Award Number: DAMD17-98-1-8271

TITLE: The Tumor Suppressor Protein TEPI/PTEN/MMAC1 and Human Breast Cancer

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REPORT DATE: June 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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The Tumor Suppressor Protein TEP1/PTEN/MMAC1 and Human Breast Cancer

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We have previously cloned a novel protein, TEP1, also called PTEN or MMAC1, based on its sequence homology to members of protein tyrosine phosphatases. PTEN/MMAC1/TEP1 has now been well established as an important tumor suppressor that is commonly mutated in a wide variety of human cancers. At molecular level, PTEN/MMAC1/TEP1 protein has been shown to function as a phosphatase for phosphatidylinositol 3,4,5-trisphosphate, thus acting as a negative regulator for PI 3-kinase signaling pathway. PTEN deletions and mutations are frequently found in advanced and highly metastatic cancers, suggesting that PTEN may also play a role in preventing tumor progression and metastasis, in addition to its role as a suppressor for tumor initiation. The initiation of metastasis is usually a result of cytoskeletal rearrangements which promote enhanced cell motility. We have previously shown that genetic deletion of the Pten gene can leads to increased levels of phosphatidylinositol-3,4,5-trisphosphate and enhanced phosphorylation of Akt. We have recently discovered that these cells also contain higher levels of the activated forms of Rac1 and Cdc42. Rac and Cdc42 are two small GTPases that have been implicated in cell motility and tumor invasion processes. Our studies have provided a molecular explanation for the increased cell motility and therefore invasion associated with loss of PTEN in human breast cancer cells.
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INTRODUCTION

We have previously cloned a novel protein, TEP1, based on its sequence homology to members in the dual specificity subfamily of protein tyrosine phosphatases [1]. The gene encoding TEP1 maps to chromosome 10q23, a locus frequently deleted in human glioblastomas and prostate cancers. The TEP1 gene was found to be identical to PTEN or MMAC1, a tumor suppressor gene independently isolated by positional cloning method [2,3]. Our group was the first to demonstrate that in vitro PTEN protein possesses an intrinsic phosphatase activity towards phosphotyrosine containing proteins [1].

PTEN has now been well established as an important tumor suppressor that is commonly mutated in a wide variety of human cancers. Somatic mutations in the PTEN gene have been found in glioblastoma, endometrial carcinoma, prostate and breast cancers, while inherited mutations have been associated with cancer predisposition syndromes such as Cowden disease and Banayan-Zonana syndrome [7]. Recent studies have suggested that the PTEN protein can dephosphorylate phosphatidylinositol-3,4,5-trisphosphate (PIP3) [8] in vitro. PIP3 is a product of PI 3-kinases and serves as second messengers for the activation of the serine/threonine kinase Akt, small GTPases Rac and S6 kinase [9].

In my original proposal, I have outlined strategies to investigate the involvement of PTEN in human breast cancers. In order to clearly and definitely define the contribution of PTEN deficiency to human breast cancers, we need to understand the cellular processes and the signaling pathways regulated by PTEN. We have made great progresses towards these goals. In the first funding year for my breast cancer grant (DAMD17-98-1-8271), we have shown that demonstrated that PTEN regulates cell cycle progression at the G1/S transition in both human tumor cells and mouse Pten knock-out cells [7,8]. Our observation of PTEN regulating cell cycle progression provides a molecular explanation for the marked over-proliferation and increase of S-phase cell population phenotype seen in Pten-/- mouse embryos [9]. Second, we have identified p27KIP1, an inhibitor of G1 cyclin-dependent kinases, as a specific downstream target for the PTEN-regulated signaling pathway [7,8]. Emerging evidence indicates that p27 serves as a critical cell cycle regulator that mediates the growth factor-dependent cell cycle progression and entry of the S-phase. The level of p27 is also a good prognosis marker for human breast cancer. Our studies raise an interesting possibility that PTEN deficiency or haploinsufficiency may lead to down-regulation of p27 which in turn promotes breast cancer progression.

