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Loss of cellular adhesion to matrix induces p53-independent expression of PTEN tumor suppressor

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Abstract

Background: The tumor suppressor gene PTEN has been found mutated in many types of advanced tumors. When introduced into tumor cells that lack the wild-type allele of the gene, exogenous PTEN was able to suppress their ability to grow anchorage-independently, and thus reverted one of the typical characteristics of tumor cells. As these findings indicated that PTEN might be involved in the regulation of anchorage-dependent cell growth, we analyzed this aspect of PTEN function in non-tumor cells with an anchorage-dependent phenotype.

Results: We found that in response to the disruption of cell-matrix interactions, expression of endogenous PTEN was transcriptionally activated, and elevated levels of PTEN protein and activity were present in the cells. These events correlated with decreased phosphorylation of focal adhesion kinase, and occurred even in the absence of p53, a tumor suppressor protein and recently established stimulator of PTEN transcription.

Conclusions: In view of PTEN's potent growth-inhibitory capacity, we conclude that its induction after cell-matrix disruptions contributes to the maintenance of the anchorage-dependent phenotype of normal cells.

Background

The tumor suppressor gene PTEN (also called MMAC1) has been found deleted or mutated in a great variety of human tumors and tumor cell lines [1–3], and its tumor suppressing function has been confirmed in several *in vitro* studies [4–10]. Mice which are homozygously deficient in wild-type PTEN die during embryonic development and harbor regions of increased cellular proliferation, whereas heterozygous mice are viable but spontaneously develop tumors of various origins [11,12].

PTEN has been shown to exhibit dual specificity protein phosphatase activity, as well as lipid phosphatase activity *in vitro*[13–18]. These enzymatic functions appear to be involved in the regulation of at least two separate signal transduction pathways. First, PTEN's protein phosphatase

activity is able to down-regulate focal adhesion kinase (FAK) phosphorylation, which leads to the inactivation of the Ras/MAP kinase pathway [19–21]. Second, its lipid phosphatase activity targets the second messenger phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3] and thereby blocks activation of the protein kinase B (PKB/Akt) pathway [11,18,22–24]. Whereas both of the above pathways are intimately involved in the control of cell growth and survival, PTEN-regulated FAK activity further appears to impinge on cell adhesion, cell migration, and cell invasion [20,21]. It therefore emerges that the loss of PTEN activity may confer increased survival ability, proliferative potential, and invasive capacity on cells, and thereby may promote progression towards a more malignant phenotype.

A characteristic phenotype of tumorigenic cells is their ability to grow anchorage-independently in suspension culture, or embedded in soft agar, without the need for attachment to the surface of a cell culture dish [25,26]. A flurry of papers has established a close link between anchorage-independent growth and the activity of several components of the cell cycle machinery, such as various cyclins, cyclin-dependent kinases (CDKs), and the CDK inhibitors $p21^{Cip1}$ and $p27^{Kip1}$[27–32]. There are indications that PTEN may be involved in these processes as well. For example, mouse embryonal stem (ES) cells with homozygous deletion of the PTEN gene exhibit increased anchorage-independent growth as compared to normal ES cells [12]. Similarly, transfer of a wild type PTEN gene into anchorage-independent human glioblastoma cells (which lack functional PTEN), results in their greatly reduced ability to form colonies in soft agar [4–6]. The interpretation of these latter findings, however, is complicated by the strong anti-proliferative effects of PTEN even in monolayer culture, which is consistently observed when the wild type version of this gene is introduced into PTEN-negative tumor cells [4,6–10,18,33]. Moreover, in human glioma and breast cancer cell lines, the ectopic expression of wild type PTEN leads to anoikis, which is apoptosis initiated by the disruption of cell matrix-interactions [23,34–36].

Because essentially all of these previous studies have analyzed PTEN function by introducing the cloned version of the gene back into PTEN-deficient cells, essentially nothing is known about the regulation of the endogenous PTEN gene in response to alterations of cell-matrix interactions. For example, it is unclear whether PTEN is constitutively active or becomes activated in response to changes in the cellular microenvironment. Here, we present our findings that in normal anchorage-dependent fibroblast cells, the expression and activity of endogenous PTEN is increased when cellular adhesion to matrix is disrupted. In parallel, phosphorylation of FAK, a known target of PTEN, is greatly reduced. In view of PTEN's potent growth-inhibitory capacity, we conclude from our study that the increased expression and activity of endogenous PTEN in response to the disruption of cell-matrix interactions contributes to the maintenance of the anchorage-dependent phenotype of normal cells.

