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Comparative analysis of primate and pig cells reveals primate-specific PINK1 expression and phosphorylation

Xiu-Sheng Chen[1,#], Rui Han[1,#], Yan-Ting Liu[1], Wei Huang[1], Qi Wang[1], Xin Xiong[1], Ying Zhang[2,3], Jian-Guo Zhao[2,3,4], Shi-Hua Li[1], Xiao-Jiang Li[1], Wei-Li Yang[1,*]

[1] *Guangdong Key Laboratory of Non-human Primate Research*, *Key Laboratory of CNS Regeneration (Ministry of Education)*, *GHM Institute of CNS Regeneration*, *Jinan University*, *Guangzhou*, *Guangdong* 510632, *China*

[2] *State Key Laboratory of Stem Cell and Reproductive Biology*, *Institute of Zoology*, *Chinese Academy of Sciences*, *Beijing* 100101, *China*

[3] *Beijing Institute for Stem Cell and Regenerative Medicine*, *Beijing* 100101, *China*

[4] *University of Chinese Academy of Sciences*, *Beijing* 100049, *China*

ABSTRACT

PTEN-induced putative kinase 1 (PINK1), a mitochondrial kinase that phosphorylates Parkin and other proteins, plays a crucial role in mitophagy and protection against neurodegeneration. Mutations in *PINK1* and *Parkin* can lead to loss of function and early onset Parkinson's disease. However, there is a lack of strong *in vivo* evidence in rodent models to support the theory that loss of PINK1 affects mitophagy and induces neurodegeneration. Additionally, *PINK1* knockout pigs (*Sus scrofa*) do not appear to exhibit neurodegeneration. In our recent work involving non-human primates, we found that PINK1 is selectively expressed in primate brains, while absent in rodent brains. To extend this to other species, we used multiple antibodies to examine the expression of PINK1 in pig tissues. In contrast to tissues from cynomolgus monkeys (*Macaca fascicularis*), our data did not convincingly demonstrate detectable PINK1 expression in pig tissues. Knockdown of *PINK1* in cultured pig cells did not result in altered Parkin and BAD phosphorylation, as observed in cultured monkey cells. A comparison of monkey and pig striatum revealed more PINK1-phosphorylated substrates in the monkey brain. Consistently, *PINK1* knockout in pigs did not lead to obvious changes in the phosphorylation of Parkin and BAD. These findings provide new evidence that PINK1 expression is specific to primates, underscoring the importance of non-human primates in investigating PINK1 function and pathology related to PINK1 deficiency.

Keywords: PINK1; Parkin; Mitochondria; Phosphorylation; Non-human primates; Pigs

INTRODUCTION

PTEN-induced putative kinase 1 (PINK1) accumulates on the outer membrane of damaged or dysfunctional mitochondria, where it phosphorylates Parkin and ubiquitin to facilitate the selective degradation of impaired mitochondria, to protect against neurodegeneration, a cellular process known as mitophagy (Eiyama & Okamoto, 2015; Geisler et al., 2010; Lazarou et al., 2015; Nguyen et al., 2016; Pickrell & Youle, 2015). While *in vitro* studies involving cultured cells subjected to mitochondrial stress have largely corroborated the role of PINK1 in mitophagy, evidence from *in vivo* studies remains limited (Chen et al., 2022; Cummins & Götz, 2018; Han et al., 2023; Whitworth & Pallanck, 2017). Experiments utilizing *PINK1* knockout (KO) mice (*Mus musculus*) and *Drosophila* have indicated an absence of altered mitophagy in the brains of these animals (Lee et al., 2018; McWilliams et al., 2018). Moreover, PINK1- and/or Parkin-deficient mice do not manifest neurodegenerative symptoms, a pathological feature observed in Parkinson's disease (PD) patients with homozygous mutations in *PINK1* or *Parkin* (Gispert et al., 2009; Goldberg et al., 2003; Kitada et al., 2009; Wang et al., 2023). Similarly, pigs lacking PINK1 and Parkin do not exhibit signs of neurodegeneration or discernible phenotypes (Wang et al., 2016; Zhou et al., 2015). These findings suggest that the function of PINK1 may be species-dependent and unique to humans.

Non-human primates share a close genetic affinity with humans. Gene-editing techniques such as CRISPR/Cas9

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[#]Authors contributed equally to this work

*Corresponding author, E-mail: weiliyang12@jnu.edu.cn

have enabled the KO of *PINK1* in monkey embryos (Chen et al., 2021; Yang et al., 2019). Through experimentation with these *PINK1* gene-targeted monkeys, we ascertained that PINK1 is selectively expressed in primate brains and loss of PINK1 leads to neuronal loss (Yang et al., 2019, 2022). However, the expression status of PINK1 in pigs remains unclear, as *PINK1* KO pigs do not exhibit neurodegeneration or notable phenotypes (Wang et al., 2016; Zhou et al., 2015). Resolving this issue is critical for confirming the primate-specific expression of PINK1 and for evaluating the utility of primate and cellular models in investigations related to PINK1 function and PD pathogenesis associated with PINK1/Parkin deficiency.

In the present study, we employed a range of antibodies to assess and compare PINK1 expression in pig and primate tissues. Notably, in contrast to expression in primate tissues, negligible levels of PINK1 were detected in pig tissues. Furthermore, analysis revealed no significant alterations in phosphorylated substrates of PINK1 in pig cells following *PINK1* knockdown, nor was conspicuous PINK1-mediated phosphorylation observed in pig brains, in contrast to the results in monkey brains. These findings highlight the importance of utilizing non-human primate models to investigate the distinct expression and function of PINK1 in the neurobiology of primates.

MATERIALS AND METHODS

Antibodies

Rabbit polyclonal antibody B2 was generated using human PINK1 sequences (175–250 aa) by Boster Biological Technology (China). The same human PINK1 sequences were used to produce a rabbit antibody (BC100–494, Novus Biologicals, USA), widely used to recognize PINK1 in cultured cells. The human PINK1 sequences were also used as antigens to generate the mouse monoclonal antibody E7B6 by Nanjing Detai Bioengineering (China). Additionally, a synthetic peptide containing the pig PINK1 protein sequences (residues 484–504) was used as an antigen to produce a rabbit polyclonal antibody (anti-pig PINK1) by Boster Biological Technology (China). Other antibodies used in the study are listed in Table 1.

Animals

The generation of *PINK1* mutant rhesus monkeys (*Macaca mulatta*) via embryonic injection of CRISPR/Cas9 has been described in our earlier research (Yang et al., 2019). In brief, fertilized rhesus monkey eggs were injected with Cas9 mRNA and *PINK1* gRNAs to target exons 2 and 4 of the primate *PINK1* gene. This targeting can result in deletion of a large fragment of *PINK1* DNA, leading to complete inactivation of the gene. Due to the mosaic targeting nature of CRISPR/Cas9, the resulting newborn monkeys carry mosaic *PINK1* mutations and exhibit reduced *PINK1* levels (knockdown) rather than complete gene KO. Monkey tissues from 3-year-old *PINK1* mutant (M6) and wild-type (WT) monkeys were obtained from our previous studies (Yang et al., 2019, 2022). Tissues from two additional WT monkeys (aged 10 and 11 years) were also used for western blotting. The monkeys were housed at Guangdong Landao Biotechnology Co., Ltd. (Guangzhou, China). All animal procedures were approved by the Institutional Animal Care and Use Committee at Guangdong Landao Biotechnology Co., Ltd. (Guangzhou, China). The study was conducted in strict compliance with the ‘‘Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Science (est. 2006)’’ and ‘‘The Use of Non-Human Primates in Research of the Institute of Laboratory Animal Science (est. 2006)’’ to ensure personnel safety and animal welfare.

