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Journal of Cellular Biochemistry 111:1390–1400 (2010)

Journal of Cellular Biochemistry

PTEN Suppression Promotes Neurite Development Exclusively in Differentiating PC12 Cells via PI3-Kinase and MAP Kinase Signaling

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ABSTRACT

As a dual-specificity phosphatase catalyzing the dephosphorylation of phosphatidylinositols and protein substrates, PTEN is critically involved in the nervous system development. However, the regulatory role of PTEN in neurite outgrowth is still controversial, and the downstream signaling events remain elusive. Here, we show that PTEN knockdown promoted the proliferation and survival but not the neurite outgrowth of rat pheochromocytoma PC12 cells when exposed to nerve growth factor (NGF). In contrast, selective PTEN silencing in differentiating PC12 cells that express nestin significantly facilitated neurite elongation. Elevated Akt and Erk1/2 phosphorylation was involved in accelerated NGF-induced neurite development of PC12 cells following PTEN knockdown. Discriminated roles of the lipid phosphatase and protein phosphatase activities of PTEN in neurite development, as well as the detailed molecular profiles affected by these phosphatase activities, were defined by restored expression of a lipid phosphatase-deficient PTEN mutant following endogenous PTEN silencing in PC12 cells. Our study suggests an overall inhibitory effect of PTEN in neurite development reconciled by a probably indispensable role of this phosphatase in the initiation of PC12 cell differentiation. J. Cell. Biochem. 111: 1390–1400, 2010.

KEY WORDS: PTEN; PC12; RNA INTERFERENCE; NEURONAL DIFFERENTIATION; NESTIN; PI3 KINASE; MAP KINASE

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a dual-specificity phosphatase frequently mutated in human cancers [Myers et al., 1997; Shen et al., 2007; Keniry and Parsons, 2008]. By catalyzing the dephosphorylation of phosphatidylinositols and varied protein substrates, PTEN is critically involved in the regulation of cell survival, proliferation, migration, and differentiation [Tamura et al., 1998; Yamada and Araki, 2001; Zhu et al., 2006; Leslie et al., 2007]. PTEN dephosphorylates the D3 position of phosphatidylinositol-3,4,5-triphosphate (PIP3), thereby opposing the action of the phosphatidylinositol-3 kinase (PI3K). PIP3 binds and activates a class of PH domain-containing proteins, for example, PDK1 and Akt/PKB, resulting in the phosphorylation and activation of Akt by PDK. Activated Akt subsequently acts on diverse cellular targets to cause proliferation and protection from apoptosis [Yamada and Araki, 2001; Chalhoub et al., 2009]. In addition to signaling via Akt, PIP3 could activate the Rho GTPase family members Rac1 and Cdc42 via recruitment of their guanosine exchange factors (GEFs), which is implicated in the rearrangement of cytoskeleton and the development of various cell protrusions, for example, NGF-stimulated neurite outgrowth of rat pheochromocytoma PC12 cells and invadopodia formation of human melanoma cells [Murga et al., 2002; Nakahara et al., 2003; van Aelst and Cline,

Abbreviations used: NGF, nerve growth factor; GEF, guanosine exchange factors; IRS, insulin receptor substrate; CNS, central nervous system; siRNA, small interfering RNA; shRNA, small hairpin RNA; PDK1, phosphoinositide-dependent kinase 1.

Lintao Jia and Shaoping Ji contributed equally to this work.

Additional supporting information may be found in the online version of this article.

Grant sponsor: Natural Sciences and Engineering Research Council of Canada (NSERC); Grant sponsor: Canadian Institutes of Health Research (CIHR).

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Received 12 April 2010; Revised 20 August 2010; Accepted 24 August 2010 • DOI 10.1002/jcb.22867 • © 2010 Wiley-Liss, Inc.

Published online 9 September 2010 in Wiley Online Library (wileyonlinelibrary.com).

2004; Kumar et al., 2005; Aoki et al., 2007]. PTEN is also involved in the regulation of mitogen-activated protein kinase (MAPK) signaling [Ihara et al., 1997; Gu et al., 1998]. Although the direct target of PTEN remains to be investigated, PTEN is responsible for the dephosphorylation of Src homology 2 domain-containing protein, Shc, and possibly insulin receptor substrate (IRS)-I, both of which are adaptors coupling tyrosine kinases to Ras signaling by recruiting the Grb–Sos complex to the plasma membrane in a tyrosine phosphorylation-dependent manner [Gu et al., 1999; Weng et al., 2001].

PTEN plays essential roles in the development of the central nervous system (CNS), as evidenced by the enlarged neuron soma size, abnormal proliferation, and histoarchitectually disorganized brain following PTEN deletion in neural stem cells, differentiated neurons, or specifically in granules of the cerebellum and dentate gyrus [Backman et al., 2001; Groszer et al., 2001; Kwon et al., 2006]. The neural stem cell passages from PTEN knockout mice exhibited a greater self-renewal capacity and enhanced capability to form neurospheres while maintaining multipotent potential in differentiation [Groszer et al., 2006]. Conversely, PTEN overexpression in rat pheochromocytoma PC12 cells caused resistance of cells to nerve growth factor (NGF)-induced neurite outgrowth [Musatov et al., 2004], which is contradictory to a previous observation that antisense oligonucleotides-mediated suppression of PTEN also reduced the development of neurite in PC12 cells [Lachyankar et al., 2000]. Although these findings might be reconciled by another report that PI3K/Akt signaling suppresses neurite branching while promoting neurite elongation [Higuchi et al., 2003], the role PTEN plays during different stages of NGF-triggered differentiation of PC12 cells remains to be resolved. In addition, while the Ras/ERK and cAMP/PKA pathways downstream of the NGF receptor TrkA are believed to predominate in the signaling network responsible for NGF-induced neurite outgrowth, the activation of PI3K, p38 MAPK, and JAK/STATs was also involved in neuritogenesis of PC12 cells [Ihara et al., 1997; Iwasaki et al., 1999; Watanabe et al., 2004; Aoki et al., 2007]. Therefore, despite the recent advances in understanding PTEN's regulatory role in cell signaling, the mechanism via which PTEN knockdown affects the phenotypes of neural cells is yet to be elucidated [Vaudry et al., 2002; Endersby and Baker, 2008]. In this study, the expression of PTEN was constitutively suppressed in PC12 cells via vector-based shRNA, and selective silencing of PTEN in differentiating PC12 cells was achieved using a Cre/loxP shRNA expression system. The effects of PTEN suppression on cell growth and neurite development were studied in the context of these genetically modified cells, and the underlying signaling alterations were addressed.

MATERIALS AND METHODS

DNA CONSTRUCTION

pSilencer 3.1-H1 (Ambion, Inc., Austin, TX) was used for expression of PTEN-targeted shRNA. The sense and antisense oligonucleotides that encode the small hairpin siRNAs were synthesized by Invitrogen (Burlington, Ontario, Canada), annealed and cloned into the above *Bam*HI/*Hin*dIII-digested vectors. The 19 nt target sequences of PTEN shRNAs are as follows: aga gat cgt tag cag aaa c (nucleotides 18–36 of rat PTEN cDNA) and gtg aag acg aca atc atg t (nucleotides 338–356 of rat PTEN cDNA). pGFP-PTEN and the lipid phosphatase activity-dead PTEN construct pBabe-puro-PTENG129E (pBBP-PTENG129E) were kind gifts from Dr. Masahito Tamura in the National Institute of Health, USA.