Results

A model to study cell regulatory events during anchorage-independent growth is the culture of cells in suspension, i.e. on HEMA-coated plates that prevent cells from attachment to the matrix of the cell culture dish [37]. Several studies have employed this approach and characterized the regulation of various cell cycle-regulatory proteins after the transfer of cells to such suspension culture [27–32]. Here, we have used this model to analyze the potential involvement of the tumor suppressor PTEN.

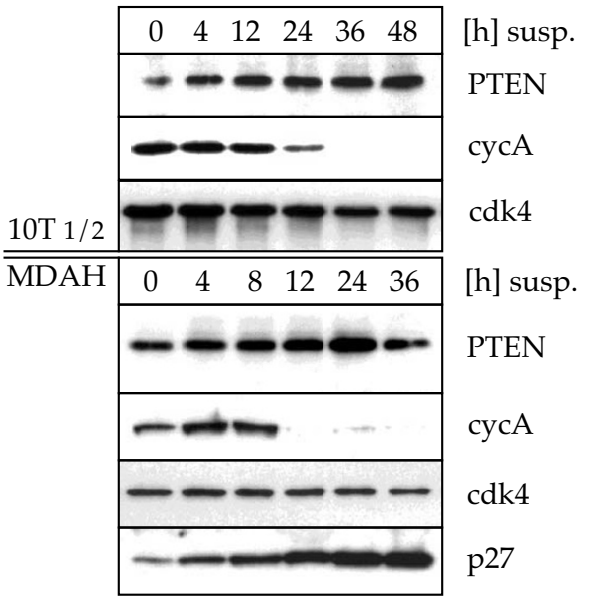

Figure 1
PTEN protein level
s in suspension culture cells MDAH or 10T1/2 cells were transferred to suspension culture conditions for the times indicated. Total cellular lysate was prepared and analyzed by Western blot with PTEN specific antibodies. In addition, different cell cycle-regulatory proteins were analyzed in parallel as indicated. The top panel shows lysates from 10T1/2 cells, whereas MDAH cells are represented in the bottom panels.

