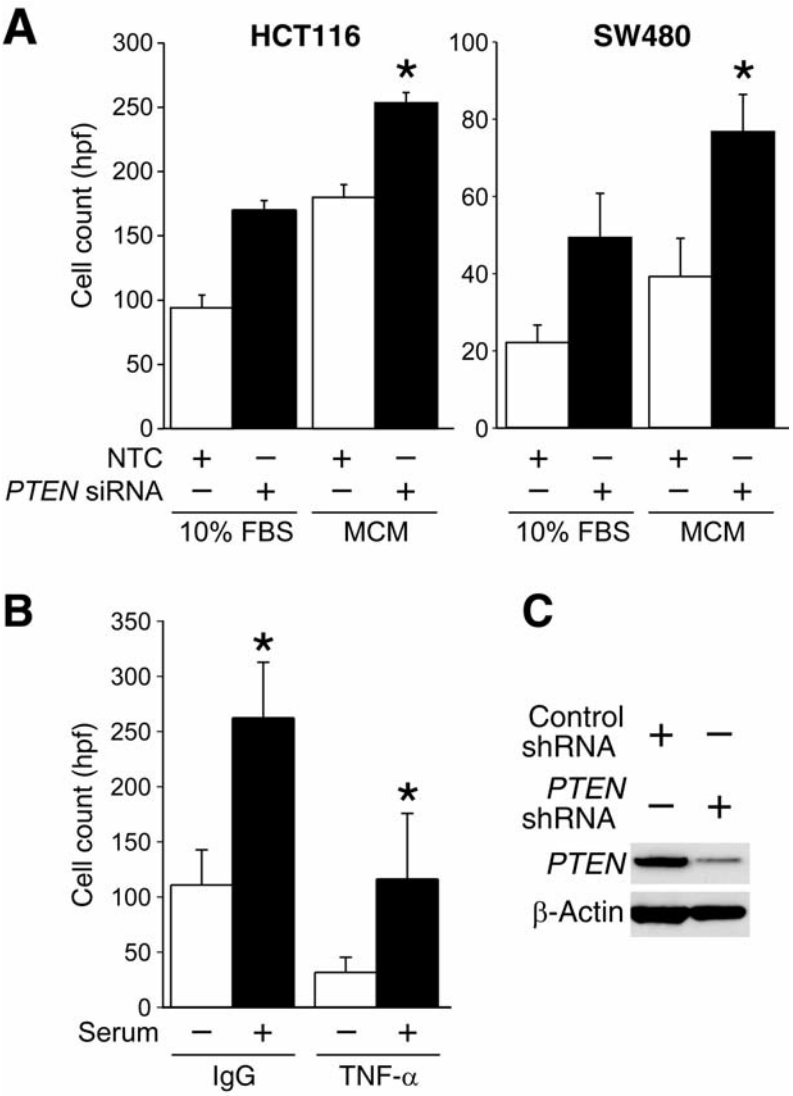

Figure 3. *MCM increases the invasion of HCT116 and SW480 cells transfected with PTEN siRNA and TNF-α antibody reduces invasion. A, A modified Boyden chamber invasion assay with thin-layered matrigel-coated Transwell chambers was performed with HCT116 and SW480 cell lines. Cells were transfected with PTEN siRNA or NTC then plated onto Matrigel coated chambers. MCM, control media (10% FBS), or serum-free media was used as the chemoattractant in the bottom well. MCM as a chemoattractant significantly increased invasion of both cell lines after the targeted inhibition of PTEN (*p<0.05). B, A modified Boyden chamber invasion assay with thin-layered Matrigel-coated chambers was performed with PTEN shRNA SW480 cells. RAW macrophages were co-cultured and SW480 cells and isotype control IgG or TNF-α antibody was added every 24 h to the control media (10% FBS), or serum-free media. C, Western blot analysis confirmed the down-regulation of PTEN in PTEN shRNA SW480 cells when compared with control; β-actin expression shows equal loading of protein.*

shRNA were plated into soft agar and analyzed after 2 weeks. When compared to controls, both *PTEN* shRNA cell lines demonstrated significantly more colony formation (Figure 5A).

