The lipid phosphatase activity of PTEN is critical for stabilizing intercellular junctions and reverting invasiveness

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To analyze the implication of PTEN in the control of tumor cell invasiveness, the canine kidney epithelial cell lines MDCKras-f and MDCKts-src, expressing activated Ras and a temperature-sensitive v-Src tyrosine kinase, respectively, were transfected with PTEN expression vectors. Likewise, the human PTEN-defective glioblastoma cell lines U87MG and U373MG, the melanoma cell line FM-45, and the prostate carcinoma cell line PC-3 were transfected. We demonstrate that ectopic expression of wild-type PTEN in MDCKts-src cells, but not expression of PTEN mutants deficient in either the lipid or both the lipid and protein phosphatase activities, reverted the morphological transformation, induced cell–cell aggregation, and suppressed the invasive phenotype in an E-cadherin-dependent manner. In contrast, overexpression of wild-type PTEN did not counteract Ras-induced invasiveness of MDCKras-f cells expressing low levels of E-cadherin. PTEN effects were not associated with marked changes in accumulation or phosphorylation levels of E-cadherin and associated catenins. Wild-type, but not mutant, PTEN also reverted the invasive phenotype of U87MG, U373MG, PC-3, and FM-45 cells. Interestingly, PTEN effects were mimicked by N-cadherin-neutralizing antibody in the glioblastoma cell lines. Our data confirm the differential activities of E- and N-cadherin on invasiveness and suggest that the lipid phosphatase activity of PTEN exerts a critical role in stabilizing junctional complexes and restraining invasiveness.

Introduction

Neoplastic progression results from the dysregulation of a series of proto-oncogenes and tumor suppressor genes. In this context, the recently discovered PTEN (phosphatase and tensin homologue deleted from chromosome 10) tumor suppressor gene has been found to be defective in a large number of human cancers, including glioblastomas, endometrial, prostate, and renal cancers, as well as melanomas (Cantley and Neel, 1999). The hallmark of malignancy is the acquisition of the invasive phenotype. This process involves breakdown of cell–cell junctions, increased motility of tumor cells, and focal proteolysis of the extracellular matrix. Among junctional complexes, the E-cadherin–catenin system exerts a critical role in the control of carcinogenesis. These complexes are subjected to inactivation by multiple mechanisms, including both genetic and epigenetic events. Accordingly, activation of the Src tyrosine kinase switches E-cadherin junctions from a strong to a weak state and induces the invasive phenotype in epithelial canine kidney MDCKts-src cells, transformed by a temperature-sensitive v-Src tyrosine kinase (pp60<sup>v-src</sup>) (Behrens et al., 1989, 1993; Takeda et al., 1995).

Several lines of evidence suggest that PTEN might participate in regulating cell–cell junctions and tumor cell invasion. The amino terminus of PTEN shows homology with tensin, a protein interacting with actin at focal adhesions, and its carboxy terminus encodes a potential PDZ binding motif. Proteins with PDZ domains, such as ZO-1, direct the assembly of multiprotein complexes, often at membrane–cytoskeletal interfaces. Furthermore, characterization of PTEN activity revealed that it is a phosphatase that acts on both phosphorylserine residues and the D3 position of phosphatidylinositol-3,4,5-trisphosphate, the product of phosphatidylinositol 3-OH kinase (PI3-kinase)* (Furnari et al., 1998; Myers et al., 1998). The PTEN mutations so far identified affect either the phosphatidylinositol phosphatase, *Abbreviations used in this paper: GFP, green fluorescent protein; PI3-kinase, phosphatidylinositol 3-OH kinase.

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or both phosphatidylinositol and protein phosphatase activities. We have previously demonstrated an involvement of the PI3-kinase signaling pathway in inducing the invasive phenotype of MDCKts-src cells (Kotelevets et al., 1998). It has also been reported that PTEN selectively dephosphorylates focal adhesion kinase, and it inhibits the motility of fibroblasts and the invasiveness of U87MG glioma cells (Tamura et al., 1998, 1999).

To delineate the involvement of PTEN in the control of tumor cell scattering and invasiveness, and to investigate the cross-talk between PTEN and the Ras and Src oncogenic pathways on the one hand, and cadherin junctional complexes on the other, we transfected MDCKras-f and MDCKts-src cells and several human, PTEN-defective tumor cell lines with vectors expressing either wild-type PTEN or PTEN mutants deficient in either the phosphoinositide phosphatase or both the protein and phosphoinositide phosphatase activities. We evaluated morphological conversion, invasion into type I collagen, cell aggregation, and the expression, composition, phosphorylation level, and subcellular localization of cadherin-containing junctional complexes.