In the second funding year, our second year research has been focused on elucidation of the potential role of PTEN in regulation of breast cancer cell migration and invasion. PTEN deletions and mutations are frequently found in advanced and highly metastatic cancers [2,3], suggesting that PTEN may also play a role in preventing tumor progression and metastasis, in addition to its role as a suppressor for tumor initiation. The initiation of metastasis is usually a result of cytoskeletal rearrangements which promote enhanced cell motility. There is growing evidence that small GTPases of the Rho family, Rac1, Cdc42 and RhoA, play a critical role in regulating the actin cytoskeleton to promote cell motility and adhesion [10]. Like all members of the Ras superfamily, Rho proteins act as molecular switches that cycle between the active GTP-bound and inactive GDP-bound states. This cycling is regulated positively by GDP/GTP exchange factors (GEFs), and negatively by GTPase activating proteins (GAPS) and guanine nucleotide dissociation inhibitors (GDIs). GEFs that activate these small GTPases all contain a pleckstrin homology (PH) domain
C-terminally adjacent to the catalytic Dbl homology (DH) domain. It has been shown that the PH domains in certain GEFs interact specifically with the membrane anchored phosphotidylinositolides, which mediate their membrane localization and activation. Overexpression of constitutively activated Rac1 and Cdc42, or their corresponding exchange factors have been shown to promote cell migration. However, whether Rac1 and Cdc42 activities are modulated by the PTEN signaling pathway is yet to be established.

In collaboration with Dr. Hong Wu’s laboratory in UCLA, we have have employed isogenic Pten+/+ and Pten-/- mouse fibroblasts to study the function of PTEN in regulation of tumor progression and metastasis. Our results demonstrate that Rac1 and Cdc42 are two of the downstream effectors of PTEN for cell motility, and that the lack of lipid phosphatase activity of PTEN is responsible for the increased cell migration phenotype of the Pten-deficient cells [11].

**BODY**

**PTEN-deficiency promotes cell motility**

![Figure 1](image)

We have compared Pten+/+ and Pten-/- mouse fibroblasts (MEF) for cell motility. By using several independent assays, we found that Pten-/- cells have increased cell motility than their wild type counterparts. In order to obtain more quantitative measurements of the migration distance, we employed a modified “wound healing” assay. In this assay, cells are first seeded on coverslips, and then the coverslips are transferred to a new plate coated with fibronectin. Upon transfer, cells migrate from the rim of the coverslip outwards onto the new plate. This method, thus, allows for accurate measurements of the migration distance over time. As shown in the Figure 1a, Pten-/- cells migrate almost twice as fast as Pten+/+ cells. Together, these results suggest that PTEN negatively regulates signaling pathways controlling cell migration.

**Increased cell motility in Pten-/- cells is due to deficiency of the PTEN lipid phosphatase activity.**

PTEN protein contains multiple structural motifs besides its phosphatase domain [1-3]. The N-terminal half of the protein shares extensive homology with tensin, a cytoskeletal protein localized to focal adhesions, and auxillin, a protein involved in synaptic vesicle transport. The C-terminal of the protein contains a motif that might bind to PDZ domain-containing proteins. In order to determine whether increased cell migration in Pten-/- cells is due to lack of the PTEN phosphatase activity or if other structural motifs may play a role, we re-introduced either wild-type (WT) or PTEN C124S, a catalytically inactive mutant, into the Pten-/- cells by retrovirus-mediated gene delivery method. We used a retroviral vector that expresses the gene of interest and the green fluorescence protein (GFP) as a bicistronic mRNA. GFP was translated using an internal ribosome entry site (IRES-GFP). This expression system allowed us to sort the GFP positive, thus PTEN expressing cells, by fluorescence activated cell sorting (FACS) following retroviral infection. As shown in Fig. 3C, WT PTEN and C124S mutant were expressed in comparable levels in the sorted
populations. The sorted populations were then subjected to cell migration assays. As shown in Fig. 1a and quantified in 1b, WT PTEN, but not C124S mutant, could fully reverse the migration phenotype of Pten+ cells, confirming that the enhanced motility is directly due to the lack of PTEN phosphatase activity.

