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Research Article

p27 small interfering RNA induces cell death through elevating cell cycle activity in cultured cortical neurons: a proof-of-concept study

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Abstract. Recent research has demonstrated that cell cycle-associated molecules are activated in multiple forms of cell death in mature neurons, and raised a hypothesis that unscheduled cell cycle activity leads to neuronal cell death. But there is little evidence that changes in endogenous level of these molecules are causally associated with neuronal cell death. Here we transfected small interfering RNA (siRNA) targeting cyclin-dependent kinase (CDK) inhibitor p27, which plays an important role in cell cycle arrest at G_1-S phase, into cultured cortical neurons. Transfection of p27 siRNA reduced neuronal viability in a time-dependent manner. p27 siRNA induced phosphorylation of retinoblastoma protein (Rb), a marker of cell cycle progression at late G_1 phase. Moreover, phosphorylation of Rb and neuronal cell death provoked by p27 siRNA were abrogated by pharmacological CDK inhibitors, olomoucine and purvalanol A. Our data demonstrate that a decrease in endogenous p27 induces neuronal cell death through elevating cell cycle activity.

Keywords. Small interfering RNA, cell cycle, p27, retinoblastoma protein, cyclin-dependent kinase inhibitors, neuronal death.

Introduction

Most neurons enter and remain in a terminally differentiated or resting state after the final cell division. However, recent research raised a hypothesis that aberrant activation of cell cycle machinery plays a pivotal role in apoptosis in postmitotic neurons after various brain insults, including cerebral ischemia, Alzheimer's disease, amyotrophic lateral sclerosis, and DNA damage [1]. Increase in cyclin D_1 and cyclin-dependent kinase (CDK) 4 expression, and phosphorylation of retinoblastoma protein (Rb), which plays an essential role in cell cycle progression in G_1 phase, are repeatedly demonstrated in these experimental models. Moreover, inhibition of G_1-phase CDKs is neuroprotective against nerve growth factor deprivation, cerebral ischemia, and β-amyloid toxicity [2]. These observations suggest that aberrant cell cycle activity is necessary for apoptosis in mature neurons. But it remains unclear whether aberrant cell cycle activity is sufficient for neuronal apoptosis.

CDK activities are positively regulated by their association with cyclins. In addition, small proteins (CDK inhibitors) associate and inhibit the cyclin-CDK complexes [3]. There are seven CDK inhibitors, and among them, p27 is highly expressed in the central nervous system and inhibits the cyclin D_1-CDK4/6 activity at G_1 phase [4]. Reduction of p27 expression level was observed in various neuronal cell death models, including cerebral ischemia [5, 6], repolarization [7], and glutamate toxicity [8]. Moreover, overexpression of p27 prevents the death of neurons caused by trophic factor deprivation [9], DNA damage [10], and proteosomal inhibition [11]. Thus, p27

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down-regulation is hypothesized as an early trigger in aberrant cell cycle progression and neuronal cell death. However, there is little evidence that decrease in p27 is directly involved in neuronal cell death. Although three independent p27-deficient mice were developed [12–14], no information is available on neuronal cell viability in the central nervous system of mice lacking p27.

RNA interference, a method of post-transcriptional gene silencing, has been shown to function across a wide range of organisms [15]. Small interfering RNA (siRNA), a 21-nucleotide double-stranded RNA intermediate, has become a powerful tool to knockdown specific gene expression by degradation of mRNA and potentially will be useful for the analysis of loss-of-function phenotypes in mammals. A number of genes have been successfully knocked-down in postmitotic neurons [16–18]. Thus, siRNA may have great advantages for exploring gene function in mammalian neuronal cultures.

In this study, we transfected p27 siRNA to primary neuronal culture from embryonic rat cerebral cortex, and showed that decrease in endogenous p27 induced neuronal cell death and phosphorylation of Rb. Moreover, the Rb phosphorylation and neuronal cell death elicited by p27 siRNA transfection were abrogated by application of pharmacological CDK inhibitors, olomoucine and purvalanol A. The results suggest that decrease in endogenous p27 expression induces neuronal cell death through elevating cell cycle activity, and provide support for the concept of aberrant cell cycle activity-induced neuronal cell death.

Materials and methods

Primary neuronal culture. Primary neuronal cultures were prepared as described previously [19] with some modifications. All animal experiments conformed to the Japanese Pharmacological Society guide for the care and use of laboratory animals and the institutional guidance, with care to minimizing the number of animals and their suffering. In brief, the cerebral cortices from Wistar rats (SLC Inc., Shizuoka, Japan) at embryonic days 17–18, were treated with 0.25% trypsin (Difco Laboratories, Detroit, MI) and 0.01% deoxyribonuclease I (Sigma Chemical Co., St. Louis, MO) at 37 °C for 30 min. The cells were suspended in Neurobasal medium (Invitrogen Corp., San Diego, CA) containing 10% fetal bovine serum (Biowest SAS, Rue de la Caille, France), and were plated at a density of 2.0×10^5 cells/cm^2 in 6-well plates (Corning Inc., Acton, MA) or on glass coverslips (Matsunami Glass Ind. Ltd., Japan) equipped with FlexiPerm (Sartorius AG, Göttingen, Germany) precoated with 0.02% (v/v) polyethyleneimine (Sigma). Cultures were kept at 37 °C in a humidified 5% CO_2/95% air. At 24 h after plating, the medium was changed to serum-free Neurobasal medium supplemented with 2% B27 (Invitrogen). Half of the culture medium was changed every 3 days. To determine the purity of our cortical cultures, they were double-immunostained with anti-neuron-specific-nuclear-protein (NeuN) antibody (Chemicon International, Inc., Temecula, CA) and Hoechst 33342 (Sigma). On day 8 *in vitro*, 94.8% of total Hoechst-positive cells were NeuN positive, indicating that the vast majority of our cultures were cortical neurons.

