Early Parkinson’s disease symptoms in α-synuclein transgenic monkeys

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Abstract
Parkinson’s disease (PD) is an age-dependent neurodegenerative disease that can be caused by genetic mutations in α-synuclein (α-syn) or duplication of wild-type α-syn; PD is characterized by the deposition of α-syn aggregates, indicating a gain of toxicity from accumulation of α-syn. Although the major neuropathologic feature of PD is the degeneration of dopaminergic (DA) neurons in the substantia nigra, non-motor symptoms including anxiety, cognitive defect and sleep disorder precede the onset of motor impairment, and many clinical symptoms of PD are not caused by degeneration of DA neurons. Non-human primate models of PD are important for revealing the early pathology in PD and identifying effective treatments. We established transgenic PD rhesus monkeys that express mutant α-syn (A53T). Six transgenic A53T monkeys were produced via lentiviral vector expressing A53T in fertilized monkey eggs and subsequent embryo transfer to surrogates. Transgenic α-syn monkeys demonstrate the specific early symptoms caused by mutant α-syn and provide insight into treatment of early PD.

Introduction
Parkinson’s disease (PD) is an age-dependent neurodegenerative disease that shows late-onset degeneration of dopaminergic (DA) neurons in the substantia nigra, which leads to a complex motor disorder featuring bradykinesia, tremor, rigidity and postural instability. Although the majority of PD patients are sporadic cases, genetic mutations in the α-synuclein (α-syn) gene also cause familial PD (1–4). Both sporadic and familial PD exhibit non-motor symptoms for many years before the onset of motor impairment (5–7) whereas motor symptoms appear when >50% of dopaminergic neurons in the substantia nigra have degenerated (8). The non-motor symptoms of PD encompass mood disorders such as anxiety and depression, psychosis, dementia, sleep disorders, and autonomic dysfunctions. As a result, PD is thought to be a multisystem neurodegenerative disorder clinically characterized by motor and non-motor dysfunction. Although non-motor...
symptoms occur prior to motor dysfunction and affect one’s quality of life, these early symptoms are often not treated or diagnosed to link to PD.

Animal models showing early PD pathology are important for identifying effective therapeutic strategy to treat PD. However, there are no transgenic rodent models that completely recapitulate the key clinical and neuropathologic features of PD (9). For example, transgenic mouse models of PD often lack the typical DA neurons seen in patient brains with PD (9). Since the genomics, brain anatomy and neuronal circuitry are significantly different between primates and rodents, species-dependent differences are likely to account for the fact that the same disease protein may induce differential pathological changes in different species. Furthermore, rodent models of PD are difficult to mimic non-motor symptoms of PD, such as depression, anxiety and emotional abnormalities. Non-human primates would serve as good animal models to uncover non-motor behavioral changes that may mimic early clinical symptoms in PD. In addition, it remains unclear whether mutant α-syn causes distinct non-motor symptoms in early stages of PD. Based on these facts, we decided to use non-human primates to investigate PD pathology. By establishing transgenic rhesus monkeys via expressing mutant α-syn in the fertilized monkey eggs, we found that mutant α-syn causes non-motor symptoms in an age-dependent manner. The non-human primate model of PD will be valuable for helping us identify early pathological events and treatments of PD.

