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Weili Yang, Jinan University
Yunbo Liu, Chinese Academy of Medical Sciences
Zhuchi Tu, Jinan University
Chong Xiao, Chinese Academy of Medical Sciences
Sen Yan, Jinan University
Xishan Ma, Chinese Academy of Medical Sciences
Xiangyu Guo, Jinan University
Xiusheng Chen, Jinan University
Peng Yin, Emory University
Zhengyi Yang, Chinese Academy of Sciences

Only first 10 authors above; see publication for full author list.

Journal Title: Cell Research
Volume: Volume 29, Number 4
Publisher: Springer Nature [academic journals on nature.com]: Hybrid Journals | 2019-04-01, Pages 334-336
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1038/s41422-019-0142-y
Permanent URL: https://pid.emory.edu/ark:/25593/v42wx

Final published version: http://dx.doi.org/10.1038/s41422-019-0142-y

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Accessed September 24, 2024 6:00 AM EDT
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Dear Editor,

PINK1 mutations cause autosomal recessive and early-onset Parkinson’s disease (PD) with selective neurodegeneration. Unfortunately, current PINK1 knockout (KO) mouse1–5 and pig models6,7 are unable to recapitulate the selective and overt neurodegeneration seen in PD patients. Furthermore, endogenous PINK1 in the mouse brain is expressed at very low levels and can only be detected via immunoprecipitation, meaning that PINK1’s function in the mammalian brain needs to be assessed using larger animals that are closer to humans. We previously used CRISPR/Cas9 to target the monkey gene in one-cell stage embryos. Using the same approach, we designed two gRNAs to target exon 2 (T1) and exon 4 (T2), which encode a kinase domain in the PINK1 gene of rhesus monkeys (Fig. 1a). CRISPR/Cas9 and PINK1 gRNAs were injected into one-cell stage rhesus monkey embryos. A T7E1 assay and sequencing of PCR products from the injected embryos showed high efficiency (61.5%) in targeting PINK1 (Fig. 1b and Supplementary information, Fig. S1). Transfer of 87 embryos to 28 surrogate rhesus monkeys resulted in 11 pregnancies (39.2%) (Fig. 1b). Eleven fetuses developed to term and were born naturally. Of these live monkeys, eight carried PINK1 mutations (M), and three were wild type (WT). However, three mutant monkeys (M1, M3 and M4) were newborn triplets that struggled to survive and died 3–4 days after birth. One WT newborn monkey also died after a difficult labor. Another mutant monkey (M2) died 7 days after birth without noticeable warning signs or symptoms. The other three mutant monkeys (M6, M7 and M8) have lived for three years; M5, however, reduced its food intake and showed weakness at the age of 1.5 years, and died 30 days after anesthesia for MRI examination.

The mosaicism of CRISPR/Cas9-mediated mutations can induce different extents of PINK1 loss and phenotypes, allowing us to examine the relation of PINK1 mutations with PINK1 expression and pathological changes. Indeed, a T7E1 assay and sequencing analysis of the targeted DNA regions revealed various types of DNA mutations (Supplementary information, Fig. S1). Importantly, we identified a large deletion (7,237 bp) between exon 2 and exon 4 in dead monkey tissues via PCR and sequencing of PCR products, as well as whole-genome sequencing (Fig. 1c and Supplementary information, Fig. S2). T7E1 analysis of several potential off-target genes in brain cortical tissues from PINK1 mutant monkeys (M1, M2, M3, and M4) and blood tissues from live PINK1 mutant monkeys (M5, M6 and M7) revealed no mutations (Supplementary information, Fig. S3a). Whole-genome sequencing of M1, M2, and M3 monkeys showed no significant mutation rates in the top 20 potential off-target genes (Supplementary information, Fig. S3b) and analysis of 2,189 possible off-target sites with up to five mismatches of the gRNA sequences in the genome also revealed no off-targeting events (Supplementary information, Fig. S3c).

The remarkable neuronal loss seen in PINK1 mutant monkeys was not reported in PINK1 KO mice1,2 or pigs,3,4 and may be differently expressed in the brain. The large deletion in PINK1 is different from point mutations found in humans and should completely eliminate PINK1 expression. To provide more evidence for the specific targeting of the PINK1 gene and the resulting phenotype, we used western blotting to assess PINK1 expression and PCR to evaluate the relative degree of the large deletion by detecting the ratio of truncated DNA resulting from this deletion to the remaining intact exon 3 DNA (Fig. 1c–e). We found that ~65%–70% of PINK1 alleles in M1 cortex and striatum and M2 cortex carry the ~7.2 kb deletion (Fig. 1f). Western blotting analysis of PINK1 mutant monkey brains also confirmed differing extents of deficiency in PINK1, neuronal proteins (NeuN, PSD95, CRMP2, and SNAP25), and doublecortin (DCX) (Fig. 1e). All our results clearly showed that M1 and M2 monkey cortical tissues had the highest degree of the large PINK1 deletion and the lowest level of PINK1 and neuronal proteins (Fig. 1g). Counting NeuN-positive cells also verified that M1 and M2 cortical and M1 striatal tissues had significantly fewer neuronal cells than WT controls (Fig. 1h, i). Moreover, we verified that PINK1 is also abundantly expressed in the human brain (Fig. 1j).