For mutagenesis to generate shRNA off-target PTEN constructs, a Stratagene (La Jolla, CA) Quickchange site-directed mutagenesis kit was used and a two nucleotides mutation was introduced into PTEN cDNA, resulting in the replacements of the 24th nucleotide "C" with "A," and 33rd nucleotide "A" with "G" without affecting the encoded amino acids. Briefly, primers Ptm1 and Ptm2 and primers Ptm3 and Ptm4 were used for the first and second round PCR, respectively, for introduction of mutations into pGFP-PTEN according the manufacturer's instruction. The double mutant was then subjected to PCR amplification using primers Pt-U and Pt-D, and the resulting 1.2 kb fragment was cloned into *Eco*RI/*Sal*I-digested pBBP-PTENG129E, generating the off-target wild-type PTEN construct pBBP-WTPTEN-M2. A 900 bp *Bgl*II/*Sal*I fragment was further subcloned from pBBP-PTENG129E into pBBP-WTPTEN-M2, which resulted in the generation of the off-target PTENG129E construct pBBP-PTENG129E-M2. The sequences of the primers are, Ptm1, cat caa aga gat cgt tag cag gaa caa aag gag ata tca aga gg; Ptm2, cct ctt gat atc tcc ttt tgt tcc tgc taa cga; tct ctt tga tg; Ptm3, gac agc cat cat caa aga gat agt tag cag gaa caa aag gag; Ptm4, ctc ctt ttg ttc ctg cta act atc tct ttg atg atg gct gtc; Pt-U, ttt gaa ttc atg aca gcc atc atc aaa gag; and Pt-D, ttt gtc gac tca gac ttt tgt aat ttg tg.

The nestin promoter-controlled shRNA-expressing system was generated based on the U6-STOP-shA1 construct kindly provided by Dr. Klaus Rajewsky (CBR Institute for Biomedical Research, Harvard Medical School, Boston, MA). The PTEN shRNA-expressing cassette U6-loxP-STOP-loxP-PTENshRNA was generated by PCR amplification of U6-STOP-shA1 using primers U6U and siD1 (targeting nucleotides 319–339 of PTEN cDNA), U6U and siD2 (targeting nucleotides 19–37) or U6U and siDN (as a mismatched control). The resulting 650 bp fragments were then ligated into a pGEM-T Easy-1 vector (Promega, Madison, WI). The sequences of the primers are: U6U, ttg cgg ccg ctc tag atc cga cgc cgc cat ctc tag; siD1, tat cga taa aaa aga tct tga cca atg gct aag tct ctt gaa ctt agc cat tgg tca aga tct tcg tat aat gta tgc tat acg aa; siD2, tat cga taa aaa aga gat cgt tag cag aaa caa agc ttt gtt tct gct aac gat ctc ttc gta taa tgt atg cta tac gaa; and siDN, tat cga taa aaa agg aat gct tca agc aaa gaa agc ttt ctt tgc ttg aag cat tcc ttc gta taa tgt atg cta tac gaa. The pNecZ construct that expresses a Cre recombinase from a nestin promoter was a gift from Dr. Toshikuni Sasaoka (National Institute for Basic Biology, Myodaiji, Okazaki, Japan). A 13.7 kb *Not*I/*Sma*I fragment of pNecZ was then subcloned to pcDNA3 to generate pcDNA3-nestin-Cre. All constructs were identified by restriction enzyme digestion and confirmed by sequencing.

CELL CULTURE AND TRANSFECTION

The rat pheochromocytoma cell line PC12 was purchased from ATCC (Manassas, VA). Cells were plated in rat tail collagen I (BD Biosciences, Mississauga, ON, Canada)-coated vessels and maintained in Dulbecco's Modified Eagle Media (DMEM) supplemented with 15% horse serum and 2.5% fetal-bovine serum (FBS) at 37°C, 5% CO_2. Cells were transfected with the stated constructs in six-well

plates or 60 mm dishes using a Lipofectamine 2000 reagent (Invitrogen), followed by selection with either 400 μg/ml G418 (Invitrogen) or 10 μg/ml puromycin according to the antibiotic resistance of the plasmids.

NEURITE INDUCTION AND MEASUREMENT

Neurite outgrowth of PC12 cells was induced by addition of 50 ng/ml 7S NGF (Invitrogen) on the first day of induction and supplementation of 50 ng/ml NGF daily afterwards. Neurite-bearing cells were defined as those with processes equivalent in length to at least two cell diameters. Neurite number and length assays were performed using an Image-Pro Plus software with mean lengths determined by an average end-to-end measurement of neurites in 10 random cells.

RT-PCR AND QUANTITATIVE RT-PCR

Total RNAs of PC12 cells were prepared with a TRIzol reagent (Invitrogen), followed by first-strand synthesis of cDNAs using the Superscript II reverse transcriptase (Invitrogen). PCR amplification of WT PTEN or double-mutated PTEN was carried out using primers Ptn-U and Ptmn-D, or Ptm-U and Ptmn-D, respectively. The cDNAs of nestin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control were amplified with primers Nes-U and Nes-D, and Gad-U and Gad-D, respectively. The sequences of the primers were as follows. Ptn-U, cca tca tca aag aga tcg tta gca ga; Ptm-U, cca tca tca aag aga tag tta gca gg; Ptmn-D, cat ctt gtg aaa caa cag tgc cac; Nes-U, gga tga tgg ctt gag agg tgc; Nes-D, cta gtc ttc ccc tga gga cca; Gad-U, cct tca ttg acc tca act ac; Gad-D, gga agg cca tgc cag tga gc. For quantitative RT-PCR (qRT-PCR), cDNA was detected using SYBR Premix Ex Taq™ (TaKaRa), and all quantifications were normalized to GAPDH. The primers used to partially verify the microarray data (Suppl. Tab. 1) were listed in Suppl. Tab. 2.

WESTERN BLOTTING

Cells were washed twice with ice-cold PBS, and total cell lysates were prepared via scraping in modified RIPA lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM PMSF and 1× protease inhibitor cocktail (Sigma)). For phosphorylation analysis, 1 mM sodium orthovanadate and 1 mM NaF were added into the RIPA lysis buffer. After protein quantification using a BCA Protein Assay Kit (PIERCE, 23227), lysates containing 20–50 μg protein was loaded and separated by 10% SDS–PAGE, transferred to PVDF (Bio-Rad), and incubated sequentially with appropriate primary antibodies and horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit secondary antibody (Cedarlane). The blots were developed using a SuperSignal West Pico Chemiluminescent kit (Pierce) or an ECL Western blotting detection system (Amersham Biosciences). The primary antibodies used for blotting included ERK1/2, phospho-ERK1/2, Akt, phospho-Akt (ser 473), phospho-TrkA, β-actin, caspase 9 (Santa Cruz), PTEN, phospho-p38 (Cell Signaling), and nestin (Chemicon) antibodies.

