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Modulation of mitochondrial function by stem cell-derived cellular components

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ABSTRACT

Huntington's disease (HD) is the most common hereditary neurodegenerative diseases, in which the loss of striatal neuron caused by the aggregation of mutant huntingtin protein (mHtt) is the main pathological feature. Our previous studies have demonstrated that human adipose stem cells (hASC) and its extracts can slow down the progression of HD *in vitro* and *in vivo*. hASC are readily accessible adult stem cells, and the cytosolic extracts contain a number of neurotrophic factors. Here, we further explored the role of the hASC extracts in neuronal death and mitochondrial function in HD. Our results showed that the hASC extracts prevent mHtt-induced cell toxicity and cell apoptosis. Moreover, the hASC extracts recovered mHtt-induced mitochondrial oxidative stress and reduced mitochondrial membrane potential. The hASC extracts blocked the interaction between p53 and mHtt, and decreased the endogenous p53 levels at both transcriptional and post-translational levels, resulting in the instability of p53 and increased neuronal survival. Taken together, these findings implicate protective roles of hASC extracts in mHtt-induced mitochondrial apoptosis, providing insights into the molecular mechanism of the hASC in the therapeutic strategy of HD.

1. Introduction

Huntington's disease (HD) is an incurable progressive autosomal dominant neurological disorder caused by expanded CAG repeats coding for glutamine in the *Huntingtin* (*Htt*) gene. Many studies have shown that aggregation of mutant Htt (mHtt) caused cell death *in vivo* [1–3] and *in vitro* [4,5]. Specially, mHtt contributes to neurodegenerative disorders through the activation of intrinsic apoptosis pathway involving mitochondria [6–8]. The lower level of mitochondrial membrane potential has been detected from both human HD patient and full-length Htt-transgenic HD mice brains compared to the normal human and wild-type mice [9]. More interestingly, N-terminal mHtt is identified on the mitochondrial membrane from YAC72 mice brain [9] and mHtt is present in mitochondrial fraction from HD *in vitro* cell model [10]. Mitochondrial dysfunction is a causative factor in most of neurodegenerative diseases [11–13]. Many events can occur during the process of mitochondria-mediated cell death including loss of mitochondrial membrane potential, imbalance of anti-apoptotic proteins and pro-apoptotic proteins. As a result, the levels of mitochondrial reactive oxygen species (ROS) are increased and other apoptotic inducing factors are released to the cytosol, which lead to the activation of pro-caspase to induce apoptosis[14,15].

p53, a tumor suppressor protein, promotes apoptosis during the development of central nervous system in response to injury and in neurodegenerative disorders [16]. In response to injury and cellular stress, p53 induces DNA damage and cell death through the transcriptional transactivation of its target genes including Bax, puma and p21 [17,18]. In addition, p53 protein levels also can be regulated through its stabilization by stress signals and its destabi-

Abbreviations: EGFP, enhanced green fluorescent protein; hASC, human adipose stem cells; HD, Huntington's disease; RT–PCR, reverse transcription polymerase chain reaction; ROS, reactive oxygen species; mHtt, mutant huntingtin protein.

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lization by protein degradation system [16,19]. Current studies have shown that p53 induces apoptosis both in transcription-dependent pathway and transcription-independent action at the mitochondria [20]. p53 plays a key role in the mitochondria-associated cellular dysfunction and behavioral abnormalities of HD mice [21]. In HD mice, mHtt binds to p53 and upregulates p53 transcriptional activity [21]. Behavior abnormalities and neurodegeneration in mHtt-transgenic mice are suppressed by the genetic deletion of p53 [21]. It has also reported that p53 is stabilized by mHtt, resulting in accumulation of p53 and increase the activity of p53 [21,22].