Recent studies have suggested that PIP3 is a major in vivo substrate for PTEN. Interestingly, PTEN G129E, a point mutation first being identified as an inherited mutation in Cowden disease kindreds, has been shown to be deficient for the phosphatase activity towards PIP3, while its activity towards synthetic protein substrates is unaffected. Using PTEN G129E mutant, we further tested whether PTEN controls cell migration through its lipid phosphatase activity or its protein phosphatase activities. As shown in Figure 1a and 1b, PTEN G129E behaved similar to the C124S mutant as they were both unable to rescue the migration phenotype in this assay. This experiment suggests that the enhanced motility of Pten+ cells is a result of the loss of PTEN phosphatase activity, in particular, its lipid phosphatase activity.

**Pten deletion results in up-regulation of Rac1 and Cdc42 activity.**

Since increased cell motility is associated with a deficiency in PTEN lipid phosphatase activity, and cells lacking Pten contain elevated levels of PIP3, we next examined whether activation of known downstream PIP3 effectors might be responsible for the increased cell migration phenotype in Pten−/− cells. Two of the most notable effectors involved in cell migration are the small GTPases Rac1 and Cdc42. Activation of Cdc42 and Rac1 has been implicated in promoting cell migration and their GDP/GTP exchange factors (GEFs) can be activated in a PIP3-dependent manner. We therefore examined whether PTEN deficiency leads to a change in the Rac1 and Cdc42 activities. To determine the endogenous levels of the GTP-bound (thus the activated) forms of Rac1 or Cdc42, we adopted an affinity-based effector-Rac1 or Cdc42 GTPase co-precipitation assay. In this assay, the p21-binding domain (PBD) of PAK1, which specifically recognizes Rac1-GTP or Cdc42-GTP forms, was expressed as a GST-fusion protein to act as a bait to trap the respective GTPases in cell lysates. To confirm the specificity of this assay, we pre-loaded Rac1 and Cdc42 present in the cell lysates with either GTPγS or GDP prior to affinity precipitation by GST or GST-PAK immobilized glutathione agarose beads. The GTP-bound forms of Rac1 and Cdc42 could form tight complexes with GST-PAK1, which could be detected by Western blot analysis with the corresponding antibodies. However, GST-PAK1 could not interact with either GTPase when they were loaded with GDP, suggesting that the affinity precipitation assay is specific and effective in assessing the activation states of Rac1 and Cdc42.

We then applied the GST-PAK precipitation assay to examine the level of endogenous GTP-bound forms of Rac1 or Cdc42 in Pten+ and Pten−/+ cells. As shown in Fig. 2 in the logarithmically growing cells, there are marked increases of the GTP-bound forms of Rac1 and Cdc42 in Pten−/− cells compared to Pten+ cells, while the total protein levels of Rac1 or Cdc42 are not affected by the PTEN status. In order to test whether Rac1 and Cdc42 were activated in a PI3-kinase dependent manner, we also treated Pten+ cells with the PI3-kinase inhibitor LY294002. We have found that similar to Akt, a downstream effector of PI3-kinase, Rac1 activity dramatically decreases upon LY294002 treatment, indicating that Rac1 activation in Pten− cells is dependent on enhanced PIP3 levels in these cells [11]. Together, these studies suggest that PTEN deficiency leads to increases of the activated forms of Rac1 and Cdc42 in Pten− cells.
Dominant negative forms of Rac1 and Cdc42 reverse the migration phenotype in Pten<sup>−/−</sup> cells.

To determine whether the elevated levels of the GTP-bound forms of Rac1 and Cdc42 in Pten<sup>−/−</sup> cells are responsible for the increased cell migration phenotype, we introduced either WT or dominant negative forms of Rac1 (N17Rac1), Cdc42 (N17Cdc42), or RhoA (N19RhoA) into Pten<sup>−/−</sup> cells by retroviral infection, again using the pMX-IRES-GFP vector. These mutants are thought to act by sequestering specific GEFs necessary for activation of Rac1, Cdc42, or RhoA, preventing their functions [10]. Since the infection efficiencies were more than 90% and the observed GFP expressions were at similar levels as analyzed by FACS, we used unsorted populations for the cell migration assay. Figure 3 shows that expression of N17Rac1 and N17Cdc42 in Pten<sup>−/−</sup> cells could reverse the cell migration phenotype by 100% and 50%, respectively. The less efficient reversion by N17Cdc42 could reflect the suggested hierarchical relationship between the Rac1 and Cdc42, where Cdc42 is thought to function upstream of Rac1. As a control for the specificity of these GTPases, we also expressed the dominant negative form of RhoA (N19RhoA). Activation of RhoA causes formation of focal adhesions and stress fibers, but since the abundance and size of these structures correlate negatively with cell locomotion, RhoA seems more likely to be involved in adhesion than motility. No effect on the migration of Pten<sup>−/−</sup> fibroblasts was observed with N19RhoA, nor with the wild type Rho GTPases. These results indicate that Rac1 and Cdc42 serve as downstream effectors of PTEN in the regulation of cell migration.

