

PC3 potentiates NGF-induced differentiation and protects neurons from apoptosis

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PC3^{TIS21/BTG2} is member of a novel family of antiproliferative genes (BTG1, ANA/BTG3, PC3B, TOB, and TOB2) that play a role in cellular differentiation. We have previously shown that PC3^{TIS21/BTG2} is induced by nerve growth factor (NGF) at the onset of neuronal differentiation in the neural crest-derived PC12 cell line, and is a marker for neuronal birth. We now observe that PC3^{TIS21/BTG2} ectopically expressed in PC12 cells synergises with NGF, similarly to the cyclin-dependent kinase inhibitor p21, potentiating the

induction of the neuronal markers tyrosine hydroxylase and neurofilament 160 kDa. Furthermore, PC3^{TIS21/BTG2} protects from apoptosis elicited by NGF deprivation in terminally differentiated PC12 cultures. Such effects might be a consequence of the arrest of cell cycle exerted by PC3^{TIS21/BTG2}, or expression of a sensitising (neurogenic) property of the molecule. *NeuroReport* 13:417–422
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INTRODUCTION

The process of neural development is the result of a regulatory network that coordinates cell cycle and neurogenic genes. Cell cycle control seems to have a role in both neural determination and terminal differentiation, as well as in neuronal survival (for review see [1]).

In this process might be involved the gene PC3 (also known as TIS21 or BTG2), originally isolated as an immediate early gene activated at the onset of the neuronal differentiation triggered by nerve growth factor (NGF) in the neural crest-derived rat PC12 chromaffin cell line [2], and prototype member of a novel family of antiproliferative genes that play a role in cellular differentiation (namely, BTG1, BTG3/ANA, PC3B, TOB, and TOB2) (for review see [3]). PC3 was also isolated in mouse NIH3T3 cells as a tetradecanoyl phorbol acetate-induced sequence, TIS21 [4] and, more recently, in human, called BTG2. This latter turned out to be induced by p53 and by p73, and to be involved in cell survival after genotoxic response [5,6].

In addition to the observation that PC3 is rapidly induced following the NGF-dependent differentiation of the PC12 cells, other evidence suggested its involvement in neuronal differentiation. PC3 mRNA was found to be transiently expressed in the ventricular zone of the neural tube [2,7], with a correlation to the period and to the area where the neuroblast becomes post-mitotic and starts to differentiate into a mature neuron [7]. PC3 is therefore a marker for neuronal birth. Subsequent studies further clarified that PC3/TIS21 identifies single neuroepithelial cells that switch from proliferative to neuron-generating division [8]. In fact,

the PC3/TIS21 mRNA and protein was found to be expressed during the G₁ phase in the subset of neuroepithelial (NE) cells of the neural tube, identified by the early neuronal differentiation marker β III-tubulin, and for a short period in the post-mitotic neuronal daughter cell [8]. Given that the change in the division mode of NE cells, from symmetrical to asymmetrical (i.e. generating one post-mitotic neuron and one NE cell), determines the onset of neurogenesis, this again correlates the expression of PC3/TIS21 to the onset of neurogenesis.

More recent data shed light on the mechanism by which PC3 inhibits cell cycle, indicating that this occurs through the inhibition of cyclin D1 transcription [9]. The key regulator of cell cycle, pRb, functions as inhibitor of G₁ entry when in a dephosphorylated state, whereas pRb phosphorylation by the cyclin-dependent kinase 4 (CDK4), associated with its partner cyclin D1, inactivates its growth-suppressing activity. Thus, a reduced expression of cyclin D1, caused by PC3, inactivates CDK4 and leads in the end to the activation of pRb, with a consequent arrest in G₁ (for review see [10]).

These data, in conjunction with our demonstration that PC3 inhibits cell proliferation also in PC12 cells ([11]; see also below) suggested that PC3 might have a role in neuronal differentiation as inducer of the growth arrest required for differentiation [7].

We evaluated this hypothesis in the same neuronal cellular system in which PC3 was cloned, and observed that, although unable to autonomously trigger differentiation of PC12 cells, PC3 potentiated the differentiation

elicited by NGF and enhanced the survival of cells undergoing apoptosis following forced de-differentiation.