To further evaluate the role of the PI3K/Akt pathway in migration, a metastatic cell line isolated from a liver metastasis after stable *PTEN* shRNA knockdown (*PTEN* shRNA mSW480 was used (15). A monolayer scratch assay was performed using this cell line and compared to control cells; increased migration of *PTEN* shRNA mSW480 cells was clearly noted compared to the non-metastatic control shRNA-transfected cells (Figure 5B; left and middle). These findings are consistent with our study using transient siRNA knockdown. Western blot analysis demonstrated effective knockdown of *PTEN* in the *PTEN* shRNA mSW480 cell line (Figure 5B; right). Increased invasion toward MCM was also noted using the *PTEN* shRNA mSW480 cells (Figure 5C). The increased migration and invasion that was observed in

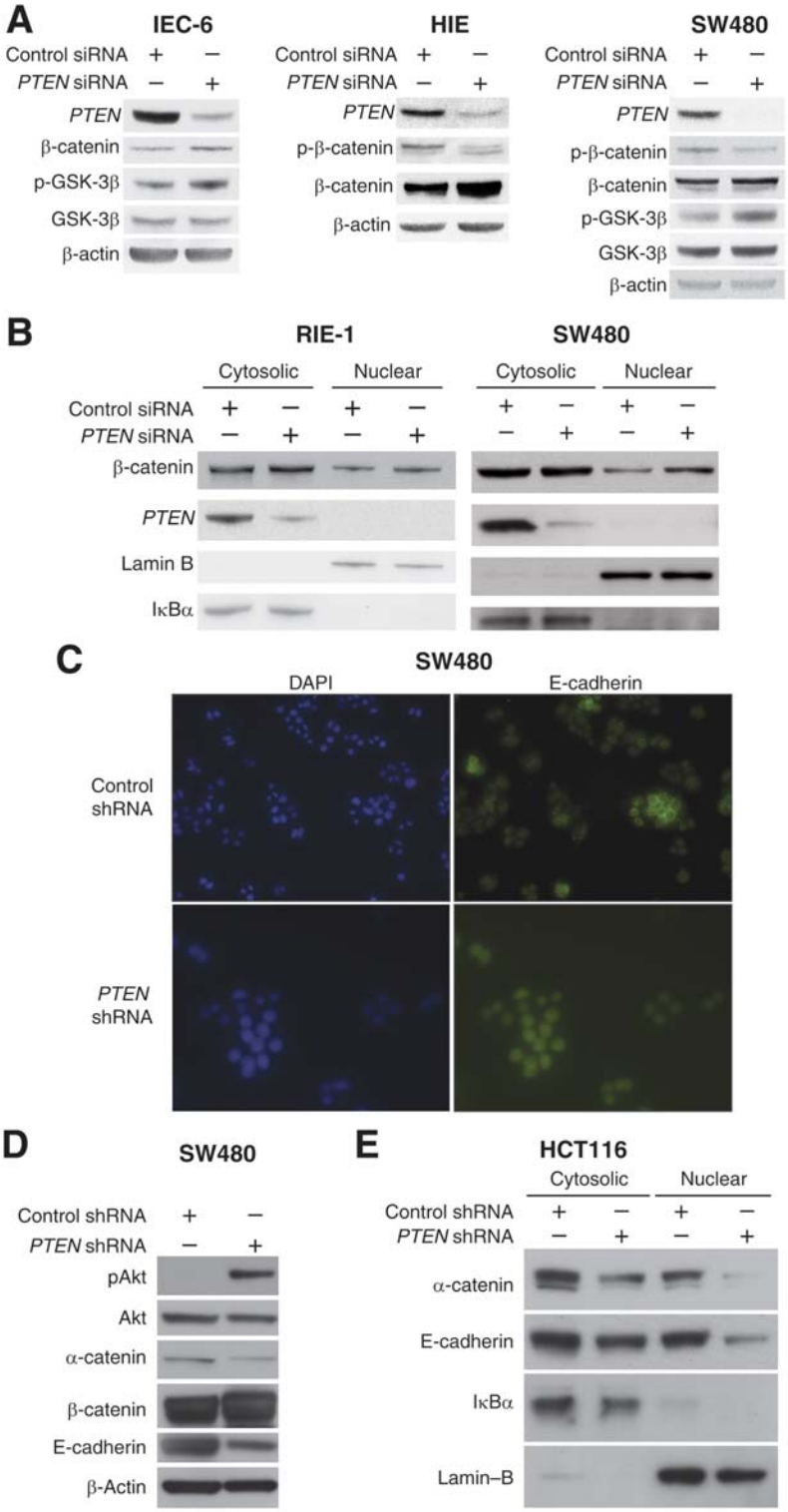

Figure 4. *PTEN knockdown increases nuclear β-catenin in RIE-1 and SW480 cell lines and decreases E-cadherin in SW480 and HCT116 cells. A, IEC-6, HIE, RIE-1, and SW480 cell lines were transfected with PTEN siRNA and NTC and cell lysates were analyzed by Western blot analysis for total protein levels of β-catenin. There was increased total β-catenin in the intestinal cell lines IEC-6 and HIE. B, Western blot analysis demonstrated increased nuclear β-catenin with PTEN knockdown in SW480 cells and RIE-1 cells. C, Immunofluorescence demonstrated a decreased amount of E-cadherin junctional staining in SW480 PTEN shRNA cells when compared with control. D-E, Western blot analysis confirmed the down-regulation of E-cadherin and α-catenin in SW480 and HCT116 PTEN shRNA cells when compared with control.*