IMMUNOCYTOFLUORESCENT MICROSCOPY

PC12 cells cultured on collagen I-coated coverslips or neurons cultured on poly-D-lysine-coated coverslips were subjected to immunofluorescent staining at indicated time. Briefly, cells were washed twice with ice-cold PBS, fixed with 4% paraformaldehyde, and permeablized via incubation with 0.5% Triton X-100. Cells were then blocked with PBS-T (0.1% Triton X-100) containing 10% goat serum, incubated with a primary antibody and an appropriate Alexa Fluor 488 or 568 Dye-labeled goat secondary antibody (Molecular Probes). Cells were ultimately observed under a fluorescent microscope. The primary antibodies used for staining included β-tubulin (Santa Cruz) [Yu et al., 2006], Tuj1 (Covance) [Su et al., 2004], nestin (Chemicon) [Zang et al., 2008], and PTEN (Chemicon) [Fu et al., 2008].

MICROARRAY AND GENE EXPRESSION COMPARISON

The PC12 cells stably expressing PTEN shRNA (PC12-PS5) were further transfected with the off-target constructs of wild-type PTEN or the lipid phosphatase activity-dead mutant, PTENG129E. Five single cell clones that exhibit comparably high expression of wild-type or mutant PTEN were obtained and propagated. Cells from each transfection were then pooled and harvested, and total RNAs were prepared using an RNeasy Mini Kit (QIAGEN). RNA samples were subjected to quality assessment by the Agilent Bioanalyzer 2100, followed by cDNA synthesis and gene expression analysis using the

TABLE I. Assessment of the Effects of PTEN's Lipid and Protein Phosphatase Activities on Gene Expressions

Logarithm values			Assessment	Designated abbreviation
$-1 < \log_2 X1 < 1$	$-1 < \log_2 X2 < 1$		Not affected by PTEN	N
	$\log_2 X2 \leq -1$ or $\log_2 X2 \geq 1$		Contrarily affected by the lipid and protein activity of PTEN	D−
$\log_2 X1 \leq -1$ or $\log_2 X1 \geq 1$	$-1 < \log_2 X2 < 1$		Exclusively affected by the lipid activity of PTEN	L
	$\log_2 X2 \leq -1$ or $\log_2 X2 \geq 1$, contrary direction		Contrarily affected by the lipid and protein activity of PTEN	D−
	$\log_2 X2 \leq -1$ or $\log_2 X2 \geq 1$, same direction	$-1 < \log_2 X3 \leq -1$	Exclusively affected by the lipid activity of PTEN	P
		$\log_2 X3 \leq -1$ or $\log_2 X3 \geq 1$, same direction	Cooperatively affected by the lipid and protein activity of PTEN	D+
		$\log_2 X3 \leq -1$ or $\log_2 X3 \geq 1$, contrary direction	Contrarily affected by the lipid and protein activity of PTEN	D−

X1, Gene expression ratios of pkw cells to pk cells; X2, gene expression ratios of pkh cells to pk cells; X3, gene expression ratios of pkw to pkh cells. pk, constitutive PTEN knockdown cells (pooled clones); pkw, pk cells further transfected to express the off-target wild-type PTEN; pkh, pk cells further transfected to express the off-target PTEN mutant with half phosphatase activity, that is, protein phosphatase activity only (PTENG129E).

Rat Genome 230 v2.0 Affymetrix array system performed at the StemCore Laboratories of the Ottawa Health Research Institute.

The expression ratios (X) between differentially modified PC12 cells were expressed as logarithms to the base 2 ($\log_2 X$). Whereas the $\log_2 X$ values ≥ 1 or ≤ -1 were considered significant difference arbitrarily, only genes with $\log_2 X$ values >2 or <-2 between the PTEN knockdown plus WT PTEN re-introduction cell group (pkw) and simply PTEN knockdown cell group (pk) were presented. The effects of the lipid and protein phosphatase activities of PTEN on the expression of a certain gene were estimated as shown in Table I.

STATISTICAL ANALYSIS

All values are reported as means ± SEM. Differences were assessed by two-tailed Student's *t*-test using the Excel software. $P < 0.05$ was considered to be statistically significant.