Human adipose stem cells (hASC) have been shown to be multipotent and possessed the ability to differentiate into multiple cell lineages. hASC are feasible source for stem cell-based therapy due to their abundance, multipotency, and ethical consideration [23–25]. Current study shows that the hASC can produce many important neurotrophic factors that are essential for neuronal growth, differentiation and survival [26]. Similarly, a recent study has shown that hASC protect the brain from traumatic brain injury-induced neurodegeneration and motor and cognitive impairments [27]. Our previous studies have demonstrated that both the hASC and the hASC extracts could slow down the progression of HD through reducing mHtt aggregation [28,29]. However, the detailed molecular mechanism whether the hASC extracts can prevent the neuronal death and mitochondrial dysfunction has not been well studied. In this study, we investigated the protective role of the hASC extracts in cell death and mitochondrial dysfunction in HD, and further explored the p53 involved mechanism.

2. Materials and methods

2.1. Cell culture

Mouse neuroblastoma cell line (N2a) were cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Scientific, MA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. Cell apoptosis assay and mitochondrial dysfunction assay

For LDH release assays, 200 μl of culture medium were collected and used. The LDH level was measured with *in vitro* Toxicology assay kit (Sigma–Aldrich, MO, USA) by spectrophotometer following the manufacturer's instructions. For CCK8 cell viability assay, cell culture medium was replaced with new medium containing 10% CCK-8. Two hours later, the culture medium was collected and the absorbance was measured at 450 nm using a microplate reader. For Annexin V/PI cell death assays, the Annexin-V-FITC and PI Apoptosis Detection Kit (BD Bisosciences) was used followed by FACS analysis (FACS Calibur, BD, CA, USA) according to the manufacturer's instructions. For JC-1 staining, after harvesting and washing with PBS, cells were stained with JC-1 (Invitrogen), MitoSoxRed (Invitrogen) or Mitotracker (Invitrogen) Red for 20 min. Cells were washed once with binding buffer and measured by flow cytometry and (or) captured by fluorescence microscopy.

2.3. Immunoblotting and immunoprecipitation

Cell lysate was lysed with RIPA lysis buffer (Biosesang Inc., Seoul, Korea) and the total protein concentration was quantified by a colorimetric detection assay (BCA Protein Assay, Pierce, USA). Equal amounts of protein lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and transferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA, USA). Interested proteins were probed by primary antibodies and corresponding peroxidase-labeled secondary antibodies, followed with detection by ECL (Millipore Corporation).

For immunoprecipitation experiments, amount of 500 μg of protein lysate from N2a cells transfected with appropriate plasmids including Htt and mHtt alone were precipitated with 1.5 μg of mouse anti-mHtt antibody. Amount of 30 μl protein A/G-Sepharose beads (American Qualex, CA, USA) were added and the immune complexes were pulled down overnight at 4 °C under rotation. Beads were washed extensively with cool lysis buffer and lysed by boiling in the presence of Laemmli buffer. Immunoprecipitates were subjected to SDS–PAGE.

2.4. Statistical analysis

Data were analyzed using Student's *t*-test. Data were expressed as mean ± S.E.M. Differences were deemed significant when $P < 0.05$. Image intensity was quantified using Nikon NIS-Elements-AR software.

3. Results

3.1. The hASC extracts alter pro- and anti-apoptotic proteins in vitro and in vivo

To investigate whether there is a protective role of the hASC extracts in HD *in vivo* and *in vitro*, we determined the cell death and the mitochondria related proteins using immunoblotting. We found that there were higher expression levels of p53, Bax and cleaved caspase-3 protein and lower expression level of Bcl2 protein in the region of striatum in R6/2 mice brain compared to the wild type mice. However, the injection of the hASC extracts to the R6/2 mice normalized these protein expressions, shown in Fig. 1A.

In addition, we also determined the above proteins in mHtt-transfected N2a cells and found that there were higher p53, Bax and cleaved caspase-3 protein expression levels in mHtt-transfected cells compared to the control (normal Htt was used as vector control here and below), but, with the treatment of the hASC extracts to mHtt-transfected cells significantly rescued these proteins to the normal levels except that of Bcl2 protein, which did not show a significant change in total lysates (Fig. 1B).