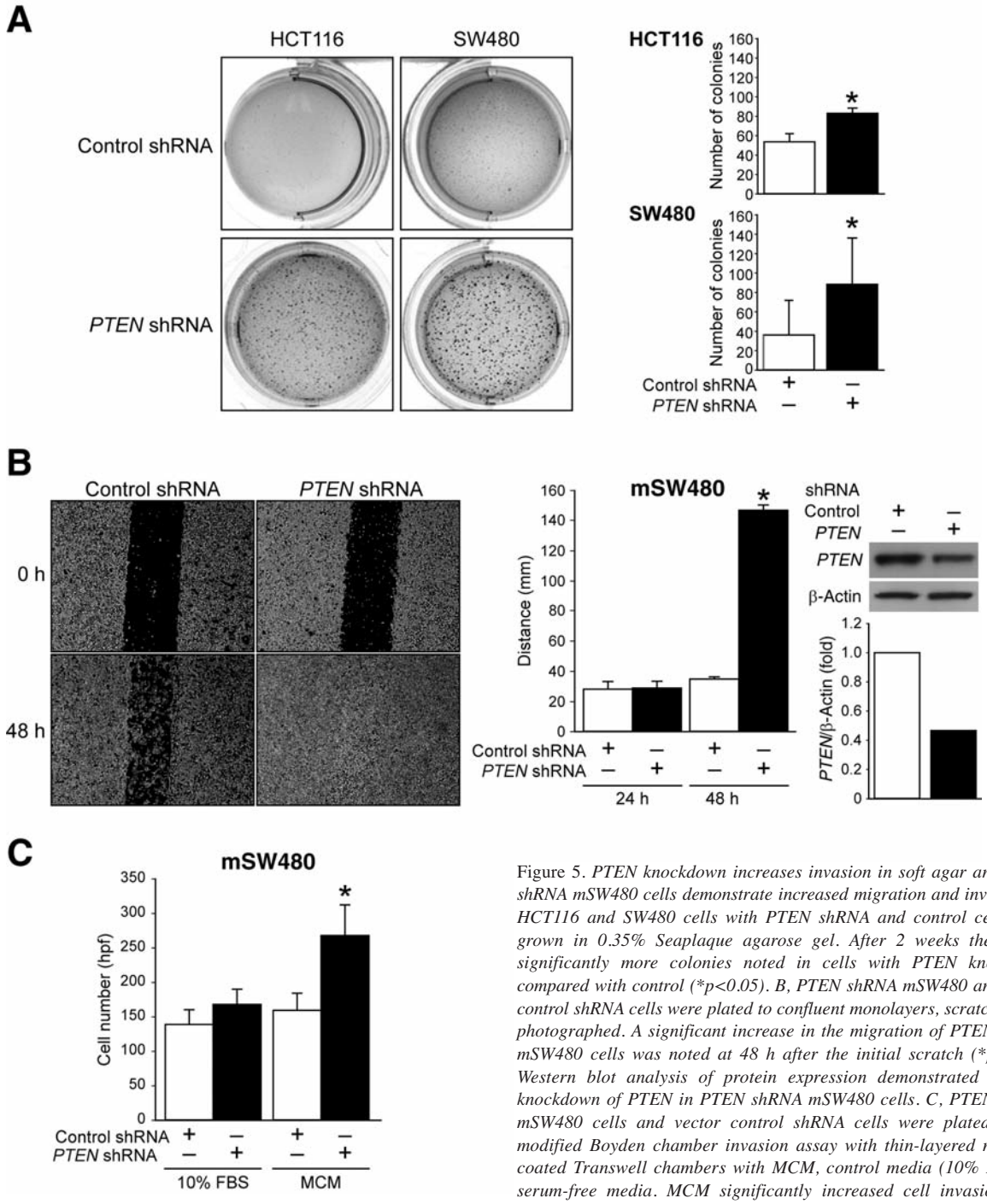

Figure 5. *PTEN knockdown increases invasion in soft agar and PTEN shRNA mSW480 cells demonstrate increased migration and invasion. A, HCT116 and SW480 cells with PTEN shRNA and control cells were grown in 0.35% Seaplaque agarose gel. After 2 weeks there were significantly more colonies noted in cells with PTEN knockdown compared with control (*p<0.05). B, PTEN shRNA mSW480 and vector control shRNA cells were plated to confluent monolayers, scratched, and photographed. A significant increase in the migration of PTEN shRNA mSW480 cells was noted at 48 h after the initial scratch (*p<0.05). Western blot analysis of protein expression demonstrated effective knockdown of PTEN in PTEN shRNA mSW480 cells. C, PTEN shRNA mSW480 cells and vector control shRNA cells were plated onto a modified Boyden chamber invasion assay with thin-layered matrigel-coated Transwell chambers with MCM, control media (10% FBS), or serum-free media. MCM significantly increased cell invasion when compared to control media (*p<0.05).*

the metastatic *PTEN* shRNA cell line with the addition of MCM suggests that the tumor microenvironment plays a role in metastatic tumor formation as well. Taken together, these experiments corroborate our findings that *PTEN* is involved in EMT-induced metastasis.

Discussion

To our knowledge, this is the first report demonstrating that the knockdown of *PTEN* leads to the induction of EMT in CRC cells. The ability of CRC cells to migrate toward the

basement membrane and then invade through its dense connective tissue matrix are key steps in the dissemination of these cells to distant sites (34). Our results demonstrate that the observed increases in migration and invasion are mediated in a PI3K/Akt-dependent manner. Furthermore, we show that through *PTEN* down-regulation and a concomitant decrease in epithelial markers, the PI3K pathway mediates EMT in CRC.

Mutations of the PI3K pathway are a common occurrence in various types of cancer (10, 35). *PTEN* is an important regulator of the cell cycle, most notably cell division, and mutations of the *PTEN* gene are found in high frequency among several cancer types (36, 37). Loss of *PTEN* appears to influence metastasis by promoting cell proliferation while suppressing apoptosis at the metastatic site (38). Decreased *PTEN* expression occurs with relatively high frequency in metastatic CRC (15, 39, 40). Recently, *PTEN* was found to be weakly expressed in primary CRCs in patients with liver metastasis; decreased *PTEN* expression was also noted with advanced stage disease and lymph node metastasis (39). Rychahou *et al*. (15) found that Akt2 overexpression in wild-type *PTEN* CRC cells led to the formation of micrometastases. However, to observe sustained metastases, *PTEN* loss was required. The down-regulation of *PTEN* was also observed in advanced stage hepatocellular carcinoma, prostate carcinoma, and melanoma (41, 42). Taken together, this suggests that *PTEN* suppression or loss in advanced stage disease contributes to tumor invasion and metastasis.

