Overexpressing TPTE2 (TPIP), a homolog of the human tumor suppressor gene PTEN, rescues the abnormal phenotype of the PTEN<sup>−/−</sup> mutant

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ABSTRACT

One possible approach to normalize mutant cells that are metastatic and tumorigenic, is to upregulate a functionally similar homolog of the mutated gene. Here we have explored this hypothesis by generating an overexpressor of TPTE2 (TPIP), a homolog of PTEN, in PTEN<sup>−/−</sup> mutants, the latter generated by targeted mutagenesis of a human epithelial cell line. Overexpression of TPTE2 normalized phenotypic changes associated with the PTEN mutation. The PTEN<sup>−/−</sup>-associated changes rescued by overexpressing TPTE2 included 1) accelerated wound healing in the presence or absence of added growth factors (GFs), 2) increased division rates on a 2D substrate in the presence of GFs, 3) adhesion and viability on a 2D substrate in the absence of GFs, 4) viability in a 3D Matrigel model in the absence of GFs and substrate adhesion 5) loss of apoptosis-associated annexin V cell surface binding sites. The results justify further exploration into the possibility that upregulating TPTE2 by a drug may reverse metastatic and tumorigenic phenotypes mediated in part by a mutation in PTEN. This strategy may also be applicable to other tumorigenic mutations in which a homolog to the mutated gene is present and can substitute functionally.

INTRODUCTION

Loss of function of tumor suppressor genes, like PTEN, can facilitate tumorigenesis and metastasis, and in nontumorigenic cell lines, can result in phenotypic changes associated with both processes [1–12]. One way to rescue loss of function of a tumor suppressor gene, is to reintroduce the normal gene, in an expression plasmid into the cells [4, 8, 13–20]. This approach shows promise, but to date has not yielded effective treatments [21, 22]. There is, however, an alternative and, possibly, less intrusive strategy that has not been explored. If the defective gene contributing to tumorigenesis has one or more functional homologs, then stimulating overexpression of one of them in mutant cells may restore the function of the defective gene. PTEN indeed has two homologs, TPTE1 and TPTE2 (TPIP), a PTEN pseudogene [23–27], a TPTE1 pseudogene [23] and seven TPTE2 (TPIP) pseudogenes [23–28] (www.ncbi.nlm.nih.gov/gene; www.uniprot.org Q6XPS3; www.ensemble.org). By over-expressing a homolog of PTEN with similar catalytic and membrane-binding domains in a cell that has lost PTEN function such as TPTE2 (TPIP), one may be able to normalize the aberrant phenotype caused by the absence of PTEN. And, if expression of the homolog could be up-regulated by a surface receptor, and a chemical or monoclonal antibody could be identified that activates that receptor, one might be able to design a drug that reestablishes PTEN function in PTEN mutants and thus suppresses tumorigenesis and metastasis.

Recently, we explored this hypothesis by testing it in a model system, the simple amoeba Dictyostelium...
discoideum, which has been used for over 65 years as an exceptional model for human development and white blood cell function [29]. D. discoideum contains an ortholog of the human PTEN gene, ptenA, and a homolog of pten, lpten. Both PtenA and Lpten contain two signature domains of human PTEN, the CDC14-protein tyrosine phosphatase domain and the lipid C2-binding domain [30]. The ptenA null mutant, ptenA, exhibits strong behavioral defects in motility, chemotaxis, aggregation and multicellular morphogenesis [31–37]. The null mutant of the homolog, lpten, behaves as a very weak phenocopy of ptenA [30]. Overexpressing full length lpten rescued all developmental and behavioral defects of the ptenA mutant [30], an observation that provided initial support for the proposed hypothesis.

Here, we have used a similar strategy to test whether overexpressing TPTE2, also known as a homolog of PTEN, in a human PTEN null mutant, PTEN+/−, generated in the nontumorigenic breast epithelial cell line, MCF-10A [10], reversed characteristics associated with the loss of PTEN function –i.e., rescued the mutant PTEN+/− phenotype. The PTEN+/− strain was generated by targeted homologous recombination, and previously demonstrated to differ from the parent strain MCF-10A in a number of phenotypic characteristics [9, 10]. The PTEN+/−-associated phenotypes explored here include 1) an increased rate of wound healing, accentuated by the removal of growth factors (GFs) from the supporting medium, 2) an increase in the rate of cell division in the presence of GFs, 3) adherence and viability on a 2D substrate in the absence of GFs, 4) viability in a 3D Matrigel model in the absence of GFs and substrate adhesion, and 5) loss of the apoptosis-associated character of annexin V binding and endocytosis [38–42]. We show that overexpressing TPTE2 (TPTE) in PTEN+/− mutant cells reverses all of the phenotypic changes associated with the PTEN mutation and in the cases of wound healing and annexin V binding, actually accentuates the normal PTEN+/− phenotype. These results add further support to the hypothesis that a receptor mediated signal that upregulates a homolog of a defective tumor suppressing gene can normalize defects in mutant cells associated with the mutation, with the potential of suppressing tumorigenesis and metastasis and stimulating apoptosis. This approach may also prove effective for other types of cancer-associated mutations, for which the mutant gene has a functional homolog that can be upregulated.

RESULTS

Generating PTEN+/− TPTE2OE strains

The human genome contains one copy of PTEN [3, 5, 43], one each of the two PTEN homologs TPTE1 and TPTE2 (TPIP) [23, 24, 26, 27], and a pseudogene [25]. PTEN produces two transcripts, PTEN and PTEN long (Figure 1A) [43, 44]. Here, we have focused on the effects of overexpressing TPTE2 (TPIP), which will be referred to hence as TPTE2, in the mutant strain PTEN+/−, the latter generated from the breast epithelial cell line MCF-10A [10]. PTEN+/− was previously shown to differ phenotypically from MCF-10A (PTEN+/+). The differences included independence of growth on growth factors, resistance to apoptosis, a decrease in susceptibility to doxorubicin and shape changes in suspension [9, 10]. TPTE2 was selected over TPTE1 (TPTE) because the former was predicted to have phosphatase activity [25, 27, 45, 46] whereas TPTE1 was predicted, based on amino acid sequence, not to have activity [26]. Amplification and sequencing of TPTE2 from cDNA preparations of MCF-10A revealed three TPTE2 transcripts, TPTE2-1, TPTE2-2 and TPTE2-3, diagrammed in Figure 1B. The variants differed at both the N- and C-terminal regions (Figure 1B). All three, however, contained the CDC14 protein tyrosine phosphatase domain involved in the conversion of PIP3 to PIP2, and the PTEN-C2 domain, a lipid-binding domain involved in localization of PTEN to the inner face of the plasma membrane (Figure 1B) [3, 25]. TPTE2-3 corresponded to TPTE2+, identified by Walker et al. [27]. All three TPTE2 variants contained four transmembrane (TM) domains in the N-terminal half of the transcript (Figure 1B). These TM’s are absent in PTEN (Figure 1A). TPTE2-1 (Figure 1B) was selected for overexpression because of its high sequence homology to PTEN, including similar relative positions of the CDC14 and C2 domains (Figure 1A). To generate TPTE2-1 overexpression strains in the PTEN mutant PTEN+/−/−, a transformation plasmid was generated that contained the TPTE2-1 cDNA fused in frame to GFP and under the control of the cytomegalovirus (cmv) promoter (Figure 1C). The TPTE2 overexpression plasmid integrated ectopically into the genome of the mutant strain PTEN+/−/−. Two independent clones of TPTE2-1 overexpressors, TPTE2+/− and TPTE2+/−, were employed in subsequent analyses.