3.2. The hASC extracts prevent mHtt-induced cell toxicity and mitochondrial dysfunction

Since we observed the alterations of cell death related proteins by mHtt and the hASC extracts, we hypothesized that the hASC extracts may have a protective role in cell death and mitochondrial dysfunction. To test this hypothesis, we evaluated the cell viability using CCK8. Our result revealed that the cell viability was significantly reduced by the overexpression of mHtt compared to the control, but it was significantly rescued after the treatment of the hASC extracts (Fig. 2A). Then, in LDH release assay cells that transfected with mHtt were significantly increased LDH release compared to the control. However, this was decreased to the normal level with the treatment of the hASC extracts (Fig. 2B). We also detected the cell death using PI & Annexin V staining. The analysis graph showed the increase of apoptotic proportion due to the overexpression of mHtt, and the decrease by the treatment of the hASC extracts (Fig. 2C and S1A).

In order to determine the hASC extracts might have a protective role in mHtt-induced mitochondrial dysfunctio, MitoSoxRed was

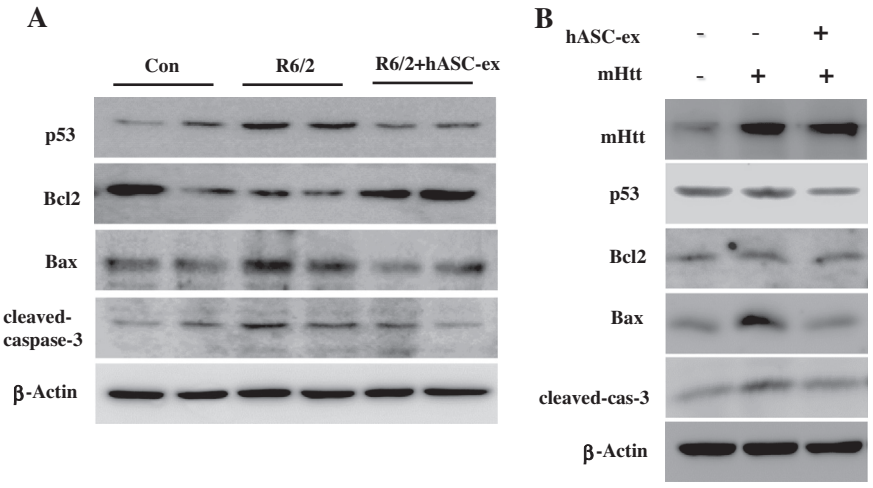

Fig. 1. The hASC extracts modulate pro- and anti-apoptotic proteins *in vitro* and *in vivo*. (A) The treatment with hASC-extracts reduced the levels of p53, Bax, and cleaved caspase-3, and increased the Bcl2 level. (B) N2a cells were transfected with Htt used as control or mHtt, and were treated with 100 μg/ml hASC extracts. At 48 h, the treatment with hASC-extracts reduced the levels of p53 and Bax. Representative experiments are shown.