Results

We used the lentiviral vector to generate transgenic rhesus monkeys via the same strategy we have used to generate transgenic Huntington’s disease and GFP monkeys (10,11). In the lentiviral vector, mutant α-syn (A53T) is linked to ECFP via F2A, which can be self-cleaved in cells to separate A53T from ECFP, and is expressed under the human ubiquitin (hUBC) promoter (Fig. 1A). This lentiviral A53T is able to effectively infect primary neuronal cells in vitro (Fig. 1B). Over the past 4 years, we injected viral A53T into 133 MII oocytes of rhesus monkeys. Of these injected oocytes, 81.8% (108 of 133) developed into 4–16-cell stage embryos, and 75 embryos were transferred into 25 surrogate monkeys, resulting in 11 pregnancies (44.0%) that yielded 15 fetuses as a result of some twin pregnancies (Fig. 2A). Of these fetuses, seven developed to full term, leading to seven live newborns (47%, 7/15), with the oldest (110217) born on 9 May 2011 (Fig. 2B). We verified that the transgene had been ubiquitously integrated into genomes in different tissues (Fig. 2C). There were also eight spontaneous miscarriages, yielding aborted fetuses at different gestation days (31–170 days). PCR analysis verified that six live monkeys (6/7) and five aborted fetuses (5/8) were positive for transgenic A53T, such that 73.3% of monkeys examined carry the transgenic A53T gene (Fig. 3A). Using quantitative PCR, we estimated the copy numbers of transgenic A53T were between 2 and 7 in these transgenic monkeys versus two copies of endogenous α-syn in wild-type monkey (Fig. 3B).

Figure 1. Expression of lentiviral A53T vector in rodent neuronal cells. (A) A53T is linked with ECFP via F2A and expressed under the human ubiquitin (hUBC) promoter in the lentiviral vector. (B) Cultured mouse striatal neurons transduced by lentiviral A53T show positive staining by anti-α-syn. Scale bar: 10 µm.
To confirm the transgene expression, we used western blotting to examine tissues from aborted transgenic and wild-type monkeys and verified the expression of A53T at the protein level in different tissues from the aborted transgenic A53T monkey (Fig. 4A). Notably, one of the transgenic A53T monkeys died of dystocia during a difficult birth. This stillborn transgenic A53T monkey allowed us to use immunohistochemistry for comparison with a wild-type stillborn monkey and to identify the expression of transgenic A53T in the substantia nigra, cortex and striatum of the developed brain (Fig. 4B and C). Using anti-α-syn antibody to perform immunohistochemistry staining, we saw a number of neurons expressing A53T in the substantia nigra, and some show punctate staining that may reflect the enrichment of transgenic A53T in synaptic terminals. Double immunostaining confirmed that neurons expressing transgenic A53T are also labeled by the antibody to NeuN, a neuronal marker (Fig. 5A). Examining the A53T transgenic monkey brain using antibodies to NeuN and GFAP did not reveal obvious degeneration in the substantia nigra, cortex and striatum. In addition, the majority of A53T transgenic neurons show diffuse staining by anti-α-syn and were negative to the antibody against S129 phosphorylated synuclein (Fig. 5B). Since aggregated synuclein in Lewy bodies is labeled by anti-S129 and the aggregation of mutant synuclein is age-dependent (12), the negative anti-S129 staining in the stillborn monkey brain suggests that transgenic A53T in the developing monkey brain is largely soluble.

All live transgenic A53T monkeys developed normally, with no distinguishable differences from age-matched non-transgenic monkeys. We saw no significant abnormalities in growth or movement, such as walking difficulty or falls, in transgenic A53T monkeys, which is consistent with the age-dependent disease progression of PD. While monitoring the behaviors of transgenic A53T monkeys using methods described previously (13), we found that the oldest A53T transgenic male monkey (110217) began to develop cognitive defects and an anxiety phenotype at the age of 2.5 years, unlike a non-transgenic male control monkey that was born at the same time and housed in a single cage under identical conditions as the A53T monkey. For the oldest male A53T transgenic monkey (2.5 years), we selected three wild-type male monkeys of the same age as controls. For the two younger female A53T transgenic monkeys (1.5 years), we selected two female (120660, 120696) and one male (120671) WT monkeys of the same age as their controls. We also included a 5-year-old GFP transgenic monkey generated in our previous study as a control (11). Three other younger A53T monkeys (<1 years) were too young for training and repeating the above tests.