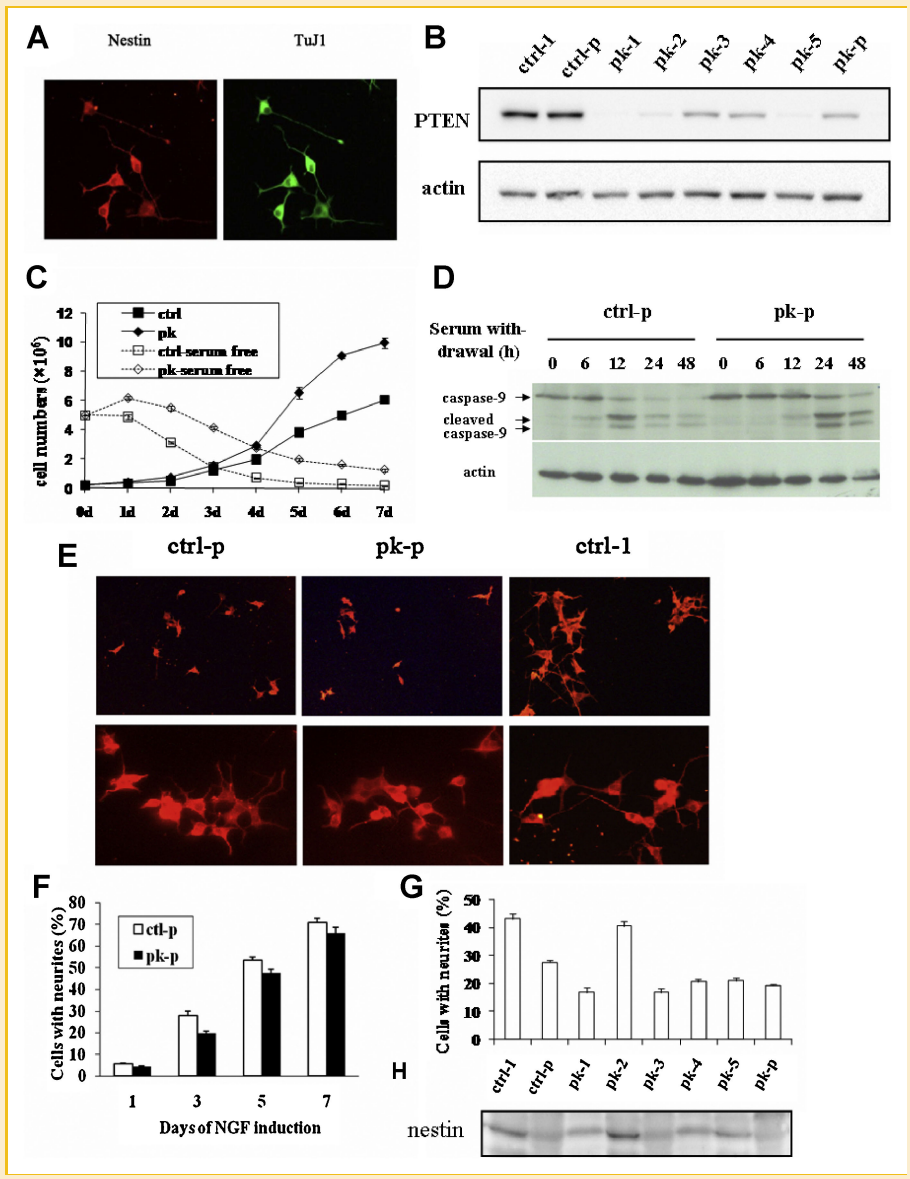

Fig. 1. PTEN knockdown in PC12 cells resulted in improved proliferation and survival, but heterogeneous responses to NGF-induced differentiation. A: PC12 cells were cultured in complete media supplemented with 50 ng/ml NGF for 5 days, and subjected to staining for the indicated markers (Magnification 400×). B–G: PC12 cells were transfected with a pSilencer3.1/H1-neo construct of PTEN shRNA (pk) or empty vector (ctrl), and stable single (ctrl-1 and pk-1 to pk-6) and pooled (ctrl-p and pk-p) clones were obtained. pk, PTEN knockdown clones. B: PTEN expression was analyzed by Western blotting. C: Control (ctrl) and PTEN knockdown (pk) cell clones were cultured in complete media or in serum-free media, and viable cells were counted at the indicated time after trypan blue staining. D: Cells cultured in serum-free media were harvested and subjected to Western blotting of caspase-9. E–G: Cells were cultured in complete media supplemented with 50 ng/ml NGF for indicated time (F) or for 3 days (E, G) with daily replenishment of NGF. Cells were then visualized by immunofluorescent staining of β-tubulin (E), and cells with neurites, that is, cells bearing neurites equivalent to or longer than two cell diameters, were counted (F, G). Data represent the means of results obtained from three random microscope fields. $^*P < 0.05$ compared with ctl-p. Magnifications in (E): upper panel, 200×; lower panel, 400×. H: PC12 cell clones was cultured in the absence of NGF, and harvested for Western blotting analysis of nestin.

RESULTS

PTEN KNOCKDOWN PC12 CELLS ARE MORE PROLIFERATIVE AND ANTIAPOPTOTIC BUT ARE HETEROGENEOUS IN THEIR RESPONSES TO NERVE GROWTH FACTOR

NGF-induced neurite outgrowth of PC12 cells is a well-documented model for neuronal differentiation studies. PC12 cells undergoing neurite outgrowth, but not undifferentiated PC12 cells establish the expression of nestin, a type VI intermediate filament protein detected specifically in neural progenitors, suggesting that nestin could also serve as a marker of differentiating PC12 cells (Fig. 1A and data not shown). In addition, unlike neural stem cells or differentiated neurons which express the intermediate filament protein, nestin, and the class III β-tubulin, Tuj1, respectively, PC12 cells undergoing neurite outgrowth showed the expression of both markers (Fig. 1A). To investigate the role of the phosphatase PTEN during this process, vector-based small hairpin RNA (shRNA) was used to knockdown the expression of PTEN in PC12 cells. As shown in Fig. 1B,C, PTEN knockdown in PC12 cells resulted in significantly increased proliferation in normal growth condition, as well as improved cell survival after serum withdrawal, which is consistent with a delayed activation of the apoptosis executioner caspase-9 (Fig. 1D). However, when exposed to NGF, the pooled PTEN knockdown cells failed to exhibit accelerated neurite outgrowth compared with the mock-transfected cells (Fig. 1E,F). To further study the effects of PTEN suppression on neurite development, we cloned the transfected cells and cultured them in the presence of NGF. Whereas the neurite outgrowth in a majority of the cell clones were comparable to that in wild-type PC12 cells, we observed accelerated neurite outgrowth and increased ratios of cells that undergo neurite outgrowth in 4 of 56 clones of PTEN knockdown cells (Fig. 1G and data not shown), and unexpectedly, in two mock-transfectant clones. Western blotting analysis excluded a direct correlation of PTEN expression levels with the neurite outgrowth variation (Fig. 1B,G), suggesting an intrinsic heterogeneity of PC12 cells in their responses to NGF induction. Thus, PTEN knockdown in PC12 cells caused accelerated proliferation and improved survival following serum withdrawal, but not increased neurite outgrowth when exposed to NGF.

ELEVATED AKT AND MAP KINASE SIGNALING IN PTEN KNOCKDOWN PC12 CELLS

In a further attempt to dissect the cell signals that account for NGF-induced neurite outgrowth, we found that PTEN knockdown caused a significant increase in phosphorylated Akt and ERK1/2 but not phosphorylated p38 MAPK or the NGF receptor TrkA (Fig. 2A), while delayed Akt dephosphorylation was observed following NGF withdrawal (Fig. 2B). Elevated NGF-induced Akt and Erk1/2 phosphorylation was detected in both cells undergoing accelerated neurite outgrowth and cells showing unchanged neurite development after PTEN knockdown (Fig. 2C). A similar increase in NGF-induced Akt and Erk1/2 phosphorylation was detected in the mock-transfected cell clone, which exhibited accelerated neurite development compared with other mock-transfected or wild-type cells (Fig. 2C). In addition, a moderate rather than dramatic increase in ERK1/2 phosphorylation was observed in PTEN knockdown cells with improved neurite outgrowth in comparison with wild-type