Alterations of the PI3K pathway have been associated with EMT in a variety of cancer types and activation of the PI3K effector protein Akt has been observed in squamous cell, renal, and bladder carcinomas (43-45). Transfection with constitutively active Akt in squamous cell carcinoma lines resulted in reduced cell cell adhesion, increased motility, and increased invasiveness *in vivo* (43). Activation of Akt in rat kidney epithelial cells was found to be important for transforming growth factor (TGF)-β1-induced EMT both *in vitro* and *in vivo* which suggests that Akt may act as an important downstream mediator of TGF-β1 (44). In bladder cancer cell lines, N-cadherin expression was found to contribute to invasion by increasing phospho-Akt levels and decreasing E-cadherin expression (45). These studies suggest that therapeutic manipulation of the PI3K pathway may control tumor cell invasion and metastasis. Currently, little is known regarding the relationship of EMT to CRC and the PI3K pathway. Previously, we showed that induction of oncogenic KRAS in CRC cells resulted in EMT; treatment with the PI3K inhibitor, LY294002, produced a spindle cell morphology, reduced expression of E-cadherin, and increased invasiveness of CRC cells, suggesting that inhibition of PI3K may enhance EMT in CRC (46). Recently, a correlation was reported between overexpression of phosphatase of regenerating liver 3, an upstream regulator of *PTEN*, and the downregulation of *PTEN* which resulted in changes consistent with EMT (37). In normal cells, *PTEN* modulates migration of mesoderm cells in the chick embryo by controlling EMT and the directional motility (47).

Macrophages are a key source of TNF-α production which is thought to promote tumor invasion and metastasis (48, 49). This hypothesis is further supported by our data showing increased invasion and metastasis with the addition of TNF-α containing MCM and, importantly, a dramatic decrease in invasion after the addition of TNF-α neutralizing antibody. Recently, TNF-α was found to stabilize Snail, a transcription factor and critical regulator of EMT in breast cancer (50). The TNF-α-induced stabilization of Snail resulted in inflammation-induced EMT, migration, invasion, and metastasis of breast cancer cells. An increase in TNF-α also promotes tumor growth and vascularity in mouse melanoma, lung cancer, and mammary tumors (51). Clinical observations have shown an association between TNF-α expression and Dukes' stages in CRC (52, 53). Furthermore, TNF-α stimulation represses E-cadherin and augments EMT induced invasion of renal cancer cells. These findings support the important role of inflammatory mediators, specifically TNF-α, in the tumor microenvironment.

The subsequent nuclear accumulation of β-catenin, which is responsible for the activation of Wnt genes, and loss of function *APC* mutations are found in almost all cases of malignant CRC transformation. The nuclear accumulation of β-catenin and the lack of nuclear β-catenin in the tumor center has provided insight into the reversible changes that occur at the invasive front of CRCs (54). Nuclear accumulation of β-catenin, occurring at the invasive front, is an important factor in EMT, and β-catenin regulation by Wnt signaling activation is implicated in the tumor microenvironment. Recently, it was noted that TNF-α from murine macrophages promoted Wnt/β-catenin signaling through inhibition of GSK3β in gastric tumor cells, yet providing another mechanistic link between inflammation and β-catenin signaling (55). Our results are consistent with the increase in nuclear β-catenin that is observed in the invasive front of CRCs (56). We noted an increase in the nuclear accumulation of β-catenin after *PTEN* knockdown in normal and neoplastic epithelial-derived cell lines. The nuclear accumulation of β-catenin at the invasive front of CRCs is an indication of EMT and the up-regulation of nuclear β-catenin after *PTEN* knockdown suggests that *PTEN* plays an integral role in EMT. Our data suggest that β-catenin and its mediated gene transcription are regulated by *PTEN*, which may represent a possible mechanism by which *PTEN* enhances intestinal cell differentiation and suppresses CRC progression.

In conclusion, we show that knockdown of *PTEN* increases the migration and invasion of CRC cells which is associated with cellular changes consistent with EMT. These

findings further corroborate recent *in vivo* and clinical studies demonstrating a correlation between *PTEN* reduction or loss and late stage cancer (15, 39, 40). The combination of *PTEN* knockdown and MCM containing TNF-α further enhanced CRC cell migration and invasion, indicating that factors from the surrounding inflammatory tumor microenvironment act in concert with genetic alterations to stimulate CRC progression.

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References

1 Baker CV: The evolution and elaboration of vertebrate neural crest cells. Curr Opin Genet Dev *18*: 536-543, 2008.

2 Diep CB, Kleivi K, Ribeiro FR, Teixeira MR, Lindgjaerde OC and Lothe RA: The order of genetic events associated with colorectal cancer progression inferred from meta-analysis of copy number changes. Genes Chromosomes Cancer *45*: 31-41, 2006.

3 Brabletz T, Jung A, Spaderna S, Hlubek F and Kirchner T: Opinion: migrating cancer stem cells – an integrated concept of malignant tumour progression. Nat Rev Cancer *5*: 744-749, 2005.