MATERIALS AND METHODS

Cell culture, cell lines and transfections: PC12 cells (originally obtained from D. Schubert, Salk Institute, 5th passage) were grown in DMEM containing 5% bovine serum and 5% horse serum (Hy Clone, Logan, Utah) in 10% CO₂.

Transfection of the plasmids was performed by the liposome technique using the Lipofectamine reagent (Invitrogen, USA), as per manufacturers instructions. The indicated amount of DNA (see figure legends), diluted in Optimem containing Lipofectamine (5 or 30 µl for 35 mm or 90 mm dishes, respectively), was added to the cultures, left to incubate for 18 h, then substituted with normal DMEM.

Plasmids, PC3 expression vectors and mutants: pSCT-βgal and pSCT-PC3 were constructed as described [9]. In particular pSCT-PC3 was obtained by cloning into 5' *Xba*I and 3' *Hind*III sites of the pSCT vector the coding region of PC3 cDNA (nucleotides 65–541, with the stop codon). pCEP-WAF1/p21 expression vector was from B. Vogelstein [12].

Immunofluorescence staining and antibodies: Transfected cells, grown on poly-lysine coated coverslips, were treated for immunofluorescence staining as described [9]. Briefly, cells were fixed for 20 min at room temperature in PBS containing 3.75% paraformaldehyde, and incubated, after PBS washes, for 2 min in 0.1 M glycine–PBS. Permeabilisation was obtained with 0.2% Triton X-100 in PBS for 4 min at room temperature and was followed, after a PBS wash, with a 60 min incubation at room temperature with one, or two where indicated, primary antibodies, diluted in PBS. A3H rabbit polyclonal [11] was diluted 1:50, anti-βgal rabbit polyclonal (Chemicon International Inc., Temecula, CA) was diluted 1:50, anti-medium-size neurofilament (160 kDa; NF-M) and anti-tyrosine hydroxylase TH-2 mouse monoclonals (TH; Sigma Chemicals) were diluted 1:20 and 1:1000, respectively, whereas the rabbit polyclonal anti-p21 (PC55, Calbiochem) was diluted 1:20. Secondary antibodies were as indicated, either FITC- or TRITC-conjugated (tetramethylrhodamine isothiocyanate, from Jackson Laboratories, USA). Cells were mounted with PBS:glycerol (3:1). Immunofluorescence was performed on a Leitz Dialux 22 microscope.

Induction of apoptosis by NGF-deprivation and morphological evaluation: Apoptosis of neuronal PC12 cell cultures was induced following a described procedure [13]. Cells cultured for 10 days with NGF in low serum (to facilitate the entry of cells in G₀ and thus the NGF-induced differentiation) were replated, transfected after 24 h by Lipofectamine with the indicated constructs, after 48 h deprived of NGF (also adding an anti-NGF antibody to remove NGF bound to the receptors), and 20 h later fixed and immunostained. Morphological evaluation of apoptosis was performed as described [14]. Briefly, cells successfully transfected were detected by monitoring βgal, revealed by fixation and incubation with anti-βgal antibody. Cells were then mounted and fluorescence was visualised as described

above. Stained nuclei were scored by blind analysis and categorised according to the condensation and staining of chromatin. Normal nuclei were identified as non-condensed chromatin dispersed over the entire nucleus. Apoptotic nuclei were identified by condensed chromatin, contiguous to the nuclear membrane, as well as by nuclear fragmentation of condensed chromatin. In each experiment 12 fields per plate of about 40 nuclei were counted.

RESULTS

The neural crest-derived chromaffin cells (and the continuous line PC12) in presence of NGF differentiate into sympathetic neurons [15]. In PC12 cells, PC3 mRNA is induced by NGF rapidly, with a 15-fold peak at 1 h, and transiently, being almost undetectable after 3 h [2]. To evaluate whether PC3 has a role in the onset and/or maintenance of neuronal differentiation, we over-expressed PC3 in PC12 cells by transient transfection, and triggered the differentiation immediately after, by addition of NGF. After 72 h we analysed the expression of two markers for neuronal terminal differentiation, TH and NF-M, by immunofluorescence staining [16,17]. TH is involved in the synthesis of dopamine and is highly induced during the differentiation of sympathetic neurons. We observed that cells positive for ectopic PC3 (detected by the antibody A3H, [11]) expressed TH very brightly above its basal background expression, with a frequency more than doubled with respect to the cells treated with NGF and transfected with βgal expression construct as control (Fig. 1, Fig. 3). Similarly, ectopic expression of PC3 was associated to a significant increase of the cytoplasmic, perinuclear staining corresponding to the NF-M protein (Fig. 2, Fig. 3). The same increase in the frequency of expression of the two neuronal markers was observed in PC12 cells ectopically expressing the CDK inhibitor p21, consistently with a previous report indicating that p21 elicits biochemical changes similar to those induced by NGF (without inducing differentiation *per se*; [18]). On the other hand, in the absence of NGF, the frequency of TH and NF-M expression in cells positive for ectopic PC3 did not differ from cells ectopically expressing βgal (Fig. 3). As a whole, this leads us to conclude that PC3 potentiates the differentiation elicited by NGF in PC12 cells, but is unable to autonomously trigger the process of differentiation.