The generation of *PINK1* KO pigs via embryonic injection of CRISPR/Cas9 and somatic nuclear transfer has been described in earlier research (Wang et al., 2016). In brief, cortical tissues from WT and homozygous *PINK1* KO pig brains (two female and one male WT; one male and one female KO) at postnatal day 1 were prepared at the Institute of Zoology, Chinese Academy of Sciences. Tissues were obtained from 7-month-old WT pigs (*n*=3 pigs) in our previous studies (Yan et al., 2018, 2023).

Tissues were obtained from WT mice (3–4 months) housed at the animal facility at Jinan University. All procedures involving animals were conducted in accordance with the Animal Care and Use Committee of Jinan University (Approval No. IACUC-20210220-06).

Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted from the brains and peripheral tissues of monkeys and pigs (*n*=3 WT monkeys and *n*=3 WT pigs). Briefly, 20–50 mg of tissue was homogenized in 1 mL of ice-cold TRIzol (Invitrogen) and solubilized at room temperature for 10 min. After this, 200 μL of chloroform was added and the mixture was briefly vortexed, followed by centrifugation at 12 000 ×*g* for 15 min at 4°C. The clear upper phase was transferred to a fresh tube without disturbing the interface. An equal volume of isopropanol was added to the clear phase and vigorously mixed. The mixtures were then placed at −20 °C for more than 4 h or overnight. RNA was precipitated at 12 000 ×*g* in a centrifuge for 15 min at 4 °C. The supernatant was removed, and the pellet was twice washed with ice-cold 75% ethanol, followed by centrifugation at 12 000 ×*g* for 15 min at 4°C. Finally, the pellets were dissolved in 50 μL of RNase-free water to dissolve RNA. Reverse transcription of RNA was performed using a PrimeScript™ RT Reagent Kit (Takara, China) with genomic DNA eraser. All procedures were conducted according to the manufacturer's instructions without modification. A QuantiNova SYBR Green PCR Kit (Qiagen, Germany) was used to perform qPCR, with three replications for each sample. Quantitative assessment of the relative levels of *PINK1* mRNA expression to actin was performed by qPCR and reverse transcription polymerase chain reaction (RT-PCR) using the primers listed in Supplementary Table S1.

Monkey and pig primary cortical astrocyte culture

Primary cortical cell cultures were prepared from postnatal day 1 piglets. Cortical tissues were isolated from a fetal monkey brain at embryonic day 90, following the method described in our previous research (Yang et al., 2022). Briefly, cortical tissues from the monkey and pig brains were dissected and cut into 1 mm^3 pieces. The dissected tissues were treated with 0.01 g/mL papain (Lyophilized LS003119) and DNase (1:50) in Dulbecco's Modified Eagle Medium (DMEM) buffer for 20–30 min at 37 °C, followed by trituration with a 1 mL pipette tip 20 times. Cells were then washed once with the tissue culture medium and centrifuged at 300 ×*g* for 5 min at room temperature. The cells were plated on glass coverslips precoated with 0.1 mg/mL poly-D-lysine and 1 g/mL laminin and grown in culture medium (DMEM 90%+fetal bovine serum

10%+penicillin-streptomycin (100×). The medium was initially refreshed after a single day, and subsequent partial medium renewals were carried out every 2 to 3 days.

Knockdown of *PINK1* in primary cultured astrocytes

Transfection of cultured monkey and pig astrocytes via electroporation was performed using a Nucleofector Kit (Lonza, Switzerland) before plating primary glial cells on poly-D-lysine coated 12-well plates. In brief, 2×10^6 cells were used for electroporation with 20 pmol of small interfering RNA (siRNA, scrambled control siRNA and *PINK1* siRNA). After 48 h, the cultured cells were collected for western blotting and qPCR. The sequences of two sets of monkey and pig *PINK1* siRNA oligos are listed in Supplementary Table S2.

Western blotting

To assess whether anti-pS65-Parkin could recognize PINK1-mediated phosphorylation of Parkin, human *PINK1* and *Parkin* plasmids were co-transfected into HEK293 cells. The cell lysates were subjected to western blotting using anti-PINK1 (BC100–494) and anti-pS65-Parkin (AF3500). The tissues and cells were homogenized in ice-cold RIPA buffer (50 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, 1 mmol/L EDTA pH 8.0, 1 mmol/L EGTA pH 8.0, 0.1% SDS, 0.5% DOC, 50 mmol/L NaF, and 1% Triton X-100) with protease inhibitor cocktail and PMSF (100 μg/mL). The lysates were sonicated and incubated on ice for 30 min, then centrifuged at 12 000 ×*g* for 10 min at 4°C. The supernatants were transferred to new tubes and added with protein loading buffer to 1×dilution. The protein samples were denatured by heating at 100 °C for 10 min. Equal amounts of proteins were loaded in sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and run at 120 V, followed by PVDF membrane transfer and staining proteins of interest by specific antibodies at 4 °C overnight. Blots were washed three times with Tris-Buffered Saline with Tween 20 (TBST) and incubated with a horseradish peroxidase (HRP)-labeled antibody at room temperature for 1 h. After thrice washing with TBST, the membranes were incubated using an Immobilon Western HRP Substrate Kit (Millipore, USA), and images were captured using a Chemiscope 6200 (Clinx Science Instruments, China). Multiple western blot experiments were performed, and the expression level of vinculin in each sample was used to adjust the loading amount for western blotting. Representative images are presented in the figures.

Statistical analysis

The RT-PCR gel electrophoresis and western blotting results were quantified via densitometry. The ratios of DNA or proteins of interest to the internal control (actin) or loading control (vinculin) were used for quantification. From multiple experiments, we selected gel results with similar levels of internal or loading controls in each sample for quantification. Data are expressed as mean±standard error of the mean (*SEM*). Statistical analysis was performed using GraphPad Prism v8.02 software.