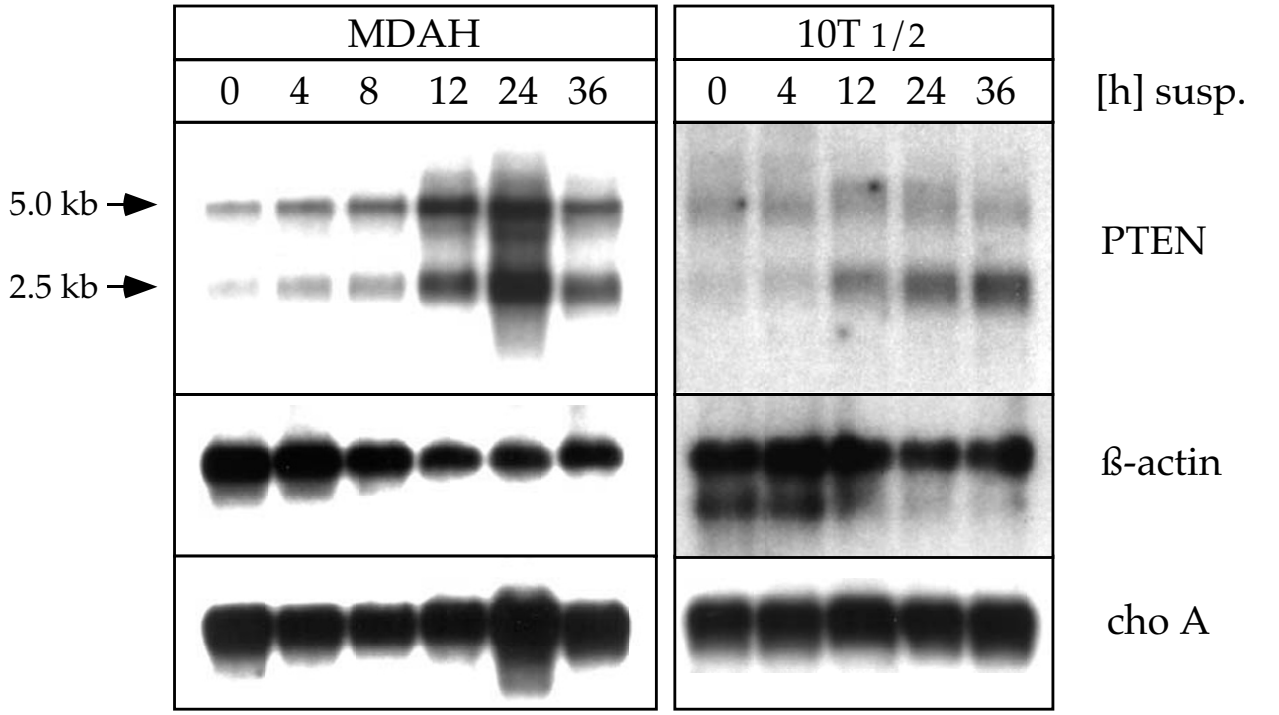

Figure 2
PTEN mRNA levels in suspension culture cells MDAH or 10T1/2 cells were transferred to suspension culture conditions for the times indicated. Poly A^+ RNA was harvested and subjected to Northern blot analysis. To detect PTEN mRNA, a radioactively labeled PTEN cDNA fragment was used. To control for the amounts of mRNA loaded in each lane, the filters were stripped and rehybridized to a probe for β-actin, as well as a probe for choA.

The murine cell line 10T1/2 and the human cell line MDAH, both of which are anchorage-dependent fibroblasts, were detached from their tissue culture dishes and cultured further on HEMA-coated plates. As shown in Figure 1, this transfer to suspension culture resulted in elevated expression of PTEN protein in each cell line. This increase was apparent within the first four hours and continued for several more hours. In parallel, the expression of cyclin A, an essential component of certain cyclin-dependent kinases and absolutely required for the progression of cells through the cell cycle [38,39], was downregulated under these conditions (Figure 1), consistent with earlier observations [27,28,31]. Furthermore, expression of the CDK inhibitor $p27^{Kip1}$ was strongly increased in suspension cells (Figure 1), similar to what has been observed in other cell types after the disruption of cell-matrix interactions [29,31,32]. Finally, as we have reported before [31], the expression of cdk4, one of the catalytic subunits of CDKs, was not significantly altered in suspension culture cells and therefore could be used as a loading control for the Western blot analysis. As expected in the case of anchorage-dependent cells, the activity of cyclin-dependent kinases was strongly reduced and cell proliferation was inhibited under these suspension culture conditions (not shown).

To analyze whether the observed induction of PTEN protein was due to elevated expression of its mRNA, Northern blot analysis was performed. In this case, increased expression was found as well (Figure 2). In MDAH cells in particular, the increase in PTEN mRNA closely correlated with the observed increase in PTEN protein and encompassed both major mRNA species of 2.5 and 5.0 kb. In the mouse cells, only the shorter mRNA species appeared to be induced. In order to determine the relative increase in PTEN mRNA levels, the Northern blots were stripped and re-hybridized with control probes for β-actin and choA, the latter a highly abundant mRNA of unknown function [40]. We consistently found that the amount of β-actin mRNA was somewhat reduced during suspension culture, whereas the amount of choA remained relatively stable. We therefore used choA as the loading control and calculated the increase in PTEN mRNA with reference to choA. Using this approach, we determined that PTEN mRNA

