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p53 signalling mediates acupuncture-induced neuroprotection in Parkinson's disease

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ABSTRACT

Parkinson's disease (PD) is a progressive neurodegenerative disorder associated with a selective loss of dopamine (DA) neurons in the substantia nigra of the midbrain. Recently, it has been demonstrated that acupuncture treatment has protective effects in PD. However, to date, the molecular mechanisms underlying acupuncture's effect on DA neuronal protection are largely unknown. In this study, we report that p53 signalling mediates the protective effects of acupuncture treatment in a mouse model of PD. We found that the acupuncture treatment in the mouse PD model results in significant recovery to the normal in the context of behaviour and molecular signatures. We found that the gene network associated with p53 signalling is closely involved in the protective effects of acupuncture treatment in PD. Consistent with this idea, we demonstrated that specific knockout of the p53 gene in the midbrain DA neurons abrogates the acupuncture induced protective effects in the mouse model of PD. Thus, these data suggest that p53 signalling mediates the protective effects of acupuncture treatment in PD.

1. Introduction

Parkinson's disease (PD) is one of the most common neurodegenerative diseases and is characterised by selective loss of dopaminergic (DA) neurons in the substantia nigra of the midbrain [1,2]. Because midbrain DA neurons play an essential role in the regulation of voluntary movement and other behaviours [3], the major symptoms of PD are behaviour disorders including resting tremors, rigidity, bradykinesia and postural instability [4]. A low percentage of PD cases result from mutations in genes such as alpha-synuclein, whereas the more common sporadic forms of PD are promoted by environmental factors [5]. The current treatments for PD patients are limited and provide temporary relief of symptoms without curing the progression of the disease.

Recently, acupuncture treatment has been used an effective alternative therapy for PD [6,7]. Several lines of evidence suggest that acupuncture treatment in PD patients and mouse models has protective effects on the DA neurons of the midbrain [8,9]. However, despite several studies that explored the effect of the treatment of acupuncture on the neuroprotective effects, the underlying molecular mechanisms of these effects are still largely unclear. It has been suggested that increased neurotropic factors plays a critical role in acupuncture-induced neuronal protection [10]. Other studies have also shown that the inhibition of inflammatory responses in the PD model mediates acupuncture effects [11]. In our previous study, we showed the changes in protein expression profiles in favour of DA neuronal survival induced by the acupuncture treatments, suggesting a feasible epigenetic network for the protective effects of acupuncture treatment in the PD mouse model [12].

In this study, we examined whether the transcriptional signalling in DA neurons plays a role in the protective effects of the acupuncture treatment in the mouse model of PD. Interestingly, we

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found that the gene network involved in p53 signalling was significantly changed as a result of the acupuncture treatment, suggesting that p53 signalling may mediate the acupuncture-induced neuroprotective effects. p53 is known to be crucial role in sensing DNA damage and in the cellular response to various stresses that affect DNA integrity even in neurons [13]. Recently, several studies have suggested that p53 dysfunction is involved in neuronal impairment in neurodegenerative disease [14]. More importantly, p53 inhibition or knockout was shown to be beneficial in cases of neuronal damage [15]. Thus, it is possible that p53 signalling may be the mediator of acupuncture treatment that contributes to the neuronal survival of midbrain DA neurons. Thus, in this work, we utilised a conditional p53 knockout PD mouse model to examine the protective effects of acupuncture treatment on DA neurons. Our observations provide a deeper molecular understanding of the acupuncture treatment and reveal the crucial role of p53 signalling in the transcriptional changes that lead to the acupuncture-induced protective effects in PD.

2. Materials and methods

2.1. Animals and MPTP treatment

Male 7-week-old C57BL/6 mice (Central Laboratories Animal Inc, Republic of Korea) weighing 23–25 g were maintained on a 12 h/12 h light/dark cycle and room temperature (23 ± 1 °C) with free access to water and food. All the experiments were approved by the Kyung Hee University Animal Care Committee for animal welfare. Experiment 1. Twenty mice were randomly assigned to four groups (each n = 5): CON (Control), MPTP (MPTP only), AP (MPTP+acupuncture treatment on the GB 34 acupuncture point), and CP (MPTP+acupuncture treatment on the control point). All groups were treated with intraperitoneal (i.p) injection of saline or MPTP (30 mg/kg, dissolved in saline; Sigma–Aldrich, MO, USA) for 5 days. Two hours after MPTP treatment, the AP group was treated with GB34 acupuncture point and the CP group was treated with control point for 12 days. Experiment 2. Fifteen wild type mice (WT, p53 +/+) and 17 p53 knock out mice (KO, p53 -/-) were randomly assigned to six groups: p53 +/+ Control (n = 5), p53 +/+ MPTP (n = 5), p53 +/+ MPTP+AP (n = 5), p53 -/- Control (n = 5), p53 -/- MPTP (n = 5), and p53 -/- MPTP+AP (n = 7). The MPTP and acupuncture treatment were the same as in Experiment 1.