To test cognitive function, we recorded the time for A53T transgenic monkeys to recognize a red box containing peanuts after training them to pick up peanuts from the red box whose position on a strip of steel was randomly switched with four other colored boxes (Fig. 6A). A shorter time to pick up the peanuts thus reflects a better capacity for learning and memory. After one training session, the control monkey began to recognize the red box and uncovered peanuts directly. However, even after 25 training sessions, the oldest A53T monkey still uncovered the boxes randomly to pick up peanuts. As a result, this A53T monkey took a significantly longer time than the control monkey to recognize and pick up peanuts in the red box (Fig. 6B, 3.06 ± 1.12 s versus 0.98 ± 0.14 s of the control, \( P = 0.00049 \), Supplementary Material, Movies S1 and S2). To rule out the influence of color discrimination, we used containers with different shapes (round, triangle, square, rectangle and pentangle) and again found that the oldest A53T monkey still took a longer time to pick out the correct container containing food (1.26 ± 0.03 versus 2.74 ± 0.44 for control versus A53T monkey, \( P = 0.0288 \)). To test fine finger coordination or dexterity, monkeys were allowed to pick up candies in holes that were in a plate rotating at 30 rpm
The oldest and two younger A53T transgenic monkeys failed more often and took longer to pick up candies than the control monkeys (Fig. 6D, Supplementary Material, Movies S3–S5), suggesting the fine finger coordination and dexterity of A53T monkeys are not as good as the control monkeys.

By monitoring monkeys via continuous video recording at different ages, we found that the oldest A53T monkey also showed stereotypic behaviors that were repetitive, unvarying actions without goal or function. The A53T monkey walked in circles more often than the control monkey (Fig. 7A; Supplementary Material, Movie S6), which is an anxiety-related stereotypic behavior (13,14). The stereotypic circling behavior of the A53T monkey was more frequent than in the control monkey (31.86 ± 0.59 times/h versus 3.86 ± 0.44 times/h, \( P = 1.49 \times 10^{-8} \)) and lasted longer (134.29 ± 1.54 s/h versus 3.00 ± 0.32 s/h for the control monkey, \( P = 4.35 \times 10^{-7} \), Fig. 7A). Nevertheless, we found no significant sleep abnormalities in this A53T monkey (Fig. 7B) or the other A53T transgenic monkeys. We know that cognitive defects and anxiety phenotypes are often seen in PD patients at early disease stages, preceding motor deficits (5,6). Thus, the behavioral phenotypes of the A53T monkey are consistent with the non-motor symptoms of PD patients at the early disease stage.

The loss of over 50% of the dopaminergic neurons is known to be required before motor abnormalities become apparent in PD patients (8). Consistent with the lack of apparent motor symptoms, MRI analysis revealed no obvious degeneration in the

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**Table 1.** Summary of live transgenic monkeys

<table>
<thead>
<tr>
<th>Animal</th>
<th>Age (years)</th>
<th>Sex</th>
<th>PCR</th>
<th>Estimated A53T copy numbers</th>
<th>Notes</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>110217</td>
<td>male</td>
<td>+</td>
<td>3.94</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>120666</td>
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<td>+</td>
<td>3.71</td>
<td>1-2</td>
</tr>
<tr>
<td>3</td>
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<td>female</td>
<td>+</td>
<td>5.6</td>
<td>3-4</td>
</tr>
<tr>
<td>4</td>
<td>99058</td>
<td>female</td>
<td>-</td>
<td>2</td>
<td>0</td>
</tr>
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<td>5</td>
<td>130996</td>
<td>female</td>
<td>+</td>
<td>7.38</td>
<td>5</td>
</tr>
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<td>7</td>
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<tr>
<td>7</td>
<td>130919</td>
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<td>+</td>
<td>4.29</td>
<td>2</td>
</tr>
</tbody>
</table>

**Figure 3.** PCR genotyping and copy numbers of transgenic A53T monkeys. (A) The PCR products from seven live transgenic A53T monkeys and one wild-type monkey were obtained with two sets of primers specific to transgenic A53T-ECFP construct. β-Actin DNA served as an internal control for normalization and the endogenous monkey α-syn gene was normalized as two copies. (B) The general information of transgenic monkeys and assessment of copy numbers of transgenic A53T in monkeys via quantitative PCR.
brains of A53T monkeys compared with the control monkey (Fig. 8). Also, analysis of blood samples of transgenic A53T monkeys did not show any significant changes in electrolytes, glucose, hemoglobin, blood cell counts, etc. compared with control monkeys.

