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Common anti-apoptotic roles of parkin and α-synuclein in human dopaminergic cells

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Abstract

Parkin, a product of the gene responsible for autosomal recessive juvenile parkinsonism (AR-JP), is an important player in the pathogenic process of Parkinson's disease (PD). Despite numerous studies including search for the substrate of parkin as an E3 ubiquitin–protein ligase, the mechanism by which loss-of-function of parkin induces selective dopaminergic neuronal death remains unclear. Related to this issue, here we show that antisense knockdown of parkin causes apoptotic cell death of human dopaminergic SH-SY5Y cells associated with caspase activation and accompanied by accumulation of oxidative dopamine (DA) metabolites due to auto-oxidation of DOPA and DA. Forced expression of α-synuclein (α-SN), another familial PD gene product, prevented accumulation of oxidative DOPA/DA metabolites and cell death caused by parkin loss. Our findings indicate that both parkin and α-SN share a common pathway in DA metabolism whose abnormality leads to accumulation of oxidative DA metabolites and subsequent cell death.

Keywords: Parkin; Apoptosis; Antisense; Knockdown; Neuroblastoma; Synuclein dopamine metabolism; Quinone

Parkinson's disease (PD) is the second most common neurodegenerative disorder primarily caused by selective loss of dopaminergic neurons in the midbrain substantia nigra pars compacta. Familial PD has been highlighted to study the mechanisms underlying neurodegeneration in PD, although only 5–10% of patients with PD are of the familial form of PD [1,2]. To date, 10 causative genes have been mapped and cloned in familial PD by linkage studies, which have significantly enhanced our understanding of the genetic mechanisms of PD [3]. Of these genes, *parkin*, the causative gene (*PARK2*) of AR-JP, representing the most prevalent form of familial PD [4], is of special interest, because it encodes an E3 ubiquitin–protein ligase [5], which

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covalently attaches ubiquitin to target proteins, designating them for destruction by the proteasome [6,7]. These findings suggest that impediment of *parkin* leads to deterioration of dopaminergic neurons and that PD, at least AR-JP, is caused by the failure of proteolysis mediated by the ubiquitin–proteasome system [8]. Since then, our knowledge about parkin has expanded, and indeed at present various putative substrates, e.g., CDCrel-1, synphilin-1, α-SN22 (O-glycosylated form of α-synuclein [α-SN]), Pael-R, and cyclin E have been identified [9–14]. Moreover, negative regulation of parkin E3 activity by parkin modification, such as nitration and phosphorylation, has been reported [15–17], but the pathophysiological role of parkin is still poorly understood.

One crucial issue that needs to be investigated is why AR-JP brains show severe neuronal loss with gliosis in the substantia nigra and mild neuronal loss in the locus coeruleus and why dopaminergic neurons in the substantia nigra are particularly vulnerable to the loss-of-function effect of parkin, though parkin is expressed ubiquitously throughout the brain. To define how the loss-of-function of parkin induces selective dopaminergic neuronal death in the midbrain, we interfered with endogenous parkin mRNA, a potentially suitable in vitro model of AR-JP for investigating the mechanism of selective dopaminergic neuronal death. To knock down the level of parkin in cells, we designed a full-length human parkin antisense (abbreviated as-parkin) using an adenovirus vector that has a high multiplicity of infection (moi) toward post-mitotic cells or cell lines which has neuronal characteristics and is an excellent tool to search for the effect of as-parkin on differentiated SH-SY5Y cells that exhibit features characteristic of dopaminergic neurons.

Here, we report that as-parkin selectively induced apoptosis of SH-SY5Y cells in a caspase-dependent manner. We also found that loss of parkin resulted in accumulation of endogenous L-3,4-dihydroxyphenylalanine (DOPA)- and dopamine (DA)-chromes derived from auto-oxidation of DOPA/DA-quinones, which mediates the toxic effect by covalently binding to the thiol group of proteins and consequently disintegrates cellular integrity and eventually causes cell death [18–20]. α-SN is a putative protein associated with membrane transport or signal transduction but of unknown function. α-SN gene mutations such as missense or multiplication cause familial autosomal dominant PD [21–27]. We found that forced expression of α-SN suppressed the loss of cell viability and accumulation of oxidative DOPA/DA metabolites caused by loss of parkin. Based on these findings, we propose that parkin and α-SN contribute to a common DA metabolic pathway; the impairment of which may lead to selective degeneration of dopaminergic neurons and consequently to PD.