siRNA preparation and transfection. We made transfection on day 8 *in vitro*. siRNAs were synthesized by Qiagen (Hilden, Germany). The target sequences were as follows. Control siRNA: AATTCTCCGAACGTGTCACGT. The 3′-sense strand was labeled with Alexa Fluor-488. Scrambled p27 siRNA: AAGACCGAGCCATTGAGGTAA. p27 siRNA: AAGCACUGCCGAGAUAUGGAA. Transfection was performed with lipofectamine 2000 (Invitrogen). Briefly, lipofectamine 2000 (5 µl/well) and siRNA (5 µl/well) were incubated at 25 °C for 5 min, and both were mixed at 25 °C for 20 min. The siRNA-lipid mixture was added to the cultures. The final amount of the siRNA was 50 and 100 pmol/well. siRNA was removed by changing the medium at 4 h after the transfection. Transfection efficiencies as determined by double staining for MAP2 and control siRNA labeled with Alexa Fluor-488 were 90.1% on average. Pharmacological CDK inhibitors, olomoucine (Sigma) and purvalanol A (Merck and Co., Inc., San Diego, CA), were added 1 h prior to the transfection, with continued treatment until assay.

Assessment of cell viability. Mitochondrial dehydrogenase activity that reduces 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to MTT formazan was used to determine cell survival as described previously [20]. In brief, the cells were incubated with 0.25 mg/ml MTT (Sigma) for 35 min at 37 °C in humidified 5% CO_2/95% air. The reaction was stopped using a solution (pH 4.7) containing 50% dimethylformamide (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 20% sodium dodecyl sulfate (SDS) (Wako). The amount of intracellular MTT formazan product was quantified spectrophotometrically using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA; Model 550) at an excitation wavelength of 570 nm and an emission wavelength of 655 nm.

Immunocytochemistry. After fixation with 4% paraformaldehyde (Wako) in phosphate-buffered saline (PBS) for 30 min at 4 °C, cells were washed and permeabilized with 0.1% Triton X-100 (Wako) in Tris-buffered saline (TBS) (25 mM Tris pH 8.0, 125 mM NaCl) for 15 min. The cultures were incubated with TBS containing 5% goat serum (Vector Laboratories Inc., Burlingame, CA) for 1 h at room temperature, and then with a primary antibody, overnight at 4 °C. The primary antibodies used

were anti-p27 (mouse, 1:200 dilution; BD Transduction Laboratories, San Jose, CA), anti-microtubule-associated protein-2 (MAP2) (rabbit, 1:1000; Chemicon), anti-NeuN (mouse, 1:1000; Chemicon). After washing, the cells were incubated with a fluorochrome-conjugated secondary antibody (Alexa 488-conjugated anti-mouse IgG, 1:1000 dilution; Alexa 568-conjugated anti-rabbit IgG, 1:1000 dilution; Molecular Probes Inc., Eugene, OR) for 1 h at room temperature. For the last 5 min, cells were incubated with the bisbenzimide, Hoechst 33342 (Sigma), a fluorescent probe intercalated into nuclear DNA. After washing, images were acquired with a cooled CCD camera (Hamamatsu ORCA II; Hamamatsu Photonics KK, Hamamatsu, Japan) and a 40× objective lens equipped with an inverted microscope (Eclipse TE300; Nikon Corp.). Those images were analyzed using an Aqua-Cosmos system (Hamamatsu Photonics).

For 5-bromo-2′-deoxyuridine (BrdU) labeling, BrdU (Sigma) was added at a final concentration of 10 μM simultaneously with siRNAs transfection. After fixation, the DNA was hydrolyzed into single strands by 2 N HCl, and the cultures were incubated with anti-BrdU antibody (mouse, 1:500 dilution; Sigma). After washing, the cells were incubated with a fluorochrome-conjugated secondary antibody (Alexa 488-conjugated anti-mouse IgG, 1:1000 dilution; Molecular Probes) for 1 h at room temperature. After further washing, images were acquired as described above.

Immunoblotting. Cells were washed with cold TBS and lysed for 30 min on ice in radioimmunoprecipitation (RIPA) buffer (10 mM Na_2HPO_4, 300 mM NaCl, 0.1% SDS, 1% NP-40, 0.1% sodium deoxycholate, 2 mM ethylenediamine-N,N,N',N'-tetraacetic acid, pH 7.0) containing 0.5 μg/ml leupeptin (Peptide Institute Inc., Osaka, Japan), 1 μg/ml pepstatin A (Peptide Institute), 1 mM NaF (Wako), and 1 mM Na_3VO_4 (Wako). Proteins were denatured by heating at 65 °C in sample buffer (10 mM Tris-HCl, pH 6.8, 10 mM dithiothreitol, 2% SDS, and 0.01% bromophenol blue) for 10 min. Protein concentration was measured using a bicinchoninic acid protein assay kit according to the manufacturer's instructions (Pierce Biotechnology, Inc., Rockford, IL). Samples (50 μg) were separated electrophoretically, then transferred to polyvinylidene difluoride membranes. The membranes were blocked with 4% nonfat milk in TBS with 0.1% Tween-20 for 2 h, then incubated overnight with primary antibodies at 4 °C. The primary antibodies used were anti-p27 (mouse, 1:1000 dilution; BD Transduction Laboratories), anti-p19 (rabbit, 1:250 dilution; Santa Cruz Biotechnology Inc., Heidelberg, Germany), anti-β-actin (mouse, 1:2000 dilution; Sigma), anti-phospho-Rb (Ser795) antibody (rabbit, 1:2000 dilution; New England Biolabs, Beverly, MA). They were subsequently probed with secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (diluted 1:1000–1:2000; Sigma). After washing, detection was performed using the enhanced chemiluminescence assay (Amersham Pharmacia Biotech, Piscataway NJ). To provide semiquantitative analysis, band densitometry analysis of the membrane was performed using scanned images of nonsaturated immunoblot films, using software (Scion Image, version Beta 4.0.2; Scion Corp., Frederick, MD). Pixel intensities of the bands obtained in each experiment were normalized using β-actin signals, then calculated as a percentage of control bands in the same membrane.