**Results and discussion**

PTEN reverses the morphological conversion induced by pp60src

To investigate the cross-talk between the PTEN and the Src and Ras oncogenic pathways, we expressed wild-type PTEN and lipid phosphatase– or phosphatase-deficient PTEN mutants in MDCK cells, transformed by either a temperature-sensitive mutant of v-Src (MDCKts-src cells) (Behrens et al., 1989, 1993; Takeda et al., 1995) or v-Ras (MDCKras-f) (Vlemingckx et al., 1991). It was previously demonstrated that PTEN mutation Δ237–239 results in a 10-fold decrease in phosphatase activity toward phosphatidylinositol (1,3,4,5)P4 without affecting protein phosphatase activity, whereas PTEN mutations Δ55–70 and C124A affect both the lipid and protein phosphatase activities of PTEN (Furnari et al., 1998; Tamura et al., 1998). PTEN expression in transformants was assessed by immunoblotting using anti-tag antibodies (anti-Xpress or anti-HA) as well as anti-PTEN antibody (Fig. 1A). In MDCKts-src cells, PTEN overexpression affected neither the phosphorylation of focal

![Figure 1](https://cjb.rupress.org/abstract/content/155/7/1130/F1.large.jpg)
adhesion kinase nor the MAPK and PI3-kinase activities (unpublished data). In contrast, the ectopic expression of wild-type PTEN, but not PTEN mutants, reduced the phosphorylation of Akt, a downstream target of PI3-kinase, as shown in Fig. 1 B and in line with previous reports for other cell lines (Myers et al., 1998).

The possible cross-talk between PTEN and Src signaling pathways was first assessed at the morphological level. At the nonpermissive temperature for Src activity (40°C), both parental MDCKts-src and MDCKts-srcPTENwt cells grew as small clusters of adherent cells with few membrane ruffles or lamellipodia. Switching the MDCKts-src cells to the permissive temperature induced a fibroblast-like morphology. The first effects were apparent 4 to 6 h after the temperature shift, as cells formed prominent lamellipodia and began to acquire the fibroblastic morphotype. 14 h after the shift, colonies of cells were almost completely dissociated (Fig. 1 C). Similar results were obtained in MDCKts-srcPTENΔ55–70 expressing inactive PTEN (Fig. 1 C). In contrast, after a 14-h incubation at 35°C, MDCKts-srcPTENwt retained a polygonal epithelioid morphotype, and cell clusters remained largely intact. In addition, wild-type PTEN delayed the scattering of MDCKts-srcPTENwt cells as compared with the parental cell line in wound healing assays (unpublished data).

Inhibition of pp60src-induced invasiveness by wild-type PTEN
Because PTEN interfered with Src-induced cell scattering, we further investigated the invasive properties of parental and PTEN-transfected MDCKts-src cells in type I collagen gels. As shown in Fig. 2 A, the parental MDCKts-src cells and those derivatives that did not express exogenous PTEN, MDCKts-src11 and MDCKts-src31, became invasive after a temperature shift to 35°C. In contrast, the six MDCKts-srcPTENwt clones that overexpressed wild-type PTEN remained noninvasive at the permissive temperature (Fig. 2 A). Overexpression of PTEN mutants in MDCKts-src cells did not revert the invasive phenotype induced by Src at 35°C, demonstrating the critical role of the PTEN lipid phosphatase activity in the control of the invasive phenotype. Besides the lipid phosphatase activity of PTEN, invasion suppression clearly involved the E-cadherin system, as inactivation of E-cadherin by DECMA-1 mAb induced the invasive phenotype in MDCKts-srcPTENwt, both at the restrictive and the permissive temperature for Src activity (Fig. 2 A). Likewise, PTEN could not counteract the invasive phenotype of MDCKras-f cells that poorly expressed E-cadherins (Vleminkx et al., 1991).

Stabilization of E-cadherin junctional complexes by PTEN
To gain further insight into the mechanisms involved in the PTEN-mediated reversal of Src-induced cell scatter-
ing and invasiveness, we used the fast aggregation assay to test whether PTEN activity affects the E-cadherin–dependent adhesion system. A prominent feature of suspended parental MDCKts-src cells is the inability to aggregate at the permissive temperature, whereas cell aggregates form readily in an E-cadherin–dependent manner at the restrictive temperature (Fig. 2 B, left). In contrast, wild-type PTEN but not mutant PTEN transfectants (Δ237–239/71, Δ55–70/3, and C124A/11) formed aggregates at the permissive temperature for Src activity (35°C). All aggregations observed here were impaired by the E-cadherin neutralizing antibody DECMA-1 (Fig. 2 B). These results confirmed the critical role of the lipid phosphatase activity of PTEN in stabilizing E-cadherin junctions and reverting cell scattering and invasiveness.