Materials and methods

Adenoviruses. We used the adenoviral plasmid (pAdEasy-1) and the shuttle vector (p-shuttle-CMV) (Q.Bio gene). Various cDNAs used were inserted into the shuttle vector. The shuttle vector plasmid was linearized with *Pme*I. Electrocompetent *Escherichia coli* BJ5183 cells were added and electroporation was performed in 2-mm cuvettes in a Gene Pulser electroporator. Cells were inoculated onto 10-cm Petri dish containing LB-agar and 50 μg/ml kanamycin. Smaller colonies were picked and grown in 2 ml LB-broth (Sigma Chemical St. Louis, MO) containing 50 μg/ml kanamycin. Recombination was confirmed with *Pac*I. Approximately 5×10^6 cells were plated onto 10-cm culture dish. Ten micrograms of plasmid DNA linearized by *Pme*I, 12 μl FuGENE6 (Roche Molecular Systems, NJ), and 500 μl OptiMEM (Gibco-BRL) were mixed and transfected, according to the protocol provided by the manufacturer. After 7–10 days, the cells were collected by scraping off the 10-cm dish together with floating cells in the culture. The supernatant was removed after low-speed centrifugation. After sonicating the pellet, the cells were resuspended into 1 ml Dulbecco's modified Eagle's medium (DMEM) and frozen to −80 °C. In the next step, 500 μl of viral lysate was used to infect 7×10^7 cells in 15-cm dish. This process was repeated 1–3 times. Viruses were purified by CsCl banding; the final yield was 10^{10} plaque forming units.

Cells and cell culture. Human neuroblastoma cells (SH-SY5Y) and HeLa cells were obtained from American Type Culture Collection. The cells were maintained in growth medium (DMEM, Sigma, supplemented with 10% fetal bovine serum [Gibco-BRL, Gaithersburg, MD], 100 U/ml penicillin, and 100 μg/ml streptomycin) at 37 °C under 5% CO_2. SH-SY5Y cells were cultured with 100 μM of all *trans*-retinoic acid in dimethyl sulfoxide (DMSO) (Sigma R-2625) for 3 to 4 days for differentiation. The cells were infected with the antisense adenovirus at 150 moi; LacZ at 150 and 5 moi, wild and mutant α-SN adenovirus at 5 moi. Cells were collected 36 h after infection, centrifuged, and analyzed.

Western blotting. Infected or control cells were lysed in Laemmli SDS sample buffer. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (NuPAGE, Invitrogen, San Diego, CA) and transferred onto polyvinylidene difluoride (PVDF) membrane. Western blotting was performed according to the ECL protocol provided by the supplier (Invitrogen, San Diego, CA) using specific antibodies of parkin and cleaved caspases (Cell Signaling Technology, Beverly, MA), α-SN (BD Transduction Laboratories, Lexington, KY), and β-Gal (Promega, Madison, WI).

Cell survival assay. Cells were infected with as-parkin or LacZ adenovirus and incubated for 48 h in 96-well plate. Cell viability was evaluated using the WST8, MTT reduction assay. Briefly, the solution of 0.1 mg/ml MTT in DMEM was added to each well and incubated for 2 h. The transmission was evaluated at 450 and 655 nm by 96-well microplate reader (Bio-Rad, Richmond, CA).

TUNEL assay. Terminal-deoxynucleotidyl transferase mediated d-UTP nick end labeling (TUNEL) assay was performed using formalin-fixed, ApopTag In Situ Apoptosis Detection Kits (Intergen, Purchase, NY). Fragmented DNA was labeled by fluorescein isothiocyanate (FITC) and observed under a fluorescence microscope.

Measurements of DOPA/DA-chromes. Thirty-six hours after infection, cells were solubilized in 500 μl of 1% Triton X-100 solution for 2 h and then centrifuged at 20,000*g* for 30 min at 4 °C. The supernatant was used as cell extract and was incubated for 3 min at room temperature. After 10% TCA protein precipitation, the generation of DOPA/DA-quinones was estimated by measuring the absorbance of the incubation supernatant at 475 nm based on the formation of DOPA/DA-chromes. The amount of DOPA/DA-chromes was calculated from a standard curve constructed using known amounts of DA and 0.01 mg/ml tyrosinase. The protein concentration in the cell extracts was determined by using the BCA Protein Assay Reagent Kit

(Pierce Chemical, Rockford, IL) with bovine serum albumin as a standard.