Proof of overexpression

TPTE2 has been found to be highly expressed in the testes, and at lower levels in spermatoocytes, the brain and stomach [26, 27, 47, 48]. It is expressed at negligible levels in other tissues [47, 48]. To demonstrate that TPTE2 was actually overexpressed in strains TPTE2+/−/− and TPTE2+/−, growth cultures were analyzed by RT-PCR. MCF-10A cells and the two PTEN+/− derivative mutants expressed TPTE2, but the PTEN+/− derivative mutants did so at approximately 60% and 40% that of MCF-10A cells (Figure 2A, 2B). TPTE2-+/− and TPTE2+/−, expressed TPTE2-1 at 3 fold and 2.5 fold, respectively the level of the PTEN+/− mutants (Figure 2A, 2B). To demonstrate that the fusion protein TPTE2-GFP was expressed, we employed indirect immunostaining, in which cells were permeablisized and fixed, treated with a mixture of two anti-GFP mAbs, DSHB-GFP-12A6 and DSHB-GFP-AC9, and then treated with the secondary fluorescent anti-
mouse IgGH+L Alexa 488 mAb. Over 80% of TPTE2-1oe and TPTE2-2oe-2 cells grown in tissue culture preparations exhibited bright diffuse staining (Figure 3A–3F), demonstrating that they expressed high levels of TPTE2-GFP throughout the cytoplasm. There was no staining in PTEN−/− mutant cells treated in a similar fashion (Figure 3G, 3H).

### Wound healing

In the model system *D. discoideum*, it was demonstrated that individual cells of the *ptenA* (human *PTEN* ortholog) mutant exhibited a variety of motility-associated defects, including a reduction in velocity, an increase in lateral pseudopod formation and an increase in turning, which affected the efficiency of chemotaxis and thus caused defects in multicellular morphogenesis [31–37]. Overexpressing the homolog of *ptenA*, lpten, in the mutant *ptenA* rescued all of these phenotypic defects [30].

Identification of similarly strong motility and chemotaxis differences between individual cells of strains MCF-10A and the *PTEN*−/− derivative strain to test whether TPTE2 overexpression could rescue mutant defects, proved difficult because of the low velocity and weak chemotactic responses of MCF-10A and PTEN−/− cells. We therefore exploited the characteristic of collective cell migration, the basis of wound healing [49–52]. It has been suggested that collective cell migration may, in fact, more accurately reflect the motile behavior of cells during tumorigenesis and metastasis [53–55]. *PTEN* had previously been implicated in this behavior [56, 57]. We therefore tested for, and identified, strong behavioral differences between the parental MCF-10A line and the two mutants, *PTEN*−/−-1 and *PTEN*−/−-2 in the wound healing process. In wound healing assays, cells are either removed from a confluent monolayer by scraping a gap (“wound”) [58, 59], or by removing an insert initially positioned in a monolayer, to generate a gap (“wound”) [60]. Cells at the two edges of

Figure 1: The *PTEN* and *TPTE2* transcripts, and integration of the *TPTE2* overexpression vector. (A) The *PTEN* and *PTEN* long transcripts. (B) The three *TPTE2* transcript variants, *TPTE2*-1, *TPTE2*-2 and *TPTE2*-3. (C) The ectopic, random integration of the construct containing the cmv promoter, *TPTE2* variant 1 (*TPTE2*-1) open reading frame, GFP tag, bGH-PA-terminator sequence and poly A tail. Box in upper right-hand corner of panel A shows color-coded domains in panels A and B.
the opposing confluent layers in both assays then fill the gap by collective cell migration [50, 58, 59]. Leader cells move into the wound and the two opposing sheets of cells collectively move in the direction of the leader cells, filling the gap [50, 61–63]. To test wound healing, we used the latter assay [60, 64]. To assess conditions that provided the most extreme differences between parental MCF-10A cells and the two derivatives PTEN\(^{-/-}\) strains, we grew the initial monolayer in DMEM+GFs, then monitored wound healing in the three media DMEM+GFs, DMEM+S,-other GFs and DMEM-GFs. In DMEM+GFs medium, there was an initial, small difference in the speed of wound healing between MCF-10A and the two PTEN\(^{-/-}\) clones, observed at 10 hours (Figures 4A, 5A–5C, respectively). However, both the MCF-10A and PTEN\(^{-/-}\) clones subsequently completed wound healing by 12.5 hours (Figures 4A, 5A–5C, respectively). Overexpressing TPTE2 in mutant clone PTEN\(^{-/-}\)-1, in the derivatives TPTE2\(^{oe-1}\) and TPTE2\(^{oe-2}\), retarded wound healing, resulting in rates not only below that of the two PTEN\(^{-/-}\) clones, but also below that of the parental strain MCF-10A cells (Figures 4A, 5D, 5E, respectively). Wound healing for the two PTEN\(^{-/-}\) TPTE2\(^{oe}\) derivatives was completed in 26 hours. These results suggested that overexpressing TPTE2\(^{oe}\) not only rescued the PTEN\(^{-/-}\)-associated trait in DMEM+GFs medium, but in this case accentuated the behavioral phenotype of the parental control, MCF-10A.

When the GFs other than serum were removed, in the medium DMEM + S,-other GFs, the rate of wound healing by MCF-10A cells was dramatically reduced (Figures 4A, 5A–5C, respectively). Overexpressing TPTE2 in mutant clone PTEN\(^{-/-}\)-1, in the derivatives TPTE2\(^{oe-1}\) and TPTE2\(^{oe-2}\), retarded wound healing, resulting in rates not only below that of the two PTEN\(^{-/-}\) clones, but also below that of the parental strain MCF-10A cells (Figures 4A, 5D, 5E, respectively). Wound healing for the two PTEN\(^{-/-}\) TPTE2\(^{oe}\) derivatives was completed in 26 hours. These results suggested that overexpressing TPTE2\(^{oe}\) not only rescued the PTEN\(^{-/-}\)-associated trait in DMEM+GFs medium, but in this case accentuated the behavioral phenotype of the parental control, MCF-10A.