RESULTS

Anti-PINK1 antibodies fail to detect pig PINK1

Detecting PINK1 in the brains of small animals, especially rodents, has proven to be challenging (McWilliams et al., 2018; Yang et al., 2022). PINK1 was previously considered to undergo rapid degradation *in vivo*, unless stabilized in damaged mitochondria to initiate mitophagy (Beilina et al., 2005; Greene et al., 2012). However, our recent studies using the commercial antibody BC100–494 revealed selective expression of PINK1 in primate brains (Yang et al., 2019, 2022). To validate these findings, it is important to employ alternative antibodies. Therefore, we generated a mouse clonal antibody (E7B6) using the same human PINK1 sequences as antigens for the rabbit antibody BC100–494 (Figure 1A). Comparative analysis of antigen sequences in PINK1 among humans, monkeys, pigs, and mice indicated high levels of conservation across these species (Figure 1B). Antibody specificity can be assessed using a knockdown approach that selectively depletes the expression of the targeted protein. In our previous study, we successfully generated *PINK1* mutant monkeys using CRISPR/Cas9 editing to target the *PINK1* gene in fertilized eggs (Yang et al., 2019). Specifically, a 3-year-old monkey (M6) was found to carry *PINK1* mutations in both exons 2 and 4, leading to a significant decrease in PINK1 expression in its brain (Yang et al., 2019, 2022). Thus, we used brain tissues from M6 as a control to validate antibody specificity in recognizing endogenous PINK1 in comparison with brain tissues from WT monkeys. Furthermore, we generated *PINK1* KO pigs via embryonic CRISPR/Cas9 injection and somatic cell nuclear transfer, enabling complete deletion of the *PINK1* gene (Wang et al., 2016). Brain tissues from homozygous *PINK1* KO newborn piglets were used to evaluate the ability of anti-PINK1 antibodies to recognize endogenous PINK1 in pig tissues. Using cortical brain tissues from WT and M6 monkeys and pig tissues, we confirmed that BC100–494 specifically reacted with PINK1 in the primate brain (Figure 1C). However, PINK1 was not detected at the corresponding molecular weight in either the pig brain or peripheral tissues (Figure 1C). We also generated a rabbit anti-human PINK1 antibody (B2), which demonstrated reactivity with transfected human PINK1 (Figure 1D, left panel) and diminished interaction with PINK1 in the cortex of the PINK1 mutant (M6) monkey (Figure 1D, right panel). This antibody also appeared to interact with bands in pig tissues, which exhibited molecular weights (MW) similar to those observed for monkey PINK1 (Figure 1D, right panel). However, these bands persisted in the PINK1 KO pig samples (Figure 1D), indicating that they are non-specific bands.

Given the potential limitation of rabbit polyclonal antibodies in recognizing pig PINK1, we developed mouse monoclonal antibodies against the same human PINK1 sequences and identified one clone (E7B6) that exhibited strong reactivity with transfected pig *PINK1* in HEK293 cells (Figure 1E). Subsequent western blotting was performed to determine whether E7B6 could detect PINK1 in pig tissues. Although E7B6 successfully recognized primate PINK1 (red arrow in Figure 1F), which was reduced in the *PINK1* mutant monkey (M6) brain, it did not detect PINK1 expression in the *PINK1* KO pig tissues (Figure 1F).

We also generated a rabbit polyclonal antibody using the C-terminal region of pig PINK1 (Figure 2A), a well-conserved region in human, monkey, and pig PINK1. Human sequences were also employed to generate the commercial antibody Ab23707 (Abcam) (Figure 2B). Although Ab23707 displayed reactivity with reduced levels of monkey PINK1 in the M6 monkey cortex, it failed to identify PINK1 in pig tissue (Figure 2C). Notably, in the pig brain tissues, immunoreactive bands were detected with Ab23707, which persisted in the

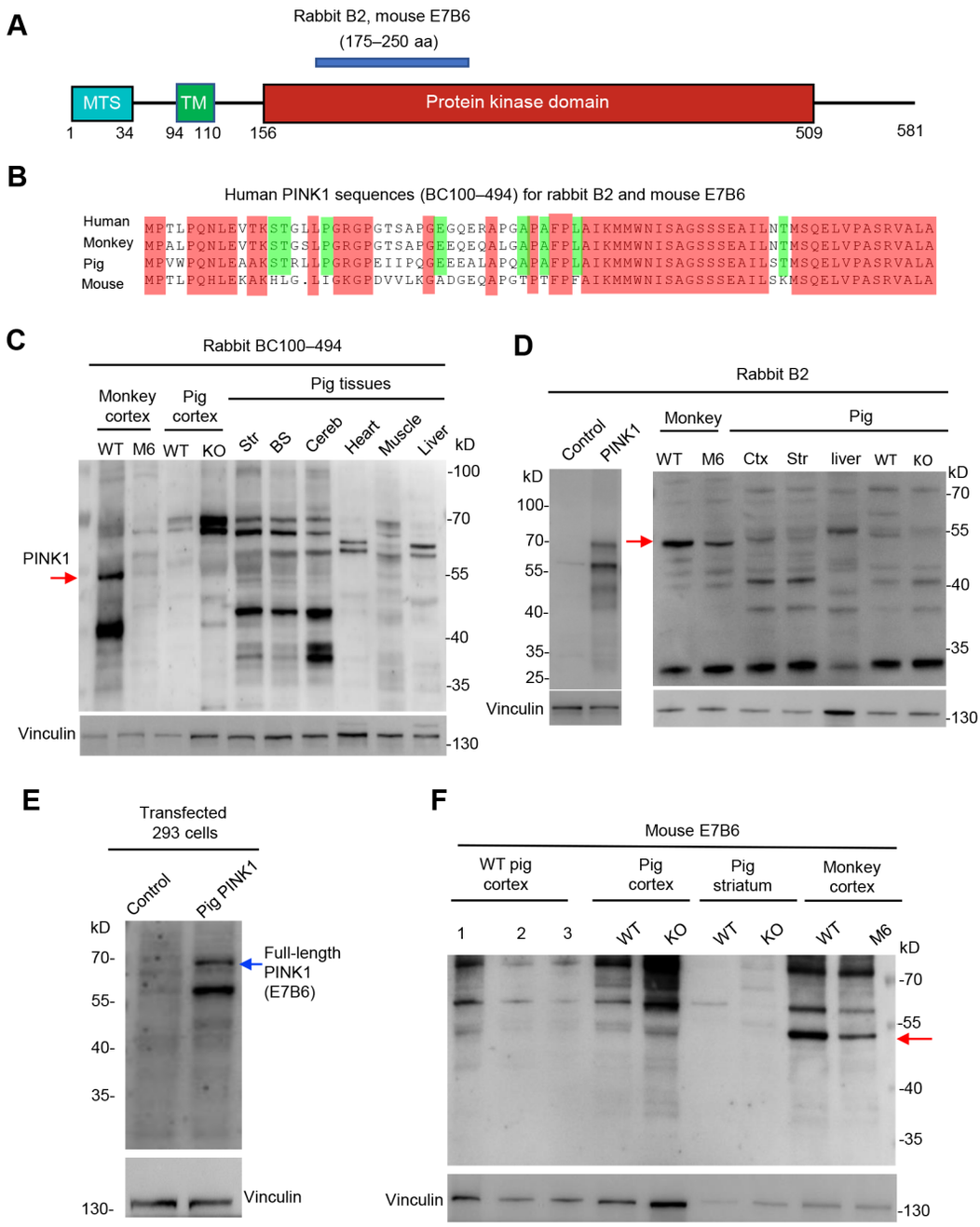

Figure 1 Undetectable PINK1 in pig tissues using antibodies targeting middle region of PINK1

A: Rabbit polyclonal B2 and mouse monoclonal E7B6 antibodies were generated using human PINK1 sequences (175–250 aa). B: Sequence similarity of PINK1 region across different species for antibody generation. Identical sequences in humans, monkeys, pigs, and mice are highlighted in red, while sequences present in humans, monkeys, and pigs but not in mice are highlighted in green. C, D: Western blot analysis of monkey and pig tissues using rabbit anti-PINK1 (BC100–494) (C) and B2 (D) antibodies. WT and KO represent brain cortex tissues from WT and *PINK1* KO pigs. M6 is monkey with *PINK1* mutations. E, F: Western blot analysis of transfected HEK293 cells (E) and monkey and pig tissues (F) using mouse monoclonal E7B6 antibody. Red arrows indicate PINK1. WT: wild-type; M6: *PINK1* mutant monkey; Ctx: cortex; Cereb: cerebellum; Str: striatum; BS: brain stem.