Statistical analysis. All data were expressed as means ± SEM. Differences between groups were examined for statistical significance using Dunnett's t test or Turkey's multiple t test. A P value less than 0.05 denoted the presence of a statistically significant difference.

Results and discussion

Antisense parkin causes loss of viability of SH-SY5Y cells

We first examined the effect of knockdown of parkin on the viability of human neuroblastoma cells (SH-SY5Y). These cells contain dopaminergic machinery and can differentiate into neuronal-like phenotypes when treated for 3–5 days with retinoic acid (RA), accompanied by arrest of cell proliferation and increased dopamine metabolism [28,29]. Infection of SH-SY5Y with full-length human as-parkin adenovirus caused deterioration of cell viability, as monitored by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. The differential SH-SY5Y cell death effect was observed in the range between 50 and 250 moi titer of as-parkin adenovirus (data not shown) and thereafter we routinely used 150 moi titer (Fig. 1A, left panel). Control β-galactosidase (β-Gal) adenovirus had no effect on cell viability, although β-Gal was highly expressed in SH-SY5Y cells (Fig. 1A, right panel). Infection of cells with as-parkin caused a marked decrease of endogenous parkin protein level in differentiated SH-SY5Y cells, without altering actin level in the cells (Fig. 1A, right panel). The effect of as-parkin was abrogated, upon co-infection with sense-parkin (data not shown).

Effect of antisense parkin is cell-type specific

Intriguingly, we found that the effect of as-parkin on cell viability was much less in undifferentiated growing SH-SY5Y cells compared with differentiated cells (Fig. 1B, left panel). In addition, as-parkin did not influence cell viability of HeLa cells derived from human adenocarcinoma of the uterine cervix, which do not express parkin protein and lack the dopamine metabolic pathway (Fig. 1B, right panel). Thus, antisense knockdown of parkin exerts its effect based on the cell type, and the effects are observed in a dopaminergic neuron-specific manner, and depend on the differentiation state of dopaminergic neurons.

Antisense parkin induces apoptotic cell death

As shown in Fig. 1C, the cells appeared clear when their morphology was compared with uninfected (control) and β-Gal expressing cells. SH-SY5Y cells infected with as-parkin adenovirus showed morphological