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![Figure 2](image-url): Expression of TPTE2 in the control strain MCF-10A, the two PTEN\(^{-/-}\) strains and the two PTEN\(^{-/-}\) strains in which TPTE2 is overexpressed. Expression was measured by RT-PCR. (A) Expression of TPTE2 as percent control (MCF-10A). (B) Representative gel of TPTE2 expression. GAPDH expression was assessed to verify uniform loading of gels.
Parental MCF-10A cells underwent only 56% closure after 48 hours (Figures 4A, 4B, 6A). In contrast, the two PTEN\(^{-/-}\) strains underwent either near or complete closure in 24 hours (Figures 4A, 4B, 6B, 6C, respectively). Overexpressing TPTE2, in TPTE2\(^{-/-}\)-1 and TPTE2\(^{-/-}\)-2, reversed rapid closure of the gap and again accentuated the characteristic of slow closure exhibited by the parental line MCF-10A (Figures 4A, 4B, 6A, 6D, 6E, respectively). Whereas closure by MCF-10A was 56% complete after 48 hours, that of the two PTEN\(^{-/-}\)TPTE2\(^{OE}\) lines was 27% and 38%, respectively (Figures 4A, 4B, 6A, 6D, 6E, respectively).

When all GFs, including serum, were omitted, in DMEM-GFs medium, MCF-10A cells underwent only 10% closure by 48 hours (Figures 4A, 7A), whereas cells of the two PTEN\(^{-/-}\) mutants underwent 100% closure by 24 hours (Figures 4A, 4C, 7B and 7C, respectively). Overexpressing TPTE2 in the two overexpression strains reinstated the MCF-10A phenotype (Figures 4A, 4C, 7A, 7D, 7E, respectively). The percent closure was 10 and 1%, respectively in the two TPTE2 overexpression lines, after 48 hours (Figures 4A, 4C, 7D and 7E, respectively). Together, the results demonstrate that the slightly lower rate of wound healing by MCF-10A cells in the presence of GFs, and the extremely slow rate in the absence of GFs were lost in the PTEN\(^{-/-}\) mutants, which undergo rapid wound healing in the presence of GFs, but reestablished in PTEN\(^{-/-}\) mutants in which TPTE2 was overexpressed.

**Rate of cell division in the presence of GFs**

It was previously demonstrated that loss of PTEN, in PTEN\(^{-/-}\) mutant cells, conferred growth factor-independent proliferation [10]. We observed, both in tissue culture and in suspension cultures in DMEM+GFs medium, that it took longer for MCF-10A cells than PTEN\(^{-/-}\) cells to reach confluence in a tissue culture dish in DMEM+GFs medium. To test whether this difference was related to a shorter cell cycle and if so, whether overexpression of TPTE2 reinstated the slower rate of parental MCF-10A cells, we measured the time between cleavage furrows for 10 dividing cells in preconfluent preparations in DMEM+GFs medium on the plastic surface of tissue culture dishes. Examples of cleavage and the times of sequential cleavage furrows are presented for representative MCF-10A, PTEN\(^{-/-}\) and TPTE2\(^{-/-}\)-I cells.

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**Figure 3:** TPTE2\(^{-/-}\)-I cells express the TPTE2-GFP fusion protein. (A–F) Examples of TPTE2\(^{OE}\) cells expressing TPTE2-GFP. (G, H) Examples of PTEN\(^{-/-}\) cells. Cells were treated with a mixture of two anti-GFP mAbs and then stained with the secondary fluorescent antibody against mouse mAb, anti H+L Alexa 488 antibody.
in Figure 8A–8C, respectively. No differences were evident in the actual cellular mechanics of cell division. However, the average interval times between cleavage furrows differed. The mean ± standard deviation of interval times for 10 MCF-10A cells and 10 PTEN−/− cells were 17.3 ± 2.9 (N = 10) and 11.6 ± 0.7 (N = 10) hours, respectively, a decrease of 33%. The difference was significant (p value 3 × 10−5). The interval time for 10 PTEN−/−TPTE2OE−1 cells was 21.0 ± 4.3 hours, which was not significantly different from that of MCF-10A cells (p value 5 × 10−2), but significantly different from that of the PTEN−/− cells (p value 9 × 10−6). These results indicate that at the cellular level in the presence of GFs, the division rate of individual PTEN−/− cells was higher than that of MCF-10A cells and that overexpressing TPTE2 in a PTEN−/− background returned the rate of division to that of parental MCF-10A cells.

**Table 1:** Wound healing in the presence and absence of growth factors

| Cell line | DMEM + GFs | DMEM + S, other GFs | DMEM − GFs |
|-----------|------------|---------------------|------------|
| **Time (hr)** | **Wound (μm)** | **% zero time** | **Time (hr)** | **Wound (μm)** | **% zero time** | **Time (hr)** | **Wound (μm)** | **% zero time** |
| MCF-10A | 0 255 ± 3 0 | 0 305 ± 35 0 | 0 326 ± 6 0 |
| 10 59 ± 21 77 | 24 137 ± 35 55 | 24 293 ± 22 10 | 24 298 ± 16 10 |
| 12.5 0 100 | 48 134 ± 37 56 | 48 298 ± 16 10 |
| PTEN−/− | 0 272 ± 4 0 | 0 271 ± 5 0 | 0 310 ± 12 0 |
| 10 0 100 | 24 0 100 | 24 0 100 | 24 0 100 |
| 12.5 0 100 | 48 0 100 | 48 0 100 |
| PTEN−/−2 | 0 303 ± 3 0 | 0 264 ± 5 0 | 0 290 ± 6 0 |
| 10 0 100 | 24 63 ± 29 76 | 24 0 100 | 24 0 100 |
| 12.5 0 100 | 48 0 100 | 48 0 100 |
| TPTE2OE−1 | 0 247 ± 7 0 | 0 288 ± 5 0 | 0 270 ± 10 0 |
| 10 199 ± 15 19 | 24 257 ± 21 11 | 24 220 ± 53 19 | 24 220 ± 53 19 |
| 12.5 182 ± 24 27 | 48 211 ± 41 27 | 48 244 ± 29 10 | 48 244 ± 29 10 |
| TPTE2OE−2 | 0 332 ± 5 0 | 0 294 ± 5 0 | 0 355 ± 7 0 |
| 10 221 ± 27 34 | 24 201 ± 51 32 | 24 355 ± 7 0 | 24 355 ± 7 0 |
| 12.5 152 ± 40 54 | 48 199 ± 94 38 | 48 351 ± 9 1 | 48 351 ± 9 1 |

**Figure 4:** Overexpressing TPTE2 in PTEN−/− cells rescues mutant wound healing characteristics. Wound healing was tested in three media in which growth factors (GFs) were manipulated: DMEM+GFs medium, (DMEM+GFs), DMEM plus serum but lacking other GFs medium, (DMEM+S-other GFs) and, DMEM minus GFs medium (DMEM-GFs). (A) Wound healing data (mean ± standard deviation, N = 3) are presented as wound width in μm over time and percent of original wound width. Total time of experiment was predicated on the rate of total wound healing in the PTEN−/− mutant for each medium. (B) Data (means) graphed for wound healing as a function of time in DMEM+S, -other GFs medium. (C) Data graphed for wound healing as a function of time in DMEM-GFs medium.
Growth, adhesion and viability in the absence of GFs