4 Jeanes A, Gottardi CJ and Yap AS: Cadherins and cancer: how does cadherin dysfunction promote tumor progression? Oncogene *27*: 6920-6929, 2008.

5 Suzuki H, Masuda N, Shimura T, Araki K, Kobayashi T, Tsutsumi S, Asao T and Kuwano H: Nuclear beta-catenin expression at the invasive front and in the vessels predicts liver metastasis in colorectal carcinoma. Anticancer Res *28*: 1821-1830, 2008.

6 Locksley RM, Killeen N and Lenardo MJ: The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell *104*: 487-501, 2001.

7 Tsianos EV and Katsanos K: Do we really understand what the immunological disturbances in inflammatory bowel disease mean? World J Gastroenterol *15*: 521-525, 2009.

8 Christofori G: New signals from the invasive front. Nature *441*: 444-450, 2006.

9 Rychahou PG, Jackson LN, Silva SR, Rajaraman S and Evers BM: Targeted molecular therapy of the PI3K pathway: therapeutic significance of PI3K subunit targeting in colorectal carcinoma. Ann Surg *243*: 833-842; discussion 843-834, 2006.

10 Bellacosa A, de Feo D, Godwin AK, Bell DW, Cheng JQ, Altomare DA, Wan M, Dubeau L, Scambia G, Masciullo V, Ferrandina G, Benedetti Panici P, Mancuso S, Neri G and Testa JR: Molecular alterations of the *AKT2* oncogene in ovarian and breast carcinomas. Int J Cancer *64*: 280-285, 1995.

11 Cheng JQ, Ruggeri B, Klein WM, Sonoda G, Altomare DA, Watson DK and Testa JR: Amplification of *AKT2* in human pancreatic cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA. Proc Natl Acad Sci USA *93*: 3636-3641, 1996.

12 Suzuki A, de la Pompa JL, Stambolic V, Elia AJ, Sasaki T, del Barco Barrantes I, Ho A, Wakeham A, Itie A, Khoo W, Fukumoto M and Mak TW: High cancer susceptibility and embryonic lethality associated with mutation of the *PTEN* tumor suppressor gene in mice. Curr Biol *8*: 1169-1178, 1998.

13 Di Cristofano A, Pesce B, Cordon-Cardo C and Pandolfi PP: *PTEN* is essential for embryonic development and tumour suppression. Nat Genet *19*: 348-355, 1998.

14 Podsypanina K, Ellenson LH, Nemes A, Gu J, Tamura M, Yamada KM, Cordon-Cardo C, Catoretti G, Fisher PE and Parsons R: Mutation of *PTEN*/Mmac1 in mice causes neoplasia in multiple organ systems. Proc Natl Acad Sci USA *96*: 1563-1568, 1999.

15 Rychahou PG, Kang J, Gulhati P, Doan HQ, Chen LA, Xiao SY, Chung DH and Evers BM: Akt2 overexpression plays a critical role in the establishment of colorectal cancer metastasis. Proc Natl Acad Sci USA *105*: 20315-20320, 2008.

16 Nassif NT, Lobo GP, Wu X, Henderson CJ, Morrison CD, Eng C, Jalaludin B and Segelov E: *PTEN* mutations are common in sporadic microsatellite stable colorectal cancer. Oncogene *23*: 617-628, 2004.

17 Abubaker J, Bavi P, Al-Harbi S, Ibrahim M, Siraj AK, Al-Sanea N, Abduljabbar A, Ashari LH, Alhomoud S, Al-Dayel F, Uddin S and Al-Kuraya KS: Clinicopathological analysis of colorectal cancers with *PIK3CA* mutations in Middle Eastern population. Oncogene *27*: 3539-3545, 2008.

18 Kim S, Domon-Dell C, Kang J, Chung DH, Freund JN and Evers BM: Down-regulation of the tumor suppressor *PTEN* by the tumor necrosis factor-alpha/nuclear factor-kappaB (NF-kappaB)-inducing kinase/NF-kappaB pathway is linked to a default IkappaB-alpha autoregulatory loop. J Biol Chem *279*: 4285-4291, 2004.

19 Kumar S and Weaver VM: Mechanics, malignancy, and metastasis: the force journey of a tumor cell. Cancer Metastasis Rev *28*: 113-127, 2009.