Effect of PTEN on E-cadherin–catenin complexes

We next compared the expression, phosphorylation level, and cellular localization of E-cadherin and associated proteins in MDCKts-src and MDCKts-srcPTENwt cells before and after a shift to the permissive temperature for Src activity. At the restrictive temperature, the E-cadherin signal was concentrated at cell–cell contacts (Fig. 3 A). Time-course studies showed that Src activation in MDCKts-src cells resulted in progressive reduction of junctional complexes, delocalization of E-cadherin from the cell surface, and loss of epithelioid morphotype (Fig. 3, B and C). In contrast, MDCKts-srcPTENwt cells preserved cell–cell junctions and E-cadherin localization for longer times (Fig. 3, D–F). The same was observed for α-catenin, β-catenin, and p120ctn (unpublished data).

Because E-cadherin activity crucially depends on its association with several cytoplasmic proteins, we analyzed the total amount of E-cadherin, and associated α-catenin, β-catenin, and p120ctn. We found that any of these expression levels were similar at both nonpermissive and permissive temperatures and also not significantly altered by PTEN expression (Fig. 4 A). In addition, Src-induced tyrosine phosphorylation of E-cadherin and associated catenins was comparable in MDCKts-src cells and their PTEN derivatives (Fig. 4 B). Thus, in the presence of wild-type PTEN, Src-induced tyrosine phosphorylation of E-cadherin junctional complexes is not sufficient to promote the disruption of adherens junctions.
Reversion of invasiveness of PTEN-defective human cell lines after restoration of PTEN expression

To further extend the effects of PTEN on the stabilization of junctional complexes and the reversion of the invasive phenotype, we restored PTEN expression in human cell lines known to be PTEN defective: glioblastoma cell lines U87MG and U373MG, melanoma cell line FM-45, and prostate carcinoma cell line PC-3 (Guldberg et al., 1997; Tamura et al., 1998; Whang et al., 1998). We first analyzed the expression patterns of epithelial E-cadherin, mesenchymal N-cadherin, and cadherin-11 (Fig. 5 A). All three cadherins were found to be expressed in PC-3 cells, which are known to be defective in Ca²⁺-dependent cell aggregation due to ßE-catenin defects (Morton et al., 1993). In contrast, the U87MG and U373MG glioblastoma cells expressed mainly N-cadherin, and FM-45 melanoma cells were featured by weak expression of N-cadherin and major expression of cadherin-11. A small amount of the latter cadherin was also identified in U373MG cells. In agreement with the results obtained from MDCKs-α¹ cells, wild-type PTEN reverted the invasive phenotype of U87MG and U373MG glioblastoma cells stably transfected by wild-type PTEN, and of FM-45 melanoma and PC-3 prostate carcinoma cells transiently transfected using vaccinia virus–mediated PTEN expression (Fig. 5, B and C). In the latter two cell lines, overexpression of mutant PTEN molecules did not revert the invasive phenotype (Fig. 5 C). These results confirm the critical role of the lipid phosphatase activity of PTEN in regulating invasiveness. Moreover, the invasive phenotype of the U87MG and U373MG glioblastoma cell lines was clearly dependent on N-cadherin activity, because it was inhibited after application of the N-cadherin blocking antibody GC-4 (Fig. 5 C), whereas it was noneffective on FM-45 cells that express mainly cadherin-11 (Fig. 5 A, and unpublished data). Nonetheless, PTEN-mediated invasion suppression was not associated with any significant change in the expression pattern of cadherins (Fig. 5 A, and unpublished data).

Several studies have demonstrated that the switch in expression from E-cadherin to either N-cadherin or cadherin-11, as observed regularly in melanoma and prostate cancer cells, might promote cell motility (Tomita et al., 2000; Li et al., 2001). Whereas E-cadherin mediates tight cell–cell adhesion, the mesenchymal N-cadherin and cadherin-11 exert a dual activity on both cell–cell interactions and cell motility and invasiveness. N-cadherin and cadherin-11 might also exert a dominant effect over E-cadherin, as demonstrated in breast cancer cells after ectopic expression of N-cadherin (Nieman et al., 1999; Hazan et al., 2000). Because the N-cadherin–mediated survival and cell motility of melanoma cell lines seem to involve the PI3 kinase pathway (Li et al., 2001), PTEN might function as a potent gate keeper to counteract invasiveness mediated by mesenchymal cadherins. In consequence, PTEN inactivation during tumor progression might favor tumor cell dissemination.

