Exosomes as a potential messenger unit during heterochronic parabiosis for amelioration of Huntington’s disease

Mijung Lee a, Wooseok Im a,b,*, Manho Kim a,c,d,**

a Department of Neurology, Biomedical Research Institute, Seoul National University Hospital, Seoul, Republic of Korea
b Institute of Women’s Life Medical Science, Gangnam Severance Hospital, Seoul, Republic of Korea
c Protein Metabolism and Neuroscience Dementia Medical Research Center, College of Medicine, Seoul National University Hospital, Seoul, Republic of Korea
d Neuroscience and dementia Research Institute, Seoul National University College of Medicine, Seoul, Republic of Korea

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A B S T R A C T

Background: Huntington’s disease (HD) starts its pathology long before clinical manifestation, however, there is no therapy to cure it completely and only a few studies have been reported for delaying the progression of HD. Recently, it has been shown that heterochronic parabiosis can modulate the neurodegenerative diseases. Despite the importance of the transportation process of positive factors during heterochronic parabiosis, there were limited understandings because the transportation process is nanoscale, which makes it difficult to identify the messenger unit. We demonstrated that heterochronic parabiosis could modulate HD in R6/2 mice model, and identified the messenger unit for transferring positive factors in the young blood serum.

Methods: R6/2 mice were surgically connected with young wild-type mice (n = 13), old wild-type mice (n = 8), or R6/2 mice (n = 6) to examine the effect of heterochronic parabiosis. Parabionts composed of 5- to 6-week-old transgenic and wild-type mice were observed for 6 weeks in a single cage. The in vitro cellular model of HD cells were treated by the blood serum of the young or old mice, and by the exosomes isolated from thereof. The in vitro cellular model of HD were developed by differentiating neural stem cells cultured from SVZ of the brain.

Results: After the heterochronic parabiosis, the weight loss and survival of HD mice was improved. Also, mutant Huntingtin aggregation (EM48 p < 0.005), improvement of mitochondria dysfunction (PGC-1a p < 0.05, p-CREB/CREB p < 0.005), cell death (p53 p < 0.05, Bax p < 0.05, Cleaved-caspase3 p < 0.05), and cognition (DCX p < 0.05) showed a near complete restoration. In addition, treating in vitro cellular model of HD by the exosomes from young blood serum improved mutant Huntingtin aggregation (EM48 p < 0.05), mitochondria biogenesis (p-CREB/CREB p < 0.005), cell death (p53 p < 0.05, Bax p < 0.005, Cleaved-caspase3 p < 0.05, Bcl-2 p < 0.05), and cell proliferation (WST-1 p < 0.005).

Conclusions: We found that the overall pathology of HD could be improved by the shared blood circulation through heterochronic parabiosis, furthermore, we demonstrated that the exosomes could be messengers for transferring positive factors, showing the potential of exosomes from young blood for the amelioration of HD.

1. Introduction

Huntington’s disease (HD) is a fatal, progressive neurodegenerative disease with an autosomal dominant inheritance, characterized by chorea, involuntary movements of the limbs and cognitive impairment (Bates et al., 2015; Khakh and Sofroniew, 2014; Reddy, 2014). HD is caused by an expanded CAG repeat in the HD gene on chromosome 4 (Bates et al., 2015; Khakh and Sofroniew, 2014; Smith et al., 2014). This mutation produced an extended N-terminal polyglutamin stretch in the huntingtin (Htt) protein leading to intracellular accumulation and aggregation of mutant huntingtin (mHtt) (Khakh and Sofroniew, 2014). Accumulation of mHtt aggregates causes striatal cell death through transcriptional dysregulation, activation of intrinsic apoptosis pathways, mitochondrial dysfunction, and alters protein-protein interactions (Aronin and DiFiglia, 2014; Ross et al., 2014). To present date, there is no ultimate cure for the disease, however, several clinical trials such as

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** Correspondence to: W. Im, Institute of Women’s Life Medical Science, Gangnam Severance Hospital, 211 Unju-ro, Seoul 06273, Republic of Korea.
** Correspondence to: M. Kim, Department of Neurology, Seoul National University Hospital, 101 Duehak-ro, Seoul 03080, Republic of Korea.
E-mail addresses: IMWOOSEOK@yuhs.ac (W. Im), kimmanho@snu.ac.kr (M. Kim).