PINK1 KO pig brain cortex (Figure 2D). Using our rabbit anti-pig PINK1 antibody, we conducted comparative analysis of PINK1 expression in monkey and pig tissues. The anti-pig PINK1 antibody succeeded in detecting reduced PINK1 protein expression in the M6 monkey brain cortex, but yielded no discernible signal in the cortex (Ctx), corpus callosum (CC), or striatum (Str) of the pig (Figure 2E). These findings obtained from the use of multiple antibodies and *PINK1* KO pig brain tissues suggest that PINK1 expression in pig tissues is either too low to be detected or altogether absent.

Expression of *PINK1* mRNAs in different species

To investigate whether the low level of PINK1 protein in pigs is attributable to transcriptional regulation, we designed RT-PCR primers to examine *PINK1* transcripts in monkey, pig, and mouse tissues (Figure 3A). After comparing the expression of *PINK1* mRNA in the cortex, striatum, and liver, ubiquitous expression of *PINK1* was observed across all three species (Figure 3B). To compare the expression of *PINK1* mRNA more rigorously in monkeys and pigs, we identified identical sequences in exons 2 and 3 of the monkey and pig mRNA

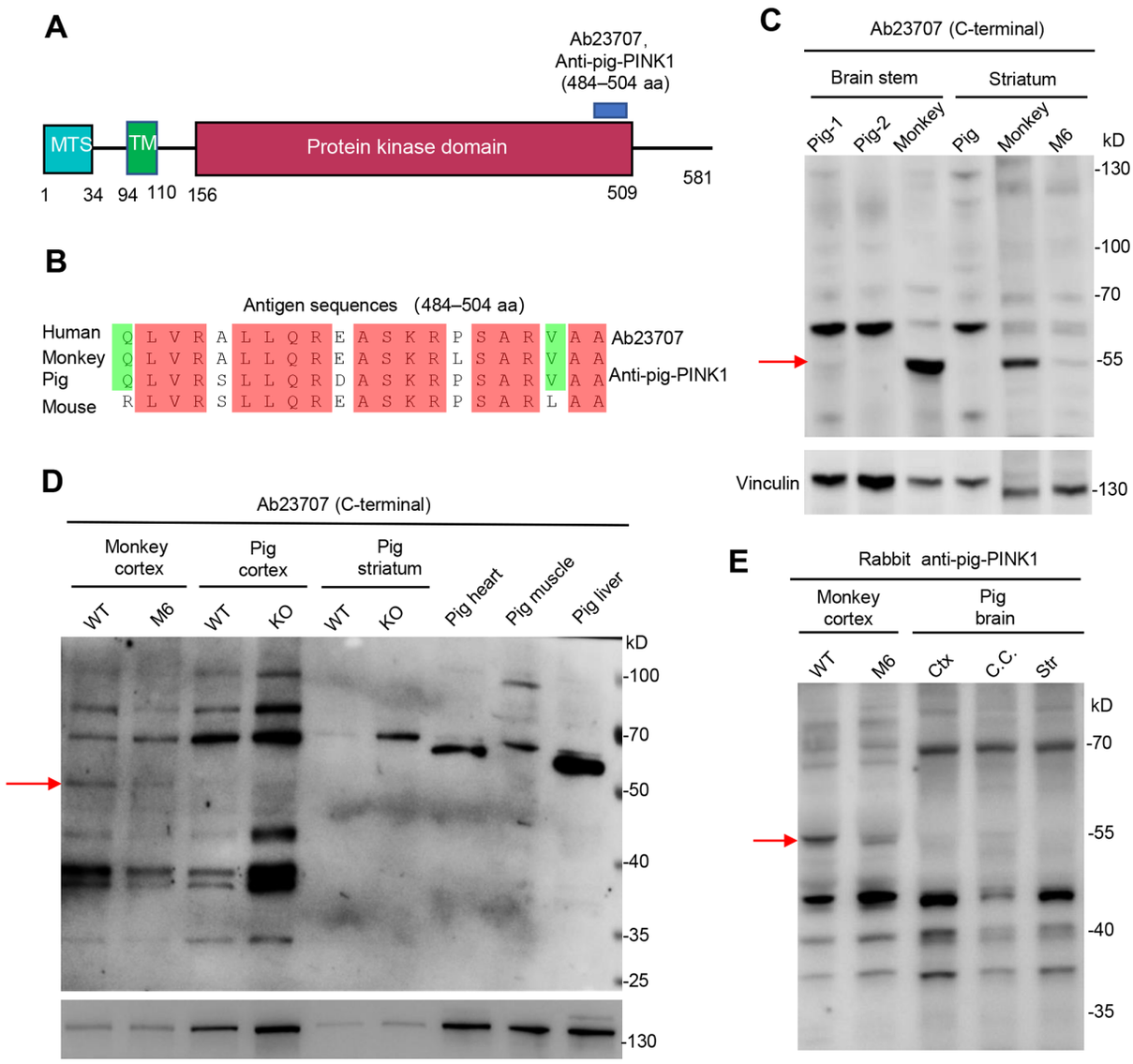

Figure 2 Undetectable PINK1 in pig tissues using antibodies targeting C-terminal region of PINK1

A: Rabbit anti-C-terminal human antibody (Ab23707) and pig-specific antibody (anti-pig-PINK1) were generated based on PINK1 amino acids 484–504. B: C-terminal PINK1 sequences (484–504) were highly conserved across species. Identical sequences in humans, monkeys, pigs, and mice are highlighted in red, while sequences present in humans, monkeys, and pigs but not in mice are highlighted in green. C: Western blot analysis of striatum and brain stem tissues of monkeys and pigs using Ab23707. Note, PINK1 protein (red arrow) was decreased in *PINK1*-mutant monkey (M6) cortex. D: Western blot analysis with Ab23707 showing no difference in immunoreactive bands between WT and *PINK1* KO pig tissues. E: Anti-pig PINK1 western blot analysis of monkey and pig brain tissues. M6 represents *PINK1* mutant monkey cortex, serving as a control to validate decreased immunoreactive signals for PINK1 (red arrows). Ctx: cortex; CC.: corpus callosum; Str: striatum.

and designed the same primers for RT-PCR under the same PCR conditions (Figure 3C). Quantitative RT-PCR revealed similar *PINK1* mRNA expression in the pig and monkey brain tissues, although lower expression of PINK1 in the pig liver (Figure 3D). Analysis of PCR products via agarose gel electrophoresis confirmed similar *PINK1* expression in the pig and monkey brain tissues, as well as reduced *PINK1* expression in the pig liver (Figure 3E, F).

Knockdown of *PINK1* in cultured astrocytes

We previously identified PINK1 expression in glial cells in monkey brains (Yang et al., 2022). Cultured astrocytes enable further examination of the reactivity of PINK1 antibodies by knocking down *PINK1* gene expression. SiRNAs can be readily transfected into cultured astrocytes to induce knockdown of target gene. Therefore, we designed a siRNA to reduce *PINK1* mRNA expression in pig and monkey astrocytes cultured and maintained *in vitro* (Figure 4A). Following transfection of *PINK1* siRNAs, a significant reduction in *PINK1* mRNA levels was observed in cultured pig astrocytes (Figure 4B, C) and cultured monkey astrocytes (Figure 4D, E). Thus, these findings indicate that transient transfection of siRNA can effectively silence gene expression in cultured astrocytes, producing a profound short-term effect.