Statistical evaluation. Data are shown as mean ± SEM. For statistical analyses, one-way analysis of variance (ANOVA) was followed by Tukey's test.

Result

To reveal directly whether decrease in p27 is a cause or a symptom of neuronal cell death, we transfected p27 siRNA into cultured cortical neurons and analyzed cell survival using MTT assay at various time points after the transfection. The expression level of p27 was unchanged until 12 h after the transfection of p27 siRNA at 100 pmol/well, but decreased thereafter at 24 and 48 h (at maximum) (Fig. 1a, b). The expression level of p27 was partially reversed at 72 h after the transfection of p27 siRNA (Fig. 1a, b). A reduction of p27 was not observed by transfection of control siRNA or scrambled p27 siRNA at any time examined (Fig. 1a, b). There was no change in the expression level of p19, another CDK inhibitor, at 48 h after transfection of p27 siRNA (data not shown). p27 siRNA reduced neuronal viability in a time-dependent manner that became apparent at 24 h after the transfection, whereas control siRNA or scrambled p27 siRNA did not affect neuronal survival at any time examined (Fig. 1c). Similar results were obtained by immunocytochemical analysis (Fig. 1d–i). In the control cultures, almost all MAP-2-positive neurons expressed p27 (Fig. 1d–f). Transfection of p27 siRNA induced apparent decrease in the fluorescence intensity of p27, and cells with low or undetectable p27 fluorescence intensity showed decreased MAP2 immunoreactivity and deformed morphology (Fig. 1g–i). The knockdown effect and reduction of neuronal survival was not observed after the transfection of p27 siRNA at 50 pmol/well (data not shown).

Next, we attempted to determine whether p27 siRNA abolishes the function of p27 as a CDK inhibitor, *i.e.* whether the reduction of p27 is causally related to the elevation of cell cycle activity. When cell cycle transits from G_1 to S phase, Rb is phosphorylated by CDKs [21]. Thus, we analyzed Rb phosphorylation on a CDK consensus site, Ser795, after the transfection of p27 siRNA using a phospho-epitope-specific antibody. Only a trace