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aiming at silencing mHtt are currently ongoing (Hickey et al., 2005; Kieburtz et al., 2018).

Heterochronic parabiosis is a surgical union of 2 organisms of different ages that leading to the formation of a vascular anastomosis and a shared circulatory system between two mice (Middeldorp et al., 2016). Recently, it has been shown that heterochronic parabiosis can modulate the HD pathologies in the qZ175 mouse model (Rieux et al., 2020).

However, previous studies have only focused on which factors of ‘young blood’ have a positive effect on neurodegenerative disease, although a thorough understanding for the transportation of positive factors from the young blood to the old during heterochronic parabiosis is necessary in order to discover and identify positive factors (Katsimpari and Rubin, 2015; Middeldorp et al., 2016; Villeda et al., 2014; Wyss-Coray, 2016). There were only vague understandings like the diffusion of blood that how the positive factors are transported, because the transportation process is nanoscale, which makes it difficult to identify the messenger unit.

We have focused on exosomes which are the smallest membranous vesicles (40-100 nm) that has cargo ability for intercellular matter ex (Allison and Suzie, 2017; Kalani et al., 2014; Singh et al., 2013). Exosomes are generated via the inward budding of endosomes, to form multivesicular bodies (MVBs) that fuse with the membranes to release exosomes into the surrounding environment (Boukouris and Mathivanan, 2015; Cho et al., 2017). Exosomes, depending on their parental origin, contain a variety of proteins, lipids, non-coding RNAs, mRNA, and miRNA, collectively termed as “cargo” contains. Due to their cargo ability, exosomes represent a novel form of intracellular communication among cells without cell-to-cell direct contact. Exosomes are selectively taken up by the surrounding or distal cells and can reprogram the recipient cells due to their active cargo content (Keller et al., 2006; Ludwig and Giebel, 2012).

To demonstrate the effect of heterochronic parabiosis on HD and to identify the messenger unit during heterochronic parabiosis, we introduced the R6/2 mice model which is another representative mouse model of HD, in addition to the previously reported qZ175 mice model (Mangiarini et al., 1996). R6/2 mice carry expanded CAG sequences on an inserted fragment of the human Htt gene (Menalled and Brunner, 2014), and they show a rapidly-emerging progressive disease phenotype that parallels physiological and behavioral features of HD including profound motor dysfunction (Curtin et al., 2016; Menalled and Brunner, 2014; Menalled et al., 2009). qZ175 mice have CAG sequences that are carried in the native murine Htt gene (Menalled et al., 2012), thus showing expanded life-span and slower disease progression relative to R6/2 mice. Therefore, R6/2 mice can show the effect of parabiosis more dramatically than qZ175 mice. However, R6/2 mice has not reported for parabiosis because they are highly vulnerable to stress and at 3 to 4 months of age, they develop trimmings and cause death by muscle loss (Li et al., 2005; Menalled et al., 2014). By minimizing the damage of parabiosis surgery, we were able to make R6/2 mice survive over 12 weeks in the case of control group, and over 17 weeks in the case of heterochronic parabiosis group. We successively produced the R6/2 animal model for the heterochronic parabiosis for the first time to the best of our knowledge. By the heterochronic parabiosis of R6/2 mice, it was once again confirmed that young blood has positive factors that could improve the HD pathology. Prior to the direct administration of the exosomes to R6/2 mice to identify the exosomes as a messenger unit of positive factors, we treated the in vitro cellular model of HD by the exosomes extracted from the young serum. Although this is an indirect approach, it is free from many environmental variables that must be considered in direct administration of exosomes, such as the storage condition of exosome which affects the activity of exosome significantly (Lee et al., 2016a). We demonstrated that the exosome has more than equivalent effect compared to serum, which indicates the potential of exosomes as a messenger unit for the positive factor of young serum during the heterochronic parabiosis. It could lead to a development of potential small molecule in exosome interventions, and a group of soluble factors in exosome targeting several pathways may help therapeuic benefits for HD.