**Discussion**

Our findings show age-dependent non-motor symptoms in A53T transgenic monkeys. Thus, like transgenic A53T mouse models, our transgenic A53T monkeys may develop more robust phenotypes as they age. Despite the lack of robust symptoms in young transgenic A53T monkeys, these monkeys are valuable for helping us identify early pathological events that are more likely to happen in PD patients because of the similarities between monkeys and humans. Importantly, non-motor symptoms in small animal models of PD are difficult to characterize; using a transgenic A53T monkey model allowed us to identify cognitive defects and anxiety-like behaviors as early phenotypes.

Non-motor symptoms, such as depression/anxiety, cognitive deficits, constipation, genitourinary problems and sleep disorders, often precede the motor symptoms of PD patients (5,15,16). These symptoms are difficult to be replicated in small animals such as rodents. Non-human primate models would allow us to investigate how mutant α-syn causes non-motor symptoms and behavioral changes that are similar to those in PD patients. In non-human primates, stereotypic behaviors, which are repetitive actions that appear to have no goal or function, are a typical sign of anxiety (13,14,17). However, we saw no sleep disturbances, another early symptom of PD, in transgenic A53T monkeys. The lack of sleep disorder suggests that such disturbance may only occur in early stages of sporadic PD patients or later when transgenic A53T monkeys are older. Alternatively, it also suggests that mutant α-syn is more likely to affect mood behavior than sleep function in the early disease stage.

Transgenic and toxin-induced PD animal models have been widely used in studying the pathogenesis and identifying therapeutics. Toxin-based animal models, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated monkeys, have given us hemi-parkinsonian primate models and resulted in new therapeutic strategies for dopaminergic neuron degeneration (18). Compared with toxin-induced PD animal models, transgenic PD models should show more stable and replicable pathological changes and phenotypes, which are critical for developing effective treatments. Injection of viral vectors...
expressing mutant α-syn into the substantia nigra of monkeys was used to study the toxicity of mutant α-syn in primate brains. The injected areas in the monkey brains show cytoplasmic α-syn aggregates, dystrophic neurites and a moderate loss of dopaminergic neurons (19,20). Recently, Lewy body (LB) extracts from PD patient brains were inoculated into the substantia nigra or striatum of wild-type macaque monkeys, resulting in intracellular accumulations of pathological α-syn and progressive axon-initiated dopaminergic nigrostriatal neurodegeneration (21). However, the viral vector injection and LB extract inoculation yield the overexpression of toxic proteins in the adult monkey brains, whereas A53T transgenic monkey brain tissues we examined were from the newborn monkey that died of difficulty birth. The lack for α-syn aggregates in the stillborn monkey brain supports the idea that α-syn-mediated aggregation and degeneration are dose and age-dependent. Since the restricted expression of mutant proteins in brain regions is difficult for assessing the clinical symptoms caused by the systemic expression of mutant proteins, systemic expression of transgenic mutant proteins in monkeys will make valuable primate models to uncover important and early symptoms as well as unique neuropathology that may not be seen in small animals. For example, a transgenic monkey model of Huntington disease (HD) displays axonal degeneration that is not seen in the HD mouse model expressing the same transgene (22).

Transgenic monkey models of PD will also be valuable for identifying biomarkers for PD. By continuously monitoring the development of neurological symptoms of these transgenic monkeys, we may identify biomarkers associated with the progressive symptoms. In addition, the transgenic PD model will be helpful for verifying important therapeutic targets in the future. For example, although induced pluripotent stem (IPS) cells from PD patients have been established to identify chemicals that can reverse the pathologic phenotypes of these IPS cells (23), validation of the effects of these chemicals in PD animal models can be achieved by using transgenic PD monkey models before their clinic trails on PD patients.