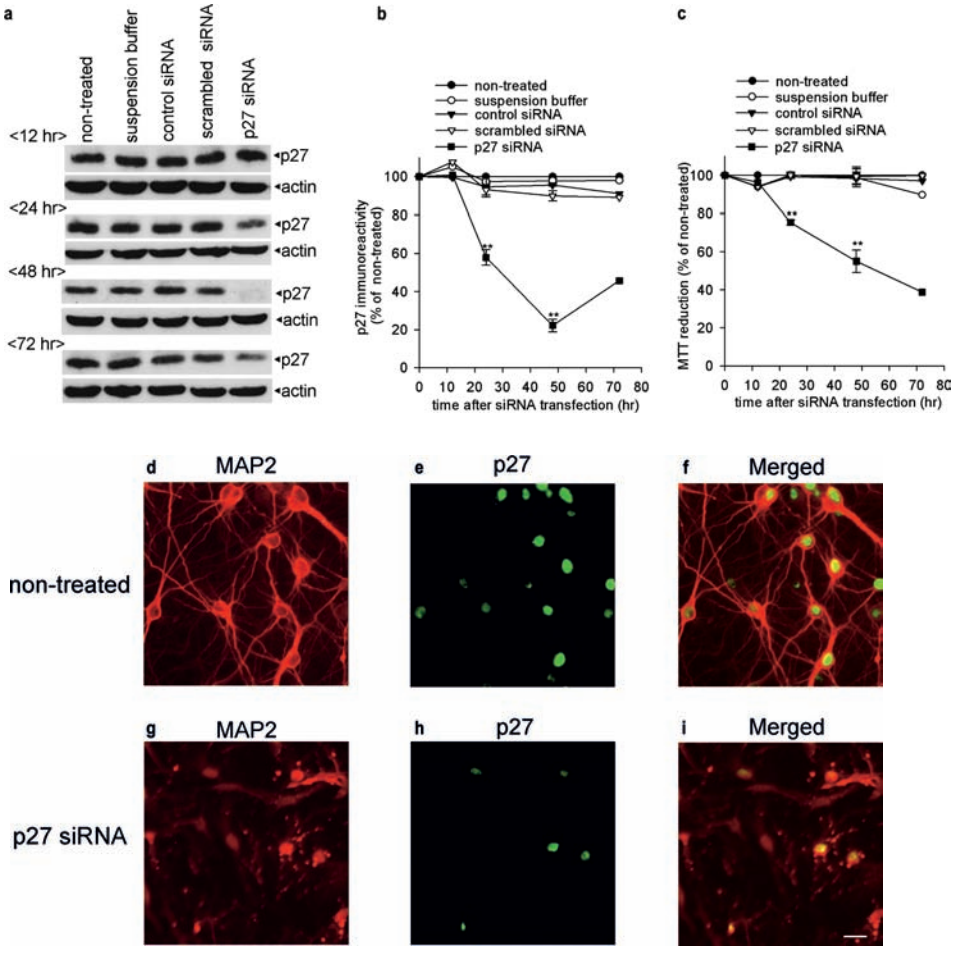

Figure 1. Transfection of p27 siRNA induces reduction of p27 and neuronal cell death. (*a*) Representative immunoblots of p27 at 12, 24, 48, and 72 h after transfection of (from left) non-transfected control (non-treated), suspension buffer to dilute siRNA, control siRNA, scrambled p27 siRNA, and p27 siRNA (each 100 pmol/well). (*b*) The pixel intensity of the p27 bands obtained in each experiment was calculated as described in Materials and methods. The results are presented as a percentage of non-treated values and represent the means ± SEM of three (two at 12, and 72 h after the transfection) independent experiments ($n = 2–3$). $^{**}p < 0.01$ *versus* non-treated: Tukey's test. (*c*) Neuronal viability was evaluated with the MTT assay at 12, 24, 48, and 72 h after the transfection. The results are presented as a percentage of non-treated values and represent the means ± SEM of three (two at 12, and 72 h after the transfection) independent experiments ($n = 2–3$). $^{**}p < 0.01$ *versus* non-treated: Tukey's test. (*d–i*) Immunoreactivity for the neuronal marker, MAP2, was visualized with Alexa-568 (red) (*d, g*); p27 was visualized with Alexa-488 (green) (*e, h*) at 48 h after the transfection. Merged picture shows that p27 was expressed exclusively in the nucleus of neurons under control condition (*f*), whereas p27 expression was decreased in the damaged neurons under the transfection of p27 siRNA (*i*). Non-treated cultures (*d–f*). Cultures transfected with p27 siRNA (*g–i*). Bar 20 μm.

level of phosphorylated Rb immunoreactivity was detected in the non-treated cultures (Fig. 2a). The expression level of phosphorylated Rb, however, increased from 24 h, reached maximum at 48 h, and returned to the level of non-treated cultures at 72 h after transfection of p27 siRNA, whereas control siRNA or scrambled p27 siRNA did not affect the phosphorylated Rb immunoreactivity at any time examined (Fig. 2a, b). BrdU incorporation is a marker for DNA synthesis to determine whether neurons reenter S phase. Only a few neurons (about 5%) were stained with anti-BrdU antibody (Fig. 3a, c). The number of BrdU-positive neurons increased significantly at 48 h after the transfection of p27 siRNA (Fig. 3b, c), whereas control siRNA or scrambled p27 siRNA did not affect the BrdU incorporation (data not shown). However, the ratio of BrdU-positive neurons was only about 10% even under the transfection of p27 siRNA (Fig. 3c).

We next investigated whether CDK activity is involved in the Rb phosphorylation and neuronal cell death induced by p27 siRNA transfection. Pharmacological CDK inhibitors, olomoucine and purvalanol A, are purine derivatives, which strongly inhibit CDK-1, 2, 4, 5 and arrest cells both at the G_1/S and the G_2/M boundaries [22]. The analysis was performed at 48 h after the transfection, when the knockdown effect of p27 siRNA was maximum (Fig. 1b). Olomoucine and purvalanol A protected neurons almost completely against cell death induced by p27 siRNA transfection (Fig. 3a). Moreover, Rb phosphorylation

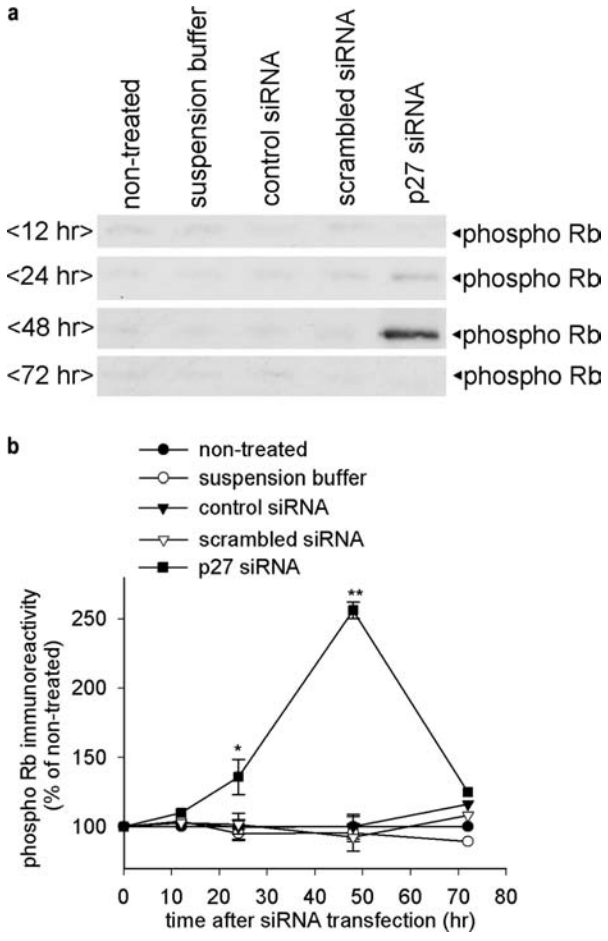

Figure 2. Transfection of p27 siRNA induces Rb phosphorylation. (*a*) Representative immunoblots of phosphorylated Rb (phospho Rb) at 12, 24, 48, and 72 h after the transfection of (from left) non-transfected control (non-treated), suspension buffer to dilute siRNA, control siRNA, scrambled p27 siRNA, and p27 siRNA (each 100 pmol/well). (*b*) The pixel intensity of the phosphorylated Rb (phospho Rb) bands obtained in each experiment was calculated as described in Materials and methods. The results are presented as a percentage of non-treated values and represent the means ± SEM of three (two at 12, and 72 h after the transfection) independent experiments ($n = 2$–3). *$p < 0.05$, **$p < 0.01$ *versus* non-treated: Tukey's test.

elicited by p27 siRNA transfection was abrogated to the level of non-treated cultures by co-treatment of olomoucine and purvalanol A (Fig. 3b). Neither drug altered the extent of reduction in p27 induced by p27 siRNA transfection (Fig. 3c). Olomoucine and purvalanol A alone did not affect the expression level of phosphorylated Rb and p27, or neuronal viability (Fig. 3).