Previous reports indicated that the TIS21/PC3 mRNA was highly and persistently induced in PC12 cells during the apoptosis elicited by NGF deprivation (analogously to what observed for c-jun), suggesting a role for this gene in the apoptotic process [19]. In considering also that PC3 activates pRb, whose function is essential for neuronal survival [20], we sought to ascertain whether PC3 played a role in this process. In fact, withdrawal of NGF from PC12 cell cultures causes their death [15,21], and the process closely resembles to programmed cell death in neurons [22,19], making this cell system a suitable model for the study of such phenomenon. Thus, we induced apoptosis in terminally differentiated PC12 cell cultures through deprivation of NGF and expressed ectopic PC3 by transfecting, according to a described protocol [13], either the empty pSCT vector, the pSCT-PC3 construct, or the antisense pSCT-PC3

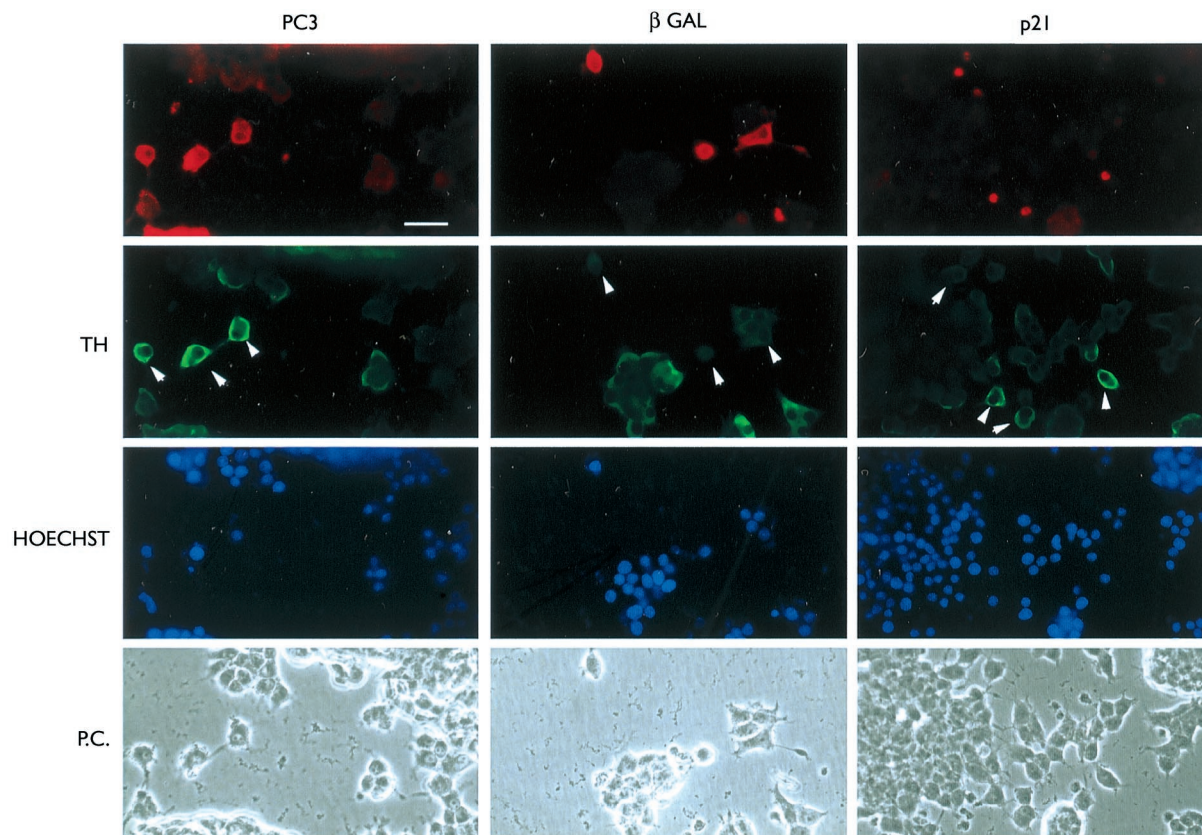


Fig. 1. PC3 potentiates the induction of TH by NGF in PC12 cells. Shown are representative immunofluorescence photomicrographs of TH expression in cultures transfected with PC3, β gal or p21. 2×10^5 PC12 cells were seeded in 35 mm dishes coated with polylysine. After 24 h cells were transfected with the expression vectors pSCT- β gal, or pSCT-PC3, or pCEP-WAF1 (2 μ g each). The following day NGF (100 ng/ml) was added and incubated for 72 h (with an intermediate change of medium). Cells were then fixed, permeabilized and stained. PC3, β gal and p21 proteins were revealed by staining with goat anti-rabbit TRITC-conjugated antibody, after incubation with anti- β gal, anti-PC3 (A3H) or anti-p21 polyclonal antibodies. TH was visualized by Mab TH-2 followed by goat anti-mouse FITC-conjugated antibody. Nuclei were detected by Hoechst 33258 dye. The corresponding phase contrast fields are shown (P.C.). Arrows indicate cells expressing the ectopic proteins. Bar = 15 μ m.

construct, in conjunction with pSCT- β gal as a transfection marker.