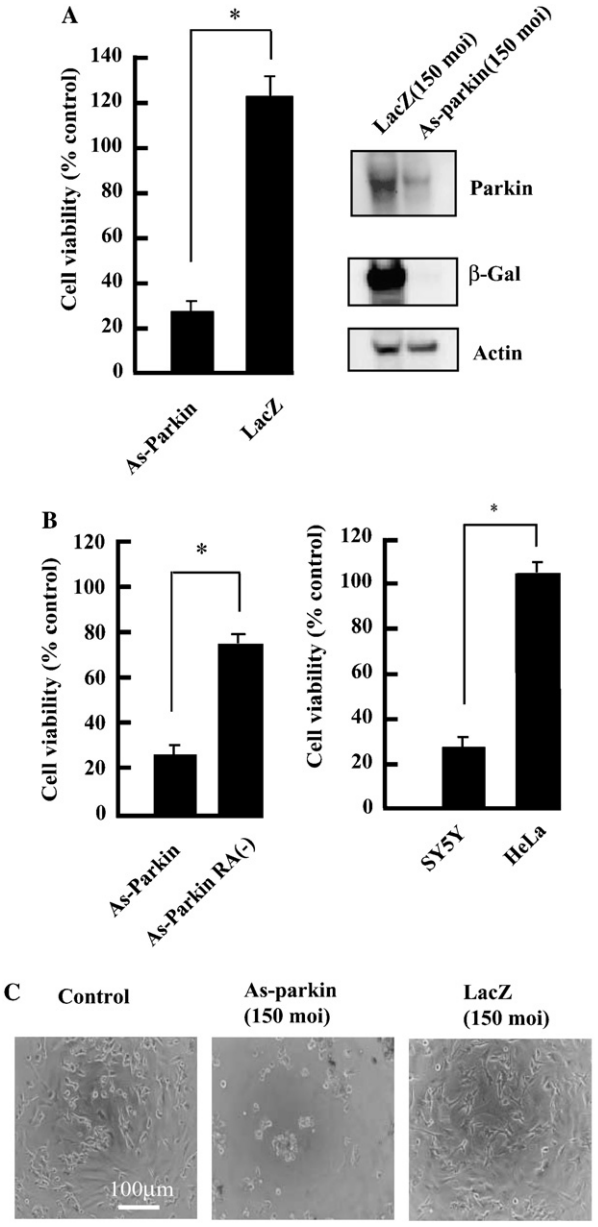

Fig. 1. Parkin knockdown is associated with loss of SH-SY5Y cell viability. (A) Effects of antisense parkin (as-parkin) and LacZ. Adenoviruses were infected for 48 h with 150 moi titers as indicated on the differentiated SH-SY5Y cells that had been pre-cultured with RA for 4 days. Cell viability was determined by the MTT assay (left panel). The results are expressed as percentage of MTT activity of uninfected cells (control). Data represent means ± SEM of 8 determinations. $*P < 0.01$ versus control group (Dunnett's t test). Cells that had been treated for 48 h with 150 moi titers of as-parkin and LacZ adenoviruses were lysed in Laemmli SDS sample buffer, and the proteins were separated by SDS–PAGE, followed by Western blotting with antibodies against parkin, β-galactosidase (β-Gal), and actin (right panel). (B) Undifferentiated SH-SY5Y cells without treatment with RA and HeLa cells were treated for 48 h with as-parkin adenovirus. The cell viability was measured and represented as indicated. (C) Morphological changes in differentiated SH-SY5Y cells upon knockdown of parkin. The cells were infected for 48 h with as-parkin and LacZ adenovirus vectors or left uninfected (control). Note the presence of apoptotic cells. Bar, 100 μm.

changes typical of apoptosis. To determine the nature of cell death induced by as-parkin, we performed TUNEL assay. As shown in Fig. 2A, as-parkin-treated SH-SY5Y cells showed nuclear condensation and fragmentation. In contrast, these changes were rarely observed in β-Gal-expressing SH-SY5Y cells. In support