It was previously demonstrated by measuring MTT reduction in cell preparations in the wells of tissue culture plates, that the viability of MCF-10A cells was dependent upon GFs, but the viability of mutant \(PTEN^{-/-}\) cells was independent [10]. We first analyzed whether overexpression of \(TPTE2\) reversed this mutant-associated characteristic, using the MTT reduction assay. In the assay MCF-10A, \(PTEN^{-/-}\) and \(PTEN^{-/-}TPTE2^{OE}\) cells were plated in the wells of a 96 well tissue culture dish containing DMEM-GFs medium. Cultures were assayed at zero, one, three and five days for MTT reduction. MCF-10A cells lost approximately 90% of MTT reduction activity after the first day (Figure 9A). In \(PTEN^{-/-}\) cultures, MTT reduction activity increased continuously over the five days of incubation, almost doubling by five days (Figure 9A). In \(PTEN^{-/-}TPTE2^{OE}\) cultures, MTT reductase activity was reduced by approximately 75% in one day, and by 100% in five days (Figure 9A), suggesting a loss in metabolic activity at a rate higher than in MCF-10A cultures (Figure 9A). Low magnification images of cells at the substratum of undisturbed preparations suggested that cells of all three cell lines adhered to the dish bottom for five days in DMEM-GFs, but that the density was higher in \(PTEN^{-/-}\) cultures (Figure 9B). In a longer analysis of MCF-10A cell cultures in DMEM-GFs medium, imaged at high magnification on the substratum, we found that spread cells were still present on the substratum, but three days after changing the medium with fresh DMEM-GF medium, cell density on the substratum decreased (Figure 9C). Low magnification images of the medium revealed that cells had released into the medium and had rounded-up (Figure 9D). Similar results were obtained for \(PTEN^{-/-}TPTE2^{OE}\) cells, but few released in \(PTEN^{-/-}\) cultures. These

![Figure 5](https://www.oncotarget.com)

**Figure 5**: Representative images in DMEM+GFs medium reveal that the \(PTEN^{-/-}\) mutant undergoes wound healing faster than parental MCF-10A cells and that overexpressing \(TPTE2\) in \(PTEN^{-/-}\) cells actually retards wound healing, accentuating the parental \((PTEN^{++})\) phenotype. (A) MCF-10A, (B) \(PTEN^{-/-}\), (C) \(PTEN^{-/-}\), (D) \(TPTE2^{OE}\), (E) \(TPTE2^{OE}\).
results together demonstrate that MCF-10A cells lose viability and release from the substratum when deprived of growth factors, but PTEN−/− derivatives are growth factors-independent, retain viability, continue to grow and remain attached to the substratum. Overexpression of TPTE2 in PTEN−/− cells reverse GF-independence, returning the phenotype to that of MCF-10A cells and actually speeding up the loss of metabolic activity.

In a final experiment to demonstrate that long term viability acquired by PTEN−/− cells is reversed by overexpressing TPTE2, cells were grown to confluency in DMEM+GFs medium, and the medium then substituted with DMEM-GFs medium and cultured for 33 additional days. The DMEM-GFs medium was replaced every seven days. At the end of this period, there were very few cells on the substratum of MCF-10A cultures, whereas PTEN−/− cultures contained cell that had formed aggregates (Figure 9E). Reintroduction of GFs after 33 days resulted in essentially no growth in the MCF-10A cultures, which were visually devoid of cells, but caused the cells in the PTEN−/− aggregates to spread and grow as polylayers (Figure 9E). Overexpression of TPTE2 and PTEN−/− cells reestablished the scenario observed for MCF-10A cells (Figure 9E).

**Viability in a 3D Matrigel matrix**

Transformed cells have been demonstrated to acquire anchorage-independent growth in soft agar [65]. Previous experiments in soft agar suggested that deletion of PTEN in MCF-10A cells did not confer this characteristic [10]. Here, however, using a 3D model [66, 67]

![Figure 6: Representative images in DMEM+S, -other GFs medium reveal that overexpressing TPTE2 in PTEN−/− cells slows the wound healing process, thus reestablishing and actually accentuating the slower rate of the parental MCF-10A cell line. (A) MCF-10A, (B) PTEN−/−-1, (C) PTEN−/−-2, (D) TPTE2oe-1, (E) TPTE2oe-2.](image-url)
of transparent Matrigel, which is composed primarily of laminin, collagen and heparin sulfate proteoglycans [68], in DMEM-GF medium, we found that PTEN<sup>−/−</sup> cells acquired anchorage independent growth and viability. When seeded in 3D Matrigel and incubated for 50 days, the great majority of MCF-10A cells died off (Figure 10A). The dramatic decrease in cells was verified by the negligible levels of staining with C<sub>12</sub>-resazurin (Figure 10A). C<sub>12</sub>-resazurin identifies metabolically active cells [69]. In marked contrast the two PTEN<sup>−/−</sup> strains formed large aggregates after 50 days, which stained with C<sub>12</sub>-resazurin (Figure 10B, 10C). Overexpression of TPTE2 reinstated the characteristics of cell death (Figure 10D, 10E). Similar results were obtained for 10 regions of duplicate preparations for each tested strain.

**Apoptosis marker**

Previous studies [10] and those described here demonstrated that deletion of PTEN results in the loss of the dependency of growth and viability on GFs and anchorage to a substratum, characteristics associated with apoptosis [38, 39]. Overexpressing TPTE2 in PTEN<sup>−/−</sup> cells reestablished the parental characteristics. We therefore tested whether MCF-10A cells and PTEN<sup>−/−</sup> cells overexpressing TPTE2 expressed apoptosis-associated annexin V binding sites and whether these sites were absent in PTEN<sup>−/−</sup> cells. This was indeed the case. The majority of MCF-10A cells grown to subconfluence in DMEM+GFs stained with fluorescently conjugated annexin V (Figure 11A). PTEN<sup>−/−</sup> cells did not bind

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**Figure 7:** Representative images in DMEM-GFs medium reveal that overexpressing TPTE2 in PTEN<sup>−/−</sup> cells reverses completely GF-independent wound healing exhibited by PTEN<sup>−/−</sup> cells, resulting in the absence of wound healing, the phenotype of parental MCF-10A cells. (A) MCF-10A, (B) PTEN<sup>−/−</sup>-1, (C) PTEN<sup>−/−</sup>-2, (D) TPTE2oe<sup>−/−</sup>-1, (E) TPTE2oe<sup>−/−</sup>-2.
annexin (Figure 11B). When TPTE2 was overexpressed in PTEN–/–, the majority again stained with annexin V (Figure 11C). Over 100 cells were analyzed for each cell line.