We observed that over-expression of PC3 significantly prevented cell death (about 40% decrease of the apoptosis induced by NGF deprivation), as judged by morphological evaluation of nuclei stained with Hoechst 33258 dye, whereas over-expression of antisense PC3 had an opposite effect (about 70% increase; Fig. 4a,b).

DISCUSSION

It is known that the decision of a cell to differentiate is often made in the G1 phase of the cell cycle; in the neuron, the progressive restriction of growth potential from stem cell to neural precursor correlates with the number of cell divisions, and a correct terminal differentiation requires correlation with the exit from cell cycle. In some cellular systems enforced cell cycle arrest achieved by over-expressing inhibitors of CDKs is sufficient to cause differentiation. Moreover, differentiation in situations of deregulated proliferation is usually abnormal or results in cell death. In PC12 cells, for instance, it has been observed that inhibition of CDKs protects differentiated cultures from apoptosis [23].

The PC12 cell system represents a model for terminal differentiation, triggered by NGF, given that chromaffin cells, derived from neural crest progenitors, are already determined to the neuronal lineage. Recently, cell cycle genes have been directly implicated in the differentiation of PC12 cells. In fact it has been shown that the concomitant inhibition of CDK2 and Cdc2, or the over-expression of Rb2/p130, a member of the Rb family, is sufficient *per se* to cause differentiation of PC12 cells [24,25]. The inhibition of CDK2 and Cdc2 in PC12 cells, which leads to the full activation of pRb-mediated arrest in G1 and to induction of differentiation, seems to stand for cell cycle arrest in itself as cause for terminal differentiation. In the case of Rb2/p130, we cannot exclude an intrinsic neurogenic activity of that molecule [25]. Recently the key cell cycle inhibitor p73 has been shown to induce differentiation of a neuronal cell line [26]. This action could involve the induction of the CDK inhibitor p21 but, given that this molecule is not *per se* inducing differentiation (at least in PC12 cells; [18]), the differentiative effect of p73 might be also cell cycle independent.