KEY RESEARCH ACCOMPLISHMENTS

Our studies in the past year have demonstrated that PTEN regulates cell motility through in phosphatidylinoside (3,4,5)-trisphosphate. Our findings are summarized as below:

1. Demonstrate that the in vivo PTEN regulates cell motility.

2. Demonstrate that the effect of PTEN on cell motility is mediated by its phosphatase activity on phosphatidylinoside (3,4,5)-trisphosphate.

3. Demonstrate that the downstream targets for PTEN in cell motility regulation are small GTPases Rac and Cdc42.

REPORTABLE OUTCOMES

Manuscripts

CONCLUSIONS

PTEN deletions and mutations are frequently found in advanced and highly metastatic cancers, suggesting that PTEN may also play a role in preventing tumor progression and metastasis, in addition to its role as a suppressor for tumor initiation. The initiation of metastasis is usually a result of cytoskeletal rearrangements which promote enhanced cell motility. We have previously shown that genetic deletion of the Pten gene can lead to increased levels of phosphatidylinositol-3,4,5-trisphosphate and enhanced phosphorylation of Akt. We have recently discovered that these cells also contain higher levels of the activated forms of Rac1 and Cdc42. Rac and Cdc42 are two small GTPases that have been implicated in cell motility and tumor invasion processes. Our studies have provided a molecular explanation for the increased cell motility and thus invasion associated with loss of PTEN in human breast cancer cells.

REFERENCES

Genetic deletion of the Pten tumor suppressor gene promotes cell motility by activation of Rac1 and Cdc42 GTPases
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Pten (Phosphatase and tensin homolog deleted on chromosome 10) is a recently identified tumor suppressor gene which is deleted or mutated in a variety of primary human cancers and in three cancer predisposition syndromes [1]. Pten regulates apoptosis and cell cycle progression through its phosphatase activity on phosphatidylinositol (PI) 3,4,5-trisphosphate (PI(3,4,5)P3), a product of PI3-kinase [2–5]. Pten has also been implicated in controlling cell migration [6], but the exact mechanism is not very clear. Using the isogenic Pten+/+ and Pten−/− mouse fibroblast lines, here we show that Pten deficiency led to increased cell motility. Reintroducing the wild-type Pten, but not the catalytically inactive Pten C124S or lipid-phosphatase-deficient Pten G129E mutant, reduced the enhanced cell motility of Pten-deficient cells. Moreover, phosphorylation of the focal adhesion kinase p125FAK was not changed in Pten−/− cells. Instead, significant increases in the endogenous activities of Rac1 and Cdc42, two small GTPases involved in regulating the actin cytoskeleton [7], were observed in Pten−/− cells. Overexpression of dominant-negative mutant forms of Rac1 and Cdc42 reversed the cell migration phenotype of Pten−/− cells. Thus, our studies suggest that Pten negatively controls cell motility through its lipid phosphatase activity by down-regulating Rac1 and Cdc42.

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Received: 23 November 1999
Revised: 4 January 2000
Accepted: 21 January 2000

Published: 24 March 2000

Current Biology 2000, 10:401–404

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Results and discussion
To test the role of Pten in cell migration, we established independent immortalized fibroblast lines from wild-type and Pten-deficient (Pten−/−) mice, using the 3T3 protocol [8]. Similar to primary mouse embryonic fibroblasts [4], the immortalized Pten−/− cell lines showed increased levels of phosphorylation of protein kinase B/Akt compared to their wild-type counterparts and were resistant to serum-deprivation induced apoptosis. In contrast to our observation with wild-type and Pten−/− embryonic stem cells [4], however, no significant differences in the rates of cell proliferation and the levels of the cyclin-dependent kinase inhibitor p27kip1 could be detected between log-phase growing wild-type and Pten−/− fibroblast cells (data not shown).