20 Mbeunkui F and Johann DJ Jr: Cancer and the tumor microenvironment: a review of an essential relationship. Cancer Chemother Pharmacol *63*: 571-582, 2009.

21 Jackson LN, Li J, Chen LA, Townsend CM and Evers BM: Overexpression of wild-type PKD2 leads to increased proliferation and invasion of BON endocrine cells. Biochem Biophys Res Commun *348*: 945-949, 2006.

22 Etienne-Manneville S: Polarity proteins in migration and invasion. Oncogene *27*: 6970-6980, 2008.

23 Rajput A, Dominguez San Martin I, Rose R, Beko A, Levea C, Sharratt E, Mazurchuk R, Hoffman RM, Brattain MG and Wang J: Characterization of HCT116 human colon cancer cells in an orthotopic model. J Surg Res *147*: 276-281, 2008.

24 Granger GA, Shacks SJ, Williams TW and Kolb WP: Lymphocyte *in vitro* cytotoxicity: specific release of lymphotoxin-like materials from tuberculin-sensitive lymphoid cells. Nature *221*: 1155-1157, 1969.

25 Carswell EA, Old LJ, Kassel RL, Green S, Fiore N and Williamson B: An endotoxin-induced serum factor that causes necrosis of tumors. Proc Natl Acad Sci USA *72*: 3666-3670, 1975.

26 Yilmaz M and Christofori G: EMT, the cytoskeleton, and cancer cell invasion. Cancer Metastasis Rev *28*: 15-33, 2009.

27 Gavert N and Ben-Ze'ev A: beta-Catenin signaling in biological control and cancer. J Cell Biochem *102*: 820-828, 2007.