Altogether, our data demonstrate that PTEN constitutes a critical effector in controlling the invasive phenotype. Because PTEN inactivation occurs in a wide range of tumors, we propose that PTEN can regulate the noninvasive phenotype via both E-cadherin–dependent and –independent pathways, according to the cellular context: (a) stabilization of E-cadherin complexes; (b) reversion of the activity and effector systems of mesenchymal cadherins; and (c) modulation of cell–matrix adhesion complexes through integrin and cytoskeleton reorganization. Development of therapeutic strategies targeting the many effector systems controlled by
PTEN may not only suppress tumor growth and induce apoptosis, but holds also the promise to restrain tumor cell dissemination and metastasis.

Materials and methods

Plasmid construction and cell transfection

Full-length wild-type PTEN cDNA and phosphatase inactive PTEN mutant Δ53–70 were generated by RT-PCR of RNA extracted from human colon crypts and the invasive glioblastoma cell line 149, respectively. The resulting PCR products were subcloned into pcDNA-His (Invitrogen), in frame with the N-terminus Xpress epitope. Expression vectors encoding wild-type PTEN or the phosphohistidineinositol phosphatase inactive PTEN mutant Δ237–239 fused with the HA epitope (Furnari et al., 1998) were provided by Dr. F.B. Furnari (University of California, San Diego, CA). Expression vectors encoding green fluorescent protein (GFP)-tagged wild-type PTEN or phosphatase inactive PTEN mutant C124A (Tamura et al., 1998) were provided by Dr. K.M. Yamada (National Institutes of Health, Bethesda, MD).

The MDCKs-ts clone 2 and MDCKr-f cells line expressing, respectively, thermosensitive v-Src and activated Ras, have been previously established by infection of MDCK cells with either a murine leukemia retroviral vector recombined with a thermosensitive v-src gene or the Harvey murine sarcoma virus (Behrens et al., 1989, 1993; Vlemmix et al., 1991; Takeda et al., 1995). MDCKs-ts clone 2 and MDCKr-f cells, and the U87 and U373 glioblastoma cell lines were transfected with PTEN expression vectors, using lipofectamine (GIBCO BRL) as previously described (Kotelevets et al., 1998). The PTEN C124A construct that lacks a selection marker was cotransfected with pcDNA3.1 plasmid (Invitrogen), conferring G418 resistance. After selection for 2 wk with 50 μg/ml G418 (Xpress-tagged constructs, PTENC124A) or 4 μg/ml puromycin (HA-tagged constructs), colonies of growing, surviving cells were randomly picked up, amplified, and evaluated for expression of the transgene by Western blotting.

For vaccinia virus–mediated transient overexpression, wild-type and mutant (Δ237–239 and Δ55–70) PTEN cDNAs were subcloned in the pE/L-GFP vector, in frame with the GFP tag (Frishknecht et al., 1999). FM-4-37 and PC-3 cells were transfected with Lipofectin (Life Technologies) and simultaneously connected with vaccinia virus strain A36R, which does not make actin tails. 10 h after transfection, PTEN cDNAs, under the control of the vaccinia virus early/late promoter, were expressed at high levels. The pE/L-GFP vector and vaccinia virus strain ΔA36R were provided by Dr. F. Frischknecht (European Molecular Biology Laboratory, Heidelberg, Germany) and B. Janssens (VIB-Ghent University, Ghent, Belgium).

Collagen type I invasion and fast aggregation assays

Single-cell suspensions were seeded on top of the type I collagen gel (Upstate Biotechnology), and cultures were incubated for 24 h at 35°C, 37°C, or 40°C. Using an inverted microscope controlled by a computer program (Vakaet et al., 1991), we counted the invasive and superficial cells in 12 fields of 0.157 mm². The invasion index was expressed as the percentage of cells invading the gel over the total number of cells (Vlemmix et al., 1991).

For the fast aggregation assay, single-cell suspensions were prepared using an E-cadherin saving procedure (Bracke et al., 1993). Cells were incubated in an isotonic buffer containing 1.25 mM Ca²⁺ and agitated in an E-cadherin saving procedure (Bracke et al., 1993). Cells were incubated in an isotonic buffer containing 1.25 mM Ca²⁺, and the invasive glioblastoma cell line 149, respectively. The resulting PCR products were subcloned into pcDNA-His (Invitrogen), in frame with the N-terminus Xpress epitope. Expression vectors encoding wild-type PTEN or the phosphohistidineinositol phosphatase inactive PTEN mutant Δ237–239 fused with the HA epitope (Furnari et al., 1998) were provided by Dr. F.B. Furnari (University of California, San Diego, CA). Expression vectors encoding green fluorescent protein (GFP)-tagged wild-type PTEN or phosphatase inactive PTEN mutant C124A (Tamura et al., 1998) were provided by Dr. K.M. Yamada (National Institutes of Health, Bethesda, MD).

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