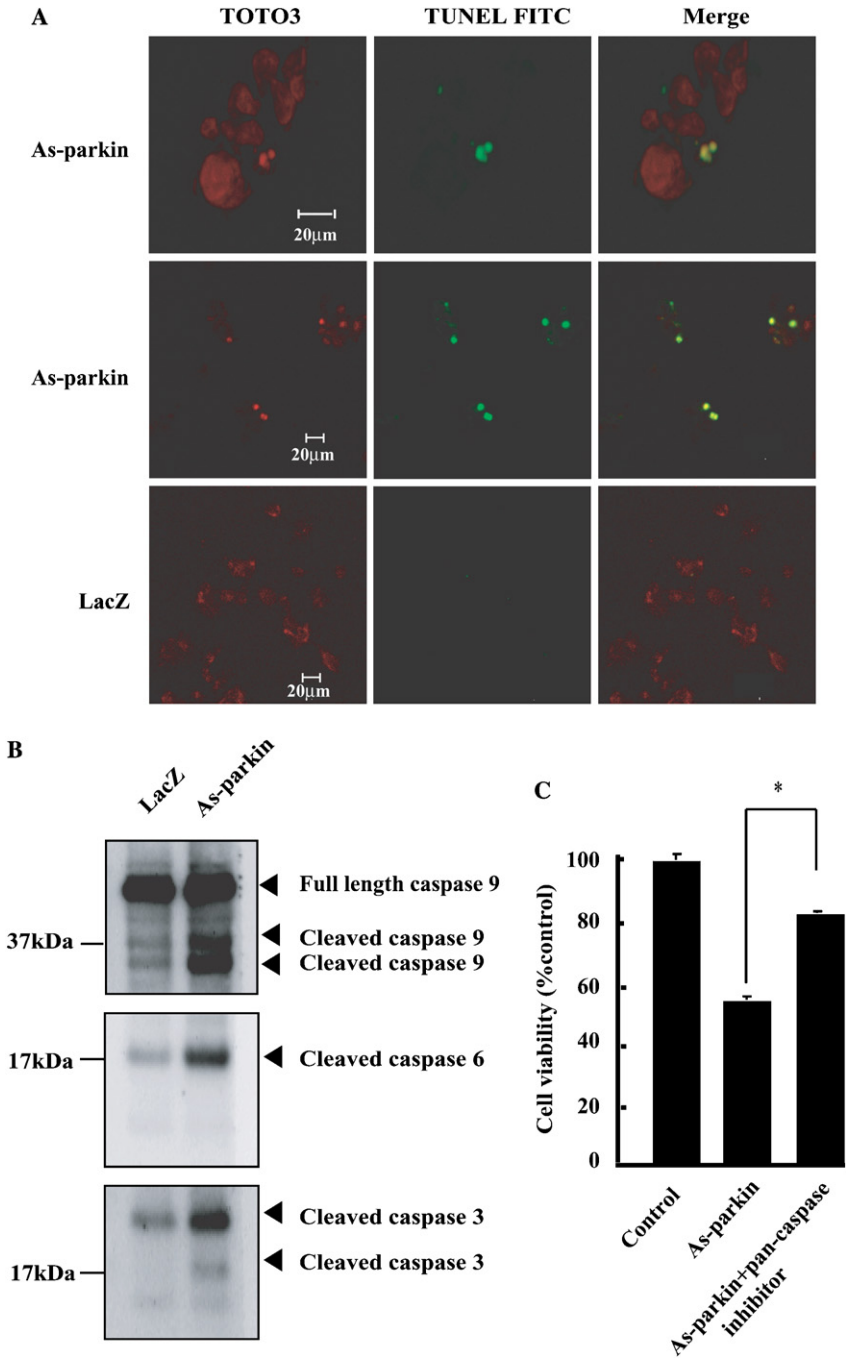

Fig. 2. Parkin knockdown induces apoptosis. (A) Detection of cells with nuclear DNA fragmentation due to parkin knockdown by TUNEL assay. Differentiated SH-SY5Y cells were treated for 48 h with as-parkin and LacZ adenoviruses (150 moi). TUNEL assay was performed to detect apoptotic cells. TUNEL-positive cells (green) were detected (TdT enzyme is labeled with FITC green fluorescence). Nuclei were counterstained with TOTO3 (red). Bar, 20 μm. (B) Activation of caspase-3, -6, and -9 by as-parkin. After infection with as-parkin and LacZ adenoviruses as for a, the cell extracts were used for Western blot analysis using antibodies against cleaved caspase-3, -6, and -9. Arrowheads on the right indicated corresponding caspases. Note that anti-cleaved caspase-3 and -6 antibodies did not react with their native forms. (C) Effects of a 'pan' caspase inhibitor on apoptosis induced by the loss of parkin. The inhibitor was added at 100 μM when cells were treated by as-parkin adenovirus as for (A). Note that the caspase inhibitor significantly blocked parkin knockdown-induced deterioration of cell viability. Data represent means ± SEM of 8 determinations. $^{*}P < 0.05$ versus control (uninfected) group (Dunnett's t test).

of the TUNEL findings, we also detected activation of caspase-3, -6, and -9 in SH-SY5Y cells under parkin knockdown (Fig. 2B). In addition, Western blot analysis showed cleaved poly(ADP)-ribose polymerase (PARP) in the course of as-parkin infection and a time-dependent increase of the 25-kDa cleaved fragment, confirming the activation of caspase(s) (data not shown). Further experiments showed that application of a pan-caspase inhibitor for 6 h before infection significantly prevented apoptotic cell death as determined by the MTT reduction assay (Fig. 2C). Taken together, these results suggest that as-parkin-induced SH-SY5Y cell death is likely to be mediated by activation of the caspase cascade.