Pten−/− fibroblasts have an increased cell motility, as shown by a classic 'wound healing' assay (Figure 1a, left panels) [9]. They were able to completely close the wound within 15 hours, whereas wild-type cells took almost 30 hours. To demonstrate that the increase in cell migration is an individual cell based and cell division-independent event, we employed a colloidal-gold based motility assay [10]. This assay revealed that Pten−/− fibroblasts could produce longer 'trails' than wild-type cells in a defined time period, indicating that Pten−/− cells indeed migrate faster than the wild-type cells (Figure 1a, middle panels). In order to obtain more quantitative measurements of the migration distance, we employed a modified 'wound healing' assay. In this assay, cells are first seeded on cover slips, and then transferred to a new plate coated with fibronectin. Upon transfer, cells migrate from the rim of the coverslip outwards onto the new plate. As shown in Figure 1a (right panels) and quantified in Figure 1b with independent cell lines, Pten−/− cells migrate almost twice as fast as wild-type cells. Moreover, careful observation of cell morphology revealed that Pten−/− fibroblasts appeared rounded and had intense cortical F-actin staining (Figure 1c). Together, these results suggest that Pten negatively regulates signaling pathways controlling cell migration.

In order to determine whether increased cell migration in Pten−/− cells is due to lack of the Pten phosphatase activity or if other structural motifs may play a role, we re-introduced either wild-type Pten or Pten C124S, a catalytically inactive mutant, into the Pten−/− cells by retrovirus infection [11]. We used a retroviral vector that expresses the gene of interest and the green fluorescent protein (GFP) as a bicistronic mRNA. GFP-positive, thus Pten-expressing, cells were sorted by fluorescence activated cell sorting (FACS) following retroviral infection. Wild-type Pten and the C124S mutant were expressed in comparable levels in the sorted populations (Figure 2c). As shown in Figure 2a
Pten-deficient fibroblasts migrate faster than wild type. (a) An equal number of wild-type (WT) or Pten\textsuperscript{−/−} fibroblasts were seeded on a fibronectin-coated plate and cultured for 24 h. Migration into the wound is shown 15 h after the wound was introduced (left panels; open arrowheads point to the boundaries of the wound at time = 0).

In the middle panel, 2 x 10\textsuperscript{5} cells per well were seeded on colloidal gold-coated 6-well dishes in duplicates. Migration of wild-type or Pten\textsuperscript{−/−} fibroblasts is shown at 24 h. The scale bar represents 10 μm. In the right panels, wild-type or Pten\textsuperscript{−/−} fibroblasts grown on glass coverslips were placed onto 5 μg/ml fibronectin-coated dishes and cultured for 15 h. Cell motility was assessed and compared using independent cell lines. Migration distances were determined by taking seven independent measurements from each coverslip. Each experiment was conducted in triplicate, and mean ± SD was calculated. The migration distance is normalized so that 100% represents migration distance of Pten\textsuperscript{−/−} cells. (c) Pten\textsuperscript{−/−} cells exhibited increased cortical actin polymerization as compared to the wild-type cells. Briefly, log-phase growing fibroblasts were cultured without serum for 20 h. After fixation in 4% paraformaldehyde, cells were permeabilized with 0.2% Triton X-100 and stained for F-actin using rhodamine-phalloidin (Molecular Probes).

Increased cell motility in Pten\textsuperscript{−/−} cells is due to the lack of lipid phosphatase activity of Pten. (a) Pten\textsuperscript{−/−} cells were infected with retroviral GFP vectors containing wild-type Pten (WT), G129E (GE), or C124S (CS) Pten mutants. Control wild-type or Pten\textsuperscript{−/−} cells were infected with viruses containing GFP only. 48 h later, GFP positive cells were sorted by fluorescence-activated cell sorting (FACS), seeded onto glass coverslips in triplicate, and grown for an additional 5 h. Cells on coverslips were then replaced onto a fibronectin-coated surface and incubated for 15 h. (b) Quantitative representation of (a). Cell migration is normalized so that 100% represents the migration distance of Pten\textsuperscript{−/−} cells infected by empty vector. (c) Pten protein levels in uninfected and infected cells after FACS sorting. Western blots of total protein extracts were probed with an affinity-purified anti-Pten antibody. Blots were reprobed with anti-FAK antibody (Santa Cruz Biotechnology) to confirm equal loading.