2. Methods

2.1. Experimental model of HD

All experimental animal procedures were performed for the Institutional Animal Care and Use Committee (IACUC, Approval number: 16-0043-C2A1) of Seoul National University Hospital, which was accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Transgenic R6/2 (B6CBA-Tg(HDexon1)62Gpb/1 J, 160 CAGs) and their WT littermates used in this study were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice used in this study were qZ175 KI heterozygous (CHDI-8100303) or WT littermates on a C57BL/6 J background strain obtained from the CHDI colony at Jackson Laboratories (Bar Harbor, ME) or bred in-house at PsychoGenics, Inc. (Tarrytown, NY). qZ175 mice, originated from the CAG 140 mice (from germine CAG expansion) were heterozygous and wild-type mice were generated by crossing heterozygous qZ175 mice on a C57BL/6 J background. The R6/2 transgenic mice model expresses exon 1 of a human mHtt and it is the most widely used animal model for studying HD. These mice were obtained by crossing ovariann transplant hemizygote females with B6CBAF1/J males. R6/2 mice and qZ175 were bred at the Seoul National University Hospital under specific pathogen-free conditions. Mice homozygous offspring of heterozygous matings were identified by polymerase chain reaction (PCR) typing of tail-tip genomic DNA. Mice were housed in groups by a 12 h light / 12 h dark cycle with ad libitum access to food and water. Body weights were recorded weekly.

2.2. Procedures for parabiosis

Parabiosis were subjected to parabiotic surgery using methods adapted from JOVE, Bunster and Meyer. Mice were anesthetized with Zoletil/Rompun intraperitoneal (i.p.) and all surgical procedures were performed under general anesthesia. Operative sides were shaved and sterilized. Lateral skin was opened from hip to shoulder and freed of attached tissue. Opposing muscle and perineum was sutured with 4-0 chromic gut (Roboz, Rockville, MD), and corresponding skin was joined with 9-mm wound clips (Fisher Scientific, Houston, TX). Transgenic R6/2 (B6CBA-Tg(HDexon1)62Gpb/1 J, 160 CAGs) disease phenotype appears at 8 weeks of age. Pairs of 5- to 6-week-old Transgenic HD and WT, sex-matched R6/2 mice were housed together in a single cage for 6 weeks. In the case of qZ175 (C57BL/6 J B6.129S1- Htt < tm1Mic/– 190JChdi) mice, WT(Young) were 8- to 10-week-old mice and WT(Old) were 28- to 30-week-old mice, which are sex- matched and housed together in a single cage for 16 weeks. Mice were housed under standard conditions (12 h light cycle from 08:00 h to 20:00 h) with ad libitum access to food and water. 100 μl of blood was collected from eyes to document joint circulation by polymerase chain reaction (PCR) of genomic DNA. At the time of killing, parabionts were anesthetized with Zoletil/Rompun intraperitoneal (i.p.) and separated through transection at the anastomosis site. Before separation, cardiac puncture was performed by obtaining 0.5-0.8 ml of blood from hearts.

2.3. BrdU administration and immunohistochemistry

To demonstrate a connected circulation between parabionts, BrdU was injected into one mice. The formation of shared blood circulation between the parabionts was tested by the injection of BrdU (150 mg/kg, Sigma-Aldrich, St. Louis, MO, USA, Cat.no. B5002) to the intraperitoneal administration of one of the parabionts after 2 weeks from surgery, and the pair was killed after 4 weeks. Mice were anesthetized and perfused through the heart with 10 ml of cold saline and 4% paraformaldehyde in
0.1 M PBS at 12 weeks of age. Brains were removed from the skull, cryoprotected in 30% sucrose at 4 °C, and sectioned by 20 μm. Free-floating sections were washed and followed by incubation in 1.5 M hydrogen chloride at 37 °C for 30 min. The sections were washed in PBS with three times and blocked with normal goat serum, then stained with the BrdU antibody (1:300, Abcam, Cambridge, MA, USA, Cat.no. ab6326). On the following day, the sections were washed in PBS with three times and incubated with Cy3 conjugated anti-rat IgG (1:100; Jackson immune Research Laboratories, Cat.no. 112–165-167) for 2 h. BrdU (red) or DAPI (blue)-stained cells were identified using an inverted microscope (BX61, Olympus Corporation, Tokyo, Japan).