2.2. Acupuncture treatment

The acupuncture treatment was performed 2 h after MPTP injection. Mice were slightly immobilised by securing their neck and applying the acupuncture treatment on the GB34 acupuncture point (Yangneungcheon, located in the depression anterior and distal to the head of the fibula) [16] or control point (in the muscle on both sides of the hips). Acupuncture point GB34 has been reported to exert clinical effectiveness for movement disorders [17] and is commonly used to treat neurodegenerative disease such as Parkinson's disease [12,18]. Acupuncture needles (15 mm in length, 0.20 mm in diameter; Haeng-lim-seo-weon Acuneedle Co., Seoul, Republic of Korea) were inserted to a depth of 3 mm in the bilateral GB34 or the control point and turned at a rate of two spins per second for 15 s. Then, the needles were immediately removed. All of the groups except the AP and CP group were immobilised for 15 s to induce the same amount of stress as the acupuncture treatment.

2.3. Whole genome expression analysis

Total RNA was isolated from the mouse midbrain with the RNeasy MiniKit (QIAGEN) according to the manufacturer's instructions. Briefly, 1 μg of total RNA was used to prepare biotinylated cRNA with an Affymetrix One Cycle cDNA Synthesis Kit according to the manufacturer's protocol. Samples were prepared for hybridisation with 15 μg biotinylated cRNA in a 1 × hybridisation cocktail according to the Affymetrix hybridisation manual. GeneChip arrays (mouse genome 430A 2.0 arrays) were hybridised in a GeneChip Hybridisation oven at 45 °C for 16 h at 60 RPM. Arrays were scanned on a GeneChip Scanner 3000, and images were extracted and analysed with GeneChip operating software v1.4. Statistically significant differences in gene expression between the different groups were confirmed using RT-PCR.

2.4. Behaviour test

Movement behaviours were measured using the rotarod test and cylinder test. On the last day, the rotarod test (MED associates Inc., VT, USA) was performed as previously described [19]. The mice were pre-trained for 2 min on an accelerating rod speed mode 1 h before the experiment. The time on the rod was recorded with a maximum of 480 s for successive rod speeds. The time to fall (Latency time) and the total running distance were analysed. The total running distance was calculated as running time × circumference of the rod. DA-deficient PD mice commonly show decreased motor function, demonstrated by fewer cylinder wall touches with the mice forelimbs [20]. The mice were localised in a plastic cylinder (20 cm tall and 12 cm diameter) and adapted for 1 min. Then, observers counted the number of wall touches for 3 min.

2.5. Immunohistochemistry

Mice were perfused transcardially with 0.05 M PBS buffer followed with 4% formalin solution. The brains were removed, post-fixed and cryoprotected. Coronal sections of the brains (40 μm thick) that encompass the entire striatum (ST) and substantia nigra (SN) were cut. Sections were incubated overnight at room temperature with a rabbit anti TH antibody (1:1000 dilution, Santa Cruz, CA, USA) then washed three times with PBST. The optical density of TH in the ST was evaluated by measuring the same area in each group. The numbers of the TH-positive cells in the SN were counted manually. The sections were photographed under a microscope (BX53; Olympus Corporation, Tokyo, Japan).

2.6. Apoptosis assay

Apoptosis in cells was evaluated by double staining with Muse™Annexin V & Dead Cell reagent (Merck KGaA, Darmstadt, Germany) and acquired on the Muse™ Cell Analyser (Merck KGaA, Darmstadt, Germany).

2.7. Statistical analysis

All statistical parameters were calculated using the GraphPad Prism 5.0 software (GraphPad Software Inc., CA, USA) and were performed using one-way ANOVA with the Newman–Keuls post-hoc test. All data were expressed as the mean ± SEM. In all of the analyses, differences were considered to be statistically significant at $p < 0.05$.