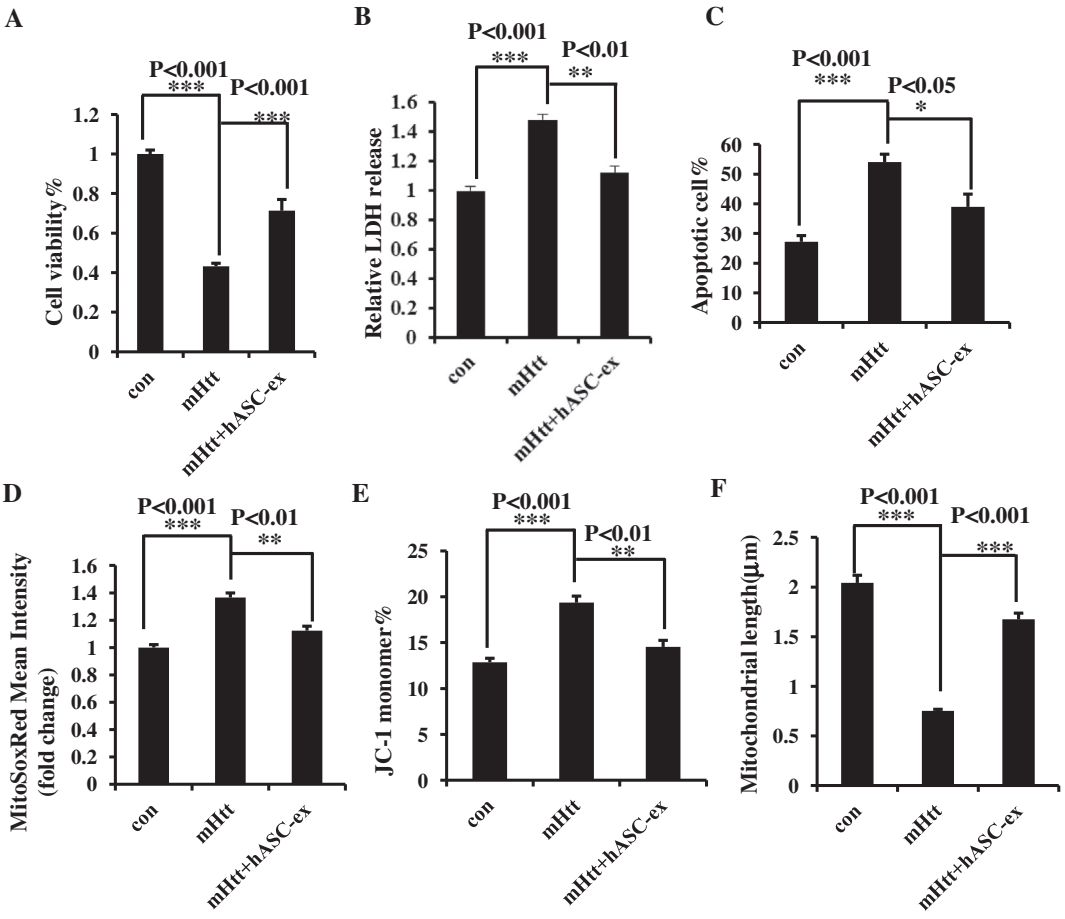

Fig. 2. The hASC extracts prevent mHtt-induced cell toxicity and mitochondrial dysfunction. N2a cells were transfected with Htt or mHtt, and were treated with 100 μg/ml of hASC extracts. (A) Transfection of mHtt reduced the cell viability and the treatment with hASC extracts attenuated the cell death induced mHtt (n = 3 each). (B) LDH assay showed that the treatment with hASC extracts reduced the LDH release induced by mHtt (n = 5 each). (C) The graph from flow cytometric analysis shows the proportion of apoptotic cells from experiments (n = 3 each). (D) mHtt transfection decreased MitoSoxRed staining and the treatment with hASC extracts increased the intensity (n = 3 each). (E) Data showed that mHtt transfection increased JC-1 monomer and the treatment with hASC extracts decreased it (n = 3 each). (F) mHtt transfection reduced mitochondrial length and treatment of hASC extracts normalized it (n = 3 each). At least 10 cells were selected in each sample and about 40 mitochondria were measured from each selected cell. Error bars represent S.E.M.

applied for mitochondrial ROS test. The graph from flow cytometry analysis showed the significant increase of MitoSoxRed due to the overexpression of mHtt and the significant decrease due to the treatment of the hASC extracts (Fig. 2D and S1B). Similarly, the mitochondrial membrane potential level was determined using JC-1 staining. JC-1 staining was analyzed by flow cytometry, which

revealed significant increase of the proportion of JC-1 monomers by the overexpression of mHtt, but it was significantly reduced again by the treatment of the hASC extracts (Fig. 2E and S1C). We then asked whether mHtt alters mitochondrial morphology. To test this possibility, N2a cells were co-transfected with EGFP-Htt or EGFP-mHtt and were analyzed by Mito-dsRed to assess the shape and length of mitochondria. Quantitation of mitochondrial length indeed demonstrated more than twofold shorter mitochondria in mHtt- versus Htt-transfected cells or mHtt-transfected cells with the hASC extracts treatment (Fig. 2F). Together, these results suggest that the hASC extracts have a protective role in mHtt-induced cell death and mitochondrial dysfunction.