28 Huang D and Du X: Crosstalk between tumor cells and microenvironment *via* Wnt pathway in colorectal cancer dissemination. World J Gastroenterol *14*: 1823-1827, 2008.
29 Brabletz S, Schmalhofer O and Brabletz T: Gastrointestinal stem cells in development and cancer. J Pathol *217*: 307-317, 2009.
30 Thiery JP: Epithelial mesenchymal transitions in tumour progression. Nat Rev Cancer *2*: 442-454, 2002.
31 Dejana E, Orsenigo F and Lampugnani MG: The role of adherens junctions and VE cadherin in the control of vascular permeability. J Cell Sci *121*: 2115-2122, 2008.
32 Guarino M, Rubino B and Ballabio G: The role of epithelial mesenchymal transition in cancer pathology. Pathology *39*: 305-318, 2007.
33 Fan D, Morgan LR, Schneider C, Blank H and Fan S: Cooperative evaluation of human tumor chemosensitivity in the soft-agar assay and its clinical correlations. J Cancer Res Clin Oncol *109*: 23-28, 1985.
34 Brooks SA, Lomax-Browne HJ, Carter TM, Kinch CE and Hall DM: Molecular interactions in cancer cell metastasis. Acta Histochem 2009.
35 Brugge J, Hung MC and Mills GB: A new mutational AKTivation in the PI3K pathway. Cancer Cell *12*: 104-107, 2007.
36 Keniry M and Parsons R: The role of *PTEN* signaling perturbations in cancer and in targeted therapy. Oncogene *27*: 5477-5485, 2008.
37 Wang H, Quah SY, Dong JM, Manser E, Tang JP and Zeng Q: PRL-3 down-regulates *PTEN* expression and signals through PI3K to promote epithelial-mesenchymal transition. Cancer Res *67*: 2922-2926, 2007.
38 Simpson L and Parsons R: *PTEN*: life as a tumor suppressor. Exp Cell Res *264*: 29-41, 2001.
39 Sawai H, Yasuda A, Ochi N, Ma J, Matsuo Y, Wakasugi T, Takahashi H, Funahashi H, Sato M and Takeyama H: Loss of *PTEN* expression is associated with colorectal cancer liver metastasis and poor patient survival. BMC Gastroenterol *8*: 56, 2008.
40 Parsons DW, Wang TL, Samuels Y, Bardelli A, Cummins JM, DeLong L, Silliman N, Ptak J, Szabo S, Willson JK, Markowitz S, Kinzler KW, Vogelstein B, Lengauer C and Velculescu VE: Colorectal cancer: mutations in a signalling pathway. Nature *436*: 792, 2005.
41 Hu TH, Wang CC, Huang CC, Chen CL, Hung CH, Chen CH, Wang JH, Lu SN, Lee CM, Changchien CS and Tai MH: Down-regulation of tumor suppressor gene *PTEN*, overexpression of p53, plus high proliferating cell nuclear antigen index predict poor patient outcome of hepatocellular carcinoma after resection. Oncol Rep *18*: 1417-1426, 2007.
42 Maehama T and Dixon JE: *PTEN*: a tumour suppressor that functions as a phospholipid phosphatase. Trends Cell Biol *9*: 125-128, 1999.
43 Grille SJ, Bellacosa A, Upson J, Klein-Szanto AJ, van Roy F, Lee-Kwon W, Donowitz M, Tsichlis PN and Larue L: The protein kinase Akt induces epithelial mesenchymal transition and promotes enhanced motility and invasiveness of squamous cell carcinoma lines. Cancer Res *63*: 2172-2178, 2003.