3. Results

3.1. Effect of acupuncture on the motor function in MPTP-induced PD mice

Previously, we showed the neuroprotective effects of acupuncture treatment in the PD mouse model [18]. To examine

the effect of acupuncture treatment in MPTP-induced PD mouse model, acupuncture was performed at GB34, and the treatment was continued at 24 h intervals for 12 consecutive days. The protective effects of acupuncture in MPTP-treated mice were examined based on the motor functions. We observed that the latency time of the MPTP-treated group was significantly decreased compared to that of the control group in Fig. 1B. However, the AP treatment in the MPTP-induced mouse model improved the latency time compared to that of the MPTP-treated group, whereas the control treatment did not produce improvement compared to the AP treatment group (Fig. 1 B). Additionally, we examined the numbers of cylinder wall touches in the rotarod test. Consistent with previous results, the MPTP-treated group showed a significant decrease in the numbers of wall touches compared to those of the control group. However, AP treatment also improved the numbers of cylinder wall touches compared to those of the MPTP group, whereas the control group did not show improvement (Fig. 1 C). To directly assess the protective effects of acupuncture on DA neurons, we performed immunofluorescence using a TH-antibody in the mouse brain substantia nigra (Fig. 1 D). The MPTP-treated mice showed significantly decreased TH-immunopositive cells compared to the control group. However,