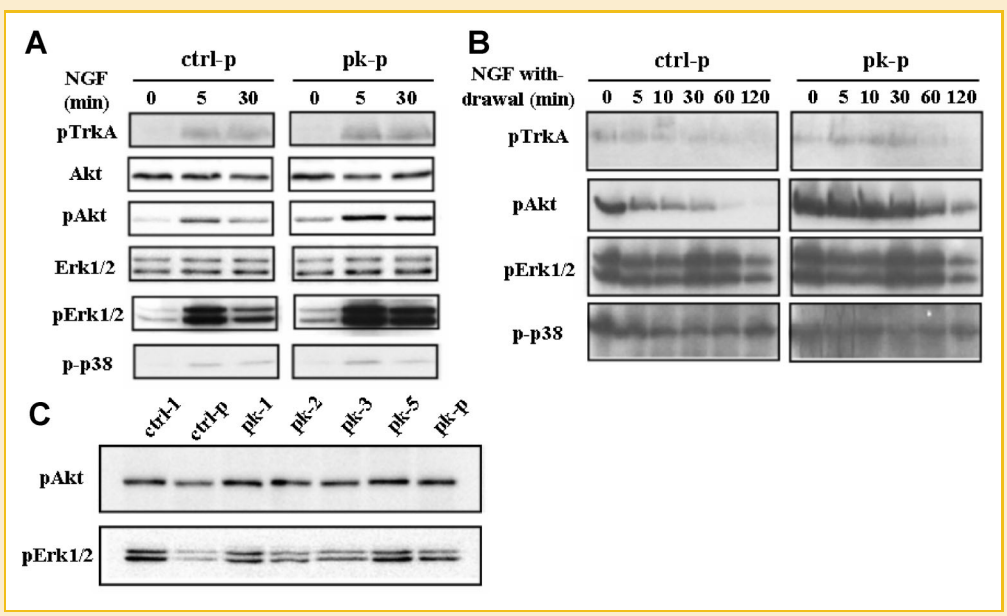

Fig. 2. Improvement of NGF-induced PI3K and MAPK signaling in PTEN knockdown PC12 cells. A,B: Pooled PC12 cell clones transfected with PTEN shRNA or empty vector were cultured in complete media and exposed to 50 ng/ml NGF for indicated time (A). Alternatively, cells were incubated in media containing 50 ng/ml NGF for 5 min, followed by NGF withdrawal and a continued culture for indicated time (B). Cells were then harvested for Western blotting analysis. C: Single and pooled PC12 cell clones transfected with PTEN shRNA or empty vector were cultured in media supplemented with 50 ng/ml NGF for 5 min. Cells were then harvested for Western blotting analysis of the indicated proteins. ctrl-1 and ctrl-p, single clone and pooled clone of PC12 cells transfected with a pSilencer3.1/H1-neo vector; pk-1 to pk-5 and pk-p, single clones and pooled clone of PC12 cells transfected with a pSilencer3.1/H1-neo construct of PTEN shRNA.

PC12 cells (Fig. 2C). These data suggest that the activation of Akt and Erk1/2 is elevated by PTEN knockdown, which is insufficient for an improvement of NGF-induced neurite outgrowth of PC12 cells.

RESTORED PROTEIN PHOSPHATASE ACTIVITY OF PTEN CAUSED ACCELERATED NEURITE DEVELOPEMNT AND ALTERED GENE EXPRESSION PROFILES

As a dual-specificity phosphatase, PTEN might regulate the signal pathways of PC12 cells and exert multifaceted effects on cell behaviors by catalyzing the dephosphorylation of either its lipid or protein substrates. To distinguish the role PTEN plays as a lipid phosphatase from the signal events mediated by its protein phosphatase, PTEN knockdown PC12 cells were further transfected with the constructs of wild-type PTEN and a G129E mutant, which lacks lipid phophatase activity but has intact protein phosphatase activity. Both constructs contain two nucleotides mutations in the target sequences of PTEN shRNA, which is enough to protect the transcripts from shRNA-directed degradation (Fig. 3A,B). Western blot analysis confirmed the enforced expression of these off-target PTEN mutants (Fig. 3C). Whereas the restored expression of wild-type PTEN inhibited the neurite development of PC12 cells after exposed to NGF for 7 days, an ectopic expression of lipid phosphatase activity-dead PTEN resulted in a significant improvement in NGF-induced neurite outgrowth in the context of PTEN knockdown PC12 cells as reflected by both the percentages of cells with neurites (Fig. 3D) and the average total neurite lengths per cell (Fig. 3E), suggesting a positive role of the protein phosphatase activity of PTEN in neurite development. Microarray analysis revealed different gene expression profiles in these transfectants. In principle, the discriminated expression levels of an individual gene in these transfectants may reflect a certain regulatory pattern of this gene by the lipid and/or protein phosphatase activities of PTEN, for example, the genes undergoing altered expression in WT PTEN—but not the G129E mutant-transfected cells when compared with the untransfected PTEN knockdown PC12 cells represent genes regulated merely by the lipid phosphatase activity of PTEN, probably via the PI3K pathways (Table II and Suppl. Tab. 1). Further studies on these genes will be beneficial to elucidating the

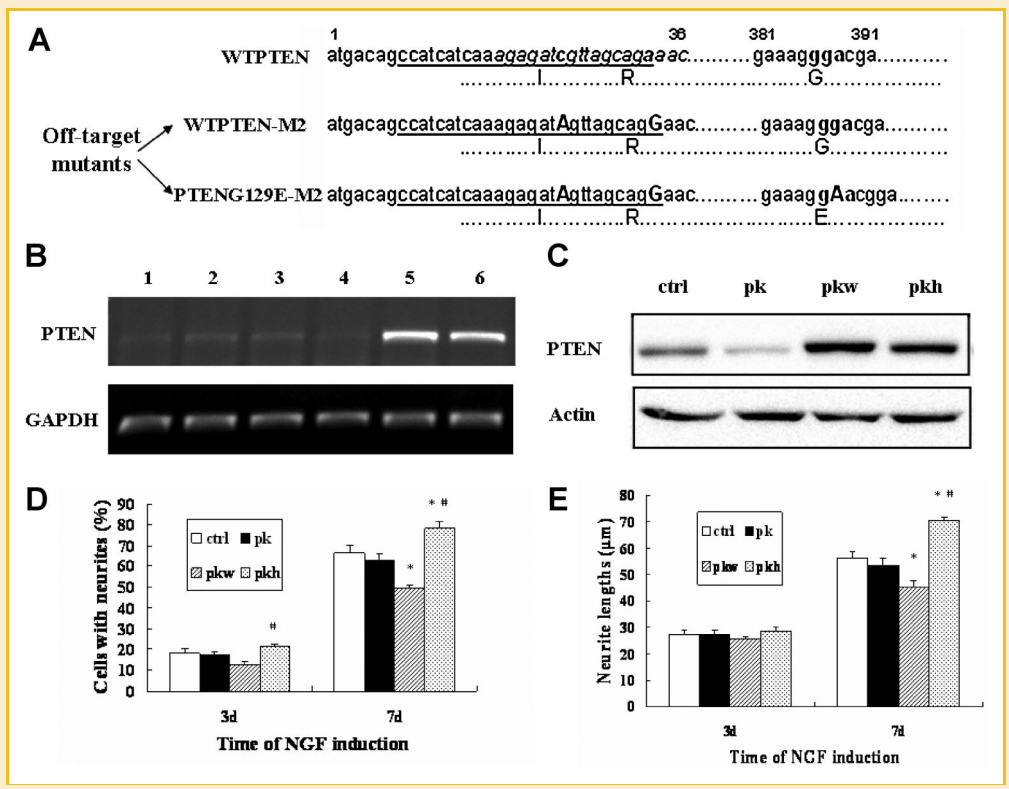

Fig. 3. Restoration of wild-type or mutated PTEN expression in PTEN knockdown PC12 cells. A: To generate off-target PTEN constructs, two nucleotides (bold capital letters) were mutated in the shRNA-targeted sequence (italic letters) without causing the alteration of the encoded amino acids. A replacement of the 129th glycine residue by the glutamic acid residue resulted in a loss of lipid phosphatase activity of PTEN. Underlined letters showed the binding sites of primers used for RT-PCR detection of mutated PTEN expression. B: PTEN knockdown PC12 cells (pooled clone, lanes 1 and 4) were transfected with the constructs of off-target wild-type PTEN (WTPTEN-M2, lanes 2 and 5) using primers ptn-U and pt-D, and lipid phosphatase-deficient mutant of PTEN (PTENG129E-M2, lanes 3 and 6) using primers ptm-U and pt-D. Stable pooled clones were obtained after selection with puromycin, and cells were subjected to RT-PCR analysis using the indicated primers. C: Pooled PC12 cell clones in (B) were subjected to Western blot analysis of the indicated proteins. D, E: Pooled PC12 cell clones in (B) were cultured in complete media supplemented with 50 ng/ml NGF for indicated time. The percentages of cells with neurites, that is, cells bearing neurites equivalent to or longer than two cell diameters, were calculated (D), and the average total neurite lengths were measured (E). Data represent the means of results obtained from three random microscope fields. ctrl, untransfected PC12 cells; pk, PTEN knockdown cells; pkw, PTEN knockdown cells further transfected with WTPTEN-M2 construct; pkh, PTEN knockdown cells further transfected with the construct of PTENG129E-M2 (protein phosphatase activity only mutant). $^{*}P < 0.05$ compared with pk; $^{\#}P < 0.05$ compared with pkw.