Upon successfully reducing *PINK1* mRNA expression in cultured pig astrocytes, we next explored whether pig *PINK1* knockdown using siRNA1 and siRNA2 in combination could reduce the detection of immunoreactive products by anti-PINK1 antibodies. Cultured monkey astrocytes were used as a control, which demonstrated a significant reduction in PINK1 protein expression following *PINK1* knockdown (Figure 5A, left panel). In contrast, the rabbit anti-human PINK1 antibody

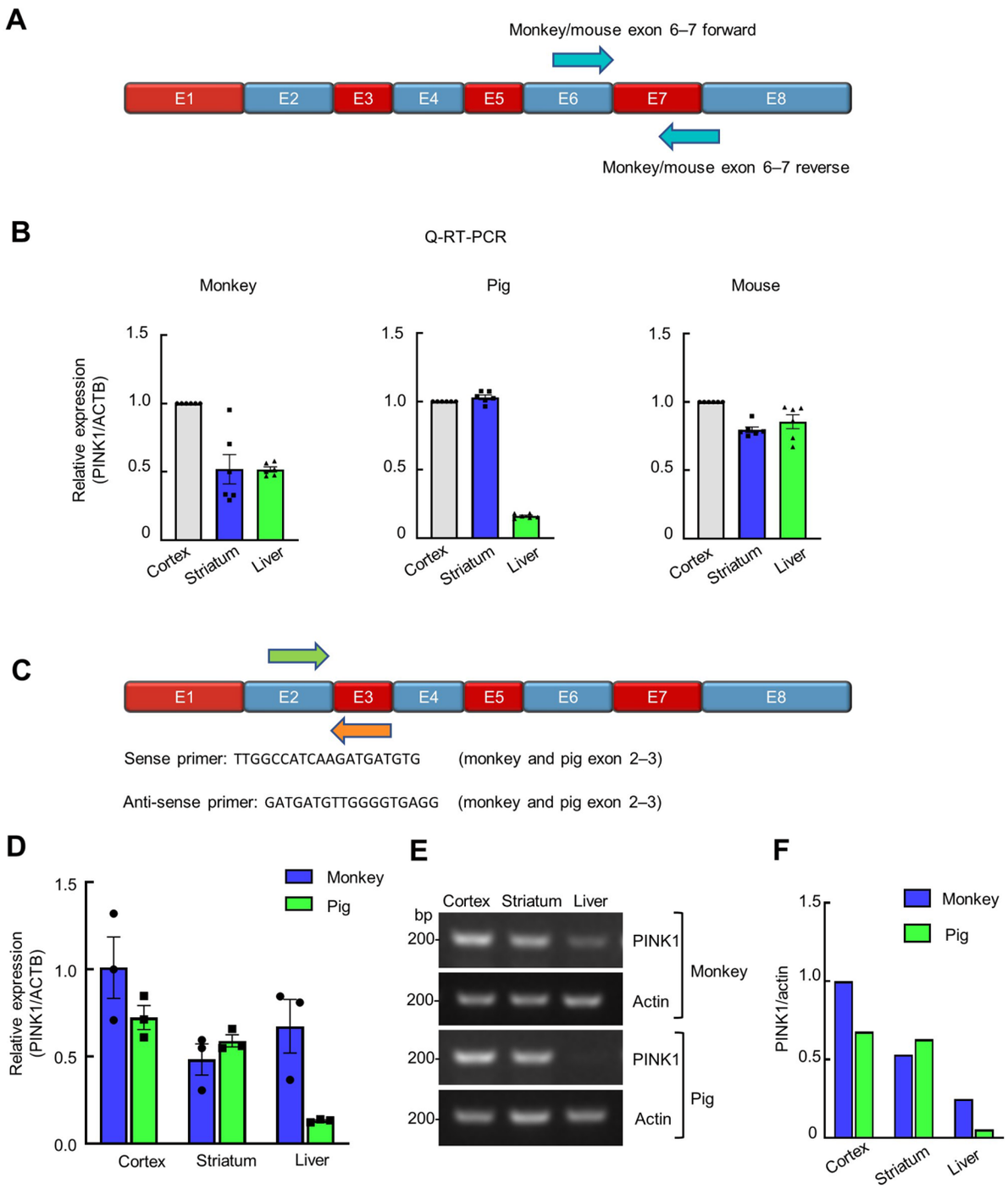

Figure 3 ***PINK1*** **mRNA expression in different species**

A: Primer locations for detecting *PINK1* mRNA in monkeys, pigs, and mice via RT-PCR. B: Quantitative RT-PCR results showing *PINK1* mRNA levels in monkey, pig, and mouse tissues. C: PCR primers with identical sequences in monkeys and pigs were used to compare *PINK1* mRNA expression. D: Quantitative RT-PCR results demonstrating similar levels of *PINK1* mRNA in monkey and pig brain tissues, but lower levels in pig liver, consistent with the findings in (B). E: Gel electrophoresis analysis of RT-PCR results indicating similar *PINK1* mRNA levels in monkey and pig brain tissues under the same PCR conditions. Actin mRNA was used as an internal control. F: Quantification of PINK1 to actin ratio (E).

(BC100–494) failed to detect any immunoreactive products in pig astrocytes with molecular weights comparable to those of monkey PINK1 subjected to siRNA knockdown (Figure 5A, right panel). Similarly, the rabbit anti-PINK1 antibody B2 failed to detect any specific bands that exhibited reduced expression in siRNA-treated pig astrocytes (Figure 5B). The mouse antibodies E7B6 (Figure 5C) and Ab23707 (Figure 5D) were also unsuccessful in recognizing reduced levels of PINK1 protein in the siRNA-treated pig astrocytes.

As PINK1 phosphorylates Parkin at serine 65 (S65), we also investigated whether the reduction of endogenous PINK1 in pig astrocytes would impact Parkin phosphorylation. We first confirmed that the anti-pS65-Parkin antibody accurately identified Parkin phosphorylation induced by transfected *PINK1* in HEK293 cells (Figure 5E). Subsequently, we employed the anti-pig PINK1 antibody, anticipated to be more reactive with pig PINK1, and the anti-pS65-Parkin antibody to examine cultured astrocytes. However, these antibodies did