DISCUSSION

PTEN is a phosphoinositide 3 (PIP3) phosphatase that regulates the level of PIP3 by counteracting the phosphorylation of PIP2 to PIP3 by the kinase PI3K [70]. PIP3 is a cytoplasmic signal, which functions primarily by activating the AKT pathway, which regulates membrane trafficking, membrane-cytoskeletal interactions and a number of other basic cellular processes [71–73], some of which are involved in tumorigenesis [74–76]. By maintaining a low level of PIP3, PTEN suppresses motility [6, 8, 30, 36, 77, 78], cell multiplication [4, 10, 12, 15], adhesion [10], aggregation [30, 36], and resistance to apoptosis [4, 10, 18]. Mutations in PTEN are found in a variety of cancers, and is considered a secondary mutation involved in metastasis and tumorigenesis [3, 5, 7, 79–81]. PTEN localizes to the plasma membrane, endoplasmic reticulum, and mitochondrial membrane [82–85]. It attaches to the plasma membrane through interactions with the CBR3 loop associated with the PTEN-C2 binding domain [85–87]. Adhesion appears to be mediated by electrostatic interactions [88]. Reversing the effects of a PTEN mutation, therefore, could suppress tumorigenesis, either inhibiting or retarding it.

Figure 8: Overexpressing TPTE2 in PTEN–/– cells slows the accelerated rate of cell division to that of parental MCF-10A cells. The time between division furrows was assessed microscopically for 10 cells of MCF-10A, PTEN–/– and TPTE2oe, and found to be 17.3 ± 2.9, 11.6 ± 0.7 and 21.0 ± 4.3 hours, respectively. (A) Representative division of MCF-10A cells. (B) Representative division of a PTEN–/– cell. (C) Representative division of PTEN–/– cells in which TPTE2 is overexpressed (TPTE2oe–/–). a, parent cell; a1 and a2, daughter cells from first division; a1-1 and a1-2, and a2-1 and a2-2, daughter cells of second division; cf arrow, cleavage furrow.
One direct approach to reversing the loss of PTEN function would be to replace the mutated gene with a normal copy. In the application of this general approach to ovarian cancer involving a mutated \( P53 \) gene, the normal \( P53 \) gene, regulated by a strong constitutive promoter in the adenovirus vector SCH58500, was used to infect mutated cells in order to reestablish \( P53 \) function. Using this general technique in clinical trials, there were indications of increased patient survival [89–91], but the general approach has not matured to a level that leads to reliable inhibition or elimination of tumors [92–95]. Recently, we considered an alternative approach. To reestablish normal function in a PTEN mutant, we hypothesized that upregulating a functional homolog of PTEN, presumably by identifying a soluble signal or monoclonal antibody that accomplishes this, might reestablish PTEN function [30]. If this approach has any validity, it would first have to be demonstrated that a homolog of the mutated gene, when up-regulated or overexpressed, reversed the alterations in phenotype caused by the loss of PTEN function in a mutated cell. Our initial attempt to explore this hypothesis was performed in the model system \( D. discoideum \) [29]. \( D. discoideum \) contains \( ptenA \), an ortholog of human PTEN, and \( lpten \), a homolog of \( ptenA \) [31, 37]. We found that overexpressing \( lpten \) rescued all of the behavioral defects associated with deletion of \( ptenA \), in the mutant \( ptenA^- \) [30]. The defects reversed by overexpressing \( lpten \) in the \( ptenA^- \) mutant included a decrease in persistence of motility and increased turning, an increase in lateral pseudopod formation, decreased chemotactic efficiency, and an inability to undergo normal multicellular morphogenesis [30].

Given these results in \( D. discoideum \), we have here applied the strategy to test whether overexpressing a homolog of PTEN in a human PTEN\(^{-/-}\) human cell line, generated by targeted mutagenesis [10], would rescue the behavioral changes caused by the mutation. In past studies, it was demonstrated that the mutant PTEN\(^{-/-}\) cell lines differed from the parent cell line by caused by the loss of PTEN function in a mutated cell. Our initial attempt to explore this hypothesis was performed in the model system \( D. discoideum \) [29]. \( D. discoideum \) contains \( ptenA \), an ortholog of human PTEN, and \( lpten \), a homolog of \( ptenA \) [31, 37]. We found that overexpressing \( lpten \) rescued all of the behavioral defects associated with deletion of \( ptenA \), in the mutant \( ptenA^- \) [30]. The defects reversed by overexpressing \( lpten \) in the \( ptenA^- \) mutant included a decrease in persistence of motility and increased turning, an increase in lateral pseudopod formation, decreased chemotactic efficiency, and an inability to undergo normal multicellular morphogenesis [30].

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Figure 9: Overexpressing TPTE2 in PTEN\(^{-/-}\) cells reverses PTEN\(^{-/-}\)-associated increases in viability, adhesion and growth on a 2D substrate in the absence of GFs. (A) MTT reduction activity of undisturbed cell preparations over five days in DMEM-GFs medium. Measurements were made on total cells in the preparation. (B) Low magnification images of MTT preparations in panel A. (C) High magnification images of MCF-10A preparations on a 2D substrate, in which DMEM-GFs medium is replaced with fresh DMEM-GFs medium at seven days and incubated three additional days. Before and after medium change is noted. (D) Low magnification image of the supernatant of an MCF-10A cell preparation on a 2D substrate in which DMEM-GFs was replaced with fresh DMEM-GFs medium at seven days and incubated three additional days. (E) Images of cell preparations grown to confluency on a 2D substrate in DMEM+GFs medium (0 days), then cultured for 33 days in DMEM-GFs medium. During the 33 days, the DMEM-GFs medium was replaced every seven days with fresh DMEM-GFs. At 33 days the medium was replaced with DMEM+GFs and incubated nine additional days.
exhibiting growth factor-independent growth, increased adherence to tissue culture plastic, and increased resistance to apoptosis in the absence of growth factors [10]. It was also demonstrated that PTEN-/- cells abnormally remodeled the cortical actin cytoskeleton to form microtentacles that adhered to a coated tissue culture dish substrate [9], a change akin to the increase in the frequency of lateral pseudopod formation by D. discoideum ptenA cells. To test our hypothesis in the human PTEN-/- cell line PTEN-/-1, a homolog of PTEN, TPTE2 was overexpressed in PTEN-/-1, generating two individual TPTE2 overexpressor cell lines, TPTE2oe-1 and TPTE2oe-2. We hypothesized that because TPTE2 harbored the conserved catalytic domain, CDC14, of PTEN, it had the potential to function in a similar fashion to mediate the dephosphorylation of PIP3 to PIP2, and because it contained the conserved membrane binding domain C2, it had the potential to bind to PTEN binding sites. We further hypothesized that overexpression of TPTE2 would overwhelm the TPTE2 binding sites, and excess TPTE2 would then bind to unoccupied PTEN binding sites in the PTEN-/- mutant, even if the avidity was lower. To this end, we first identified phenotypic characteristics of the PTEN-/- mutant that differed markedly from the parental strain MCF-10A. The PTEN-/- characteristics we selected included 1) accelerated wound healing in the presence of GFs; 2) independence of wound healing on GFs; 3) a decrease in the rate of cytokinesis in the presence of GFs; 4) adhesion and viability in the absence of GFs; 5) anchorage-independent growth in the absence of GFs; and 7) loss of annexin V binding sites, a characteristic associated with apoptosis (Table 1). We demonstrate that overexpression of TPTE2 in PTEN-/- cells, in the cell lines TPTE2oe-1 and TPTE2oe-2, reversed all of the changes associated with the PTEN-/- mutation and for at least one case.