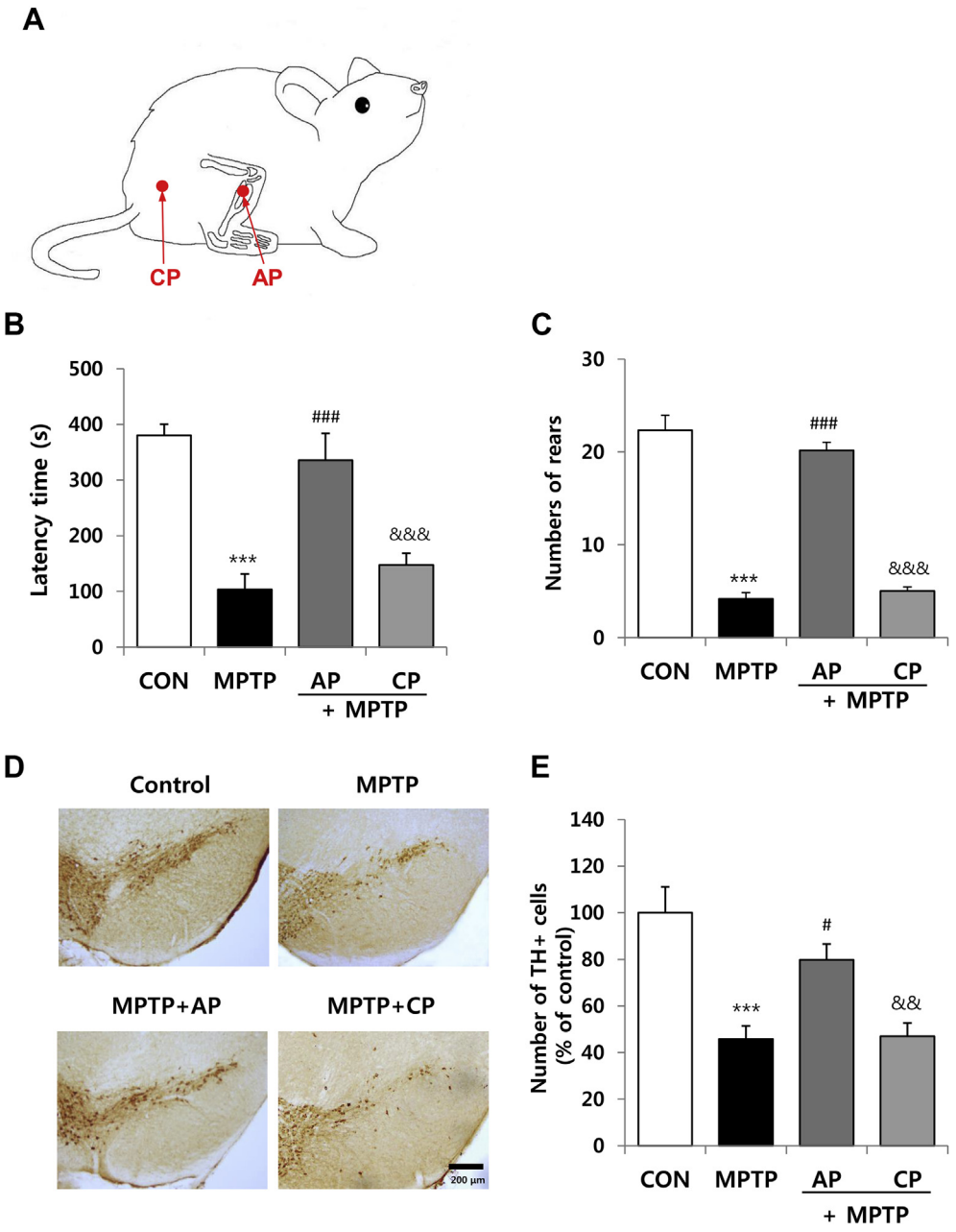

Fig. 1. Protective effects of acupuncture treatment in MPTP-induced PD mice. (A) The acupuncture was performed on the GB34 acupuncture point (AP) and control point (CP) of the mice. (B-C) The behaviour test was performed 12 days after the saline or MPTP (30 mg/kg) injection. In the rotarod test (B) and cylinder test (C), acupuncture treatment results in recovery of movement behaviours of mouse model of PD. Statistical analysis was performed using one-way ANOVA followed by Newman–Keuls post hoc tests ($^{***}p < 0.001$ compared to the control group; $^{\#\#\#}p < 0.001$ compared to the MPTP group; $^{\&\&\&}p < 0.001$ compared to the AP group). CON, control; MPTP, MPTP only; AP, MPTP+acupuncture treatment on the GB34 acupuncture point; CP, MPTP+acupuncture treatment on the control point. (D) Immunostaining of midbrain sections from 8-week-old controls, MPTP, MPTP+AP and MPTP+CP treated mice for tyrosine hydroxylase (TH). (Scale bars, 200 μm). (E) Quantification of Fig. 1D. Data represents means ± SEM, ANOVA-test, $^{*}p < 0.05$, $n = 5$.

acupuncture treatment on the MPTP-treated mice showed significantly increased TH-positive neurons (Fig. 1 D and E). Therefore, taken together, these results suggested that acupuncture treatment is effective at improving motor function and protects the DA neurons in the MPTP-induced mouse model of PD.

3.2. P53 signalling mediates acupuncture-induced neuronal protection

Acupuncture-induced neuronal protection may be caused by changes in the epigenome, including changes in protein expression [12]. Because we have shown the protein expression profiles associated with DA neuronal survival in response to the acupuncture treatments in the PD mouse model, we hypothesised that acupuncture treatment induce epigenetic changes associated with DA neuronal protection. Thus, to assess the changes in the gene expression pattern induced by acupuncture treatment, we analysed the transcriptome of MPTP-induced midbrain DA neurons after acupuncture treatment and compared the expression profiles to the control cells. Remarkably, acupuncture treatment resulted in dramatic changes in global gene expression in MPTP-treated midbrain DA neurons and recovery to normal into gene expression pattern (Fig. 2A, Sup. Table1). The differential expression of these genes was confirmed by quantitative real time PCR (Fig. 2 C).

We identified 76 genes whose expression significantly changed (≥1.5-fold) after acupuncture treatment, and GO-term analysis across the two independent experimental sets identified the changes in the gene signatures associated with signalling transduction in the acupuncture-treated midbrain DA neurons (Fig. 2 B,

