PTEN-induction in U251 glioma cells decreases the expression of insulin-like growth factor binding protein-2

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Abstract

PTEN is a tumor suppressor gene whose loss of function is observed in ~40–50% of human cancers. Although insulin-like growth factor binding protein-2 (IGFBP-2) was classically described as a growth inhibitor, multiple recent reports have shown an association of overexpression and/or high serum levels of IGFBP-2 with poor prognosis of several malignancies, including gliomas. Using an inducible PTEN expression system in the PTEN-null glioma cell line U251, we demonstrate that PTEN-induction is associated with reduced proliferation, increased apoptosis, and a substantial reduction of the high levels of IGFBP-2 expression. The PTEN-induced decrease in IGFBP-2 expression could be mimicked with the PI3-kinase inhibitor LY294002, indicating that the lipid phosphatase activity of PTEN is responsible for the observed effect. However, the rapamycin analog CCI-779 did not affect IGFBP-2 expression, suggesting that the PTEN-induced decrease in IGFBP-2 expression is not attributable to decreased mTOR signalling. Recombinant human IGFBP-2 was unable to rescue U251-PTEN cells from the antiproliferative effects of PTEN, and IGFBP-2 siRNA did not affect the IGF-dependent or -independent growth of this cell line. These results suggest that the clinical data linking IGFBP-2 expression to poor prognosis may arise, at least in part, because high levels of IGFBP-2 expression correlate with loss of function of PTEN, which is well known to lead to aggressive behavior of gliomas. Our results motivate translational research regarding the relationship between IGFBP-2 expression and loss of function of PTEN.

Keywords: PTEN; IGFBP-2; IGF-I

Insulin-like growth factor binding protein-2 (IGFBP-2) is the second most abundant IGFBP in the circulation and is found in a variety of human fluids and tissues [1]. Classically, IGFBP-2 was considered to be a growth inhibitor, as it had a well-defined role in sequestering the mitogens insulin-like growth factor (IGF)-I and IGF-II, therefore preventing binding and subsequent activation of mitogenic and anti-apoptotic pathways downstream of the IGF-I receptor (IGF-IR) [1]. However, increasing evidence indicates that under certain conditions, IGFBP-2 can act as a growth stimulator, and both IGF-dependent and -independent mechanisms have been proposed (for a review, see [2]). For example, transfection of an IGFBP-2 expression vector into the androgen-dependent prostate cancer cell line LNCaP resulted in faster progression to androgen-independence after androgen withdrawal [3]. Furthermore, IGFBP-2 has been shown to act as an IGF-independent mitogen for prostate [4] and adrenocortical tumors [5]. Clinical evidence that high levels of IGFBP-2 expression are associated with neoplastic growth is provided by studies which show that serum levels of IGFBP-2 are elevated in individuals with various types of

cancers including central nervous system [6], ovarian [7], lung [8], colon [9], and prostate [10,11]. As well, several reports have associated higher expression levels of IGFBP-2 within tumors with worse prognosis, including 4 independent reports showing a positive correlation between IGFBP-2 expression and grade of gliomas [12–15].

The tumor suppressor gene *PTEN* encodes a 403-amino acid phosphatase which has two distinct activities. First, it acts as a lipid phosphatase which dephosphorylates position 3 of the second messenger phosphatidylinositol(3,4,5)triphosphate (PIP_3) and therefore inhibits the phosphatidylinositol (PI)-3 kinase signalling pathway [16]. Second, PTEN is a dual-specificity protein phosphatase, capable of dephosphorylating serine, threonine, and tyrosine residues [16]. The PTEN gene is deleted or mutated in ~40–50% of human cancers [17–19], and loss of function of PTEN is known to be correlated with the aggressive neoplastic behavior of gliomas [25,26]. However, there have been no published reports examining the relationship between IGFBP-2 expression and PTEN status.

We wished to determine if IGFBP-2 expression is modulated by PTEN expression in an inducible PTEN expression vector system and investigate whether IGFBP-2 acts as a growth promoter and/or anti-apoptotic agent for PTEN-null tumors.

Materials and methods

Cell culture. The PTEN-null human glioma cell line U251 cells transfected with a tetracycline-inducible PTEN expression vector system were obtained from Dr. M.M. Georgescu (University of Texas) and cultured in DMEM (Invitrogen) supplemented with 10% FBS, 0.5 mg/ml G418, and 10 μg/ml blasticidin. Under these conditions, the expression of PTEN had been previously shown to be non-leaky [20]. PTEN expression was induced by the addition of 1 μg/ml doxycycline (Sigma Chemical, St. Louis, MO).