Antisense parkin increases DOPA/DA metabolites

Next we examined the effect of α-SN on the viability and DOPA/DA-chrome level in differentiated SH-SY5Y cells with a reference to parkin loss. These experiments were based on previous studies describing abnormal DA metabolism in α-SN-deficient mice [30] and α-SN binding to DA-quinones [31]. For this purpose, we constructed adenovirus vectors expressing α-SN, and first tested its effect on the expression of parkin. α-SN and its PD-linked mutants (Ala30Pro and Ala53Thr) had no effect on the levels of parkin, irrespective of the treatment of as-parkin (Figs. 4A and B). It is of note that α-SN did not express at significant levels in SH-SY5Y cells under present conditions. Then, we investigated the effect of α-SN on the as-parkin-induced loss of cell viability. As shown in Fig. 4A, infection of SH-SY5Y cells by both adenovirus vectors expressing α-SN and as-parkin caused marked reduction of their cellular chrome levels and resulted in amelioration of as-parkin-induced deterioration of cell viability (Fig. 4B). Intriguingly, coinfection of cells with wild-type α-SN and as-parkin adenoviruses abrogated as-parkin-induced accumulation of DOPA/DA-chrome. However, α-SN mutants (Ala30Pro and Ala53Thr) and β-Gal expression did not reduce the generation of DOPA/DA-chrome by as-parkin. Thus, it seems that the α-SN-induced suppression of apoptosis was associated with a reduction in the DOPA/DA-chrome level in α-SN expressing SH-SY5Y cells. These results suggest that α-SN inhibits apoptosis induced by parkin knockdown by blocking the generation of DOPA/DA-chromes; i.e., DOPA/DA-quinones.

Antisense parkin-induced extensive apoptosis of differentiated dopaminergic SH-SY5Y cells but limited apoptosis of undifferentiated SH-SY5Y cells and no apoptosis of HeLa cells, indicating cell-type specificity. With regard to the cell-specific vulnerability, an important factor seems to be dopamine (DA) metabolism, which is a peculiar feature of dopaminergic neurons. Indeed, the differentiated SH-SY5Y cells retain a high DA metabolic pathway [28,29]. DA is a molecule prone to oxidation and it contributes to the generation of reactive oxygen species, which when in abundance can cause oxidative injury of various cellular components[18,20]. Indeed, abnormally high levels of these free radicals in dopaminergic neurons have been implicated as environmental factors causing not only sporadic PD but also AR-JP [32,33]. We tested the effects of as-parkin infection on the level of endogenous DOPA- and DA-chromes (DOPA/DA-chromes), which are derived from DOPA- and DA-quinones, respectively, whose metabolites could originate from cytosolic DOPA or DA oxidation [18,20]. Thus, the amounts of DOPA/DA-chromes reflect those of endogenous DOPA/DA-quinones. DOPA/DA-chrome levels were significantly high in parkin knockdown cells whereas there was no change in β-Gal expressing ones (Fig. 4A). These findings suggest that parkin knockdown-induced apoptosis is mediated by an increase in DOPA/DA-chromes.

Recently, four groups independently reported the generation of a mouse model that lacks the *parkin* gene, which display certain abnormalities of dopamine metabolism [34–37]. However, these parkin knockout mice had only subtle phenotypes exhibiting a largely normal gross brain morphology. Based on the pathologic findings, all the parkin null mice showed no neuronal loss in the SN. This is in marked contrast to our in vitro system described in this study, in which parkin knockdown induced activation of the caspase cascade and apoptosis of dopaminergic SH-SY5Y cells. Why do parkin knockout mice lack the abnormalities seen in AR-JP patients? One plausible explanation is the presence of a putative molecule(s) that suppresses the defect induced by loss-of-function of parkin, and the abundant presence of such molecule(s) in the brain should be linked to the pathogenesis of PD. Here, we propose that α-SN is the molecule that compensates for the loss of parkin, since α-SN prevented apoptotic cell death induced by as-parkin. In this regard, Western blot analysis showed that the dopaminergic SH-SY5Y cells did not express α-SN at significant levels (Fig. 3A, lanes 2 and 4), which is in marked contrast to the high abundance of dopaminergic neurons in vivo [38]. Regardless of the compensatory role of α-SN for the loss-of-function of parkin in the AR-JP, α-SN probably cannot cope with the accumulation of toxic molecules in the absence of parkin and thus apoptotic neuronal death perhaps occurs gradually, leading to degeneration of dopaminergic neurons and consequently the development of early-onset PD. We provide the first evidence for the anti-apoptotic role of α-SN and its involvement in the common pathway of parkin.