It has been suggested that Pten negatively regulates cell migration by directly dephosphorylating p125\textsuperscript{FAK} and changing mitogen-activated protein (MAP) kinase activity [6,13]. In order to test whether p125\textsuperscript{FAK} phosphorylation and MAP kinase activation are also affected by the loss of Pten, we examined the tyrosine phosphorylation status of these proteins. Whole cell lysates from log-phase growing wild-type or Pten\textsuperscript{−/−} fibroblasts were immunoprecipitated with 4G10 anti-phosphotyrosine antibody and western blotted with anti-FAK antibody (Figure 3a, upper panel), or were immunoprecipitated with anti-FAK antibody and western blotted with 4G10 (Figure 3b, lower panel). In contrast to what would be expected if Pten could directly dephosphorylate p125\textsuperscript{FAK}, no difference in tyrosine phosphorylation of p125\textsuperscript{FAK} could be detected in Pten\textsuperscript{−/−} fibroblast lines compared to wild-type cells. The activation status of MAP kinases was not affected by the Pten deletion either, but the level of Akt phosphorylation was significantly increased in Pten\textsuperscript{−/−} fibroblast cell lines (data not shown), similar to what we have observed previously in Pten\textsuperscript{−/−} embryonic stem cells [4]. These results suggest that the enhanced cell motility caused by Pten deficiency may be mediated by effectors other than p125\textsuperscript{FAK} and MAP kinases.

and quantified in Figure 2b, wild-type Pten, but not the C124S mutant, could fully reverse the migration phenotype of Pten\textsuperscript{−/−} cells, confirming that the enhanced motility is directly due to the lack of Pten phosphatase activity.

Recent studies suggested that PI(3,4,5)P\textsubscript{3} is a major substrate for Pten both in vitro [2] and in vivo [3,4]. Interestingly, Pten G129E is deficient for the phosphatase activity towards PI(3,4,5)P\textsubscript{3}, while its activity towards synthetic protein substrates is unaffected [12]. Using the Pten G129E mutant, we further tested whether Pten controls cell migration through its lipid phosphatase activity or its protein phosphatase activities. Pten G129E behaved similarly to the C124S mutant as they were both unable to rescue the migration phenotype in this assay. This experiment suggests that the enhanced motility of Pten\textsuperscript{−/−} cells is a result of the loss of Pten phosphatase activity, in particular, its lipid-phosphatase activity.
As increased cell motility is associated with a deficiency in Pten lipid-phosphatase activity, and cells in which Pten is genetically deleted contain elevated levels of PI(3,4,5)P₃ [3,4], we next examined whether activation of known downstream PI(3,4,5)P₃ effectors might be responsible for the increased cell migration phenotype in Pten⁺⁻ cells. Activation of Cdc42 and Rac1 has been implicated in promoting cell migration [7] and their GDP/GTP exchange factors (GEFs) can be activated in a PI(3,4,5)P₃-dependent manner [14,15]. We therefore examined whether Pten deficiency leads to changes in the Rac1 and Cdc42 activities. In this assay, the p21-binding domain of PAK1 was expressed as a GST-fusion protein. GST-PAK1 can specifically recognize Rac1-GTP or Cdc42-GTP forms, but not GDP-bound forms, suggesting that the affinity precipitation assay is specific and effective in assessing the activation states of Rac1 and Cdc42 (Figure 3b). We then examined the level of endogenous GTP-bound forms of Rac1 or Cdc42 in Pten⁺⁻ cells and wild-type cells. As shown in Figure 3c, there are marked increases of the GTP-bound forms of Rac1 and Cdc42 in logistically growing Pten⁺⁻ cells compared to wild-type cells, although the total protein levels are not affected by the Pten status. As PI(3,4,5)P₃ levels were highly sensitive to growth conditions [4], we also examined the Rac1 and Cdc42 activities in unfavorable confluent culture conditions. There is a notable ~30% increase in Rac1-GTP content and a ~50% increase in Cdc42-GTP content compared to the wild-type cells (Figure 3d). When similar assays were performed using suspended cell cultures which lack the adherent stimuli, ~60% and ~130% increases of Rac1-GTP and Cdc42-GTP forms, respectively, were observed (Figure 3d, suspension). The extent of elevation in the endogenous Cdc42 and Rac1 activities in Pten⁺⁻ cells were consistent when independent cell lines were used, and reintroducing wild-type Pten into Pten⁺⁻ cells led to a decrease in the GTP-bound forms of Rac1 and Cdc42 (data not shown).
Recent experiments demonstrated that a correlation exists between activation of the activity of PI 3-kinase and the activities of Rac1 and Cdc42 [14–17]. However, whether PI 3-kinase functions downstream or upstream of Rac1 and Cdc42 remains unclear. To test whether Rac1 and Cdc42 were activated in a PI 3-kinase dependent manner, we treated \textit{Pten}\textsuperscript{−/−} cells with the PI 3-kinase inhibitor LY294002 or wortmannin. Figure 3e shows that the activities of Rac1 and Cdc42 dramatically decrease upon treatment with PI 3-kinase inhibitors, indicating that similar to Akt, Rac1 and Cdc42 activation in \textit{Pten}\textsuperscript{−/−} cells is downstream of PI 3-kinase.