Discussion

The findings presented in this report show that transfection of p27 siRNA elicited cell death, Rb phosphorylation, and increase in number of BrdU-positive cells in cultured cortical neurons. Because both Rb phosphorylation and neuronal cell death following p27 siRNA transfection were abrogated by pharmacological CDK inhibitors, elevation of cell cycle activity could be an important hallmark of neuronal cell death induced by p27 siRNA transfection.

The knockdown effect of p27 siRNA reached maximum at 48 h, and partially recovered at 72 h after transfection. The partial restoration of p27 expression level at 72 h after transfection of p27 siRNA may be due to degradation of the siRNA or new synthesis of p27 at the protein level. This is consistent with the observation that RNA interference activity induced by synthetic siRNA duplexes is transient and persists for approximately 3–7 days in cultured mammalian cells [23]. The differences in the duration of knockdown effects may depend on the nature of cell types and the stability of the targeted proteins. Our current findings indicate that the reduction of endogenous p27 level induced by p27 siRNA transfection is transient in cultured cortical neurons.

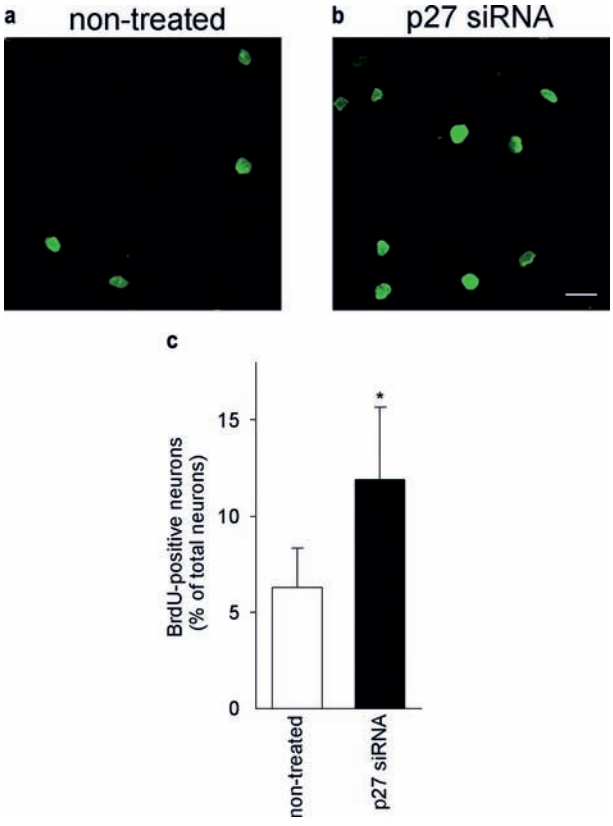

Figure 3. Transfection of p27 siRNA increases the number of BrdU-positive neurons. (*a, b*) Immunoreactivity for BrdU was visualized with Alexa-488 (green) at 48 h after the transfection. Non-treated culture (*a*). Culture transfected with p27 siRNA (*b*). Bar 20 μm. (*c*) The number of BrdU-positive and MAP2-positive cells (judged as BrdU-positive neurons) was counted. The results are presented as a percentage of total number of neurons per field (2.98×10^4 μm^2) and represent the means ± SEM in four different wells from two independent experiments. *$p < 0.05$ *versus* non-treated: Tukey's test.