3.3. The hASC extracts affect the correlations between mHtt and p53

It has been well established that mHtt affects p53 protein level *in vivo* and *in vitro*. This was further confirmed by our data that the hASC extracts down-regulate p53 protein, and we next questioned whether mHtt affects p53 stability and if so whether hASC extracts are also involved. Therefore, after overexpressing the mHt in N2a cells, these were subjected to cycloheximide (CHX) chase assay with various time points for 0.5, 1 and 2 h, respectively. Indeed, mHtt was significantly increased the half-life of endogenous p53, however, with the treatment of the hASC extracts, the half-life was markedly decreased, indicating that the hASC extracts reduce the mHtt-induced stabilization of p53 at the post-translational level (Fig. 3A). Moreover, it is well known that mHtt induces apoptosis and mitochondrial dysfunctions through interacting with p53 and modifying its post-translation [21]. To further determine the interaction between mHtt and p53 in N2a cells and whether the hASC extracts may affect the interaction between mHtt and p53, we applied immunoprecipitation assay. We found that mHtt interacts with p53 in N2a cells but this physical association is disrupted by the treatment of the hASC extracts (Fig. 3B). Interestingly, the treatment with the hASC extracts decreased the p53 protein expression levels in a time-dependent manner (Fig. 3C). To further investigate whether the p53 transcriptional activity was affected by mHtt and the hASC extracts, we performed quantitative PCR and found that not only p53 but also its target genes Bax and puma mRNA levels were up-regulated by mHtt, and were down-regulated by the treatment of the hASC extracts (Fig. 3D). Taken together, the hASC extracts reduce mHtt-induced p53 stability and distract interaction between p53 and mHtt through both transcriptional and post-translational mechanisms.

3.4. The hASC extracts have protective role in HD in vitro model cells

To test whether hASC extracts have a protective role in HD, we have established HD *in vitro* model cells using neural stem cells obtained from HD mice brain. In this cell, significant mHtt aggregation was detected (Supplementary Fig. S2). To determine whether the hASC extracts have a similar protective role in HD *in vitro* model cells, we determined the cell death and the mitochondria-related proteins using immunoblotting. Our result showed that there were lower expression levels of p53, Bax and cleaved caspase-3 protein and higher expression level of Bcl2 protein in HD *in vitro* cells with the treatment of the hASC extracts compared to the cells without the treatment of the hASC extracts (Fig. 4A). The Annexin V staining was performed for cell apoptosis assay and our result showed that there were more apoptotic cells in