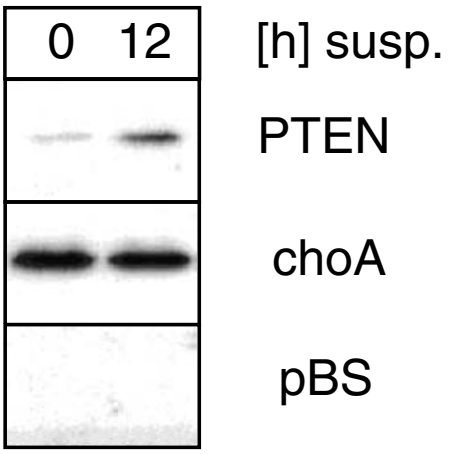

Figure 3
PTEN mRNA transcription in suspension culture cells MDAH cells were transferred to suspension culture conditions for 12 hours. To analyze the transcription of the PTEN gene, nuclei were harvested and nuclear run-off analysis was performed essentially as described [64]. As a control, the radiolabeled RNA was also hybridized to a DNA sequence representing the choA gene, as well as to non-gene-specific sequences from the plasmid pBluescript (pBs).

was increased up to 5-fold in MDAH cells and 4-fold in 10T1/2 cells. This induction was comparable to the increase observed in Western blot analysis and therefore indicated that the levels of PTEN protein were elevated due to the increased expression of PTEN mRNA. Although clearly induced in both cell lines, the kinetics of PTEN induction in 10T1/2 and MDAH cells were somewhat different at later time points; i.e., there was a slight reduction of PTEN mRNA and protein in MDAH cells at 36 hours, possibly indicating some cell type-specific differences.

By using nuclear run-off analysis, we further determined that the induction of PTEN was regulated at the transcriptional level, i.e. the transcription of the PTEN gene was significantly higher in cells that were transferred to suspension culture conditions (Figure 3).

We next analyzed whether the elevated quantity of PTEN protein would indeed be reflected in increased phosphatase activity in suspension culture cells. In order to establish whether PTEN protein phosphatase activity could be reliably measured in vitro, we first transfected PTEN-negative U87 cells with an expression vector harboring PTEN cDNA. As a control, the cells were also transfected with empty vector. Then, PTEN was immunoprecipitated from the respective cellular lysates and the antigen-antibody complex was analyzed for protein phosphatase activity. As shown in Figure 4A, only cells transfected with PTEN cDNA exhibited significant enzymatic activity. Non-transfected U87 cells, or cells transfected with vector alone, did not exhibit protein phosphatase activity above background levels.

After having established that PTEN protein phosphatase activity could be determined specifically, we transferred MDAH cells to suspension culture conditions and measured PTEN activity at various times afterwards. As shown in Figure 4B, there was an increase in PTEN activity that could be detected as early as four hours after detachment and reached its maximum at around 12 hours. It is noticeable that the activity at the onset of the experiment (0 hours, cells attached to tissue culture plates) was higher than background, which likely indicates some basal activity of PTEN in attached cells. This basal level activity was not detectable in PTEN-negative U87 cells (compare Figure 4A).

Focal adhesion kinase (FAK) is a known substrate for PTEN and has been shown to be dephosphorylated by this phosphatase in vitro and in vivo [20,21,34]. We therefore determined whether the increased PTEN phosphatase activity would correlate with decreased phosphorylation of FAK in our cells. This was indeed the case. As shown in Figure 5, the detachment of MDAH cells from their matrix resulted in decreased tyrosine-specific phosphorylation of FAK. The overall amount of FAK protein in these cells did not change under these conditions, indicating that the loss of tyrosine phosphorylation was not caused by reduced amounts of protein.

In light of a recent report establishing the tumor suppressor p53 as a transcriptional activator of PTEN expression [41], we investigated whether this protein would affect the observed induction of PTEN in our cell system. As MDAH cells themselves are p53-negative, we used MDAH cells stably transfected with a tetracycline-regulated p53 gene, called TR9-7 [42]. These TR9-7 cells were pre-treated with or without tetracycline in monolayer culture. After the induction of p53 was maximal, the cells were transferred to suspension culture conditions and analyzed for their expression of PTEN. As shown in Figure 6, the degree of PTEN induction was essentially the same in the absence or presence of p53, indicating that p53 did not affect the induction of PTEN protein under these conditions. In parallel, MDAH cells were also treated with tetracycline and transferred to suspension culture. In this case as well, tetracycline treatment had no effect on the induction of PTEN, confirming that tetracycline by itself did not affect PTEN expression (Figure 6). In conclusion, MDAH cells (p53-negative), 10T1/2 cells (p53-positive), and TR9-7 cells (high or low levels of p53) all exhibited similarly in-