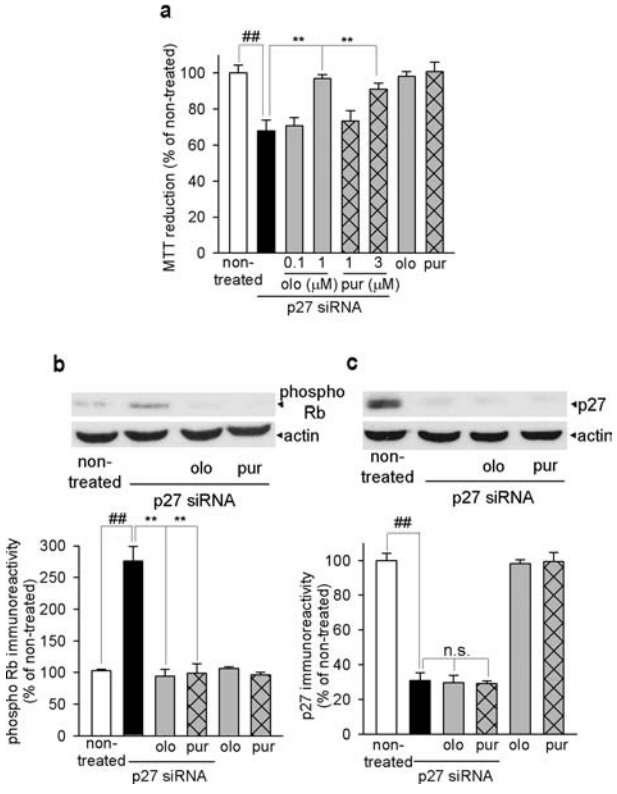

Figure 4. Pharmacological CDK inhibitors block Rb phosphorylation and neuronal cell death elicited by p27 siRNA transfection. CDK inhibitors, olomoucine (olo, 1 μM) and purvalanol A (pur, 3 μM), were added 1 h prior to the transfection of p27 siRNA (100 pmol/well), with continued treatment until assay. (*a*) At 48 h after the transfection of p27 siRNA, neuronal viability was evaluated by the MTT assay. The results are presented as a percentage of non-treated values and represent the means ± SEM of at least four independent experiments ($n = 3$–4). $^{\#\#}p < 0.01$ *versus* non-treated, $^{**}p < 0.01$ *versus* p27 siRNA: Tukey's test. (*b*) At 48 h after the transfection of p27 siRNA, expression level of phosphorylated Rb was analyzed by the immunoblotting probed with anti-phosphorylated Rb (phospho Rb) antibody. The results are presented as a percentage of non-treated values and represent the means ± SEM of at least four independent experiments ($n = 4$–5). $^{\#\#}p < 0.01$ *versus* non-treated, $^{**}p < 0.01$ *versus* p27 siRNA: Tukey's test. (*c*) At 48 h after the transfection of p27 siRNA, expression level of p27 was analyzed by the immunoblotting. The results are presented as a percentage of non-treated values and represent the means ± SEM of at least four independent experiments ($n = 4$). $^{\#\#}p < 0.01$ *versus* non-treated, not significant (n.s.) *versus* p27 siRNA: Tukey's test.

Decrease in p27 expression is an early sign of, and hypothesized to elicit, neuronal cell death, although direct evidence for this concept is lacking. Neuronal viability has not been investigated in detail in the central nervous system of mice lacking p27 [12–14]. We clearly demonstrated in the current study that transfection of p27 siRNA decreases p27 expression level and induces neuronal cell death. Since control siRNA and scrambled p27 siRNA did not reduce the p27 expression and cell viability, the observed neurotoxicity is not likely a nonspecific effect of the p27 siRNA. However, ~40% neurons still survived at 72 h after the transfection of p27 siRNA. We can not exclude the possibility that some neurons were not transfected with the p27 siRNA. The differences in susceptibility to p27 siRNA transfection are likely due to heterogeneity in the neuronal population of cultured cortical cells. Alternatively, there may be neurons that survive when p27 is not expressed. In that case, other molecules may compensate for the pro-survival function of p27. One of the candidate molecules is a CDK inhibitor p19, since mice lacking both p27 and p19 exhibit ectopic neuronal apoptosis in the cerebral cortex, suggesting that p27 and p19 play redundant, cooperative, roles in neuronal survival [24]. In the current study, p19 expression level was not altered by transfection of p27 siRNA, indicating that our experimental model does not mimic the p27 and p19 double knockout model. Taken together, our findings show that reduction in p27 is sufficient to induce cell death in at least a portion of cultured cortical neurons, and underscores the biological significance of p27 down-regulation in the neuronal cell death models where decrease in p27 is observed. To investigate the *in vivo* function of p27, we are currently undertaking a detailed analysis of central nervous system in the p27 knockout mice.

CDK inhibitors including p27 suppress CDK activity to induce cell cycle arrest in proliferating cells. In the current study, transfection of p27 siRNA induced the phosphorylation of Rb, which was completely abrogated by the pharmacological CDK inhibitors, olomoucine and purvalanol A. Moreover, p27 siRNA increased the number of BrdU-positive neurons. Since Rb phosphorylation by activated CDK is a prerequisite for the cell cycle progression to late G_1 phase and BrdU incorporation is a marker for DNA synthesis in S phase, our results indicate that p27 siRNA abolishes the function of p27 as a CDK inhibitor and induces cell cycle progression in postmitotic neurons. Since control siRNA and scrambled p27 siRNA did not elicit the Rb phosphorylation and BrdU incorporation, the cell cycle progression may be a specific result of the p27 knockdown. Neurons of the central nervous system express multiple CDK inhibitors [4]. The transient knockdown of only such CDK inhibitors, *i.e.* p27, elicited aberrant cell cycle activity, indicating that p27 plays a pivotal role in regulating cell cycle progression in neurons. Previous observations in a variety of systems indicated that aberrant cell cycle activity is necessary for cell death in postmitotic neurons [9–11, 25–29]. Our current study demonstrated that p27 siRNA transfection induced not only cell cycle activity but also neuronal cell death, both of which were completely abrogated by the pharmacological CDK inhibitors. These results suggest that aberrant cell cycle activity elicited by the p27 siRNA is sufficient for and causally related to cell death in cultured cortical neurons.

Although we found an increase in the number of BrdU-positive neurons after the transfection of p27 siRNA,

only about 10% of total neurons were BrdU positive, even under the transfection. As shown in Fig. 1c, about 60% of neurons died after p27 siRNA transfection. The majority of neurons probably died before actually entering the S phase under p27 siRNA transfection. This notion was consistent with the conclusions in other neuronal cell death models, including mild cerebral ischemia [5], glutamate toxicity [8], and β-amyloid toxicity [25]. However, other researchers reported that under serum starvation or inhibition of phosphatidylinositol 3-kinase activity, Chinese hamster ovary (CHO) cells synchronized by mitotic shake-off undergo apoptosis after progressing into S phase, as determined by [^{3}H]thymidine incorporation, another measure of DNA synthesis [30, 31]. These discrepancies reflect the differences in cell types, transformed cell lines such as CHO cell or terminally differentiated neurons. Taken together, our data suggest that p27 siRNA induces cell death before neurons progress into S phase.

Recent discoveries suggest that p27 has other activities that are unrelated to its function as a CDK inhibitor. The unexpected functions include cytoplasmic regulation of nuclear import, cell motility, and possibly transcriptional regulation in the nucleus [32–34]. These observations were made in cancerous and proliferating cells, and there is no evidence that these functions are also at work in neurons. However, we cannot exclude the possibility that transfection with p27 siRNA abolished these activities of p27, if any, in neuronal cells, since the protein level of p27 was decreased to a considerable extent in the present study. Our current findings that Rb phosphorylation and neuronal cell death induced by p27 siRNA transfection were completely abrogated by the pharmacological CDK inhibitors suggest that knocking down of the novel functions of p27 is not related with the neurotoxicity of p27 siRNA.