To date, several studies have demonstrated that α-SN exerts protective effects against various cellular stresses such as oxidative damage and related apoptosis of neurons [39,40]. Considering the reason why muta-

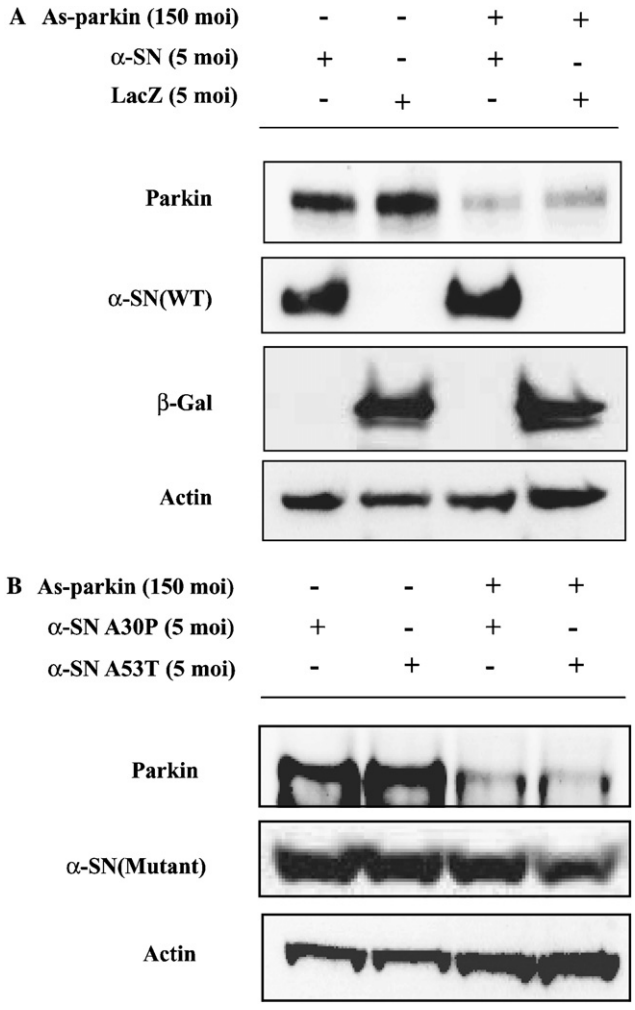

Fig. 3. Expression levels of parkin, α-SN, and LacZ. The expression levels of parkin, wild, and mutant α-SN, and LacZ in SH-SY5Y cells treated for 48 h with adenoviruses were monitored by Western blot analysis.