PC3 is known to induce cell cycle arrest in PC12 cells and other cell types (for review see [3]). In fibroblast we have

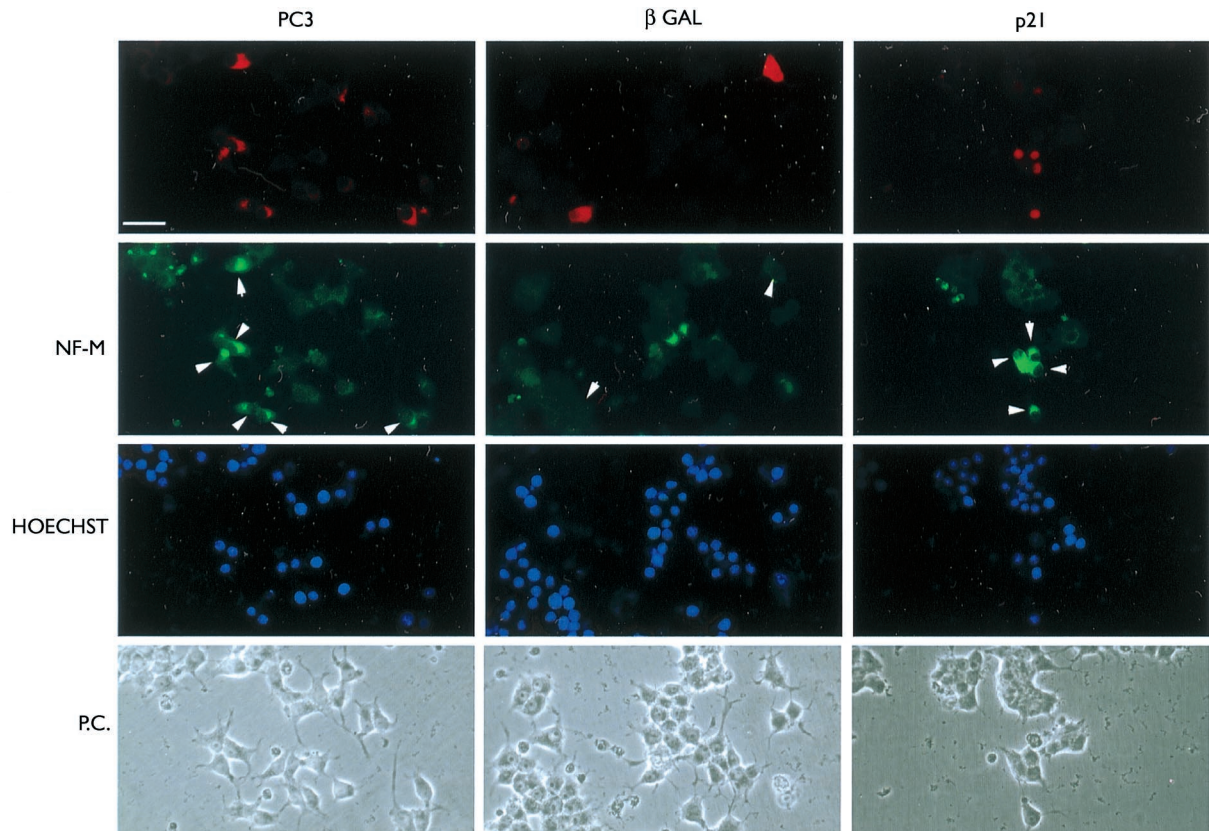


Fig. 2. PC3 potentiates the induction of neurofilament medium size (NF-M) by NGF in PC12 cells. Shown are representative immunofluorescence photomicrographs of NF-M expression in cultures transfected with PC3, β gal or p21. Cells were treated, fixed and stained as indicated in Fig. 1, except for the use of anti-NF-M mouse monoclonal, revealed by goat anti-mouse FITC-conjugated antibody. Nuclei were detected by Hoechst 33258 dye. The corresponding phase contrast fields are shown (P.C.). Arrows indicate cells expressing the ectopic proteins. Bar = 15 μ m.

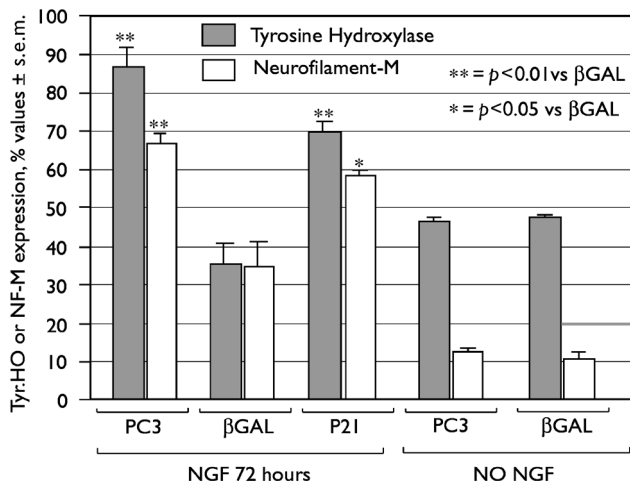


Fig. 3. Quantitative analysis of the synergistic effect of PC3 on the induction of TH and NF-M proteins by NGF. Values represent the percentage of cells positive for either TH (grey bars) or NF-M (white bar) immunoreactivity, after 72 h treatment with NGF (left side) or without treatment (right side), detected between cells ectopically expressing PC3, β gal or p21 (as indicated). Means \pm s.e.m. are from three independent experiments. At least 100 cells were counted for each group. The statistical analysis indicated was performed by Student's *t*-test.