To formally prove that the elevated endogenous activities of Rac1 and Cdc42 in \textit{Pten}\textsuperscript{−/−} cells are responsible for the increased cell migration phenotype, we introduced either wild-type or dominant negative forms of Rac1 (N17Rac1) and Cdc42 (N17Cdc42) into \textit{Pten}\textsuperscript{−/−} cells by retroviral infection. These mutants are thought to act by sequestering specific GEFs necessary for activation of Rac1 and Cdc42, preventing their functions. Figure 4 shows that expression of N17Rac1 and N17Cdc42 in \textit{Pten}\textsuperscript{−/−} cells could reverse the cell migration phenotype by 100% and 50%, respectively. The less efficient reversion by N17Cdc42 is not due to the lower expression level (data not shown), but could reflect the suggested hierarchical relationship between Rac1 and Cdc42, where Cdc42 is thought to function upstream of Rac1 [7]. As a control for the specificity of these GTPases, we also expressed the dominant negative form of RhoA (N19RhoA), a GTPase involved in focal adhesion and stress fiber formation [18,19]. No effect on the migration of \textit{Pten}\textsuperscript{−/−} fibroblasts was observed with N19RhoA, or with the wild-type Rho GTPases (Figure 4). These results indicate that Rac1 and Cdc42 serve as downstream effectors of Pten in the regulation of cell migration.

In summary, we show that inactivation of the \textit{Pten} tumor suppressor gene promotes cell motility in fibroblasts. In contrast to previous reports that Pten negatively regulates cell migration by directly dephosphorylating p125\(^{FAK}\) and changing MAP kinase activities, we demonstrate genetically that the tumor suppressor Pten controls cell motility by down regulating Rac1 and Cdc42 GTPases, and this negative regulation is dependent on the lipid phosphatase activity of Pten. In combination with our previous work and other studies, we suggest that Pten exerts its tumor suppressor function not only at the stage of tumor initiation, but also in tumor progression and metastasis.

Supplementary material
Supplementary material including additional methodological details is available at http://current-biology.com/supmat/supmatin.htm.

Acknowledgements
We thank H. Herschman, O. Wittke, C. Sawyer, K. Shuai, X. Liu for critical reading of the manuscript. We thank X.L. Liu and H.L. Lodish of MIT for kindly providing pMX-IREs-GFP vector, and Jing Gao and Nadia Gavrilova for technical assistance. R.L. is supported by the Deutsche Forschungsgemeinschaft and a Carolan Seed grant (to H.W.). H.S. is a Pew Scholar in the Biomedical Sciences. H.W. is an Assistant Investigator of the Howard Hughes Medical Institute and V Foundation Scholar. This work was supported by the V Foundation and a Carolan Seed grant (to H.W.); American Cancer Society (RPG-97-146) and National Institutes of Health grant (GM53943 to Y.Z.); Department of the Army (DAMD 17-95-1-8271) and National Institutes of Health grant (CA77695 to H.S.).

References