Figure 10: Overexpression of TPTE2 in the PTEN-/- mutant reverses the mutant-acquired capacity of cells to form aggregates and survive for extended periods in the absence of anchorage in a 3D Matrigel model, in DMEM-GFs medium. Cells were seeded in transparent 3D Matrigel in DMEM-GFs medium and incubated for 50 days, then analyzed by light microscopy and by staining with C12-resazurin, which identifies metabolically active cells.
actually accentuated the normal characteristic expressed in parental MCF-10A cells (Table 1).

**Mechanistic model**

Based upon the conserved domains in the PTEN homologs TPTE2, a mechanistic model of how TPTE2 overexpression compensates for the loss of PTEN function, is developed in Figure 12. In parental cells, PTEN (black arrows) occupies membrane binding sites (blue cups) through interactions mediated by the PTEN-C2 domain (Figure 12A). At these sites, PTEN catalyzes the dephosphorylation of PIP3 to PIP2, which keeps in check the concentration of PIP3, thus suppressing PIP3-activated pathways, most notably the AKT pathway [4, 10, 96, 97], resulting in the “normal” (control) phenotype (Figure 12A). TPTE2 (red arrows), which we have shown here is expressed in parental MCF-10A cells, binds to TPTE2 membrane receptors (yellow cups) in the endoplasmic reticulum [27] (Figure 12A). The selective binding of TPTE2 to these receptors is presumably facilitated in part by the four transmembrane

![Figure 11: Overexpression of TPTE2 in the PTEN–/– mutant reverses the mutant-acquired loss of annexin V binding, the latter a characteristic associated with apoptosis. Three pairs of representative images, light microscopic and fluorescent, are provided for annexin V-stained preparations.](image)

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Table 1: The mutant-acquired PTEN–/– characteristics reversed by overexpression TPTE2

| Characteristic                                           | MCF-10A | PTEN–/– | PTEN–/– TPTE2oe |
|----------------------------------------------------------|---------|---------|-----------------|
| Wound healing, +GFs<sup>a</sup>                         | +++     | +++     | ++              |
| Wound healing, +S, –other GFs<sup>a</sup>               | ++      | +++     | +               |
| Wound healing, –GFs<sup>a</sup>                         | –       | +++     | –               |
| Division rate, +GFs<sup>a</sup>                         | ++      | +++     | +               |
| Viability, on 2D substrate, –GFs<sup>a</sup>, undisturbed| –       | +++     | –               |
| Adhesion on 2D substrate and long term viability, –GFs<sup>a</sup> | –       | +++     | –               |
| Viability in 3D matrix, –GFs<sup>a</sup>                | –       | +++     | –               |
| Expression of annexin V surface binding site, +GFs<sup>a</sup> | +++     | –       | +++             |

<sup>a</sup>+GFs: plus growth factors. +S, –other GFs: plus serum, minus growth factors.

<sup>b</sup>“Relative level” represents a comparison to the maximum measured amongst the three genotypes. Maximum is ++++, descending (+++, ++, +) to zero (–).
domains (TMs) not present in PTEN (Figure 1), and, possibly, by the PTEN-C2 domain as well. In the PTEN−/− mutant, PIP3 levels increase due to the absence of PTEN dephosphorylation, a result of the absence of PTEN binding to PTEN sites (Figure 12B). Increased PIP3 lends to activation of the AKT pathway that results in the mutant phenotype (Figure 12B). We have shown that TPTE2 is expressed in PTEN−/− cells, but at levels approximately half that of parental cells. We presume that in PTEN−/− cells, TPTE2 would still target its normal binding sites and, in so doing, not replace PTEN at PTEN binding sites (Figure 12B). We assume that the avidity of TPTE2 to its own binding sites would be higher than its avidity to PTEN binding sites. However, when TPTE2 is overexpressed in a PTEN−/− background, TPTE2 saturates TPTE2 binding sites, and excess TPTE2 then binds to the unoccupied PTEN binding sites (Figure 12C). It is assumed that at the PTEN binding sites, TPTE2 substitutes functionally for PTEN, resulting in a reduction in the level of PIP3, thus reinstating the control cell phenotype (Figure 12C). There are of course alternative explanations for the results obtained. For instance, the 50% reduction in TPTE2 expression caused by deletion of PTEN may be basic to the PTEN−/− phenotype (i.e., the decrease in TPTE2 function results in the PTEN−/− phenotype), and overexpression of TPTE2 rescues the phenotype simply by restoring normal TPTE2 function, not by substituting for PTEN. This alternative explanation seems less plausible since it suggests that a 50% reduction in TPTE2 expression results in the PTEN−/− phenotype. Unfortunately, there have been no reports of a TPTE2−/− null mutant generated by targeted mutation. Our attempts to delete TPTE2 by targeted mutation have so far failed, possibly either as a result of the high level of redundancy, which includes two TPTE genes, TPTE1 and TPTE2, one TPTE1 pseudogene, and seven TPTE2 pseudogenes [23–28] (www.ncbi.nlm.nih.gov/gene; /www.uniprot.orgQ6xP23.www.ensemble.org), or the fact that the TPTE2 genes map to C-heterochromatin regions [98], which are less accessible to integration and, therefore, less amenable to targeted mutagenesis [99, 100].

**MATERIALS AND METHODS**

**Growth and maintenance of cell lines**

Two independent PTEN−/− mutants were previously generated by targeted mutagenesis, clone 1 (PTEN−/−-1) and clone 2 (PTEN−/−-2). Both contained targeted disruption...
of exon 2 of both PTEN alleles [10]. The parental strain of the mutants, MCF-10A, a non-tumorigenic human cell line derived from normal breast epithelium [101], the two derivative PTEN\(^{-/-}\) strains and the two TPTE2-overexpressing lines generated here from PTEN\(^{-/-}\)-I (TPTE2\(^{OE\text{-}I}\), and TPTE2\(^{OE\text{-}2}\)) were cultured in DMEM/F12 (DMEM) medium (Life Technologies, Carlsbad, CA, USA) supplemented with horse serum (S), human recombinant EGF (EGF), insulin (I), hydrocortisone (HC) and cholera toxin (CT), all obtained from Sigma Aldrich (St. Louis, MO, USA), and a penicillin-streptomycin mixture, obtained from Thermo-Fischer (Grand Island, NY) [10]. This fully supplemented medium will be referred to as “DMEM+S, otherGFs medium”, “GFs” referring to the full complement of growth factors. Medium containing serum, but lacking the other GFs (otherGFs: E, I, HC, CT) will be referred to as “DMEM+S,-otherGFs medium”, and medium lacking all growth factors will be referred to as “DMEM-GFs medium”. In the case of “DMEM+S,-otherGFs medium”, growth factors were charcoal-stripped (Valley Biomedical, Winchester, VA) from serum prior to its addition to DMEM medium.