Olomoucine and purvalanol A, pharmacological CDK inhibitors, did not affect the extent of p27 reduction produced by p27 siRNA transfection. The finding allows two interpretations. First, the CDK inhibitors did not afford neuroprotection by compromising the efficacy of p27 knockdown. Second, decrease in p27 is upstream of CDK activation. However, in the etoposide-induced neurotoxicity, where decrease in p27 is observed, flavopiridol, another pharmacological CDK inhibitor, reverses the p27 down-regulation and affords neuroprotection [35]. This report indicates that decrease in p27 is a downstream effect of CDK activation in etoposide-treated neurons. The discrepancy may reflect the differences in methods to reduce p27 level. In our experimental conditions, the p27 level was reduced by degradation of mRNA, and reduction of endogenous p27 permitted elevation of CDK activity. In the etoposide-induced apoptotic model, however, activated CDK2 likely degrades p27 through phosphorylation at Thr187 [36], which can be blocked by the CDK inhibitor. These results suggest that whether p27 functions upstream or downstream of CDK activity may dependent on the methods to induce cell death.

Our current findings strongly suggest that p27 is a pro-survival factor in cultured cortical neurons. Reduction of endogenous p27 induces CDK activation, which phosphorylates Rb and progresses the cell cycle through late G1 phase (partly S phase). The aberrant cell cycle activity eventually induces neuronal cell death. This report provides the first direct evidence for the causal relationship between the decrease in p27 and neuronal cell death via aberrant cell cycle activity. It will be of importance to extend the implications of this study to innovate methods to protect neurons in animal models and human diseases where unscheduled cell cycle activity is suggested.

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1 Becker, E. B. E. and Bonni, A. (2004) Cell cycle regulation of neuronal apoptosis in development and disease. Prog. Neurobiol. 72, 1–25.
2 Greene, L. A., Biswas, S. C. and Liu, D. X. (2004) Cell cycle molecules and vertebrate neuron death: E2F at the hub. Cell Death Differ. 11, 49–60.
3 Sherr, C. J. and Roberts, J. M. (1995) Inhibitors of mammalian G_1 cyclin-dependent kinases. Genes Dev. 9, 1149–1163.
4 Lee, M. H., Nikolic, M., Baptista, C. A., Lai, E., Tsai, L. H. and Massague, J. (1996) The brain-specific activator p35 allows Cdk5 to escape inhibition by $p27^{Kip1}$ in neurons. Proc. Natl. Acad. Sci. USA 93, 3259–3263.
5 Katchanov, J., Harms, C., Gertz, K., Hauck, L., Waeber, C., Hirt, L., Priller, J., von Harsdorf, R., Bruck, W., Hojrtnagl, H., Dirnagl, U., Bhide, P. G. and Endres, M. (2001) Mild cerebral ischemia induces loss of cyclin-dependent kinase inhibitors and activation of cell cycle machinery before delayed neuronal cell death. J. Neurosci. 21, 5045–5053.
6 Kuan, C. Y., Schloemer, A. J., Lu, A. G., Burns, K. A., Weng, W. L., Williams, M. T., Strauss, K. I., Vorhees, C. V., Flavell, R. A., Davis, R. J., Sharp, F. R. and Rakic, P. (2004) Hypoxia-ischemia induces DNA synthesis without cell proliferation in dying neurons in adult rodent brain. J. Neurosci. 24, 10763–10772.
7 Padmanabhan, J., Park, D. S., Greene, L. A. and Shelanski, M. L. (1999) Role of cell cycle regulatory proteins in cerebellar granule neuron apoptosis. J. Neurosci. 19, 8747–8756.
8 Nishiyama, N., Yasa, N., Yamada, M. K., Ikegaya, Y. and Matsuki, N. (2002) Histone deacetylase inhibitor protects neurons from glutamate toxicity by inhibiting cell cycling. Proceedings of the 32nd Society for Neuroscience Annual Meeting, November 2–7, 2002, Orlando, CA. Abstract, 428.20.
9 Park, D. S., Levine, B., Ferrari, G. and Greene, L. A. (1997) Cyclin dependent kinase inhibitors and dominant negative cyclin dependent kinase 4 and 6 promote survival of NGF-deprived sympathetic neurons. J. Neurosci. 17, 8975–8983.
10 Park, D. S., Morris, E. J., Padmanabhan, J., Shelanski, M. L., Geller, H. M. and Greene, L. A. (1998) Cyclin-dependent kinases participate in death of neurons evoked by DNA-damaging agents. J. Cell. Biol. 143, 457–467.
11 Rideout, H. J., Wang, Q. H., Park, D. S. and Stefanis, L. (2003) Cyclin-dependent kinase activity is required for apoptotic death but not inclusion formation in cortical neurons after proteasomal inhibition. J. Neurosci. 23, 1237–1245.
12 Nakayama, K., Ishida, N., Shirane, M., Inomata, A., Inoue, T., Shishido, N., Hori, I., Loh, D. Y. and Nakayama, K. (1996)