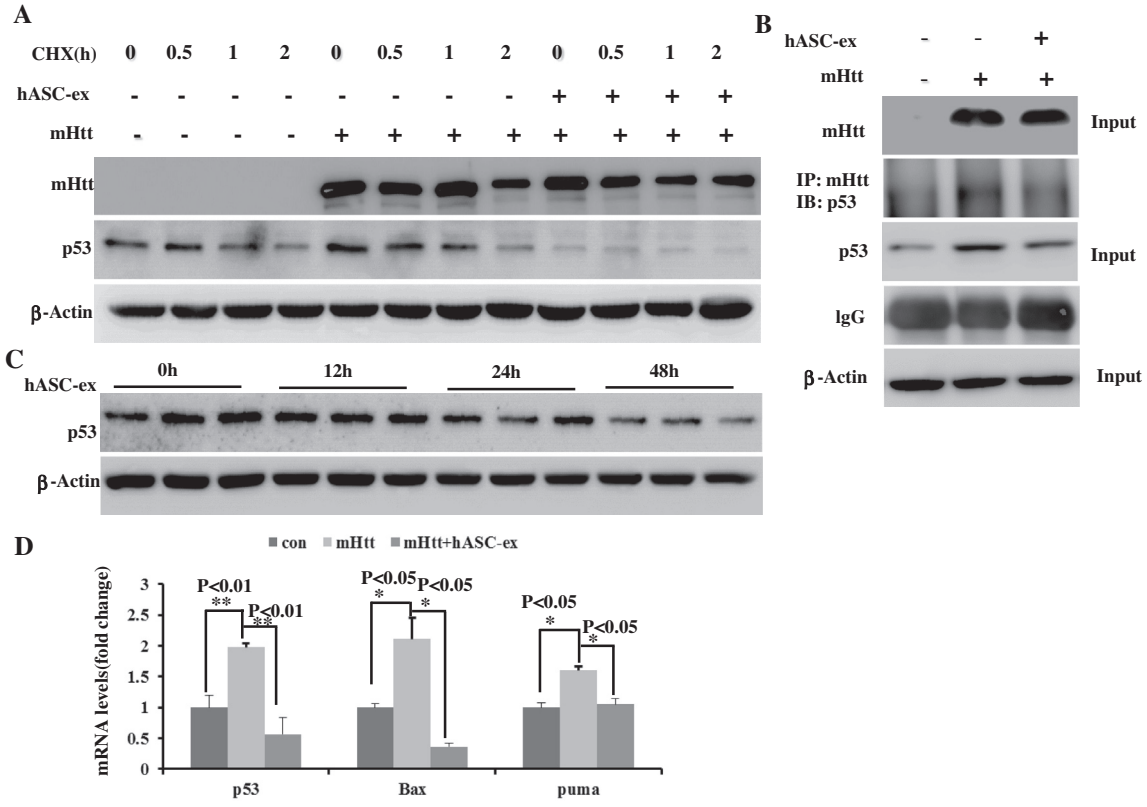

Fig. 3. The hASC extracts affect the correlations between mHtt and p53. N2a cells were transfected with Htt, mHtt, and mHtt with the treatment of 100 μg/ml hASC extracts. (A) After 48 h, cells were treated with cycloheximide (CHX) with the final concentration of 10 μg/ml for 0, 0.5, 1 and 2 h, respectively. (B) After 48 h, cell lysate was immunoprecipitated by anti-mHtt antibody and immunoblotted with anti-p53. (C) N2a cells were treated with 100 μg/ml hASC extracts for 0, 12, 24 and 48 h, respectively. (D) Cells were subjected in quantitative RT-PCR analysis and the p53, Bax and puma mRNA levels were normalized to GAPDH ($n = 4$ each). Error bars represent S.E.M.