demonstrated that this occurs through inhibition of CDK4 activity, which is responsible for the primary phosphorylation event leading to inactivation of pRb, and thus to progression of the cell cycle [9]. The same functional inhibition of CDK4, and of CDK2 and Cdc2 as well, is exerted by p21, that from a functional point acts in parallel with PC3. Therefore these molecules, both potentiating the differentiation exerted by NGF, although unable to initiate autonomously that process, share functional similarity. Moreover, NGF has been shown to cause arrest of PC12 cells in G1 phase, a decrease of CDKs expression and activity, with consequent activation of pRb, and an increase of cyclin D1 and p21 levels [27,28]. These latter changes are elicited by NGF only in presence of serum, and do not seem to be required for differentiation ([27,28]; B. Rudkin, personal communication).

A possible interpretation of our data is that PC3, and p21 as well, potentiates neuronal differentiation as the consequence of its ability to arrest cell cycle, sensitising the cell to respond more readily to the differentiative stimulus by NGF. Alternatively, PC3 might also be endowed with neurogenic properties that in PC12 cells do not find the proper context, such as appropriate target genes.

PC3 appears to play a direct role in PC12 cell survival, given its ability to rescue from death elicited by NGF deprivation a significant fraction of cells. This action is in

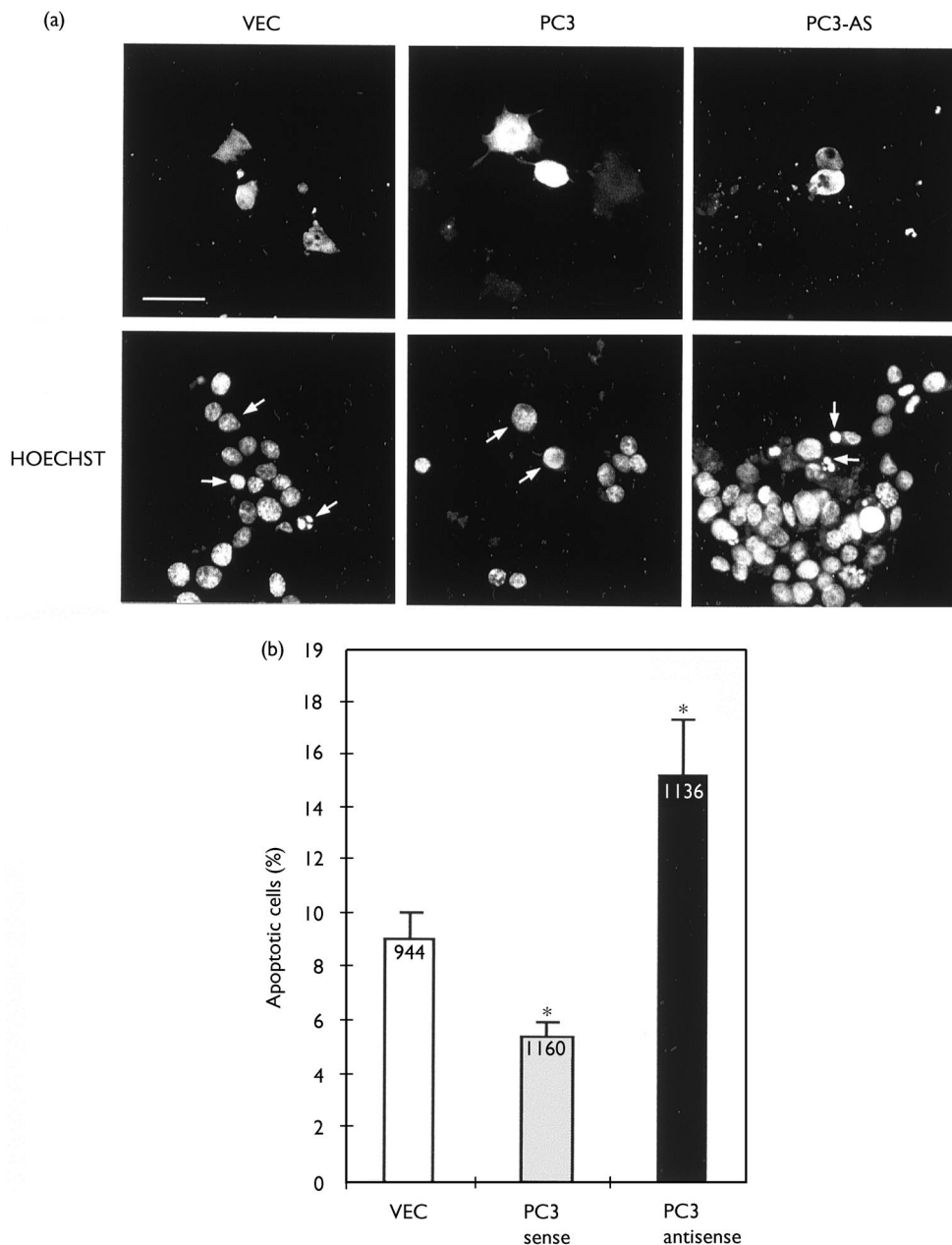


Fig. 4. PC3 prevents the apoptosis induced by deprivation of NGF. 2×10^6 cells were plated in 90 mm collagen-coated dishes, and shifted after 24 h to DMEM with 1% heat-inactivated horse serum containing 100 ng/ml NGF, for 10 days (changed three times per week). Cell cultures were then replated onto 35 mm plates coated with polylysine and laminin, adding NGF to the low-serum DMEM. After 24 h the cells were transfected by Lipofectamine with constructs indicated below. After 48 h PC12 cultures were washed once in PBS, and incubated in NGF-free medium, containing anti-NGF polyclonal antibody (Sigma) diluted 1:400. Twenty hours after NGF withdrawal, cells were fixed for immunostaining. (a) Representative immunofluorescence photomicrographs of