44 Kattla JJ, Carew RM, Heljic M, Godson C and Brazil DP: Protein kinase B/Akt activity is involved in renal TGF-beta1-driven epithelial mesenchymal transition *in vitro* and *in vivo*. Am J Physiol Renal Physiol *295*: F215-225, 2008.
45 Wallerand H, Cai Y, Wainberg ZA, Garraway I, Lascombe I, Nicolle G, Thiery JP, Bittard H, Radvanyi F and Reiter RR: Phospho-Akt pathway activation and inhibition depends on N-cadherin or phospho-EGFR expression in invasive human bladder cancer cell lines. Urol Oncol (Epub ahead of print).
46 Shao J, Evers BM and Sheng H: Roles of phosphatidylinositol 3'-kinase and mammalian target of rapamycin/p70 ribosomal protein S6 kinase in K-Ras-mediated transformation of intestinal epithelial cells. Cancer Res *64*: 229-235, 2004.
47 Leslie NR, Yang X, Downes CP and Weijer CJ: PtdIns(3,4,5) P(3)-dependent and -independent roles for *PTEN* in the control of cell migration. Curr Biol *17*: 115-125, 2007.
48 Karin M and Greten FR: NF-kappaB: linking inflammation and immunity to cancer development and progression. Nat Rev Immunol *5*: 749-759, 2005.
49 Karin M: Nuclear factor-kappaB in cancer development and progression. Nature *441*: 431-436, 2006.
50 Wu Y, Deng J, Rychahou PG, Qiu S, Evers BM and Zhou BP: Stabilization of snail by NF-kappaB is required for inflammation-induced cell migration and invasion. Cancer Cell *15*: 416-428, 2009.
51 Li B, Vincent A, Cates J, Brantley-Sieders DM, Polk DB and Young PP: Low levels of tumor necrosis factor alpha increase tumor growth by inducing an endothelial phenotype of monocytes recruited to the tumor site. Cancer Res *69*: 338-348, 2009.
52 Csiszar A, Szentes T, Haraszti B, Zou W, Emilie D, Petranyi G and Pocsik E: Characterisation of cytokine mRNA expression in tumour-infiltrating mononuclear cells and tumour cells freshly isolated from human colorectal carcinomas. Eur Cytokine Netw *12*: 87-96, 2001.
53 Chuang MJ, Sun KH, Tang SJ, Deng MW, Wu YH, Sung JS, Cha TL and Sun GH: Tumor-derived tumor necrosis factor-alpha promotes progression and epithelial mesenchymal transition in renal cell carcinoma cells. Cancer Sci *99*: 905-913, 2008.
54 Brabletz T, Jung A, Dag S, Reu S and Kirchner T: β-Catenin induces invasive growth by activating matrix metalloproteinases in colorectal carcinoma. Verh Dtsch Ges Pathol *84*: 175-181, 2000.
55 Oguma K, Oshima H, Aoki M, Uchio R, Naka K, Nakamura S, Hirao A, Saya H, Taketo MM and Oshima M: Activated macrophages promote Wnt signalling through tumour necrosis factor-alpha in gastric tumour cells. EMBO J *27*: 1671-1681, 2008.
56 Brabletz T, Jung A, Reu S, Porzner M, Hlubek F, Kunz-Schughart LA, Knuechel R and Kirchner T: Variable beta-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. Proc Natl Acad Sci USA *98*: 10356-10361, 2001.

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