For the live monkeys, MRI and video monitoring studies revealed that 1.5-year-old adult monkeys with PINK1 mutations showed significantly decreased gray matter density in the cortex (Supplementary information, Fig. S4a–c). M5 and M6 monkeys also displayed decreased movement despite no alteration in sleep behavior (Supplementary information, Fig. S4d, e, Movie S1). The M5 monkey lived up to 1.5 years and died 30 days after MRI examination. T7E1 assay revealed PINK1 mutations in its brain and peripheral tissues (Supplementary information, Fig. S5a). Immunohistochemical studies showed reduced density of NeuN-positive neuronal cells and increased GFAP staining in the cortex and striatum of the M5 monkey compared with a 1.5-year-old WT monkey (Supplementary information, Fig. S5b). Because M5 monkey brain tissues were not isolated immediately after death for electron microscopic (EM) examination, we euthanized the symptomatic M6 monkey at the age of three years for EM to provide ultrastructural evidence for neurodegeneration. Analysis of M6 monkey brain genomic DNA also revealed the large deletion between PINK1 exon 2 and exon 4 in various tissues (Supplementary information, Fig. S2b), and western blot analysis showed significantly decreased PINK1 expression in the cortex and substantia nigra compared with an age-matched WT monkey (3-year-old) (Fig. 1k). EM revealed degenerated neurons in the cortex, substantia nigra and striatum, as characterized by their electron-dense cytoplasm with no clear organelles and no identifiable nuclear membrane (Fig. 1l). Interestingly, in those degenerated neurons, the mitochondrial morphology is indistinguishable from WT monkey neurons.

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The remarkable neuronal loss seen in PINK1 mutant monkeys was not reported in PINK1 KO mice1,2 or pigs,3,4 and may be...
associated with the primate-specific expression and function of PINK1. However, most patients with PINK1 mutations do not show the same severe phenotypes as PINK1 mutant monkeys. The differences in neurodegeneration and phenotypes between PINK1 mutant monkeys and patients are very likely due to different types of PINK1 mutations. Most PINK1 mutations in humans are homozygous point mutations in one exon of the PINK1 gene, with a few cases of heterozygous large deletions. The single locus mutations and heterozygous deletion may cause a partial loss of PINK1 expression or function and heterogeneity in PINK1
function, leading to different ages of onset with the earliest being 5 years, and various phenotypes in patients. On the other hand, the CRISPR/Cas9-mediated large deletion with other mutations can completely eliminate PINK1 expression and function, resulting in more severe phenotypes and neuropathology, as seen in some dead newborn monkeys (M1 and M2). It is possible that in humans, the complete loss of PINK1 leads to lethality during early development, therefore only those mutations causing a partial loss of PINK1 function are seen. Indeed, the mosaicism of CRISPR/Cas9-mediated mutations also led to live PINK1 mutant monkeys that showed a partial loss of PINK1 and less severe neurodegeneration. Mounting evidence suggests that PINK1’s function is diverse\(^{10}\) and that its dysfunction is involved not only in PD, but also in cancers and other diseases.\(^{11,12}\) Generation of PINK1 mutant monkeys revealed the critical function of PINK1 in the primate brain and will provide a new tool to investigate the diverse functions of PINK1 and the pathogenesis related to PINK1 dysfunction.

**ACKNOWLEDGEMENTS**

This work was supported by the National Key Research and Development Program of China Stem Cell and Translational Research (2017YFA0105102, 2017YFA0105201) and the State Key Laboratory of Molecular Developmental Biology, China. We thank Yi Hong for electron microscopic examination; Jinqian Gao, Qiang Sun, Haiquan Shang, Hua Zhu, Mu Liu, Tao Zhu and Ling Zhang for animal care and behavioral analysis; Baogui Zhang, Ming Song and Yanli Liu for MRI image acquisition; Shang-Hsun Yang for technical advice; Giovanni Coppola, Zhushui Qin and Luxiao Chen for large deletion and off-target analysis of the whole genome sequence; and the Chinese Academy of Sciences-Institute of Automation Center for Advanced Imaging for MRI experiments and data analysis. We thank Cheryl Strauss for editing the manuscript.

**AUTHOR CONTRIBUTIONS**

W. Y., S. L., and X.-J. L. designed the experiments. W. Y., Z. T., C. X., X. M., S. Y., X. G., X. C., P. Y., and S. Y. performed the experiments. Z. Y. and T. J. performed imaging analysis. Y. L. and C. Q. provided the monkey facility. W. Y. and X.-J. L. wrote the manuscript. S. L. edited the manuscript.

**ADDITIONAL INFORMATION**

Supplementary information accompanies this paper at https://doi.org/10.1038/s41422-019-0142-y.

**Competing interests:** The authors declare no competing interests.

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