Materials and Methods

Antibodies

Primary antibodies from commercial sources used in this study include: mouse anti-α-syn (Thermo Scientific, MS-1572-P1ABX), mouse anti-GAPDH, rabbit anti-ECFP (Clontech Laboratories, Inc.), rabbit anti-S129 synuclein (ab59264, Abcam). All secondary antibodies were purchased from Jackson Immunoresearch and Invitrogen.

Lentiviral preparation

We used the lentiviral vector to express mutant α-syn (A53T), which is linked to ECFP via a self-cleaving 2A peptide (F2A). F2A can be self-cleaved in cells and has been widely used to separate the transgenic protein from the reporter such as ECFP in cells (24). The A53T-F2A-ECFP is expressed under the human ubiquitin (hUBC) promoter. To generate this lentiviral vector, PCR product of A53T (cDNA plasmid, provided by Dr Ken Nakamura at UCSF) was in-frame fused with F2A-ECFP fragment and inserted into pFUGW lentiviral vector. This vector was used to infect HEK293 cells for generating viruses. Lentiviral A53T viruses at 10^8 viruses/ml were produced by The Viral Vector Core at Emory University and used for embryonic injection.

Production of rhesus monkeys

All animal procedures were approved in advance by the Institutional Animal Care and Use Committee of Kunming Biomed
The lentivirus-mediated transgenic techniques in rhesus monkeys have been described elsewhere (10). In brief, adult females were hormone-stimulated and their oocytes were recovered for in vitro fertilization and culture. Lentiviral solution was loaded into the injection needle by micropipette, and the viral solution was injected into the perivitelline space of oocytes. After virus injection, the oocytes were fertilized by intracytoplasmic sperm injection and cultured for developing embryos in vitro until embryo transfer. We injected high-titer lentiviruses (10^9 viruses/ml), which express mutant (A53T) α-syn under the control of the human polyubiquitin-C promoter, into the perivitelline space of 133 MII oocytes and obtained 81.8% (108 out of 133) of the zygotes that developed into embryos at the 4–16-cell stage. Surrogate females at synchronized reproductive cycles were identified based on their hormone profiles. We transferred 75 embryos into 25 surrogates. Of those surrogates, 11 became pregnant (44.0%; 11 out of 25), and 7 live newborns were delivered at full term.

**Monkey behavior assays**

We selected three wild-type male monkeys at 2.5 years of age as controls for the oldest male A53T transgenic monkey (110217) and two wild-type female and one wild-type male monkeys at 1.5 years of age as controls for two younger female A53T transgenic monkeys (120666 and 120676). We also included a GFP transgenic monkey at 5 years of age as a control, which was generated in our previous study (10). To analyze animal behaviors, the animals were video recorded and examined blindly by two or more individuals.

**Cognitive function**

Cognitive impairment is a common non-motor symptom in PD patients (5). To test the cognitive function of the transgenic A53T monkey, the monkey was given five covered boxes (40 mm x 40 mm, 20 mm deep) with different colors that were at different positions on a wood strip. Only the red box contained peanuts,
and the five boxes were randomly changed for their positions every time. For cognitive test, we trained the monkeys 7 days for them to recognize peanuts in the boxes. At 8th–10th day, we trained them to recognize the specific boxes with different colors or shapes that contain peanuts and then used the results from 11th days for analysis. To quantify this cognitive function, we measured the times for monkeys to find the peanuts among the five boxes by examining monkeys in 8–12 experiments (one experiment a day). To rule out color discrimination, boxes with different shapes (round, triangle, square, rectangle and pentangle) were also presented to monkeys to test their ability to learn to recognize the correct box containing food.

Fine finger coordination
Monkeys were trained to pick up candies in a rotating plate in which 32 small holes (25 mm × 10 mm, 8 mm deep) contained candies while the plate was spinning at 30 rpm. After 10 times training, the performance of monkeys reached a steady level. The performance for 8–9 further times in the next consecutive 8–9 days was then recorded by video camera. The numbers of failures to immediately pick up the candies from the rotating plate, which reflect the fine coordination and dexterity of finger movement, were recorded and used for statistical analysis.