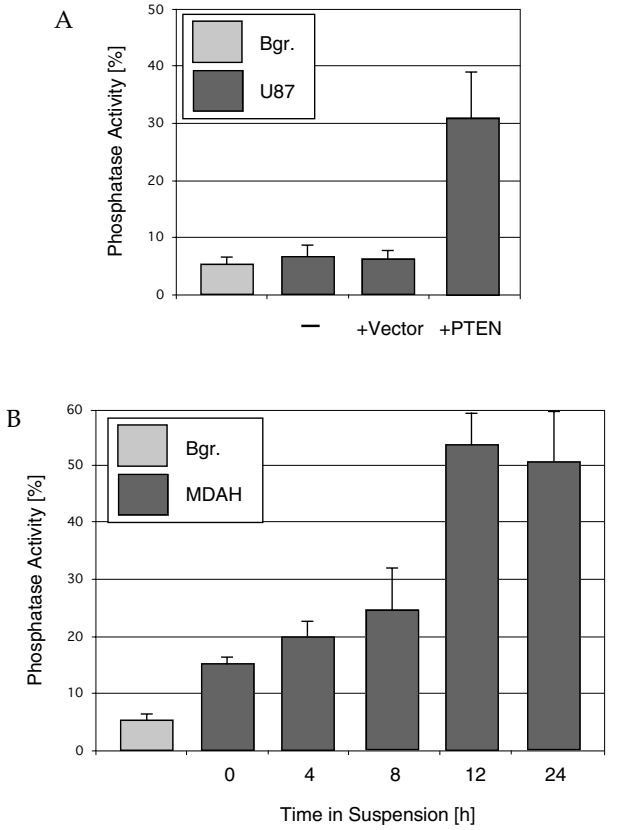

Figure 4
Protein phosphatase activity of PTEN (A) In order to determine the specificity of the PTEN phosphatase assay, PTEN-negative U87 glioblastoma cells were transiently transfected with pCMV-PTEN expression vector (PTEN) or with pCMV-blue vector without PTEN cDNA insert (Vector). As a further control, non-transfected U87 cells were used in parallel (U87). 24 hours after transfection, cellular lysates were harvested and subjected to immunoprecipitation with PTEN specific monoclonal antibodies. The collected antigen was assayed for tyrosine phosphatase activity as described in Materials and Methods. Shown is the amount of radiolabeled phosphate released from the substrate (mean of three experiments the quantity of phosphate released in the absence of added antigen. (B) In order to determine PTEN phosphatase activity in suspension cells, MDAH cells were transferred to suspension culture conditions for the times indicated. Total cellular lysates were prepared and subjected to immunoprecipitation with PTEN specific monoclonal antibodies. The collected antigen was assayed for tyrosine phosphatase activity as described in Materials and Methods. Shown is the amount of radiolabeled phosphate released from the substrate (mean of three experiments ± sd). released in the absence of added antigen.

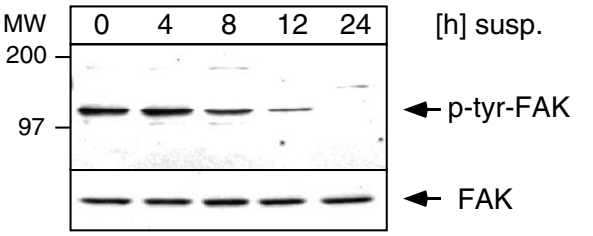

Figure 5
Phosphorylation status of focal adhesion kinase in suspension cells MDAH cells were transferred to suspension culture conditions for the times indicated. Total cellular lysate was prepared and analyzed by Western blot with antibodies specific for the tyrosine-phosphorylated form of focal adhesion kinase (p-tyr-FAK) or for the overall levels of focal adhesion kinase protein (FAK) as indicated.

creased expression levels of PTEN in response to the disruption of cell-matrix interactions. Therefore, we conclude that the observed induction of PTEN occurs independent of p53.