IGF-I and recombinant human IGFBP-2 were obtained from Protigen Incorporation (Mountain View, CA) and R&D Systems (Minneapolis, MN), respectively. The rapamycin analog CCI-779 was obtained from Wyeth (Pearl River, NY).

siRNA treatment. IGFBP-2 SMARTpool siRNA (four pooled siRNA duplexes) and a negative control lamin siRNA were purchased from Dharmacon (Lafayette, CO). U251 cells were plated at 100,000 cells/well in 6-well plates in regular growth media and were allowed to attach overnight. The following day, the appropriate siRNA was diluted in serum-free DMEM and was added to pre-diluted oligofectamine (Invitrogen) according to the manufacturer's protocol. After formation of oligofectamine:siRNA complexes, cells were washed twice with PBS and 1 ml serum-free DMEM was added to each well along with 250 μl of the oligofectamine:siRNA complex. Cells were incubated for up to 72 h and then assayed for growth effects and protein expression changes.

Cell proliferation assay. We used a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay to determine cell proliferation (cell viability). Cells (1.0×10^5/well) were plated in 6-well plates in regular growth media. After the appropriate treatment time (as indicated in figure legends), MTT (Sigma Chemical, St. Louis, MO) was added to a final concentration of 1 mg/ml, and the reaction mixture was incubated for 3 h at 37 °C. The resulting crystals were dissolved in 0.04% HCl in isopropanol and the absorbance was read at 562 nm. The data are presented in bar graphs as means plus or minus standard error of the mean. Each experiment was performed in triplicate. To assess the statistical significance of observed differences, we used Student's *t* test. All tests were two-sided, and *P* values less than 0.05 were considered to be statistically significant.

Western blotting. After each treatment, whole cell lysates were obtained using RIPA buffer (0.1 mM dibasic sodium phosphate, 1.7 mM monobasic phosphate, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, and apoprotin at 0.2 U/ml). Conditioned media were collected and concentrated ~20-fold using Centricons (Millipore, Bedford, MA).

Fifty micrograms of protein from cell lysates or concentrated conditioned media was resolved electrophoretically on denaturing 10–12% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were probed with antibodies specific for cleaved PARP (Biosource, Camarillo, CA), phospho-Akt (New England Biolabs, Beverly, MA), PTEN (Santa Cruz, San Diego, CA), phospho-p70s6k (Santa Cruz, San Diego, CA), or IGFBP-2 (Upstate, Lake Placid, NY). The position of protein was visualized with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, San Diego, CA). To confirm equal loading, membranes with cell lysates were stripped and re-probed using an antibody specific for α-tubulin (Santa Cruz, San Diego, CA), and membranes with concentrated conditioned media were stained with Ponceau Red solution (Sigma Chemical, St. Louis, MO) directly after the gel was transferred to the membrane.

Flow cytometry to assay cell cycle distribution and apoptosis. The treatment conditions for cell cycle analysis were similar to those used for the MTT assay. After the appropriate treatment period (as listed in the figure legends), adherent cells were collected using trypsin–EDTA and floating cells were collected by centrifugation. The cells were combined and washed twice with ice-cold phosphate-buffered saline (PBS) and fixed in 70% ethanol at −20 °C overnight. For cell cycle analysis, the cells were washed twice with ice-cold PBS and resuspended in propidium iodide buffer (PBS, 0.1% Triton X-100, 0.1 mM EDTA, 0.05 mg/ml ribonuclease A, and 50 μM propidium iodide). After 30 min at room temperature, the cell cycle distribution was determined by flow cytometry with a FACSCalibur (Becton–Dickinson, Franklin Lakes, NJ). The proportion of cells in the hypodiploid (sub-G1) area was considered to be apoptotic.

Results and discussion

In order to determine if IGFBP-2 expression is influenced by the functional status of PTEN, we utilized the U251-PTEN cell line, which was created by Radu et al.[20] by introducing a tetracycline-inducible PTEN expression vector into the PTEN-null human glioma cell line U251. As seen in Fig. 1A, addition of doxycycline to U251-PTEN cells resulted in the strong induction of PTEN expression at all time points assayed (19, 24, and 48 h). Furthermore, the induction of PTEN resulted in a significant decrease in the expression of IGFBP-2 (Fig. 1A). Western blot analysis of concentrated conditioned media confirmed our observed decrease in the expression of IGFBP-2 after the addition of doxycycline to U251-PTEN cells (Fig. 1B). This result provides new evidence that IGFBP-2 expression is regulated by PTEN. To verify that the decrease in IGFBP-2 expression in our system was an effect of PTEN-induction and not due to doxycycline alone, we treated parental (untransfected) U251 cells with 1 μg/ml doxycycline and did not observe any change in IGFBP-2 expression (Fig. 1B).