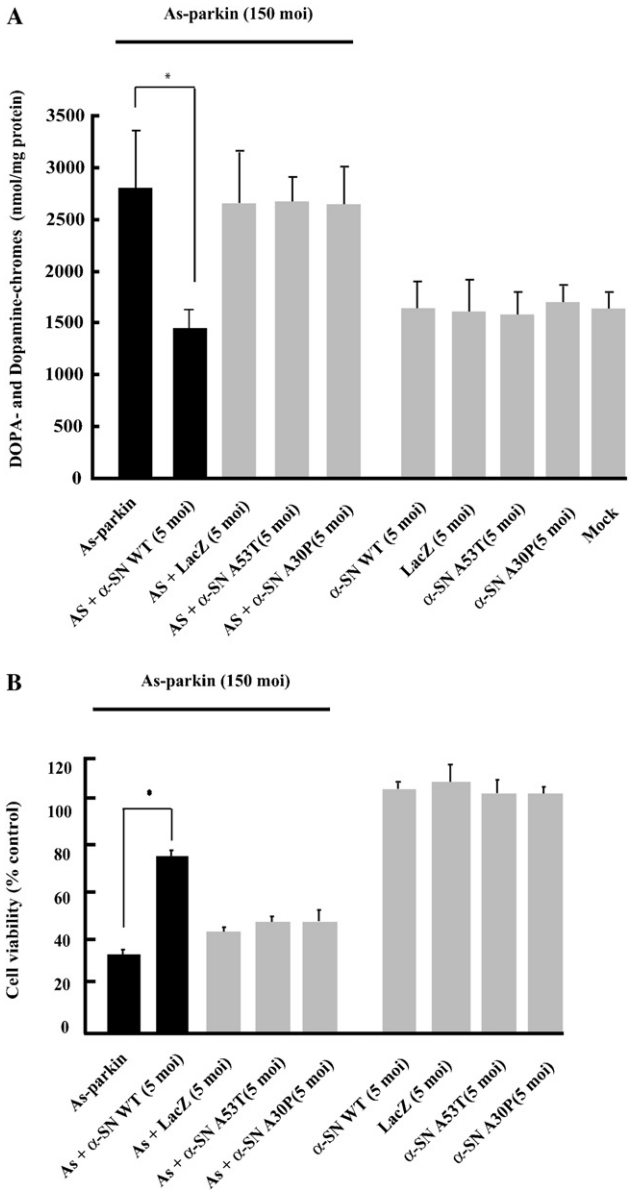

Fig. 4. α-Synuclein inhibits parkin knockdown-induced apoptosis and accumulation of DOPA- and DA-quinones. (A) Cellular level of DOPA/DA-chromes. After the differentiated SH-SY5Y cells were treated for 36 h with as-parkin, wild, and mutant α-SN, LacZ, and adenoviruses, cellular DOPA/DA-chromes were measured. Note the profound decrease of DOPA/DA-chromes in α-SN-expressing SH-SY5Y cells. Data are means ± SEM of 10 determinations. $^{*}P < 0.05$ versus control group (Turkey's multiple t test). (B) Effects of overexpression of wild and mutant α-SN on as-parkin-induced deterioration of cell viability. Differentiated SH-SY5Y cells were treated for 48 h with as-parkin adenovirus. Cells were coinfected with LacZ and α-SN adenovirus (5 moi) and at 150 moi titers of as-parkin adenovirus. The cell viability was measured and represented as in Fig. 1A (left panel).

tion of α-SN causes familial PD, it is clear that this type of disease is due to the gain-of-toxic function of the α-SN mutants with missense mutations, differing from the neuroprotective roles of the wild-type α-SN. In addition, α-SN proteins with disease-causing missense mutations tend to generate protofibrils [31,41], suggesting that protein misfolding including α-SN plays a key role in the pathogenesis of PD. In contrast, at high concentrations, it oligomerizes to β-pleated sheets known as protofibrils (i.e., fibrillar polymers with amyloid-like characteristics). Indeed, multiplication of α-SN has been reported in the autosomal dominant form of PD, indicating that overproduction of this protein affects the cellular damages. In this regard, there is a discrepancy between the protective role of α-SN in the present study and combination of PD and α-SN multiplication. This could be explained by appropriate physiological level of synuclein [40]. Thus, in patients with α-SN multiplication, the copy numbers of this gene may be related to the clinical severity of PD; patients with triplicate α-SN show dementia with Lewy bodies [24]; while those with duplicate levels do not show dementia [26,27].

It remains unclear why dopaminergic neurons of the substantia nigra are selectively vulnerable to the loss of parkin in AR-JP patients. In the present study, we provided a clue for this enigmatic puzzle. Considering the specificity of the lesions in PD, it is possible that the high oxidative state associated with DA metabolism may cause deterioration of dopaminergic neurons. The mechanism underlying increased oxidative stress may involve DA itself, because oxidation of cytosolic DOPA/DA may be deleterious to neurons. Indeed, DA causes apoptotic cell death as evident by morphological nuclear changes and DNA fragmentation [42–44]. In this regard, we showed here that as-parkin directed loss of parkin leads to abnormality of DOPA/DA metabolism, which resulted in the generation of DOPA/DA-quinones in SH-SY5Y cells. Thus, DA and its metabolites seem to exert cytotoxicity mainly by generating highly reactive quinones through auto-oxidation. On the other hand, the toxicity of DOPA and DA is due to the generation of reactive oxygen species that could disrupt cellular integrity, causing cell death. However, the reason for the production of oxidative DOPA/DA-metabolites following loss of parkin is not clear at present.

Our results showed for the first time that loss of parkin leads to death of differentiated dopaminergic cells in vitro. This cell-based experiment enhances our understanding of the pathophysiology of PD and could be potentially useful for drug screening. Our results also showed that α-SN and parkin are involved in DA metabolism and that aberrant regulation of DA is accompanied by accumulation of oxidative DOPA/DA metabolites.

Acknowledgments

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