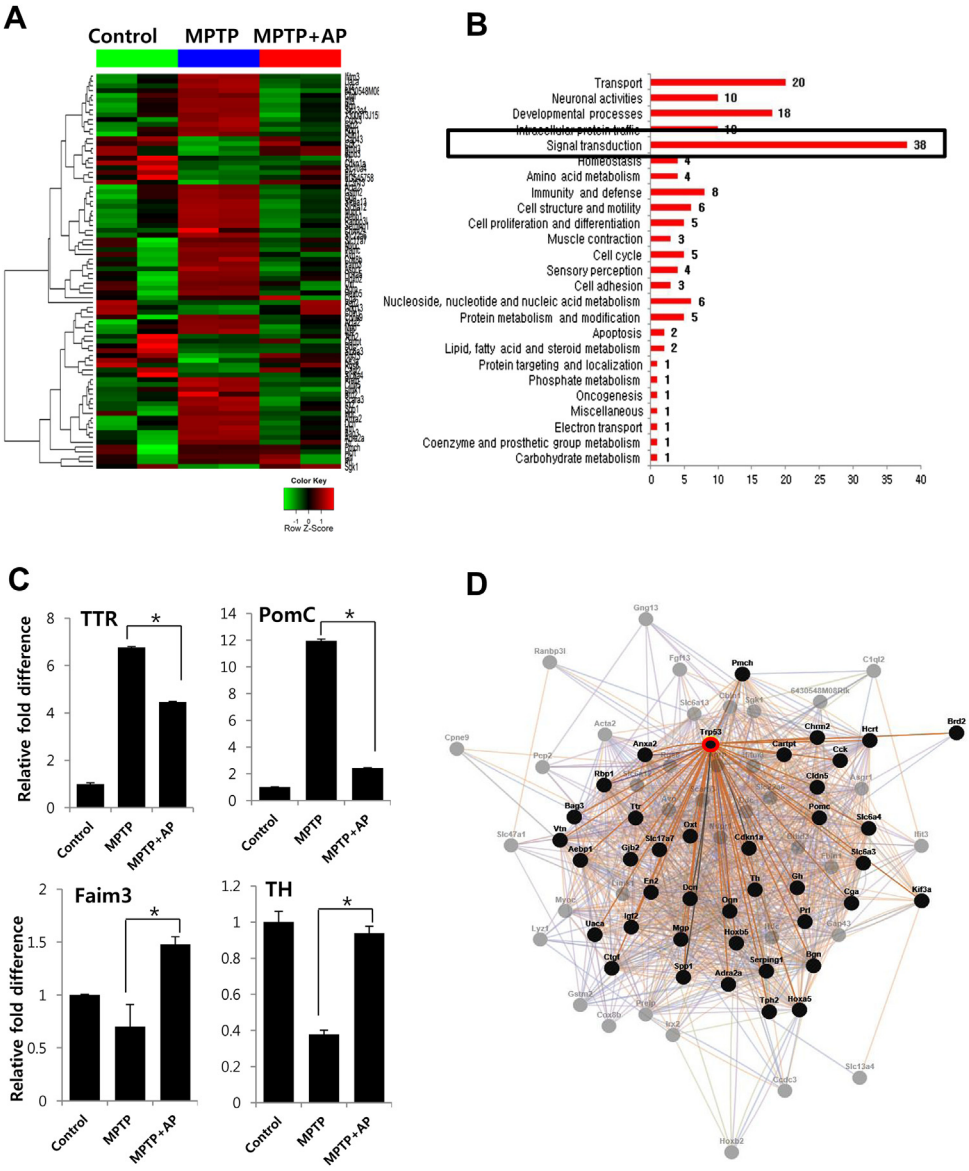

Fig. 2. p53 signalling mediates acupuncture-induced neuronal protection. (A) Heat map from microarray data of global gene expression pattern in control, MPTP and acupuncture-treated midbrain DA neurons. (B) Go-analysis of differentially expressed genes in the MPTP & acupuncture. (C) Quantitative real time PCR of differentially expressed genes: TTR, Pomc, Faim3, and TH. Data are normalised to GAPDH and are shown as the means ± SEM, Student t-test, $^{*}p < 0.05$, n = 3. (D) Transcriptional network showing hubs of multiple genes within acupuncture induced differentially expressed genes. The each circle reflects the differentially expressed genes by acupuncture treatment. The line indicates the interaction of transcriptional regulation.

Sup. Fig. 1a, Sup. Table1). This suggests that the specific changes in signalling mechanisms may be the dominant factor in acupuncture-induced neuronal protection. Next, to facilitate a comprehensive understanding of the differential gene expression in complex genomes, we combined the differentially expressed genes, and we sought to determine whether the specific signalling network might be involved in the acupuncture-induced neuronal protection. The target interactions are depicted with the distance of each circle reflecting the degree of gene expression. Surprisingly, we found that the p53 signalling network is significantly involved in acupuncture-treated DA neurons (Fig. 2 D). We found that 40 of 76 differentially expressed genes are involved in the p53 signalling network (Fig. 2 D). Moreover, all up-regulated genes in acupuncture treatment are closely associated with p53 signalling in the gene network (Sup. Fig. 2a). Taken together, our data reveal that the p53 signalling is highly involved and is likely to be important in the acupuncture-induced survival of midbrain DA neurons.