cells treated as outlined above, transfected with either 1.5 μ g pSCT empty vector (VEC), 1.5 μ g pSCT-PC3 (PC3), or 1.5 μ g pSCT-PC3 antisense (PC3-AS), and with 0.5 μ g pSCT-βgal as transfection marker. Transfected cells are visualized by anti-βgal antibody, followed by goat anti-rabbit FITC-conjugated secondary antibody; nuclei are stained by Hoechst 33258 dye. Arrows indicate the transfected cells. (b) Percentage of cells showing apoptotic nuclear morphology, after treatment as in panel (a). Means \pm s.e.m. are from three independent experiments. The total number of cells positive for transfection, analyzed in the three experiments, is indicated at the top of the bar. * $p < 0.05$ vs vector-transfected cell (Students t-test).

line with the hypothesis that neuronal apoptosis results from the failed attempt to activate cell cycle in terminally differentiated neurons [29], and is consistent with a previous report indicating that the apoptosis resulting from genotoxic stimulus is increased in mouse ES cells deprived

of PC3/TIS21 [5]. Two pathways are essentially activated in neurons by NGF withdrawal, one triggered by the c-Jun N-terminal kinase (JNK), the other by CDK4 and CDK6 as a result of cyclin D1 increase [29], with consequent inactivation of pRb. The final target of both pathways might be p53

that could activate death genes as BAX (for review see [30]) or cell cycle arrest genes such as p21 or PC3.

CONCLUSION

PC3 is an NGF-inducible immediate early gene, whose induction by NGF in PC12 cells is independent from the presence or absence of serum (unlike p21 [11]) and is transient. In fact its expression is barely detectable in differentiated cells. Thus, considering also the present data, PC3 might play a physiological role at the onset of PC12 cells differentiation, acting as transient signal for activation or repression of down stream target genes of cell cycle or other pathways. A role of PC3 in the maintenance of differentiation seems less likely. Similarly, PC3 might promote survival after NGF deprivation by inhibiting the re-entry into cell cycle, as a result of its ability to prevent pRb inactivation, possibly by reduction of cyclin D1 levels.

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