TABLE II. Representative Genes Regulated by the Lipid and Protein Phosphatase Activities of PTEN in PC12 Cells

Name	Accession no.	Descriptions	X1		X2		Comment
			M	R	M	R	
Cdh2	NM_031333.1	Cadherin 2, type 1, N-cadherin (neuronal)	32	22	−6.5	−3	D−
Sycp3	NM_013041.1	Synaptonemal complex protein 3	14.9	8	2.8	3.5	D+
Nf1	NM_012609.1	Neurofibromatosis type 1	13.9	29	5.3	13	D+
PDE1C	AF328797.1	Phosphodiesterase 1C	13	3.3	1.9	1.2	L
Pik3r1	D64048.1	Phosphatidylinositol 3-kinase p45 subunit	11.3	8.5	1.1	2.6	L
mGluR1b	Y18810.1	Metabotropic glutamate receptor type 1	11.3	17	1.2	1.0	L
Bid3	NM_057130.1	BH3 interacting (with BCL2 family) domain, apoptosis agonist	7.5	5.3	1.1	1.9	L
Ptpro	D45412.1	Brain-enriched membrane-associated proteintyrosine phosphatase (BEM)-1	7.0	3.8	−1.1	1.6	L
Bmp3	NM_017105.1	Bone morphogenetic protein 3	−4.3	−7.5	1.4	−1.7	L
IGF-1	M15481.1	Insulin-like growth factor I	−5.7	−11	−3.5	−5.2	P
FGFR-1	S54008.1	Fibroblast growth factor receptor 1beta-isoform	−8.6	−6.1	−1.6	−3.4	L
Ptprd	L19181.1	Receptor-linked protein tyrosine phosphatase	−9.2	−5.5	−9.2	−4.7	P
Htr5b	L10073.1	5-Hydroxytryptamine receptor; serotonin receptor	−10.6	−9.0	−5.3	−2.7	D+
Nrg1	U02315.1	Neu differentiation factor	−13	−7.7	−6.5	−5.3	D+
eek	X59290.1	Eph and elk-related kinase	−15	−19	1.3	−1.5	L

M and R, folds of gene expression levels in microarray and qRT-PCR assays, respectively.

signaling events downstream of PTEN during the proliferation and differentiation of PC12 cells.

EXPRESSION OF NESTIN IN PC12 CELLS CORRELATES WITH A CONCURRENTLY IMPROVED SENSITIVITY TO PTEN KNOCKDOWN IN NEURITE ELONGATION

Consistent with our observation that nestin may serve as a marker of differentiating PC12 cells, a minority of the PC12 cell population, which showed accelerated neurite elongation driven by PTEN knockdown, expressed a relatively high level of nestin before NGF induction (Fig. 1H, clone pk-2). To ask whether PTEN plays an identical role during neurite initiation and elongation stages, we used the nestin promoter and a Cre/loxP system to achieve conditional PTEN knockdown in differentiating PC12 cells that express nestin. The bacteriophage P1 Cre recombinase, once expressed from the nestin promoter, removed the loxP-flanked transcriptional stop signal of PolIII promoter between the U6 promoter and shRNA coding sequence, which thus allowed the expression of shRNAs (Fig. 4A). Indeed, a significant inhibition of PTEN was observed 6 days following NGF addition (Fig. 4B). The selective suppression of PTEN in modified PC12 cells that already developed neurites was also confirmed by immunofluorescent staining (Fig. 4C). Unlike those PC12 cells that constitutively express PTEN shRNA, conditional PTEN knockdown in these nestin-positive PC12 cells resulted in improved neurite outgrowth and elongated neurites in a long-term NGF induction of PC12 cells (Fig. 4D,E). In addition, conditional knockdown of PTEN increased NGF-induced phosphorylation of Akt and Erk1/2 (Fig. 4F). PD98059, a specific inhibitor of MEK1, the MAPK kinase, and Ly294002, an inhibitor of PI3K, significantly impaired neurite outgrowth in both control and conditional PTEN knockdown PC12 cells, in which the average neurite lengths became comparable in cells treated with the same inhibitor regardless of their PTEN expression (Fig. 4G). These results, together with the observation in constitutive PTEN knockdown PC12 cells, suggest that PTEN suppression exclusively in differentiating PC12 cells, that is, cells that have already initiated neurite development, promotes the elongation of NGF-induced neurites via combined Akt and MAPK signaling, which is in contrast to a more complicated role of PTEN during the initiation of neurite development.

DISCUSSION

Neuronal differentiation and morphogenesis are well-organized events exquisitely regulated by a complex signaling network involving the conserved PI3K and MAPK pathways and probably a crosstalk between them [Marino et al., 2003; Endersby and Baker, 2008; Chalhoub et al., 2009]. As a tumor suppressor and a dual-specificity phosphatase, PTEN plays multiple roles in negatively regulating cellular signals that lead to survival, proliferation, and differentiation [Yamada and Araki, 2001]. Recent studies have revealed that PTEN deficiency in neural stem cells resulted in improved self-renewal capacity and neuronal differentiation potential, whereas the role of PTEN in the differentiation of PC12 cells, a model cell line for studies on neurite growth, is controversial [Lachyankar et al., 2000; Musatov et al., 2004; Groszer et al., 2006]. Here, we show that constitutive suppression of PTEN in PC12 cells promoted cell proliferation and survival, but not NGF-triggered neurite outgrowth. In contrast, we observed accelerated neurite elongation of PC12 cells, which express normal PTEN during neurite initiation but subjected to PTEN silencing following expression of nestin, a putative marker of differentiating PC12 cells. These results suggest that PTEN might exert a dual effect on PC12 cell differentiation. Although PTEN plays an overall inhibitory role in neurite elongation, it might also facilitate the neural orientation of