**Generating PTEN\(^{-/-}\)-TPTE2\(^{OE}\) strains**

RNA of the parental strain MCF-10A was isolated as previously described in detail [30, 102], using Trizol (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s protocol. A 3’ RACE system with reverse transcriptase (Life Technologies, Carlsbad, CA, USA) was used to generate cDNA using 4 \(\mu\)g of total RNA and the primer AUP. The three identified TPTE2 variant cDNAs (TPTE2 variant 1 accession number KX610659, TPTE2 variant 2 accession number KX752070 and TPTE2 variant 3 accession number NM_199254) were amplified from the cDNA template using primers specific for the 5’ and 3’ ends of the TPTE2 transcript. Primers were designed using information provided by the EMBL (http://wwwensembl.org) for the TPTE2-I variant. cDNA was amplified using the primers TPTE2-1FW (5’-TCCACCCACCCATGACATTTAGTTCGACTG-3’), and TPTE2-1RT (5’-ATGAAAGTCC-3’), to amplify a 1.135bp fragment. PCR was performed using the Roche Expand Long Template Kit (Roche, Indianapolis, IN). Primers used to amplify a 1.135bp TPTE2-GFP fusion fragment were ATG120TPTE2 5’-AGTGGACACATTATGTTCGACTTCTACG-3’ ending at position 131 of the TPTE2 transcript and through 40 cycles, each consisting of 20 seconds at 92\(^\circ\) C, 30 seconds at 95\(^\circ\) C and 4 min at 70\(^\circ\) C. This was followed by a final elongation step of 10 min at 72\(^\circ\) C. Fragments were gel extracted (Qiagen, Ventura, CA, USA) and cloned into the plasmid pCR4.0 (LifeScience, Carlsbad, CA) for sequencing. Sequencing was performed at the Roy J. Carver Center of Genomics in the Department of Biology of the University of Iowa (http://biology.uiowa.edu/ccg). The TPTE2 variant TPTE2-1 was used for insertion into the transforming vector. The 5’-3’ primers were designed to begin at nucleotide 1383 in order to obtain in-frame fusion with GFP in the plasmid pcDNA3.1/CT-GFP TOPO (LifeScience, Carlsbad, CA). The stop codon was omitted to ensure that the TPTE2 and GFP sequences were in-frame and produced as a fusion protein. PTEN\(^{-/-}\) cells were grown in OptiMEM medium (Life Science, Carlsbad, CA) in 24 well tissue culture plates overnight. FuGene (Promege, Madison, WI) was employed to transfect cells with 1 \(\mu\)g/ml of plasmid containing the TPTE2-I-GFP fusion, according to the manufacturer’s instructions. PTEN\(^{-/-}\) cells were subjected to a G418 (Sigma-Aldrich, St. Louis, MO, USA) kill curve. The concentration of G418 used for selection was 150 \(\mu\)g/ml. Two TPTE2\(^{OE}\) clones were selected for further analysis using G418, TPTE2\(^{OE\text{-}I}\) and TPTE2\(^{OE\text{-}2}\). Note that both produced TPTE2-GFP transcripts.

**RT-PCR**

RNA was prepared as described in the previous section, using 2-step LongRange RT-PCR (Qiagen, Ventura, CA, USA). For reverse transcription, an Oligo-dT primer provided by the manufacturer was employed. Amplification was performed using the Roche Expand Long Template Kit (Roche, Indianapolis, IN). Primers used to amplify a 1.135bp TPTE2-GFP fusion fragment were ATG120TPTE2 5’-ATGGAACACATTATGTTCGACTTCTACG-3’ ending at position 131 of the TPTE2-I cDNA and GFP Reverse(CT-GFP) 5’-GGTAAAGTCCCTTGATGTACGCT-3’ and produced as a fusion protein. To analyze expression levels by RT-PCR, RNA was isolated as described above. Reverse Transcription was performed using Quantitec RT-PCR (Qiagen, Ventura, CA, USA). One \(\mu\)g of total RNA was denatured at 65\(^\circ\) C for 5 min, incubated in gDNA wipeout buffer for 2 min at 42\(^\circ\) C and immediately placed on ice. Reverse transcription was performed for 30 min at 42\(^\circ\) C with an Oligo-dT primer. The reaction was terminated by incubation at 95\(^\circ\) C for 3 min. Fifty ng of cDNA was used for amplification of a 200 bp TPTE2 fragment. The primers were RTfw 5’-TTGTTTGCCCTCCCTTATG-3’ and RTRv 5’-TCACATCATCATAAGAGTGAGGACCG-3’. GAPDH was used as a loading control, using the primers GAPDH Fw 5’-CATCTTCTTTCGGTGCTG-3’ and GAPDHrv 5’-CTAATGAGGCAAGGTGAGTCT-3’, to amplify an 891 base pair fragment. PCR was performed as described above, with minor changes. Primer elongation was performed for 1 min through 30 cycles for a GAPDH control fragment and through 40 cycles for the TPTE2 fragment. Gel-Electrophoresis was performed using a 3% agarose gel in TBE buffer. Images were taken with UV light under subsaturating conditions. Gel band image analysis was performed using Adobe Photoshop. To measure TPTE2 expression, integrated pixel density of the band area was determined. The integrated density is
the sum of the value of the pixels in a band, divided by the area of the band. TPTE2 expression was normalized to GAPDH expression determined in the same manner.

**Immunostaining TPTE2-GFP**

To immunostain the fusion protein in overexpression strains, cells were washed 3 times in TBS pH7.6/0.05% Tween 20 to remove growth media. Cells were fixed in 3.8% formaldehyde (Sigma Aldrich, St.Louis, MO) for 12 min. Fixed cells were washed 3 times in tris-buffered saline (TBS) pH7.6 containing 1% BSA (ACROS, Geel, Belgium), 0.05% Tween 20, to remove growth media. Fixed cells were washed 3 times in the TBS-Tween 20 buffer. Preparations were incubated for 2 hours in TBS pH7.6/1% BSA, 0.05% Tween 20 and 0.1% Triton X-100. Cells were then washed 3 times in the TBS-Tween 20 buffer. One ml of a 1:1 mixture of the anti-GFP monoclonal antibody (mAb) DSHB-GFP-12A6 and the anti-GFP mAb DSHB-GFP-4C9 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA 52245, USA [103], containing 10 µg of mAbs in TBS (pH7.6) plus 1% BSA, was added and cells incubated overnight. Excess primary antibody was removed by washing the cells 3 times with TBS (pH7.6) plus 0.05% Tween 20. The affinity-purified fluorescent anti-IgG H+L Alexa 488 antibody (Jackson Immunoresearch, Westgove, PA, USA) was used as a secondary antibody. It was diluted 1:300 in TBS (pH 7.6) plus 0.05% Tween 20, added and the preparation incubated for 2 hours. Cells were then washed 3 times in TBS (pH7.6) plus 0.05% Tween 20, and analyzed using a Nikon TE2000 inverted epifluorescent microscope (Nikon Instruments, Melville, NY, USA). Images were taken with a with an EOS Rebel T3i/EOS 600D camera (Canon, Lake Success, NY, USA).