Since PTEN has both lipid and protein phosphatase activities, we wished to clarify the mechanism by which PTEN downregulates IGFBP-2 expression. The lipid phosphatase activity of PTEN is responsible for inhibiting activation of the PI-kinase/Akt pathway [16], and in our

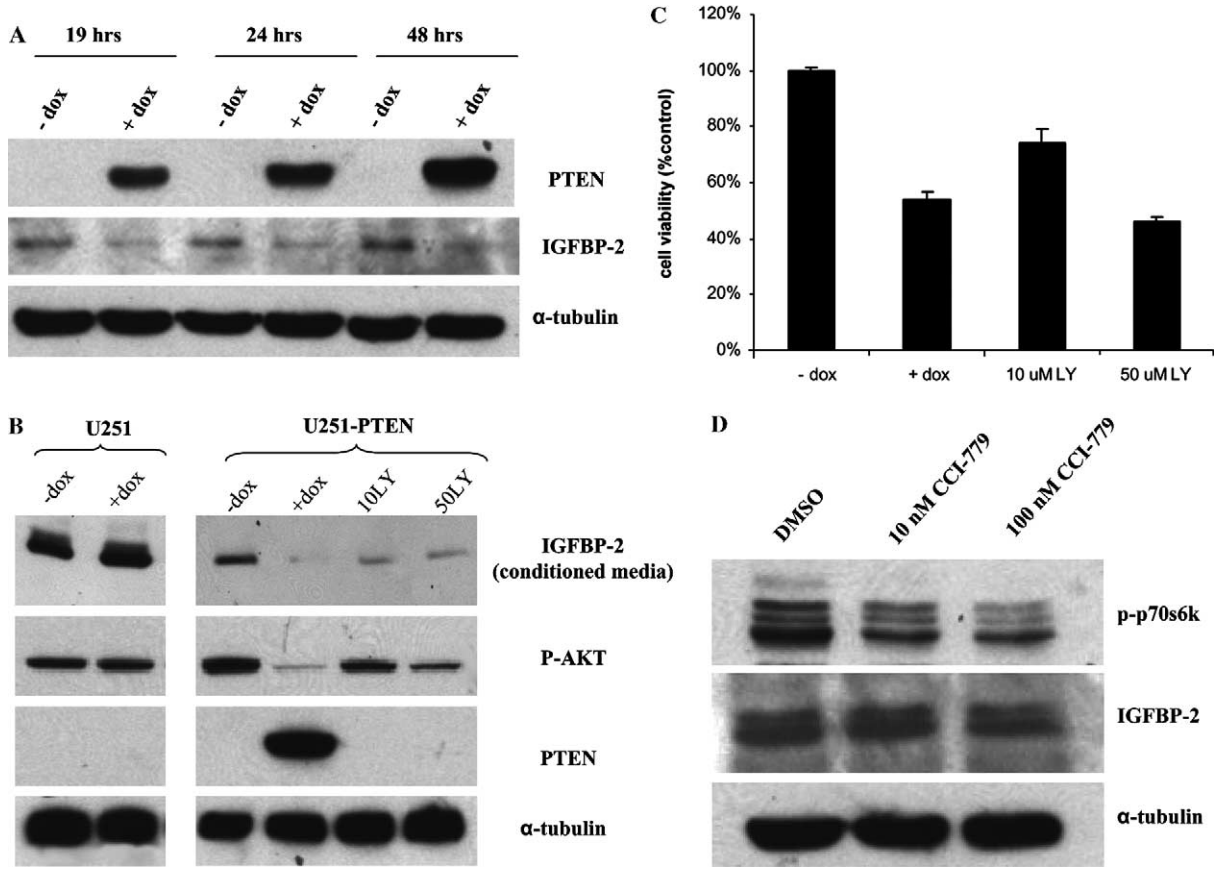

Fig. 1. PTEN-induction decreases IGFBP-2 expression in U251-PTEN cells. (A) Western blots on U251-PTEN whole cell lysates treated with or without 1 μg/ml doxycycline (dox) for 48 h in serum-free DMEM. (B) Western blot on U251-PTEN or U251 concentrated conditioned media and whole cell lysates treated with dox or the PI3-kinase inhibitor LY294002 in serum-free DMEM for 48 h. (C) MTT assay for U251-PTEN cell proliferation after 48 h with identical treatments as (B). All changes in cell viability were statistically significant when compared to control ($P < 0.05$). (D) Western blots on U251-PTEN whole cell lysates treated with the mTOR inhibitor CCI-779 or DMSO control, in the absence of doxycycline, for 48 h in serum-free DMEM.