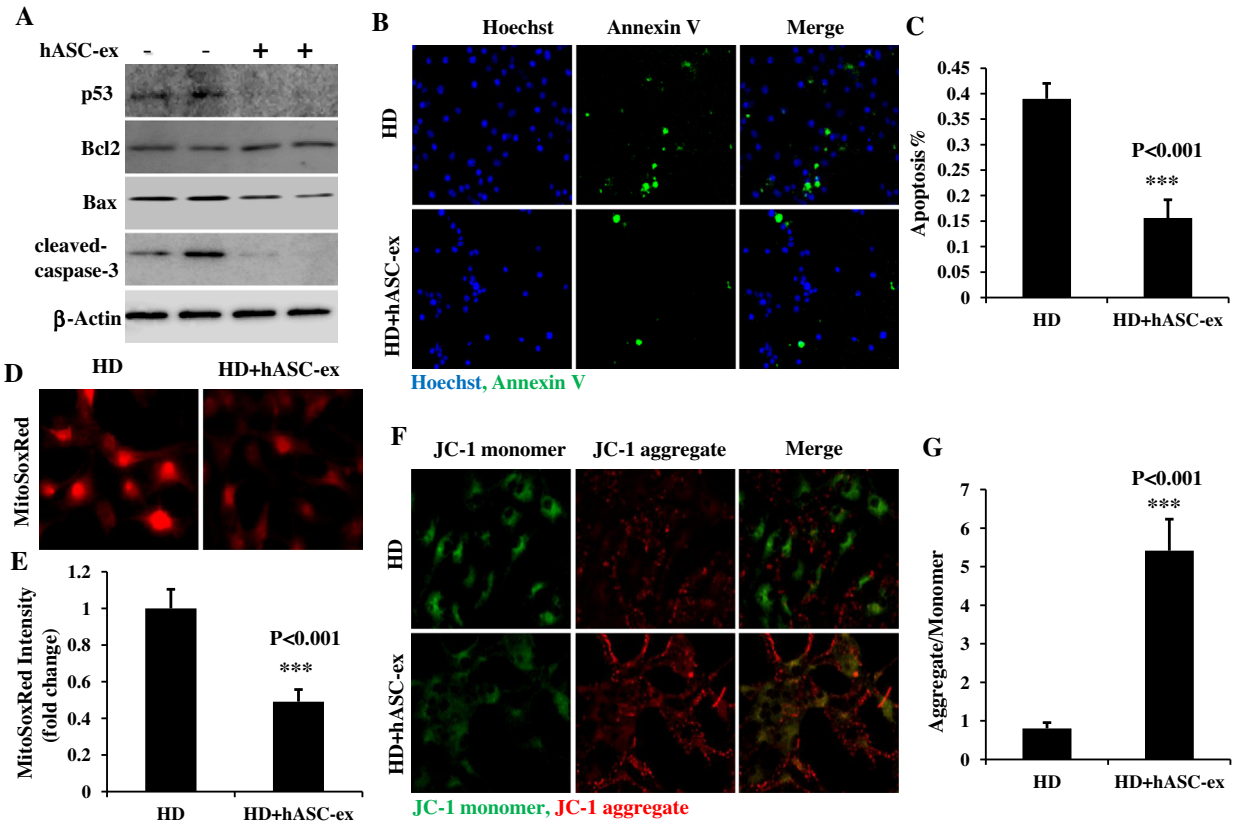

Fig. 4. The hASC extracts prevent mHtt-induced cell death and mitochondrial dysfunction in HD *in vitro* model cell. HD cells were treated with 20 μg/ml hASC extracts for 3 days. (A) The treatment with hASC-extracts reduced the levels of p53, Bax, and cleaved caspase-3, and increased the Bcl2 level. (B) Cells were subjected in Hoechst and Annexin V staining (FITC). (C) The apoptosis indicated by the rate of the numbers of Annexin V-positive cells to Hoechst-positive cells was reduced by the treatment with the hASC extracts in HD cells ($n = 4$ each). (D) Cells were subjected in MitoSoxRed staining. (E) MitoSoxRed intensity was reduced by the treatment with hASC extracts in HD cells ($n = 4$ each). (F) Cells were subjected in JC-1 staining. (G) The ratio of JC-1 aggregate to monomer was significantly increased by the treatment with hASC extracts ($n = 4$ each). Error bars represent S.E.M.

HD cells compared to the control, but the treatment with the hASC extracts significantly reduced cell apoptosis (Fig. 4B and C). Similarly, mitochondrial ROS levels were determined using MitoSoxRed staining. The result indicated that the treatment with the hASC extracts significantly reduced the red intensity compared to the none-treatment (Fig. 4D and E). JC-1 staining for mitochondrial membrane potential assay was also performed and our result revealed that the treatment with the hASC extracts significantly reduced the green intensity (JC-1 monomer) and induced red intensity (JC-1 aggregate) (Fig. 4F). The ratio of aggregate to monomer significantly increased by the treatment of the hASC extracts compared to the no treatment group (Fig. 4G), indicating a recovery of mitochondrial membrane potential by the treatment of the hASC extracts. Therefore, the hASC extracts prevent cell death and mitochondrial dysfunction in HD *in vitro* model cells.