Discussion

In light of the close correlation between the anchorage-independent phenotype and the tumorigenicity of transformed cells, it is important to fully understand the cellular mechanisms that are involved in cell growth arrest after the disruption of cell-matrix interactions. Many previous studies in this area have focused on the contribution of various components of the cell cycle machinery. Collectively, they have established that the expression of cyclin A and cyclin D, in combination with the activity of the cyclin-dependent kinase inhibitors $p21^{Cip1}$ and $p27^{Kip1}$, is a crucial determinant of anchorage-dependent cell growth culture [27–32]. However, while the above elements clearly are essential executioners of cell cycle progression, it is conceivable that other elements, directly or indirectly, might be involved in anchorage-dependent growth control as well. In this regard, a report from our laboratory has indicated a role for the serine/threonine specific protein phosphatase type 2A (PP2A) [43].

In this current study, we investigated the response of the PTEN tumor suppressor to changes in cell-matrix interactions of anchorage-dependent human and mouse fibroblast cells. Previous studies by others had shown that the ectopic expression of PTEN in anchorage-independent tumor cells greatly reduced their ability to grow in soft agar [4–6]. In this latter situation, however, the forced expression of ectopic PTEN effectively impairs cellular proliferation in general, even under two-dimensional culture conditions where the cells are attached to substratum

[4,6–10,18,33,44]. It was therefore difficult to discern from these experiments how effectively and selectively PTEN participates in anchorage-dependent growth control. As an alternative to the forced expression of ectopic PTEN in anchorage-independent, PTEN-negative tumor cells, our study has focused on the regulation of endogenous PTEN in anchorage-dependent mouse and human fibroblasts. We found that upon detachment, both cell lines exhibited increased levels of PTEN expression, due to the transcriptional activation of the PTEN gene. The increased levels of PTEN protein resulted in strongly increased intracellular PTEN phosphatase activity. Thus, our results revealed a close correlation between the disruption of cell-matrix interactions and the subsequent activation of the PTEN phosphatase. In light of the well-established growth-inhibitory effects exerted by increased levels of this phosphatase, it is reasonable to conclude that this activation of PTEN significantly contributes to the anchorage-dependent phenotype, i.e., to the inhibition of cell proliferation after detachment from matrix.

It should be noted that the fibroblast cells lines we used remain fully viable after detachment and transfer to suspension culture [31,43]. This is in contrast to most epithelial cells which undergo anoikis, i.e., apoptotic cell death after the disruption of cell-matrix interactions [45]. It is of interest that some anchorage-independent tumor cells, most of which are of epitheloid origin, become susceptible to anoikis after the introduction of exogenous PTEN [23,34–36]. These observations are in line with the established ability of PTEN to down-regulate the phosphatidylinositol 3-kinase (PI3-K)/PKB survival pathway [11,46]. The absence of anoikis in our two cell lines may reflect inherent cell type specific differences, i.e., the superior ability of fibroblasts to survive under suspension culture conditions. One could speculate that increased levels of PTEN might favor growth arrest in fibroblasts versus apoptosis in epitheloid cells. Furthermore, it appears that the specific experimental or physiological conditions of cellular attachment or detachment might influence the precise function of PTEN in these processes. For example, it was shown recently that the reduction of PTEN expression levels by antisense oligonucleotides in a colon carcinoma cell line generated differential effects on cell adhesion, depending on whether the cells were kept under static or hydrodynamic conditions of fluid flow [47].

One of the established in vivo substrates of PTEN, FAK, is known to play a major role in growth-regulatory signal transduction initiated by cell surface integrin receptors [48,49]. As we observe a correlation between increased PTEN activity and decreased levels of FAK phosphorylation (compare Figure 4B and Figure 5), it is likely that the dephosphorylation of FAK in response to the disruption of cell-matrix interactions is accomplished by increased PTEN activity. Such a scenario would plausibly explain some of PTEN's growth-inhibitory effect. Additional growth-inhibitory effects of increased PTEN activity are likely to occur through the stimulation of the cell cycle inhibitor $p27^{Kip1}$. This protein acts as inhibitor of cyclin-dependent kinases (the "cell cycle engine" [50]), and its elevated expression has been consistently demonstrated in different cell types after the disruption of cell-matrix interactions (compare Figure 1 and [31,32,51,52]). Furthermore, $p27^{Kip1}$ is an established target of PTEN signaling, i.e., its activity has been found increased after the forced expression of exogenous PTEN [53–56]. In combination with the data presented in this manuscript, it therefore appears that PTEN contributes to anchorage-dependent growth control by a two-fold approach: the dephosphorylation of the signaling molecule FAK in combination with the stimulation of the cell cycle inhibitor $p27^{Kip1}$.