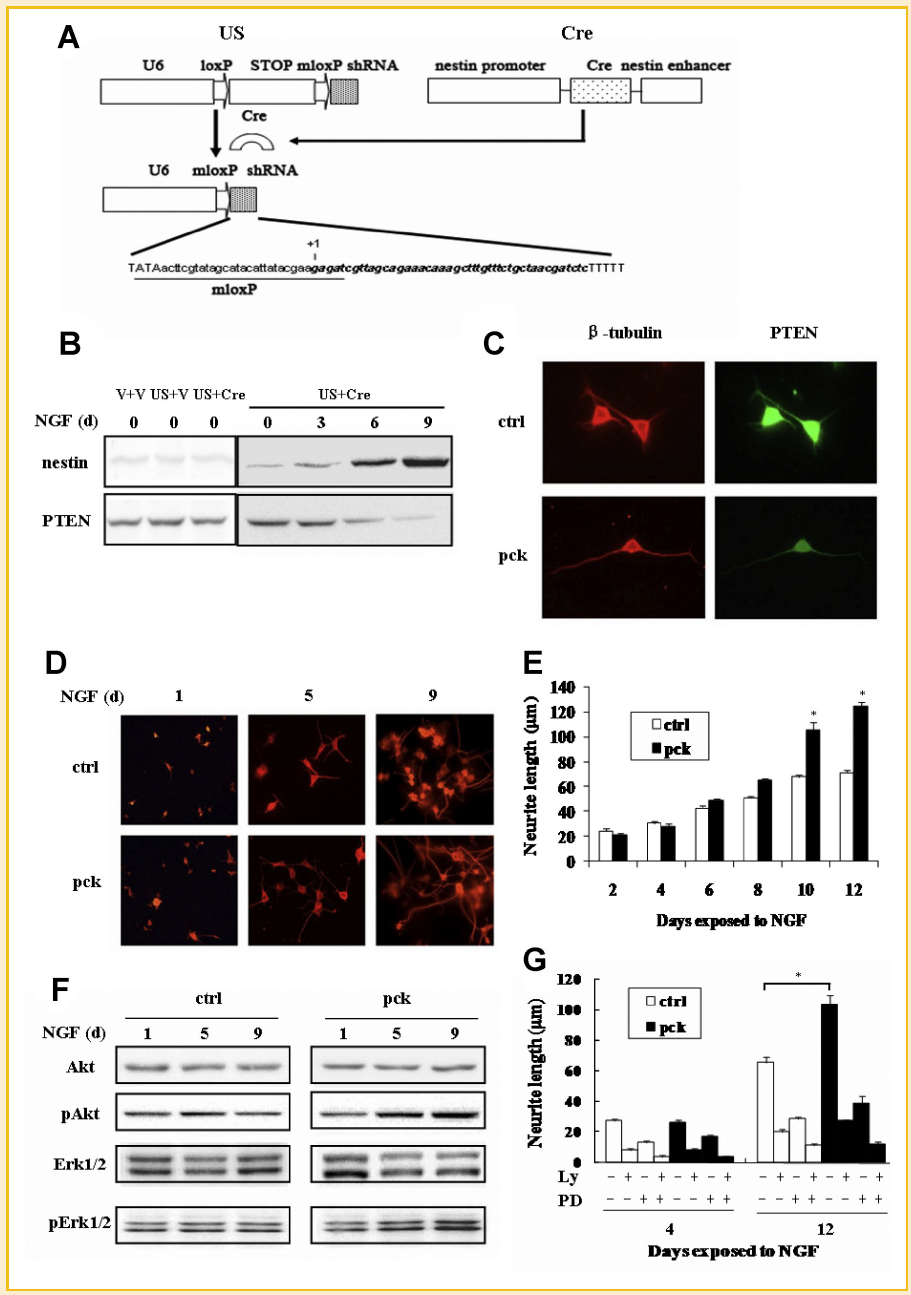

Fig. 4. Conditional PTEN knockdown in differentiating PC12 cells promotes NGF-induced neurite elongation in a long-term culture. A: A schematic diagram of the conditional shRNA expression system comprised of the pGEM-TEasy-1-U6-loxP-STOP-loxP-PTENshRNA (US) and pcDNA3-P_{nes}-Cre-E_{nes} (Cre) constructs. The bacteriophage P1 recombinase Cre expressed from a nestin promoter and enhancer removes the polIII RNA transcriptase stop sequence between the wild-type and mutant loxP sites, resulting in the transcription of the small hairpin RNA (shRNA) targeted to PTEN. The mutant loxP (mloxP) site is different from the wild-type one in the last four nucleotides overlapping with the shRNA coding region, which does not interfere with Cre-catalyzed DNA recombination as proved by published data [Oberdoerffer et al., 2005]. B–G: PC12 cells were co-transfected with the US construct plus void pcDNA3 (US + V or ctrl), or, to generate conditional PTEN knockdown (pck) cells, co-transfected with Cre and US constructs at a molar ratio of 1:10, followed by selection with 400 ng/ml G418. Pooled cell clones were cultured in media supplemented with (left) or without (right) 50 ng/ml NGF for indicated time before Western blotting analysis (B), immufluorescent staining of β-tubulin (D, magnification 200×) and measurement of neurite lengths (E, F). V + V, cells co-transfected with void pGEM-TEasy-1 and pcDNA3 vectors. Data represent the mean ± SD values of three independent experiments, each of which measured the mean total neurite lengths of five random cells. Alternatively, cells were cultured in media containing 50 ng/ml NGF for 8 days before immufluorescent staining of the indicated proteins (C, magnification 400×). $^{*}P < 0.05$ compared with ctrl. G: Cells were cultured in media containing 50 ng/ml NGF with or without the PI3K inhibitor Ly294002 (Ly, 12.5 μM) or/and MAPK inhibitor PD98059 (PD, 20 μM). NGF and inhibitors, or as a control, DMSO, were replenished daily. Data represent the mean ± SD values of three independent experiments, each of which measured the mean total neurite lengths of five random cells. $^{*}P < 0.05$.

PC12 cells during the initiation of neurite development (Fig. 5). Our findings are in agreement with a previous report that the expression of PTEN is upregulated by NGF in PC12 cells and that suppression of PTEN with antisense RNA actually inhibited the neurite outgrowth of NGF-treated PC12 cells [Lachyankar et al., 2000].

It is believed that divergent signal pathways downstream of the NGF receptor, TrkA, are responsible for the neuritogenesis of PC12 cells [Ihara et al., 1997; Iwasaki et al., 1999; Watanabe et al., 2004; Aoki et al., 2007]. While attenuating PI3K signaling by dephosphorylating the PI3K product PIP3, PTEN also catalyzes the dephosphorylation of a growing list of protein substrates, resulting in the inactivation or activation of the protein and possibly downstream signaling, which ultimately affect the expression profiles of genes related to neurite development [Gericke et al., 2006; Endersby and Baker, 2008]. Consistent with the previously reported involvement of PI3K and MAPK signaling in neurite outgrowth [Vaudry et al., 2002], we found a concomitant increase in Akt and Erk phosphorylation in conditional PTEN knockdown PC12 cells, which displayed improved neurite outgrowth. While further investigations are needed to define the mechanisms underlying PTEN knockdown-induced upregulation of MAPK signaling, PTEN is reported to negatively regulate the MAP kinase signaling by blocking an insulin-stimulated phosphorylation of the adaptor protein IRS-1 in a breast cancer model, and by directly dephosphorylating another adaptor Shc in a glioblastoma cell line U87MG [Gu et al., 1999; Weng et al., 2001]. Thus, our findings revealed that combined Akt and MAPK signaling, which is regulated by phosphatases like PTEN, plays an indispensable role in NGF-induced neurite development of PC12 cells.