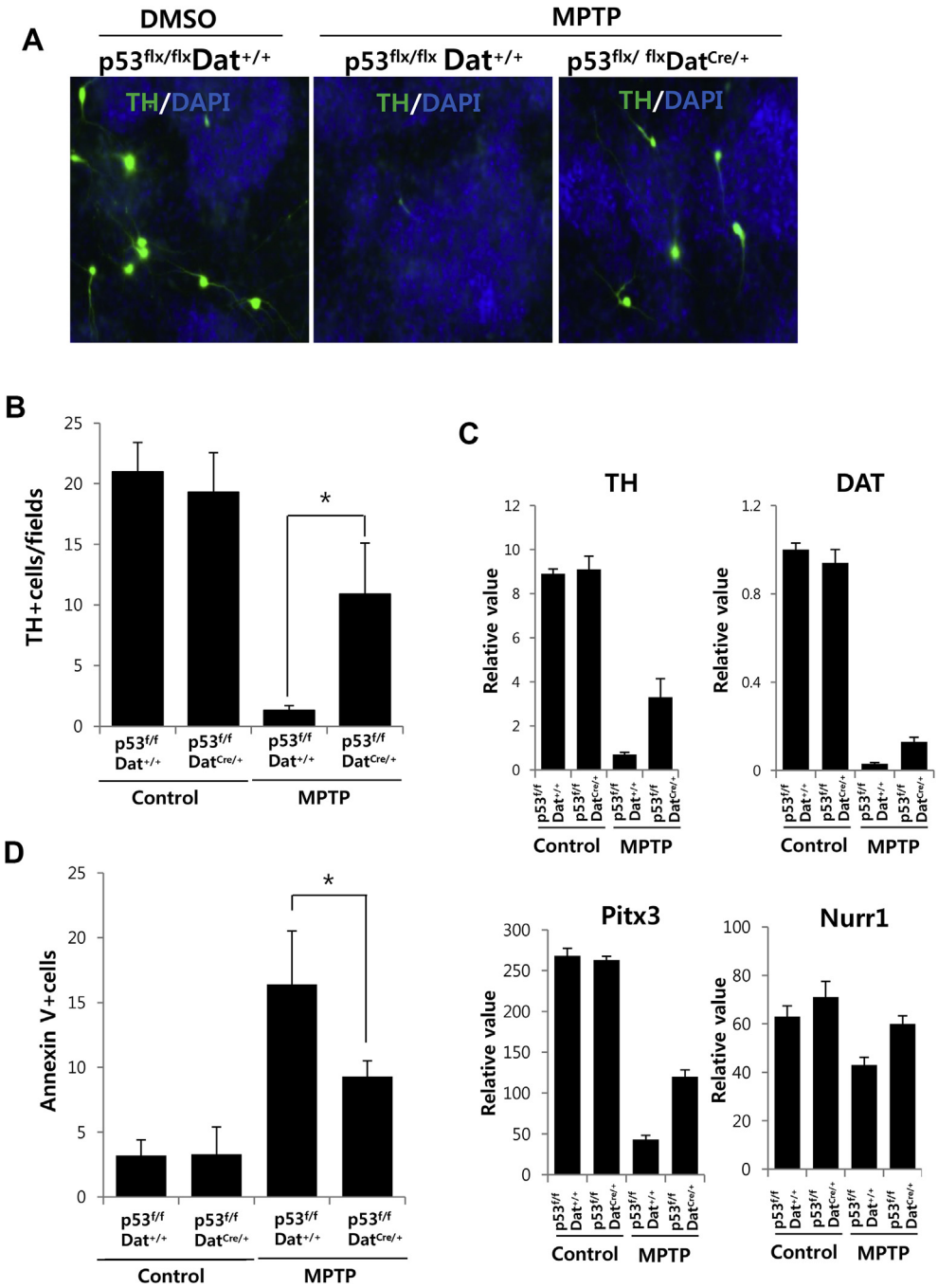

Fig. 3. Function of p53 in the survival of midbrain DA neurons. (A) Immunostaining of TH + DA neurons in MPTP-treated p53 knockout DA neurons ($p53^{flox/flox}$: $DAT^{CRE/+}$). Flx indicated flox. Scale bar = 50um. (B) Quantification of Fig. 3(A). 10 different fields were randomly selected and counted from three independent experiments. Each bar represents the means ± SEM. Student t-test, *p < 0.05, n = 3 (C) Quantitative real time PCR of DA neuronal marker genes, TH, DAT, Pitx3 and Nurr1 in MPTP-treated p53 knockout DA neurons ($p53^{flox/flox}$: $DAT^{CRE/+}$). Data are normalised to GAPDH and are shown as the means ± SEM, Student t-test, *p < 0.05, n = 3. (D) The number of annexin V stained cells in MPTP-treated p53 knockout DA neurons ($p53^{flox/flox}$: $DAT^{CRE/+}$). Cell apoptosis based on annexin V stained cells. Each bar represents the means ± SEM. Student t-test, *p < 0.05, n = 3.

3.3. The functional role of p53 signalling in acupuncture-induced DA neuronal protection

To further investigate the functional role of p53 signalling in acupuncture-induced DA neuronal protection, we investigated the knockout effect of p53 signalling on the survival of midbrain DA neurons. To selectively knockout p53 in the midbrain DA neurons, we prepared primary midbrain DA neurons from $p53^{flox/flox}$: $DAT^{CRE/+}$postnatal day 1 mice, which lead to Cre mediated deletion of p53 in post-mitotic DA neurons. Five days after plating, MPTP was introduced into the culture medium, and 3 days later, TH positive cells were counted. The TH + DA neurons almost disappeared, which is consistent with the neurotoxicity of MPTP in the DA neurons.

Interestingly, we observed that p53 knockout significantly increased the survival of MPTP-treated DA neurons, as confirmed by the immunofluorescence of DA neuronal genes such as TH (Fig. 3 A and B). Furthermore, to examine whether the acupuncture-mediated protection of DA neurons occurs through the inhibition of apoptosis, we measured the relative amounts of annexin-V-positive cells. Annexin-V and PI positive–stained cells showed nuclear DNA fragmentation that had resulted from apoptotic cell death. FACS analysis of annexin-V-positive cells revealed that MPTP treatment significantly increased the number of annexin-V-positive cells (Fig. 3D). However, p53 knockout significantly attenuated the effects of MPTP, suggesting that p53 knockout protects DA neurons against MPTP-induced apoptotic death (Fig. 3 D). In addition, quantitative RT-PCR analysis shows that DA neuronal marker genes, including TH, ChAT, DAT, Nurr1 and Pitx3 were marginally elevated in MPTP treated p53 knockout DA neurons (Fig. 3 C). These results indicated that p53 is critical for the survival and maintenance of midbrain DA neurons.