4. Discussion

We have previously demonstrated that the injection of the hASC, the hASC cultured medium and the hASC extracts into mice striatum slowed down the progression of HD, including reduction of the mHtt aggregation, promoting cell survival [28,29]. In this study, we observed a series of novel observations, indicating the protective role of the hASC extracts in mitochondrial dysfunction and apoptosis. Specially, mHtt-induced increase of both p53 protein and mRNA, and its target mRNA levels were rescued by the treatment of the hASC extracts. We found that mHtt stabilizes p53 protein, but the treatment of the hASC extracts attenuates p53 stability resulting in the decrease of p53. Taken altogether, these findings above indicate a protective role of the hASC extracts through downregulation of p53 in mitochondrial and apoptotic mechanism in HD.

Previous study has shown that the hASC-conditioned medium prevented mHtt-induced cell death [29]. In this study, we found that the hASC extracts prevent mHtt-induced cell death including the recovery of mHtt-induced apoptotic and anti-apoptotic protein expression levels both *in vivo* and *in vitro*. These results indicate the paracrine effects of hASC, which promote cell survival. Recently, we reported that the hASC extracts slowed down the progression of HD through reducing aggregation of mHtt [30]. In this study, we found that the hASC extracts prevent mHtt-induced loss of mitochondrial membrane potential, increase of mitochondrial ROS level. Consistently, HD *in vitro* model cells showed that the hASC extracts not only reduced aggregation of mHtt in HD cells, but also prevented the cell death and mitochondrial dysfunction induced by aggregation of mHtt.

Consistent with previous observations, p53 was up-regulated both in R6/2 mice and mHtt-transfected cells. Interestingly, we found that the treatment of the hASC extracts have led to the significant reduction of p53 protein both *in vivo* and *in vitro*, indicating the essential role of p53 in mHtt-induced cell death and mitochondrial dysfunction. Therefore, we further investigated how the hASC extracts regulate p53 expression, and it was found that p53 can be stabilized by mHtt. However, the treatment with the hASC extracts significantly reduced the half-life of p53, which leads to the failure of p53 accumulation. Hence, we proposed that this is an important reason why the hASC extracts can prevent mHtt-induced cell death and mitochondrial dysfunction. Many

studies have demonstrated that p53 can transcriptionally and non-transcriptionally induce apoptosis via mitochondrial mechanism [31–33]. Together, with our result showed that p53 and its target which includes Bax and puma mRNA levels were induced by mHtt and reduced by treatment of the hASC extracts. This suggests that the hASC extracts prevent mHtt-induced cell death and mitochondrial dysfunction via both transcriptional (i.e. Bax and puma) and non-transcriptional mechanisms, and the non-transcriptional mechanism bears directly on mitochondria.

Previous researches reported that stem cells were employed for the therapy of regenerative medicine, in which stem cells mostly contribute to the positive results due to their bystander mechanism. Our recent studies have shown that both the hASC and the hASC extracts slowed down the progression of HD through neurotrophic factors produced by hASC, which are obviously consistent with this point [28,29]. However, the exact factors or molecules that produced by the hASC, which may have beneficial effects in mHtt-induced cell death and mitochondrial dysfunction are still unknown. We proposed that the protein factors including cytokines and growth factors could be the key molecules, and the therapeutic effect of the hASC extracts disappears by heat inactivation [34]. In the current study, heated hASC extracts failed to prevent mHtt-induced cell death, mitochondrial ROS and loss of mitochondrial membrane potential (Supplementary Fig. S3), which suggests that protein components can be important aspects. On the other hand, the remaining questions are the identities of the exact factors from the hASC extract. Further studies will be necessary to address whether the unknown key factors have a positive function on HD separately or cooperatively.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.129.

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