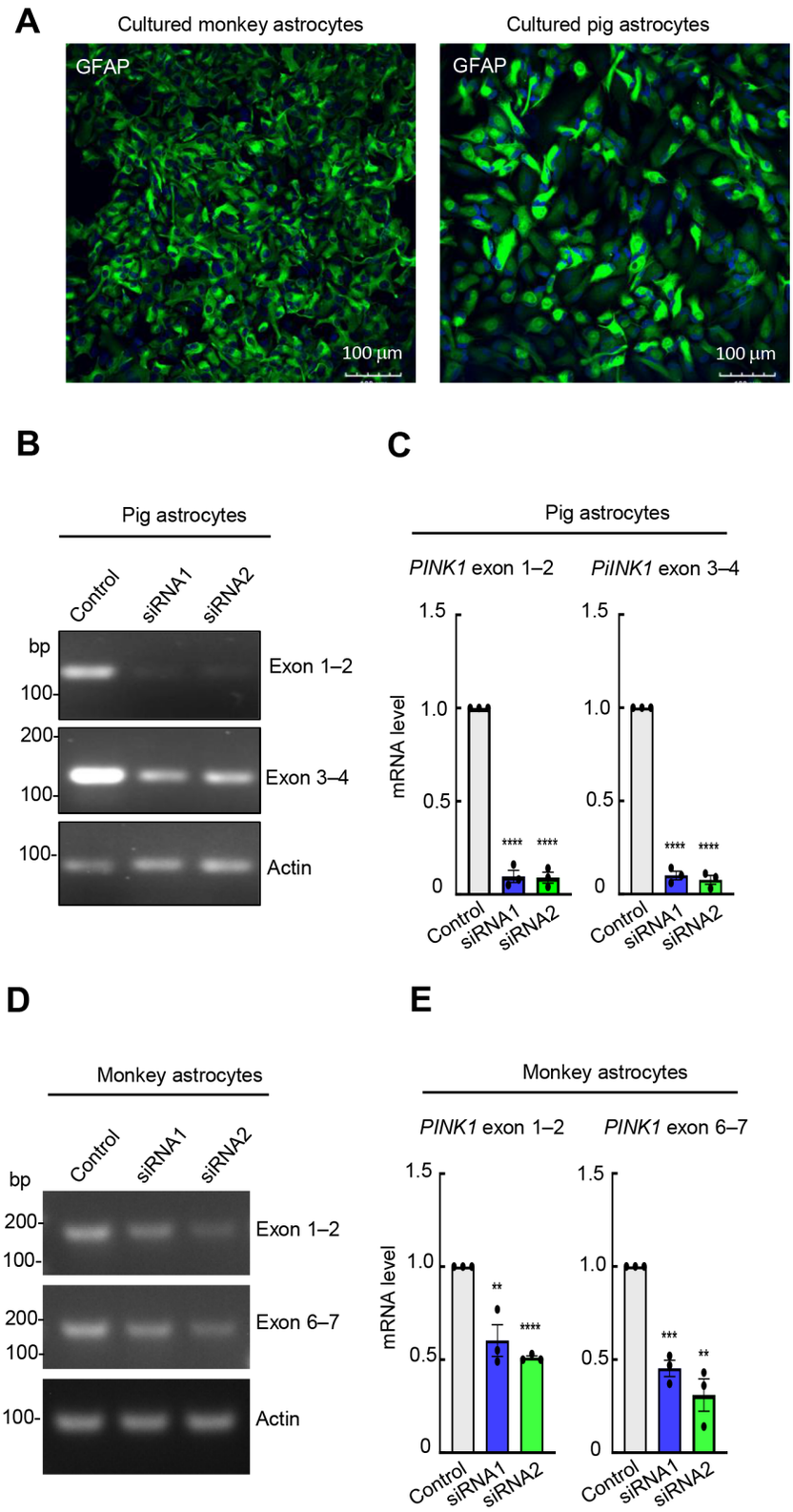

Figure 4 PINK1 mRNA expression in pig tissues and siRNA-mediated knockdown in cultured astrocytes

A: Cultured pig and monkey astrocytes used for knockdown experiments. B: Gel electrophoresis analysis of RT-PCR results showing reduction in pig *PINK1* mRNA by siRNA treatment. C: Quantitation of pig *PINK1* mRNA in control and siRNA-treated pig astrocytes. D, E: Gel electrophoresis (D) and quantitation (E) analyses of monkey *PINK1* mRNA in control and siRNA-treated monkey astrocytes. Results are represented as means±*SEM* from three independent experiments. **: $P<0.01$; ***: $P<0.001$; ****: $P<0.0001$.

not reveal any significant differences between the control and *PINK1* knockdown pig astrocytes (Figure 5F). In contrast, *PINK1* knockdown in cultured monkey astrocytes led to a decrease in immunoreactive products detected when assessed using the same anti-pig PINK1 and anti-S65-Parkin antibodies (Figure 5G). The observed disparities between pig and monkey astrocytes imply that the endogenous expression of the PINK1 protein in pigs is very low, making the

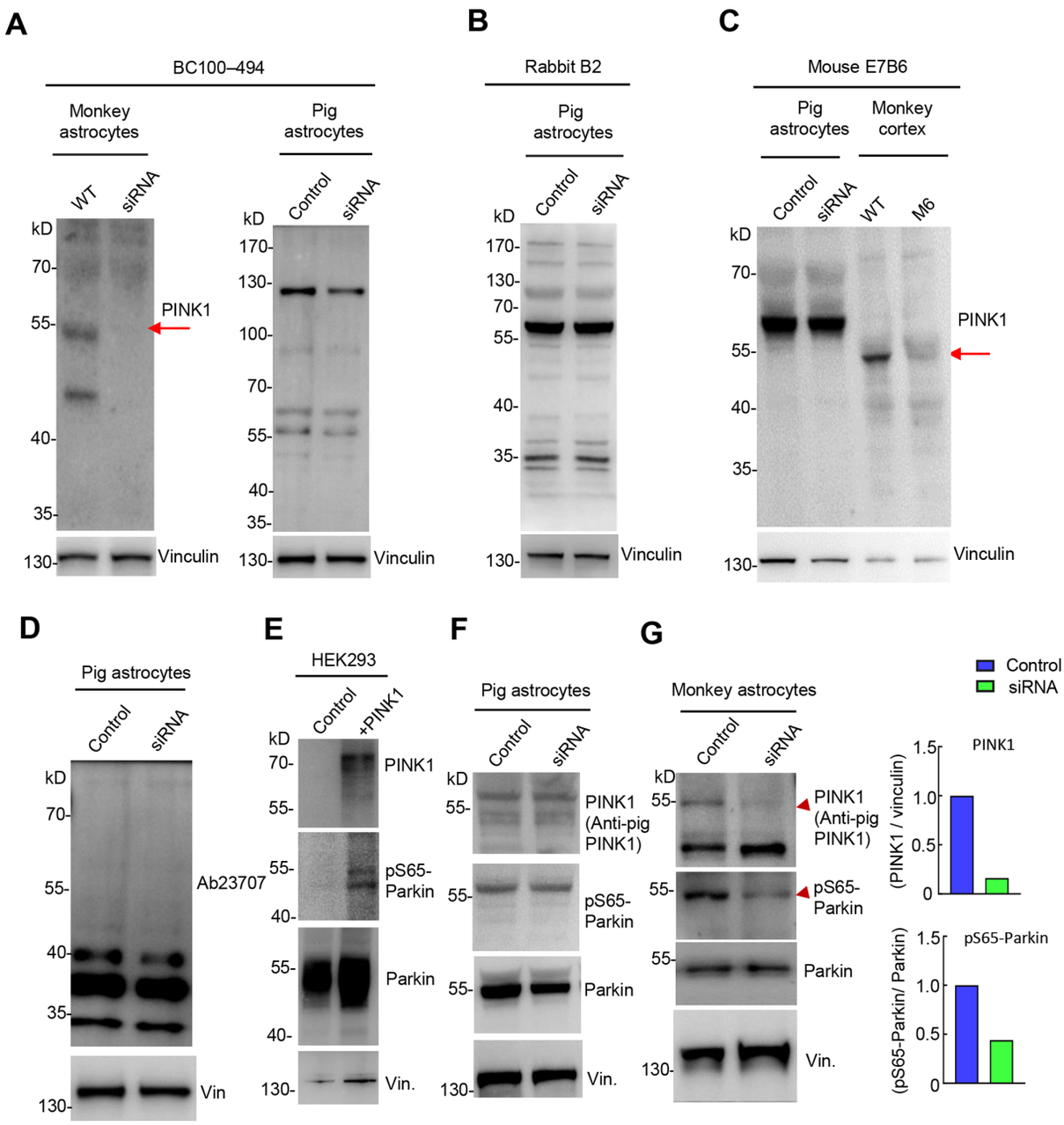

Figure 5 Undetectable PINK1 in cultured pig astrocytes subjected to siRNA-mediated knockdown