system, PTEN-induction resulted in decreased levels of activated (phosphorylated) Akt (P-Akt) (Fig. 1B). Therefore, we wished to verify if the lipid phosphatase activity of PTEN is responsible for inhibiting IGFBP-2 expression. An indirect method to determine this was to investigate the effect of the PI3-kinase inhibitor LY294002 on IGFBP-2 expression. As seen in Fig. 1B, treatment with LY294002 resulted in decreased IGFBP-2 expression in both U251 and U251-PTEN cells, suggesting that PTEN downregulates IGFBP-2 expression via its lipid phosphatase activity. As expected, both PTEN-induction and LY294002-treatment reduced the proliferation of the U251-PTEN cell line (Fig. 1C). In order to determine if inhibition of mTOR signalling, which is downstream in the Akt pathway, can decrease IGFBP-2 expression, U251-PTEN cells were treated with the rapamycin analog CCI-779 [21] in the absence of doxycycline and IGFBP-2 expression was assayed after 48 h. As seen in Fig. 1D, although CCI-779 treatment resulted in decreased levels of phospho-p70s6k (p-p70s6k), the downstream phosphorylation target of mTOR, it had no effect on IGFBP-2 expression, suggesting that the mTOR pathway is not involved in the regulation of IGFBP-2 expression.

Classically, IGFBP-2 was considered to be a growth inhibitor, as it had a well-defined role in sequestering the mitogens IGF-I and IGF-II, therefore preventing binding and subsequent activation of mitogenic and anti-apoptotic pathways downstream of the IGF-IR [1]. However, increasing evidence indicates that under certain conditions, IGFBP-2 can act as a growth stimulator, and both IGF-dependent and -independent mechanisms have been proposed. Hypothesized mechanisms for IGF-dependent growth stimulatory effects include IGFBP-2 acting as a chaperone, presenting the IGFs to the IGF-IR, or IGFBP-2 acting by binding to and increasing the half-life of the IGFs. Several proposed mechanisms have been suggested for the IGF-independent growth effects of IGFBP-2, and for a review, see [2].

To determine if IGFBP-2 acts as a growth promoter and/or anti-apoptotic agent in U251-PTEN cells, we attempted to rescue cells from PTEN-induced growth inhibition with recombinant human IGFBP-2 (rhBP-2). Due to the presence of IGFBP-2 in FBS, we wished to perform these experiments under serum-free conditions. Radu et al. [20] have previously shown that PTEN induction in U251-PTEN cells is associated with G1 arrest, however

we found that under serum-free conditions, PTEN expression results in the induction of apoptosis, as evident by propidium iodide staining (Fig. 2A) and PARP cleavage (Fig. 2B). Treatment with 0.5 μg/ml rhBP-2 did not protect U251-PTEN cells from PTEN-induced apoptosis (Fig. 2B) or growth inhibition (Fig. 2C), which suggests that the clinical data linking IGFBP-2 expression to poor prognosis may arise, at least in part, because high levels of IGFBP-2 expression are simply correlated with loss of function of PTEN. Our observation that 0.5 μg/ml rhBP-2 did not increase proliferation under control (no doxycycline) conditions is consistent with this hypothesis (Fig. 2C). Alternatively, since rhBP-2 did not spontaneously enter the cytoplasm (Fig. 2B, comparing lane 1 to lane 3 and lane 2 to lane 4), these results did not rule out an anti-apoptotic role for intracellular IGFBP-2. A role for intracellular IGFBP-2 in various biological processes has been suggested [2,22] and recently, an intracellular binding partner of IGFBP-2 named IIp45 has been isolated in glioma cells [23], but its physiological role requires further clarification. Therefore, we wished to investigate if intracellular IGFBP-2 may have an anti-apoptotic role in our system.