3.4. p53 knockout mice abrogated acupuncture induced DA neuronal protection

To extend these findings to the intact rodent central nervous system, we prepared $p53^{flox/flox}$: $DAT^{CRE/+}$mice leading to the specific deletion of p53 in post-mitotic midbrain DNs in adult mice. These mice display normal midbrain DNs, as quantified by TH immunostaining (Fig. 4 C and Sup. Fig. 3a). Behavioural studies showed that the latency time and running distance were

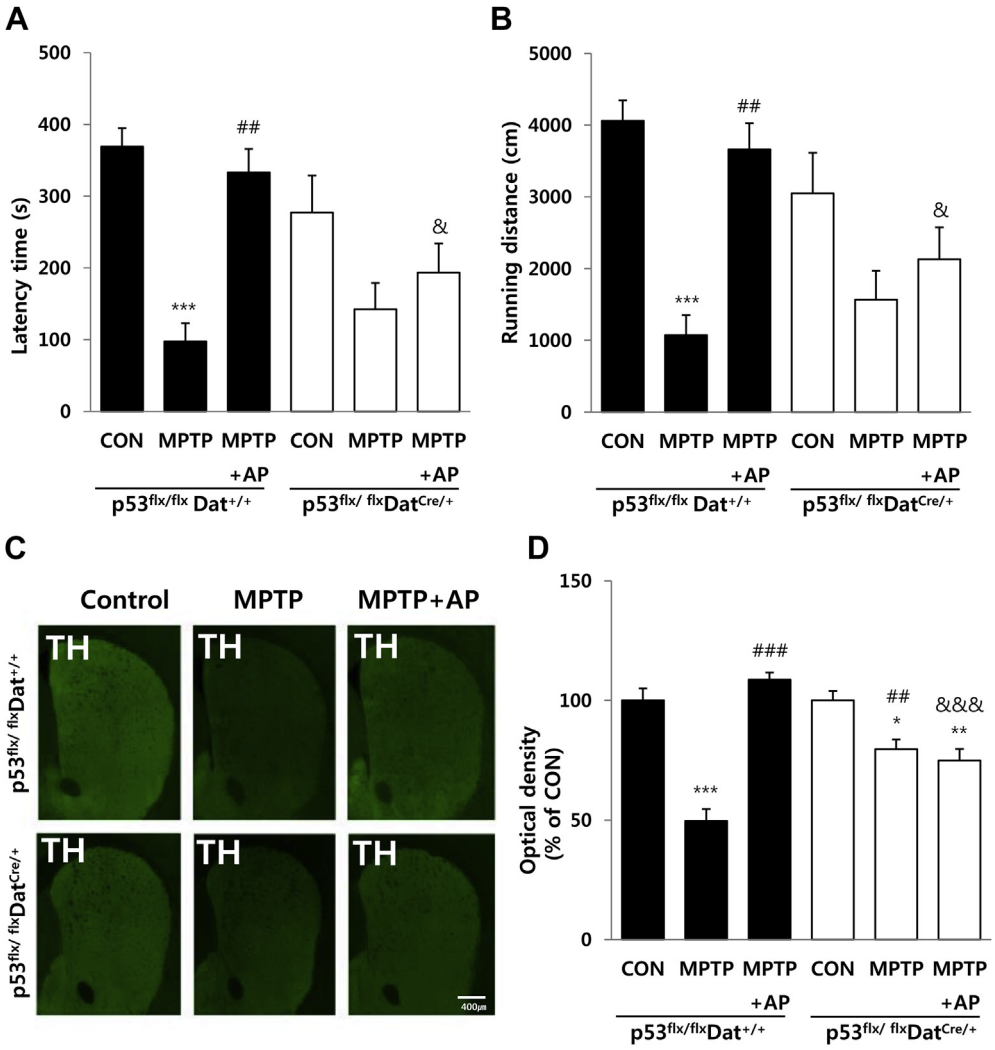

Fig. 4. p53 knockout abrogated acupuncture induced DA neuronal protection. (A), (B) The latency time (A) and running distance (B) of rod fall. Acupuncture treatment in the p53 conditional knockout ($p53^{flox/flox}$: $DAT^{CRE/+}$) mice showed reduced motor function compared to the control ($p53^{flox/flox}$: $DAT^{+/+}$) group. Statistical analysis was performed using one-way ANOVA followed by Newman–Keuls post hoc tests. $^{***}p < 0.001$ compared to the CON ($p53^{flox/flox}$: $DAT^{+/+}$) group; $^{\#\#}p < 0.01$ compared to the MPTP ($p53^{flox/flox}$: $DAT^{+/+}$) treated group; $^{\&}p < 0.05$ compared to the AP ($p53^{flox/flox}$: $DAT^{+/+}$) group. (C) Immunostaining of the TH positivity in the striatum of control and $p53^{flox/flox}$: $DAT^{Cre/+}$ mice treated with MPTP and acupuncture. (D) Quantification of optical density shown in Fig. 4 (C).