**Wound healing assay**

A wound healing assay was employed that consisted of a two well culture dish in which the wells were separated by an insert, which, when removed, formed a gap (“wound”) (IBIDI, Madison, WI, USA). The assay monitored collective directional migration into the gap between the opposing confluent layers of cells [60]. Experiments were performed in triplicate.

**Assessment of the rate of cell division**

Cells at a concentration of $10^5$ per ml were inoculated into DMEM+GFs medium in a tissue culture dish and incubated in 5% CO$_2$ at 37° C. Individual cells, which distributed independently on the dish bottom, were monitored through an Olympus CK2 inverted microscope equipped with a XCD-V50 digital camera (Sony, San Diego, CA, USA). Images were obtained every 45 seconds. The time of cell division was taken as the period between the formation of cleavage furrows. Ten dividing cells of each line were analyzed. Images were obtained as previously described.

**MTT reduction assay**

The MTT reduction assay was performed according to procedures previously described [104, 105]. In brief, $3 \times 10^4$ cells in 150 µl of DMEM-GFs medium from cultures grown to near confluency in DMEM+GFs, were plated into wells of a 96 well tissue culture plate (Midwest Scientific, Valley Park, MO, USA). After 0, 1, 3 and 5 days 20 µl of 5 mg/ml MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Life Technologies, Carlsbad, CA, USA), were added. The plates were then incubated subsequently for 3.5 hour at 37° C in the absence of light. Microscopic images, were then taken using an Olympus CK2 inverted microscope equipped with a digital XCD-V50 camera (Sony, San Diego, CA, USA), and then150µl of a MTT solvent containing 5mM HCl, 0.2% Nonidet P-40 (NP40, Amresco, Solon, OH) in isopropanol was immediately added to each culture. The 96 well plates were then shaken for 15 min at 100 rpm protected from light at room temperature. The absorbance was determined using a Spectra max Plus 384 multiwell plate reader (Molecular Devices, Sunnyvale, CA, USA) at 590 nm. All cultures were compared to day 0. Experiments were performed in triplicate.

**Viability in the absence of growth factors on a 2D substrate**

Cells were first grown in DMEM+GFs medium to confluency in the wells of a 24 well tissue culture dish. In a first approach, the DMEM+GFs medium was gently replaced with DMEM-GFs medium (zero hours), cultured undisturbed for seven days, the DMEM-GFs medium gently replaced at seven days with fresh DMEM-GFs medium and cultured three additional days. Microscopic images were taken at high magnification at seven days before and after fresh medium replacement, and at 10 days. Microscopic images were also taken of supernatant at low magnification at 10 days. In a second, long term approach, DMEM+GFs medium was replaced with DMEM-GFs, and the DMEM-GFs medium gently replaced at 9, 18 and 24 days. At 33 days, the DMEM-
GFs medium was replaced with DMEM+GFs medium and incubated for nine additional days. The cell preparations were monitored microscopically with time and images obtained as described for the wound healing assay.

**Cell cultures a 3D Matrigel model**

Cells grown in DMEM+GFs medium were randomly dispersed in a 3D Matrigel model in DMEM-GFs, as described previously in detail [66, 67, 106–108]. In brief, a 30 mm glass insert at the bottom of a customized culture dish was coated with Matrigel previously hydrated in DMEM-GFs for 20 min at 5°C. This preparation was then incubated at 37°C for one hour to cause gelation. Then 5 × 10⁶ cells, grown in DMEM+GFs medium, were resuspended in 250 µl of DMEM-GFs medium, added to 500 µl of (5%) hydrated GF-reduced Matrigel (Corning, Life Science, Corning, NJ) at 5°C and the cell-Matrigel mixture distributed atop the basal cushion of Matrigel and incubated at 37°C in 5% CO₂ for 30 min, which caused Matrigel gelation. After gelation, cells were dispersed randomly throughout the 3D gel. At 50 days C₁₂−Resazurin (Life Technologies, CA) was added directly to the dishes at a final concentration of 500 nM and incubated for 1 hour. Metabolically active cells reduce nonfluorescent C₁₂−Resazurin to fluorescent C₁₂−resorufin. Preparations were imaged with a Nikon TE2000 inverted fluorescence microscope (Nikon Instruments, Melville, NY, USA) equipped with an EOS Rebel T3i/EOS 600D camera (Canon, Lake Success, NY, USA).

**Annexin V binding**

Cells were seeded on a 30mm glass bottom dish (Cellvis, Mountian View, CA, USA) and grown for 2 days in DMEM+GFs. Cell death was assessed using Annexin V CF 488A conjugate (Biotium, Fremont, CA, USA) on unfixed cells according to the manufacturer’s instructions. In brief, media was first removed and supernatant examined and substrate microscopically imaged. Cells on the substratum were then washed two times in 1X binding buffer (Biotium, Fremont, CA, USA). Cells were incubated in staining solution containing binding buffer and 1.25 µg/ml Annexin V conjugate for 30 min at room temperature. Stained cells were washed two times with binding buffer. Cells were imaged using a Nikon TE2000 inverted epifluorescent microscope (Nikon Instruments, Melville, NY, USA). Images were taken with an EOS Rebel T3i/EOS 600D camera (Canon, Lake Success, NY, USA) within 1 hour, as recommended by the manufacturer.

**CONCLUSIONS**

We have explored the hypothesis that loss of function resulting from a mutation or an epigenetic effect, of a gene that suppresses metastasis and/or tumorigenesis, may be reversed by up-regulating or overexpressing a functional homolog of that gene in the mutated cell. We previously demonstrated that this could be accomplished in a model system for a mutant of a PTEN ortholog [30], and here we have demonstrated it in a human epithelial cell line for a targeted PTEN mutant. These results support the possibility that this strategy may have the potential to be translated into a therapy for tumorgenesis. The next step in this strategy is to screen for a compound or antibody that, through interaction with a receptor, up-regulates TPTE2 in PTEN−/− cells. This general approach may also be applicable to other cancer-associated genes with known homologs.

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**CONFLICTS OF INTEREST**

None.

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