Anxious behavior examination
Non-motor symptoms, such as depression/anxiety, cognitive deficit, constipation and sleep disorders, often precede motor symptoms in PD patients (5,15,16). In non-human primates, stereotypic behaviors can include constantly walking in circles in the cage, sucking on a finger or toe, and self-grasping (12,13,17). We analyzed the monkey’s general behavior by taking a 1-h video record every day for 7 days.

Sleep observation
The oldest A53T monkey (110217) and its age-matched control (110215) were examined. Monkeys were video recorded from 11 pm to 6 am. The wake-up times and duration of each wake-up were measured by two observers blinded to the genotypes of the monkeys. Three experiments were performed: each experiment monitored the sleep behavior of monkeys for 5–6 consecutive days.

PCR genotype and quantitation of copy numbers
To determine the integration of transgene, two sets of primers derived from the transgene construct (primers-1: 5′-TTAGGCACCTTTTGAAATGTAATCA-3′ and 5′-GCCACGTGTGTCACACATTGCAC-3′) and specific to ECFP sequences (primers-2: 5′-AAGCAAGGGTGTGGCAGAAG-3′ and 5′-CAGGTCAGGGTGGTCAGAAGCA-3′) were utilized. Genomic DNA (100 ng) of different tissues were used for PCR conditions that were set as: 96°C for 5 min and then 96°C for 45 s, 57°C for 45 s, 72°C for 45 s, for 35 cycles followed by extension at 72°C for 7 min. Wild-type monkey genomic DNA was used as a negative control.

To quantify the copy numbers of the transgenic A53T gene, we adopted the method described previously (25). A53T-alpha-synuclein vector was first diluted and mixed with wild-type monkey genomic DNA at mole ratio 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64. These mixed samples were used to generate a standard curve based on their Ct values. All samples were run at the same time with the standard samples and normalized to β-actin, which served as an internal control. The actual transgene copy number was calculated using the formula derived from the standard curve when endogenous α-syn was deemed as two copies per cell. Quantitative PCR was performed on CFX96 Real-Time...
PCR Detection System (Bio-Rad) with sense primer 5′-AAAGGC CAAGGGAGGTGTG-3′ and antisense primer 5′-TAACACCT CTTTGTCTCTTCTG-3′. PCR conditions were at 95.0°C for 60 s, and repeated 95.0°C, 5 s and 60.0°C, 20 s for 39 cycles. Melt curve was generated at 70.0–92.4°C with increments of 0.4°C every 5 s.

Western blotting and immunohistochemical studies

For western blots, brain tissues were homogenized in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA pH 8.0, 1 mM EGTA, pH 8.0, 0.1% SDS, 0.5% DOC, and 1% Triton X-100) with 1× protease inhibitor (Sigma, P8340). The tissue lysates were diluted in 1× SDS sample buffer (62.6 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.01% bromophenol blue) and sonicated for 10 s after incubation at 100°C for 5 min. The total lysates were resolved in a 4–20% Tris-glycine (Invitrogen) and blotted to a nitrocellulose membrane. The western blots were developed using the ECL Prime kit (GE Health Care/Amersham Biosciences).

Methods for immunohistochemistry were described previously (9,26). Monkey brain tissues were fixed by 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.2. Brains were removed, cryoprotected in 30% sucrose at 4°C and sectioned at 40 μm using a freezing microtome. Light micrographs were taken using a Zeiss (Oberkochen, Germany) microscope (Axiovert 200 MOT) equipped with a digital camera (Orca-100; Hamamatsu, Bridgewater, NJ, USA).

Statistical analysis

Differences between two groups were evaluated by Student’s t-test. One-way analysis of variance (ANOVA) with the Bonferroni post hoc correction was performed to determine pairwise comparisons amongst multiple data sets. For monkey behavioral analysis, at least six experiments were performed, and the results were expressed as the mean ± SEM. A P-value of <0.05 was considered significant.

Supplementary Material

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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