Conclusions

In view of PTEN's potent growth-inhibitory capacity, we conclude that its induction after cell-matrix disruptions contributes to the maintenance of the anchorage-dependent phenotype of normal cells. The underlying processes involve the stimulation of expression of $p27^{Kip1}$ and the dephosphorylation of FAK.

Materials and Methods

Materials

HEMA (poly-HEMA; poly(2-hydroxyethyl methacrylate) was obtained from Sigma (St. Louis, MO) and dissolved in ethanol at 10 mg/ml.

Cell lines and culture

C3/10T1/2 mouse fibroblasts were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD). MDAH human fibroblasts from Li Fraumeni patients (p53-negative), and the same cells stably transfected with a tetracycline-regulated p53 expression vector (TR9-7) [42], were obtained from W.R. Taylor and G.R. Stark (Cleveland Clinic Foundation, Cleveland, OH). The U87 glioblastoma tumor cell line has been described [57] and was obtained from Webster K. Cavenee (UC San Diego, La Jolla, CA).

All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. For the disruption of cell-matrix interactions, cells grown as a monolayer were either trypsinized or scraped off the culture dish and dispersed by pipetting. Then one half was seeded back into a culture dish for re-attachment, the other half was cultured in HEMA-coated plates which prevented the attachment of cells [37].