To determine if downregulating intracellular IGFBP-2 expression effects U251-PTEN cell proliferation, cells in serum-free media were treated with 50 nM IGFBP-2 SMARTpool siRNA, which is composed of 4 siRNA duplexes targeting various regions of the IGFBP-2 mRNA. A lamin A/C siRNA was used a negative control, as lamin A/C is an endogenous housekeeping gene whose expression is non-essential over the short duration of silencing experiments (Dharmacon, Lafayette, CO). As seen in Fig. 3A, treatment of U251-PTEN cells with 50 nM IGFBP-2 siRNA resulted in a significant decrease in IGFBP-2 protein expression in cell lysates after 48 h. A similar decrease in IGFBP-2 expression was observed in the conditioned media (Fig. 3B). However, despite the ability of the IGFBP-2 siRNA to potently decrease IGFBP-2 expression, it had no effect on cell viability, as determined by the MTT assay after 72 h treatment (Fig. 3C). These results suggest that IGFBP-2 does not contribute to the viability of U251-PTEN cells. However, since our experiments were performed in serum-free media and U251 cells do not produce autocrine IGF-I and IGF-II [24], we wished to determine if downregulating IGFBP-2 expression may effect IGF-dependent growth in this system. U251-PTEN cells were treated in serum-free media with 50 nM IGFBP-2 siRNA for 24 h, and then 50 ng/ml recombinant human IGF-I was added to the culture media for an additional 48 h. As seen in Fig. 3C, addition of IGF-I to lamin siRNA treated cells resulted in a ~25% increase in cell viability. A similar increase in viability was observed when IGF-I was added to IGFBP-2 siRNA treated cells, indicating that the lower levels of IGFBP-2 did not effect the sensitivity of the cells to IGF-I. Therefore, decreasing IGFBP-2 expression with siRNA did not effect the IGF-dependent or -independent growth of U251-PTEN cells.

In summary, we have shown that IGFBP-2 expression in U251 glioma cells is regulated by PTEN functional status, but IGFBP-2 does not effect the IGF-dependent or -independent growth of this cell line. These results justify further