significantly decreased after MPTP treatment ($p < 0.01$, Fig. 4A and B). However, acupuncture treatment in the MPTP-induced p53 knockout mice did not significantly increase the latency and running behaviour (Fig. 4 A and B).

Furthermore, to directly assess the protective effects of acupuncture on the survival of DA neurons in the MPTP-induced PD mouse model, we performed immunofluorescence using a TH-antibody in the mouse brain substantia nigra (Sup. Fig. 3a). Consistent with previous results, the MPTP-treated control mice showed significantly decreased TH-immunopositive cells compared to the non-treated group (Sup. Fig. 3b). However, The MPTP-treated p53 knock mice showed slightly decreased TH-positive cells compared to the control group. Moreover, we observed that acupuncture treatment with subsequent MPTP injections in control mice showed significantly increased TH-positive fibres, whereas the acupuncture treatment in MPTP-treated p53 knockout mice showed marginally increased TH-positive cells and fibres (Fig. 4 C and Sup. Fig. 3a and b). Taken together, these results suggest that p53 signalling mediates acupuncture-induced DA neuronal protection in the mouse model of PD.

4. Discussion

Parkinson disease is one of the most common neurological diseases and is associated with behaviour disorders [21]. Acupuncture is frequently used to treat this disease in mice and humans [22], but the exact epigenetic mechanism underlying its clinical efficacy is still unclear. Previous studies have demonstrated that acupuncture may stimulate the release of pain-killing natural chemicals, relax tense muscles, or inhibit the conduction of pain through counter-irritation [23]. We also found that acupuncture treatment in the MPTP mouse model resulted in differential protein expression profile that eventually led to DA neuronal survival [18]. However, the signalling mechanisms that mediate the acupuncture effect in the midbrain DA neurons remain unclear in vitro and in vivo. In this study, we demonstrated that p53 signalling mediates the acupuncture-induced DA neuronal protection.

Our data indicate that p53 signalling plays a critical role in acupuncture-induced neuronal protection. We found that significant changes in the p53 signalling network is required for the acupuncture-induced neuronal protection and the functionality of the DA neurons in the mouse model of PD. The inhibition of p53 has been found to protect against neuronal death in MPTP-treated primary DA neurons. Moreover, the protective effect of the acupuncture treatment in vivo was significantly decreased in p53 inhibition, as shown by the functional efficiency of the behaviour tests. Thus, these results support a model in which acupuncture-induced neuronal protection is mediated by the p53 signalling pathway. It is known that p53 can either induce DNA repair or cell death depending on the nature and extent of stress and damage [24]. Interestingly, several studies suggested that dysfunction of p53 is involved in neuronal impairment in neurodegenerative disease [25]. More importantly, p53 inhibition has been shown to produce beneficial effects in cases of neuronal damage [26]. In addition, p53 can directly affect the level of mitochondria and induce cell death [27]. For example, its pharmacological inhibition has been studied for a long time as a potential target in neurodegenerative disease including Alzheimer and Parkinson's disease [28]. Thus, it is possible that p53 signalling may be the significant effector of acupuncture treatment that contributes to the neuronal protection.

Importantly, we are the first to show that acupuncture treatment in the PD mouse model results in the midbrain DA neurons that highly resemble control midbrain DA neurons in the context of molecular features. Globally, the gene expression state in acupuncture-treated DA neurons is highly similar to that of control midbrain DA neurons. Moreover, the behaviour properties and the expression levels of marker genes shown in acupuncture-treated PD models are almost indistinguishable from those of the control group. These results demonstrated that acupuncture treatment efficiently induces transcriptional changes in DA neurons that may lead to improved motor function. Thus, these results have significant implications for acupuncture-based therapeutic interventions for PD.

In conclusion, our study demonstrates that acupuncture treatment leads to transcriptional changes in favour of the survival of DA neurons and that p53 signalling mediates acupuncture-mediated DA neuronal protection. Thus, our results suggest that acupuncture treatment as alternative medicine can be considered in efforts to treat PD patients.

Conflict of interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.105.

Transparency document

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