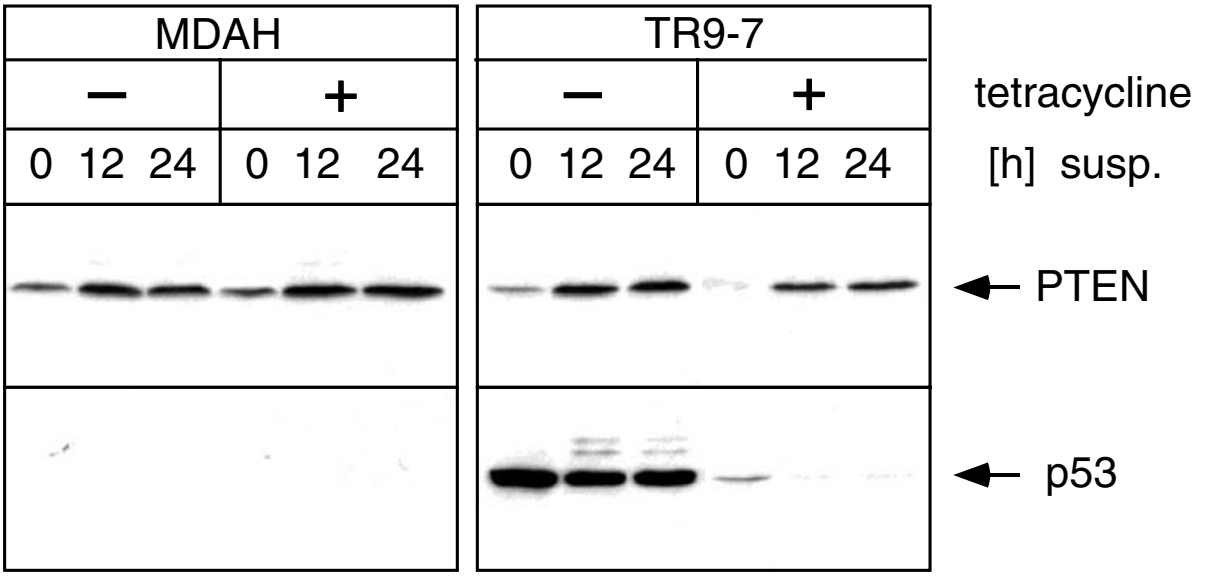

Figure 6
PTEN expression in cells with tetracycline-regulated p53 MDAH cells (which are p53-negative), or the same cells harboring a tetracycline-regulated p53 gene (TR9-7), were kept in monolayer culture in the absence or presence of tetracycline (1 μg/ml) for 24 hours. TR9-7 cells contain the tet-off system, i.e., in the presence of tetracycline, p53 is off (not expressed), whereas in the absence of tetracycline, p53 is expressed [42]. After 24 hours of incubation with or without tetracycline, the cells were transferred to suspension culture in the continued presence or absence of tetracycline. At 0, 12 and 24 hours after the onset of suspension culture, cellular lysates were prepared and analyzed by Western blot for the expression levels of PTEN as indicated. In order to verify proper control of p53 by tetracycline, the amount of p53 protein in each lysate was analyzed as well (shown in the bottom panel). Furthermore, to establish that the elevated levels of p53 were indeed functional, the increased expression of one of its target genes, $p21^{Cip1}$ [65], was confirmed by Western blot analysis; in this case, $p21^{Cip1}$ was found to be highly expressed in TR9-7 cells in the absence of tetracycline, but not in TR9-7 cells in the presence of tetracycline, nor in MDAH cells with or without tetracycline (not shown).

PTEN phosphatase assays

Phospho-tyrosine phosphatase assays were performed similarly to previously described protocols [14,58]. For the preparation of tyrosine-phosphorylated substrate, 7×10^6 HTC-IR cells [59] were incubated with medium containing insulin (50 nM/ml) and lysed with RIPA buffer. Insulin receptor was immunoprecipitated with specific antibodies, collected with protein A sepharose, and incubated with $polyGlu_4Tyr_1$ peptides (Sigma, St. Louis, MO) in the presence of [γ-^{32}P]-ATP [14]. After completion of the kinase reaction, the mix was centrifuged and the phospho-peptide-containing supernatant precipitated with 20% TCA (w/v). After washing, the phospho-peptide was solubilized in 30 mM Tris pH 8.0, and aliquots were dried onto DE81 paper (1×1 cm).

For the phosphatase assays, PTEN was immunoprecipitated from cellular lysates using anti-PTEN mouse monoclonal antibodies [44], and incubated with the substrate on DE81 paper for 5 min. at room temperature. The reaction was stopped by adding 75 mM H_3PO_4 (5 ml). Both the released as well as the retained radioactivity was determined with a scintillation counter.

RNA analysis

Total RNA was isolated using the guanidium thiocyanate method [60], followed by poly A extraction using oligo dT beads [61]. Equal amounts of each RNA sample were separated on formaldehyde/agarose gels and transferred onto nitrocellulose membranes. For hybridization, specific riboprobes were generated using T7 RNA polymerase according to manufacturer's instructions. The hybridization was carried out essentially as described [62]. After hybrid-

ization, the membranes were washed twice at 80°C in 0.2× SSPE and 0.5% SDS for 30 minutes, and subsequently exposed to Kodak X-AR autoradiographic film. After exposure, the filters were stripped and rehybridized in order to confirm that equal amounts of RNA were loaded in each lane. For this purpose, two probes were used; one was β-actin, the other was choA, which is clone A of a group of highly expressed mRNAs from Chinese hamster ovary (cho) cells [40]. The quantitation of the hybridized blots was performed using the AMBIS Radioanalytic Imaging System (Analytical Development Corporation, Colorado Springs, CO).

Western blot analysis

Total cell lysates were prepared by lysis of cells with RIPA buffer [63]. Thirty μg of each sample was processed by Western blot analysis as described [31]. Antibodies against cell cycle-regulatory proteins as well as those against focal adhesion kinase were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibodies against PTEN were generated and used as described previously [44]. The secondary antibodies were coupled to horseradish peroxidase, and were detected by chemiluminescence using the SuperSignal™ substrate from Pierce (Rockford, MD).

Authors' Contributions

R-CW cultured the cells and performed the expression analysis of PTEN. XL performed some of the Western blots and the in vitro phosphatase activity studies. MB performed experiments for the revised version of the manuscript. AHS conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

Acknowledgements

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