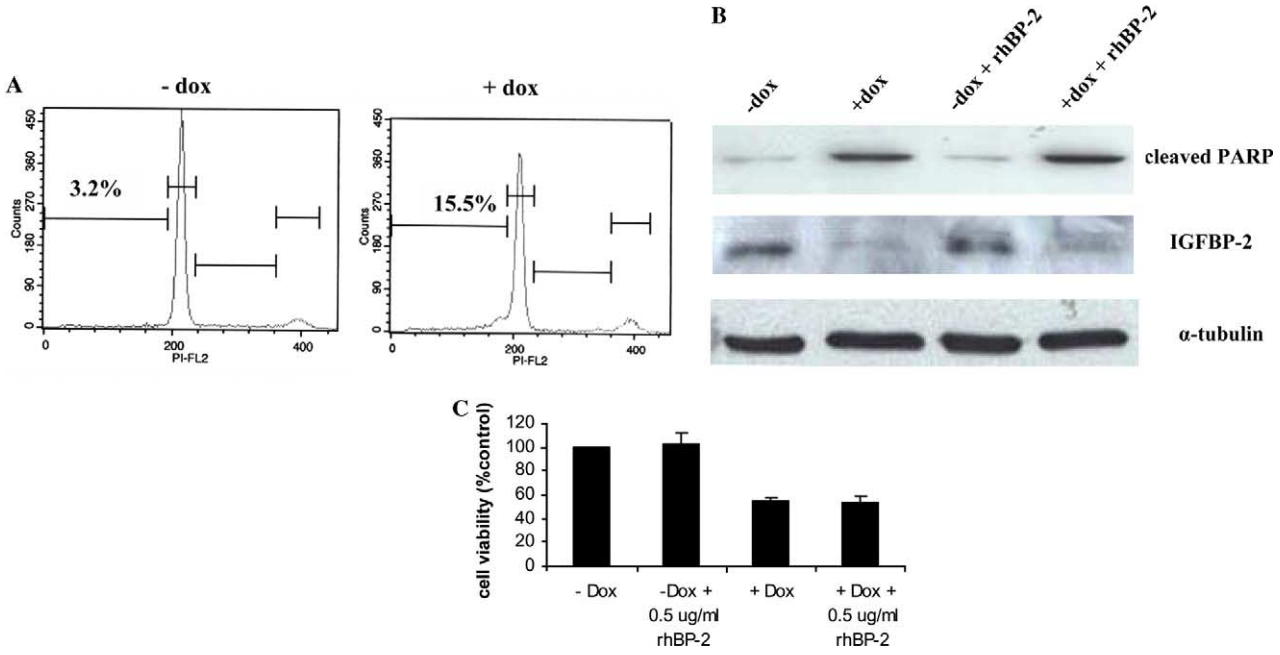

Fig. 2. Recombinant IGFBP-2 does not protect U251-PTEN cells from PTEN-induced growth inhibition and apoptosis. (A) Cell cycle analysis of U251-PTEN cells treated with or without dox for 48 h in serum-free DMEM (as described under Materials and methods). Cells in the sub-G1 peak were considered to be apoptotic. (B) Western blots on U251-PTEN whole cell lysates treated for 48 h with 1 μg/ml doxycycline (dox) with or without the addition of 0.5 μg/ml recombinant human IGFBP-2 (rhBP-2) in serum-free DMEM. (C) MTT assay for U251-PTEN cell proliferation after 48 h with identical treatments as (B). All changes in cell viability were statistically significant when compared to control ($P < 0.05$).

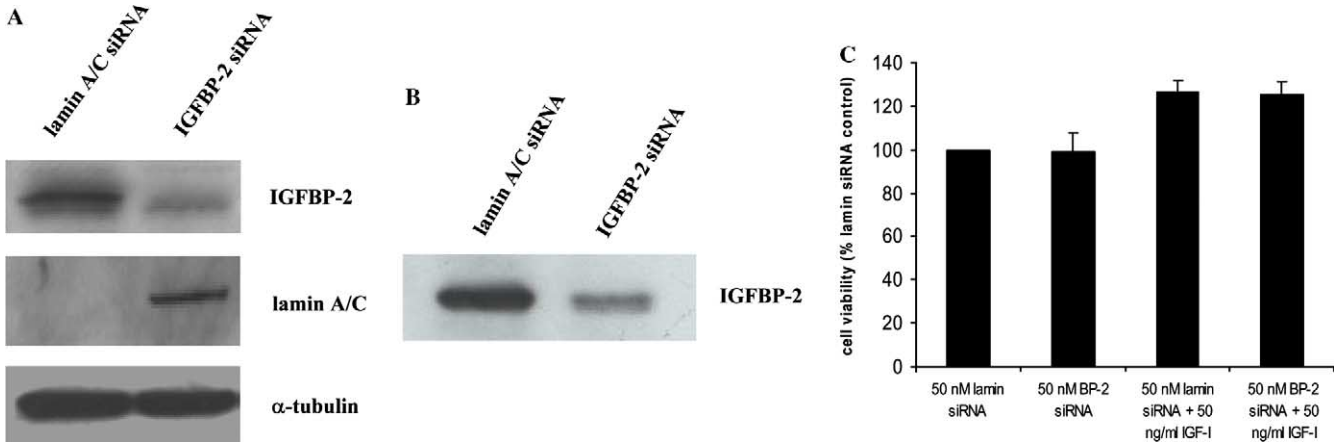

Fig. 3. IGFBP-2 siRNA does not effect the IGF-dependent or -independent growth of U251-PTEN cells. (A) Western blots on U251-PTEN whole cell lysates treated for 48 h with 50 nM IGFBP-2 siRNA or 50 nM lamin siRNA as a negative control in serum-free DMEM. (B) Western blot on U251-PTEN concentrated conditioned media treated for 48 h with 50 nM IGFBP-2 siRNA or 50 nM lamin siRNA as a negative control. (C) MTT assay for U251-PTEN cells treated for 72 h with 50 nM IGFBP-2 or lamin siRNA. After 24 h, 50 ng/ml IGF-I was added to some wells for the remaining 48 h. No statistically significant change in cell viability was observed between cells exposed to IGFBP-2 siRNA and cells exposed to lamin siRNA control. Addition of IGF-I to lamin siRNA and IGFBP-2 siRNA treated cells caused in each case a ~25% increase in cell viability compared to the viability in the presence of the siRNA and the absence of IGF-I ($P < 0.05$).

research to determine PTEN status of IGFBP-2 over-expressing human cancers. They suggest that the well-documented positive correlation of glioma grade with IGFBP-2 expression [12–15] may be at least partly accounted for by the association of IGFBP-2 overexpression with loss of function of the tumor suppressor PTEN, which is well known to lead to aggressive neoplastic behavior of gliomas [25,26].

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