A: BC100–404 western blot analysis showing reduced PINK1 in siRNA-treated monkey astrocytes, but no difference between control and siRNA-treated pig astrocytes. B: Rabbit B2 was unable to detect a difference between control and siRNA-treated pig astrocytes. C, D: Mouse E7B6 (C) and rabbit Ab23707 (D) did not detect PINK1 in cultured pig astrocytes, although E7B6 detected reduced PINK1 in *PINK1* mutant monkey cortex (M6). E: Transfection of full-length human PINK1 increased phosphorylation of transfected Parkin, recognized by anti-pS65-Parkin (AF3500). F: Anti-pig PINK1 and anti-pS65-Parkin (AF3500) did not reveal altered levels of PINK1 and pS65-Parkin in siRNA-treated pig astrocytes. G: PINK1 and pS65-Parkin were reduced in cultured monkey astrocytes treated with *PINK1* siRNA. Ratios of PINK1 to vinculin and pS65-Parkin to total Parkin are presented in right panel. siRNA1 and siRNA2 were used in combination.

suppression of *PINK1* mRNA ineffective in altering PINK1-mediated phosphorylation.

Differential PINK1-mediated phosphorylation in monkey and pig brains

To confirm the cultured glial cell findings, we performed western blot analysis to compare PINK1-mediated phosphorylation in pig and monkey brain tissues. PINK1 phosphorylates various proteins (Yang et al., 2022), including Parkin and ubiquitin. To further explore PINK1-mediated phosphorylation in pig tissues, we used specific antibodies that can detect and recognize PINK1 substrates in pig brains. We compared the expression levels of PINK1, phosphorylated Parkin (p-S65-Parkin), and phosphorylated Bad, as identified previously (Wan et al., 2018; Yang et al., 2022). Western blot analysis demonstrated significantly lower or undetectable levels of p-S65-Parkin, p-S112-Bad, and p-S136-Bad, despite the presence of their total proteins, in the pig striatum compared to the monkey striatum (Figure 6A). Additionally, quantification of the ratios of phosphorylated to total proteins confirmed the very low levels of PINK1-mediated phosphorylation in the pig striatum (Figure 6B). To validate these findings, we utilized *PINK1* KO pig brain tissues. As

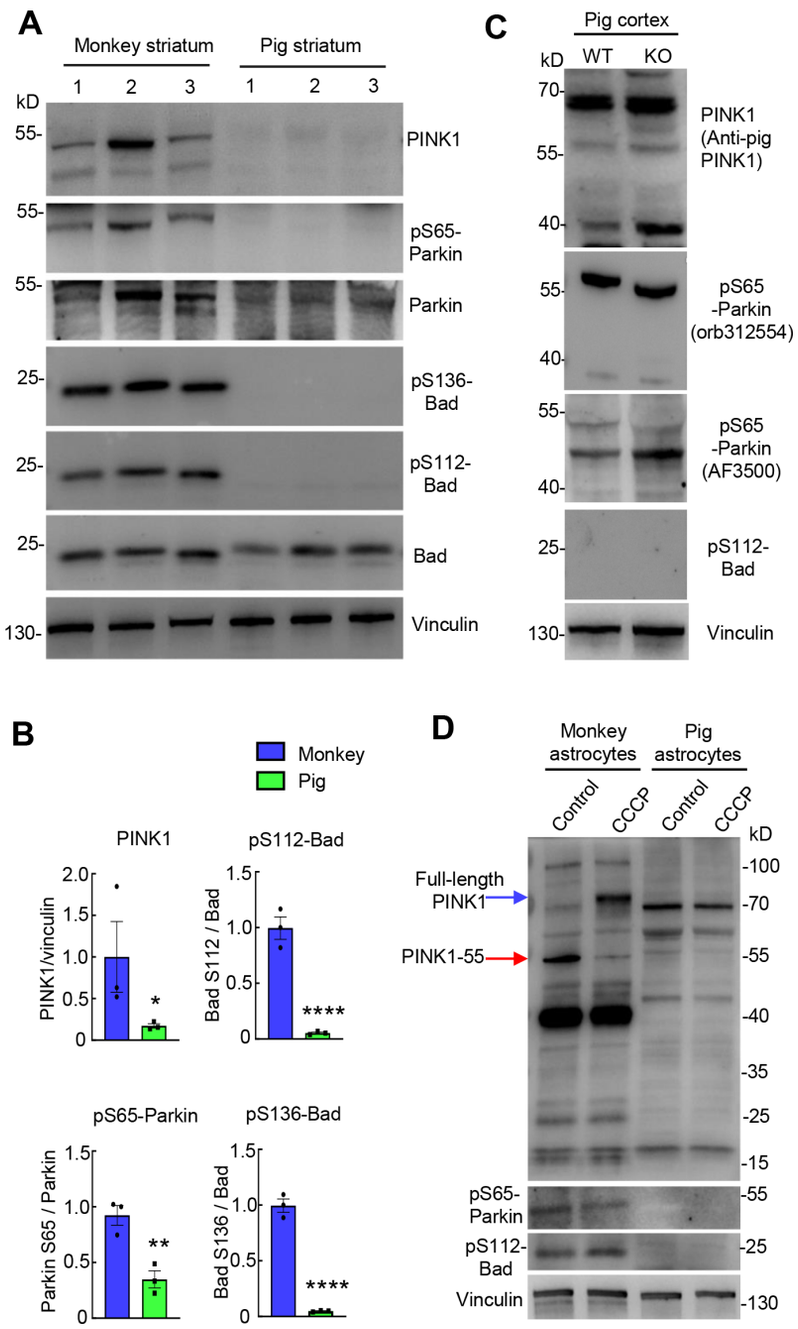

Figure 6 Differential PINK1-mediated phosphorylation in monkey and pig brain tissues

A: Western blot analysis of monkey and pig striatum with antibodies against PINK1 (rabbit B2), p-S65-Parkin, total Parkin, p-S136-Bad, p-S112-Bad, total Bad, and vinculin. Striatum tissues were obtained from three WT adult monkeys and three WT adult pigs. B: Quantification of ratios of phosphorylated to total proteins on blots in (A). Data are represented as mean±*SEM* (*n*=3). **: *P*<0.01; ***: *P*<0.001; ****: *P*<0.0001. C: Western blot analysis of WT and *PINK1* KO pig brain cortical tissues with anti-pig PINK1 antibody, two antibodies against phosphorylated Parkin (orb312554 and AF3500), and anti-p-S112 Bad and anti-vinculin antibodies. Note, no obvious alterations in these proteins were observed in *PINK1* KO pig brains. D: Cultured monkey and pig astrocytes were treated with CCCP (10 μmol/L for 16 h). Western blot analysis with anti-PINK1 (BC100–494), anti-pS65-Parkin (AF3500), and anti-pS112-Bad revealed increased levels of full-length PINK1 and reduced PINK1-55 in cultured monkey astrocytes after CCCP treatment, without an obvious increase in Parkin and Bad phosphorylation. No alterations in PINK1 or phosphorylation of Parkin and Bad were observed in cultured pig astrocytes after CCCP treatment.

shown in Figure 1, the rabbit BC100–494, rabbit B2, and mouse E7B6 antibodies could not detect differential expression of PINK1 between the WT and KO brain cortices. Western blot analysis employing the pig PINK1 antibody revealed no differences in immunoreactive bands between the WT and homozygous KO samples (Figure 6C). Furthermore, probing the same samples with antibodies against phosphorylated Parkin and Bad showed no differences between the WT and KO tissues (Figure 6C).