Mice lacking $p27^{Kip1}$ display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. Cell 85, 707–720.
13 Fero, M. L., Rivkin, M., Tasch, M., Porter, P., Carow, C. E., Firpo, E., Polyak, K., Tsai, L. H., Broudy, V., Perlmutter, R. M., Kaushansky, K. and Roberts, J. M. (1996) A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in $p27^{Kip1}$-deficient mice. Cell 85, 733–744.
14 Kiyokawa, H., Kineman, R. D., Manova-Todorova, K. O., Soares, V. C., Hoffman, E. S., Ono, M., Khanam, D., Hayday, A. C., Frohman, L. A. and Koff, A. (1996) Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of $p27^{Kip1}$. Cell 85, 721–732.
15 Campbell, T. N. and Choy, F. Y. M. (2005) RNA interference: Past, present and future. Curr. Issues Mol. Biol. 7, 1–6.
16 Krichevsky, A. M. and Kosik, K. S. (2002) RNAi functions in cultured mammalian neurons. Proc. Natl. Acad. Sci. USA 99, 11926–11929.
17 Dalby, B., Cates, S., Harris, A., Ohki, E. C., Tilkins, M. L., Price, P. J. and Ciccarone, V. C. (2004) Advanced transfection with Lipofectamine 2000 reagent: primary neurons, siRNA, and high-throughput applications. Methods 33, 95–103.
18 Davidson, T. J., Harel, S., Arboleda, V. A., Prunell, G. F., Shelanski, M. L., Greene, L. A. and Troy, C. M. (2004) Highly efficient small interfering RNA delivery to primary mammalian neurons induces microRNA-like effects before mRNA degradation. J. Neurosci. 24, 10040–10046.
19 Shitaka, Y., Matsuki, N., Saito, H. and Katsuki, H. (1996) Basic fibroblast growth factor increases functional L-type Ca^{2+} channels in fetal rat hippocampal neurons: Implications for neurite morphogenesis *in vitro*. J. Neurosci. 16, 6476–6489.
20 Abe, K. and Matsuki, N. (2000) Measurement of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction activity and lactate dehydrogenase release using MTT. Neurosci. Res. 38, 325–329.
21 Liu, D. X. and Greene, L. A. (2001) Neuronal apoptosis at the G1/S cell cycle checkpoint. Cell Tissue Res. 305, 217–228.
22 Knockaert, M., Greengard, P. and Meijer, L. (2002) Pharmacological inhibitors of cyclin-dependent kinases. Trends Pharmacol. Sci. 23, 417–425.
23 Elbashir, S. M., Harborth, J., Weber, K. and Tuschl, T. (2002) Analysis of gene function in somatic mammalian cells using small interfering RNAs. Methods 26, 199–213.
24 Zindy, F., Cunningham, J. J., Sherr, C. J., Jogal, S., Smeyne, R. J. and Roussel, M. F. (1999) Postnatal neuronal proliferation in mice lacking Ink4d and Kip1 inhibitors of cyclin-dependent kinases. Proc. Natl. Acad. Sci. USA 96, 13462–13467.
25 Copani, A., Condorelli, F., Caruso, A., Vancheri, C., Sala, A., Stella, A. M. G., Canonico, P. L., Nicoletti, F. and Sortino, M. A. (1999) Mitotic signaling by β-amyloid causes neuronal death. FASEB J. 13, 2225–2234.
26 Ino, H. and Chiba, T. (2001) Cyclin-dependent kinase 4 and cyclin D1 are required for excitotoxin-induced neuronal cell death *in vivo*. J. Neurosci. 21, 6086–6094.
27 Park, D. S., Farinelli, S. E. and Greene, L. A. (1996) Inhibitors of cyclin-dependent kinases promote survival of post-mitotic neuronally differentiated PC12 cells and sympathetic neurons. J. Biol. Chem. 271, 8161–8169.
28 Park, D. S., Morris, E. J., Bremner, R., Keramaris, E., Padmanabhan, J., Rosenbaum, M., Shelanski, M. L., Geller, H. M. and Greene, L. A. (2000) Involvement of retinoblastoma family members and E2F/DP complexes in the death of neurons evoked by DNA damage. J. Neurosci. 20, 3104–3114.
29 Park, D. S., Morris, E. J., Greene, L. A. and Geller, H. M. (1997) G1/S cell cycle blockers and inhibitors of cyclin-dependent kinases suppress camptothecin-induced neuronal apoptosis. J. Neurosci. 17, 1256–1270.
30 Hulleman, E., Bijvelt, J. J. M., Verkleij, A. J., Verrips, C. T. and Boonstra, J. (2004) Identification of a restriction point at the M/G1 transition in CHO cells. Cell. Mol. Life Sci. 61, 600–609.
31 Opstal van, A. and Boonstra, J. (2006) Inhibitors of phosphatidylinositol 3-kinase activity prevent cell cycle progression and induce apoptosis at the M/G1 transition in CHO cells. Cell. Mol. Life Sci. 63, 220–228.
32 Besson, A., Gurian-West, M., Schmidt, A., Hall, A. and Roberts, J. M. (2004) $p27^{Kip1}$ modulates cell migration through the regulation of RhoA activation. Genes Dev. 18, 862–876.
33 Coqueret, O. (2003) New roles for p21 and p27 cell-cycle inhibitors: a function for each cell compartment? Trends Cell Biol. 13, 65–70.
34 Iancu-Rubin, C. and Atweh, G. F. (2005) $p27^{Kip1}$ and stathmin share the stage for the first time. Trends Cell Biol. 15, 346–348.
35 Di Giovanni, S., Movsesyan, V., Ahmed, F., Cernak, L., Schinelli, S., Stoica, B. and Faden, A. I. (2005) Cell cycle inhibition provides neuroprotection and reduces glial proliferation and scar formation after traumatic brain injury. Proc. Natl. Acad. Sci. USA. 102, 8333–8338.
36 Tsvetkov, L. M., Yeh, K. H., Lee, S. J., Sun, H. and Zhang, H. (1999) $p27^{Kip1}$ ubiquitination and degradation is regulated by the SCF^{Skp2} complex through phosphorylated Thr187 in p27. Curr. Biol. 9, 661–664.