As a dual-specificity phosphatase, PTEN plays wide regulatory roles in varied signal pathways involved in neuronal differentiation [Groszer et al., 2006]. However, while the function of PTEN may vary in different phases of neurite development, the lipid and protein phosphatase activities of PTEN can also play discriminated roles during these processes, for example, the initiation of neurite outgrowth. In contrast to the almost unchanged neurite development following PTEN knockdown in PC12 cells, an enforced overexpression of PTEN significantly suppressed the outgrowth of neurites in a prolonged induction with NGF, which reflects an inhibitory role of PTEN in neurite elongation, and is thus consistent with a previous observation using the same neuronal differentiation model [Musatov et al., 2004]. Meanwhile, using a lipid phosphatase activity-deficient mutant of PTEN, we found that restoring the protein phosphatase activity of PTEN in PTEN knockdown cells significantly promoted neurite outgrowth of these cells, suggesting that the lipid phosphatase and protein phosphatase activities of

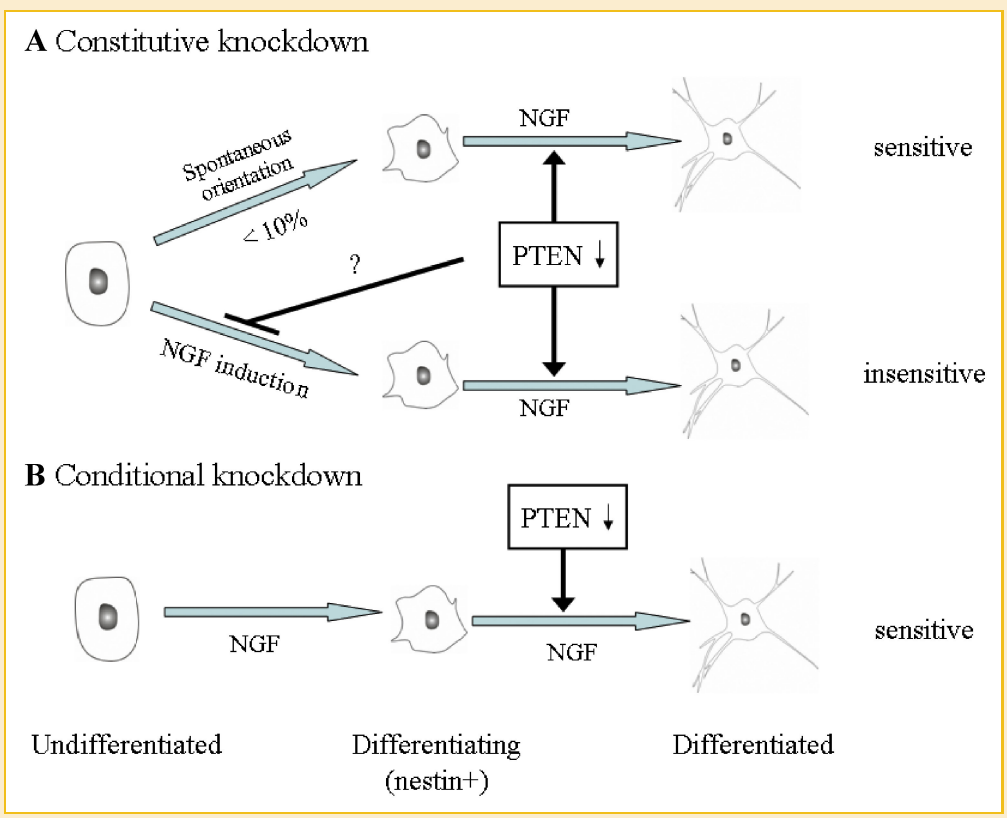

Fig. 5. The effect of PTEN knockdown on NGF-induced neurite development of PC12 cells. Upon induction with NGF, cells undergo initiation of differentiation as characterized by nestin expression, and were then subjected to neurite elongation. A: In a small population of cells, which have already experienced spontaneous neural orientation (initiation of neurite development), constitutive knockdown of PTEN promotes neurite elongation, whereas most other cells are insensitive to PTEN knockdown probably due to a contrary role of PTEN in neurite initiation and elongation. B: Selective suppression of PTEN in nestin-expressing cells, referred to as differentiating cells, accelerates the elongation of neurites.

PTEN play different roles during the initiation of neurite development and the elongation of these neurites in PC12 cells exposed to NGF.

NGF-induced neurite outgrowth in PC12 cells share common morphological and biochemical changes with the differentiation of neural stem cells [Vaudry et al., 2002; Higuchi et al., 2003]. However, they differ in many aspects: (1) upon exposure to NGF, PC12 cells undergo proliferation before exiting from the cell cycle and differentiating [Myers et al., 1997]; (2) the expression of nestin, a neural stem cell marker, is switched on and continues even when long neurites were developed in PC12 cells, whereas nestin is undetectable when neural stem cells undergo differentiation (Fig. 4B) [Zimmerman et al., 1994]; and (3) PTEN is upregulated following NGF addition, which is in contrast to a downregulation of PTEN during neural stem cells differentiation [Lachyankar et al., 2000]. In addition, our study also demonstrated that PC12 cells themselves might also be heterogeneous in their responses to NGF. Unlike a majority of the cloned PC12 cells that showed normal neurite development in response to NGF, cell clones that exhibited intriguingly accelerated neurite outgrowth could be identified in both parental PC12 cells and cells that constitutively expressed PTEN shRNA, suggesting that a possible intrinsic transition independent of PTEN might account for the enhanced sensitivity to NGF treatment in these cells (Fig. 5).

The past decade has witnessed the striking application of RNA interference (RNAi) in both in vitro and in vivo studies on gene function [Rao and Wilkinson, 2006]. In utilization of the well-defined Cre/loxP DNA recombination system, approaches to achieving target gene knockdown either in specific cell types or in an inducible manner are now available [Oberdoerffer et al., 2005; Rao and Wilkinson, 2006]. Based on a previously reported conditional shRNA system [Oberdoerffer et al., 2005], our study achieved the knockdown of PTEN selectively in differentiating PC12 cells using a nestin promoter. As exampled by our studies on the changing role of PTEN during NGF-induced PC12 cell differentiation, these temporally or spaciously specific siRNAs/shRNAs provide useful tools in precisely exploring the function of the interested genes in a particular tissue or a certain stage of differentiation. In addition, to further probe the respective roles of the lipid phosphatase and the protein phosphatase activities of PTEN, off-target mutants were obtained by generating oligonucleotide mutations in the shRNA recognition site of PTEN mRNA. The restoration of PTEN's protein phosphatase activity was thus achieved by introducing an off-target lipid phosphatase-deficient PTEN mutant into PC12 cells following shRNA-mediated silencing of endogenous PTEN. A subsequent microarray screening of the differentially expressed genes in these modified cells offered clues to coupling or excluding the expression of a specific gene to the lipid phosphatase activity of PTEN. This ''out and splitted in'' analysis strategy may provide a versatile approach to studying the roles of multifunctional proteins or proteins with varied subcellular locations provided that the appropriately mutated or truncated versions of the proteins are available. Taken together, our studies provide new insights into the signaling network regulated by PTEN and a complicated role of this phosphatase during the differentiation of neuronal phenotype cells.

ACKNOWLEDGMENTS

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) and Canadian Institutes of Health Research (CIHR) to X. Z.

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