Cultured human cell lines do not express detectable levels of endogenous PINK1. However, when subjected to mitochondrial stress, these cells exhibit full-length PINK1 expression (Geisler et al., 2010; Narendra et al., 2010). Thus, we treated cultured astrocytes with carbonyl cyanide m-

chlorophenyl hydrazone (CCCP) to induce mitochondrial stress. Results showed that monkey astrocytes displayed an increase in full-length PINK1 and a decrease in PINK1-55 (Figure 6D). Interestingly, no significant increase was observed in pS65-Parkin or pS112-Bad after full-length PINK1 expression, suggesting that PINK1-55 carries out the same phosphorylation function as full-length PINK1. However, pig astrocytes did not show any increase in PINK1 expression, pS65-Parkin, or pS112-Bad (Figure 6D). The mechanism by which PINK1-55 is converted to full-length PINK1 after mitochondrial stress in cultured monkey astrocytes warrants further investigation. These findings suggest that the regulation of PINK1 expression is species- and cell-type dependent and may involve complex mechanisms.

DISCUSSION

Large animals often exhibit gene expression and regulatory mechanisms more akin to humans than do small animals. However, our study revealed that PINK1 protein expression was significantly lower or undetectable in pigs compared to its expression in primates. These findings suggest that PINK1 protein expression may be primate-specific, which has significant implications for understanding the unique function of PINK1 in these species and its relevance to the pathogenesis of PD.

First, our results indicated that *PINK1* mRNA expression was not regulated by species-dependent transcription, as evidenced by the comparable *PINK1* transcription levels in monkeys, pigs, and mice. In addition to transcriptional regulation, protein levels are influenced by several processes, including protein synthesis and turnover. Various mechanisms are known to regulate protein synthesis, including alternative mRNA splicing, ribosomal mRNA binding, and protein translation. Additionally, diverse mechanisms involving proteases and the ubiquitin-proteasome system are involved in protein stability and degradation. Post-translational modifications, such as phosphorylation and ubiquitination, also affect the stability and functionality of PINK1 (Durcan & Fon, 2015; Liu et al., 2017). Therefore, our findings highlight the need for a more comprehensive examination of the regulatory landscape governing PINK1 protein expression in non-human primate brain tissues to clarify its primate-specific regulation.

Second, our results elucidated the selective impact of *PINK1* mutations on neurodegeneration in primate brains. Notably, previous studies have indicated that pig models with CRISPR/Cas9-mediated deletion of the pig *PINK1* gene display no neurodegeneration or obvious neurological phenotypes (Wang et al., 2016; Zhou et al., 2015). Similarly, the absence of neurodegeneration in rodent models with PINK1 deficiency has long been perplexing, as none of these models demonstrate a decrease in PINK1 protein levels (Gispert et al., 2009; Goldberg et al., 2003; Kitada et al., 2009; Wang et al., 2023). *In vitro* studies of cultured cells have shown that the PINK1 protein is unstable and undergoes rapid degradation unless stabilized in damaged mitochondria (Beilina et al., 2005; Greene et al., 2012), supporting its role in mitophagy (Lazarou et al., 2015). While human *PINK1* transfection generates full-length PINK1 (about 70 kDa) and several cleaved products, further supporting this notion, *in vivo* evidence substantiating this theory remains scarce (Cummins & Götz, 2018; Lee et al., 2018; McWilliams et al., 2018; Whitworth & Pallanck, 2017). Specifically, under physiological conditions, endogenous full-length PINK1 is not detectable in cultured cells unless severe mitochondrial damage is induced. Additionally, endogenously expressed PINK1-55 in the primate brain is shorter than the full-length PINK1 in the absence of mitochondrial stress. Consequently, the mechanisms underlying the *in vitro* and *in vivo* expression of PINK1 require further in-depth investigation.

Our earlier studies demonstrated that PINK1-55 functions as a kinase (Yang et al., 2022). Therefore, deficiencies in PINK1-55 functionality or its expression, caused by various mutations, are likely to contribute to PD pathogenesis. Considering the unique expression of PINK1-55 in primate brains, non-human primates are an ideal choice for investigating the function of PINK1 and its role in PD.

The selective presence of the PINK1 protein in primate brains implies that the evolutionary maintenance of PINK1 expression and function may be unique to primates, which could account for the occurrence of *PINK1/Parkin* mutation-mediated PD exclusively in humans (Pickrell & Youle, 2015). We searched and compared the homology of PINK1 mRNA sequence across different species on the NCBI website, and the results revealed that the *PINK1* gene coding-region sequences in mice and pigs exhibit only 82.6% and 86.1% homology with humans, respectively, compared to 97% sequence homology between monkeys and humans. Therefore, the notable differences in *PINK1* genes among mice, pigs, and humans may contribute to the selective expression of PINK1 in primates. It is possible that the mitophagic role of PINK1 is conserved across species under certain circumstances, such as mitochondrial damage or stress. Considering that PINK1 is also expressed in primate brains under physiological conditions, our study highlights the importance of investigating the physiological functions of PINK1 in addition to its well-known mitophagic activity.

Third, our study highlighted the importance of selecting animal models to investigate disease pathogenesis. While mouse models have proven invaluable, yielding a wealth of information and insights into a variety of human diseases, it remains important to recognize that small animal models often fail to fully recapitulate key neuropathological features observed in human patients. For instance, genetically-modified mouse models of neurodegenerative diseases, such as Alzheimer's, PD, and Huntington's, do not manifest overt and selective neurodegeneration (Chen & Zhang, 2022; Crook & Housman, 2011; Dawson et al., 2018; Levine et al., 2004; McGowan et al., 2006).

In addition to substantial variances in genomics, anatomy, and neural circuitry across species, marked disparities also exist in brain development and aging processes between small animals and primates. For instance, rodent brains develop rapidly within a month, in contrast to primate brains, which require more than 6 months for full development (Otani et al., 2016; Yin et al., 2022). Furthermore, mice typically have a lifespan of only 2–3 years, whereas macaques can live for more than 20 years. As such, when key pathological features cannot be replicated in small animals, it is crucial to consider large animals with greater similarity to humans as alternative models for human disease studies.

This study contributes additional complexity to the regulatory landscape of PINK1 protein expression. Our results suggest that PINK1 expression is notably and uniquely present in primate brains but is either minimally expressed or entirely absent in pig brains. Furthermore, this research emphasizes the importance of *in vivo* studies to further

elucidate the function of PINK1, thereby enhancing our understanding of how deficiencies or mutations in this critical protein contribute to the early onset of PD.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

W.L.Y. and X.J.L. designed and supervised the research experiments. X.S.C., R.H., Y.T.L., W.H., Q.W., and X.X. performed the experiments and analyzed the data. Y.Z. and J.G.Z. provided pig *PINK1* KO tissues. S.H.L. provided advice. X.J.L. and W.L.Y. wrote the manuscript. All authors read and approved the final version of the manuscript.

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