2.7. Analysis of mHtt aggregation in cells

mHtt aggregation was quantified by fluorescent immunocytochemistry. Cells were stained with Em48 antibody (1:400, Millipore, Billerica, MA, USA, Cat.no. MAP5374) after fixing with 4% paraformaldehyde. The cells were then counterstained with DAPI (1:300, Sigma, Deisenhofen, Germany, Cat.no. 28718–90-3). For the fluorescence staining analysis, we performed three independent experiments and over 500 cells are counted in each group (Lee et al., 2016c). Em48 (red) or DAPI (blue)-stained cells were counted using an inverted microscope (BX61, Olympus Corporation, Tokyo, Japan).

2.8. Tissue preparation and fluorescent immunohistochemistry

For immunohistochemistry, mice were anesthetized and perfused through the heart with 10 ml of cold saline and 4% paraformaldehyde in 0.1 M PBS at 12 weeks of age. Brains were removed from the skull, cryoprotected in 30% sucrose at 4 °C, and sectioned 20 μm. Free-floating sections were washed and blocked with normal goat serum, then stained with the Em48 antibody (1:300, Millipore, Billerica, MA, USA, Cat.no. MAP5374). On the following day, the sections were washed in PBS with three times and incubated with Cy3 conjugated anti-mouse IgG (1:100; Jackson immune Research Laboratories, Cat.no. 115–175-166) for 2 h. EM48 (red) or DAPI (blue)-stained cells were identified using an upright microscope (Ni-E, Nikon Corporation, Tokyo, Japan) (Hoffman et al., 2015; Im et al., 2013).

2.9. DCX and BrdU immunohistochemistry

Free-floating sections were washed and followed by incubation in 1.5 M hydrogen chloride at 37 °C for 30 min. After the sections were washed in PBS with three times and blocked with normal goat serum, then stained with the DCX antibody (1:300; abcam, Cambridge, MA, USA, Cat.no. ab18723) and BrdU antibody (1:300, Abcam, Cambridge, MA, USA, Cat.no. ab6326). On the following day, the sections were washed in PBS with three times and incubated with FITC conjugated anti-rabbit IgG and Cy3 conjugated anti-rat IgG (1:100; Jackson immune Research Laboratories, Cat.no. 711–097-003, Cat.no. 112–165-167) for 2 h. DCX (fitc) and BrdU (red) or DAPI (blue)-stained cells were identified by using an inverted microscope (BX61, Olympus Corporation, Tokyo, Japan).

2.10. Protein extraction and western blot analysis

Brains of R6/2 mice and zQ175 were isolated, immediately frozen on liquid nitrogen, and stored at –80 °C until protein extraction. Cultured HD cells were washed and harvested in PBS (phosphate buffered saline, WelGene, Daegu, Korea, Cat.no. LB 004–01) using a cell scraper. Protein extracts were prepared using RIPA buffer (Radio immunoprecipitation assay buffer, Thermo-Scientific, Waltham, MA, USA, Cat.no. 89900) containing freshly added protease inhibitor and phosphatase inhibitor (Roche, NJ, USA, Cat.no. 4693116001). The protein content was determined using a BCA (Bicinchoninic acid assay) protein assay kit (Pierce, Rockford, IL, USA, Cat.no. 23225, 23,227). Forty micrograms of protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 4–15% Novex NuPage Bis-Tris gel, Invitrogen, Mount Waverley, Australia) and 10% or 15% SDS-PAGE and transferred onto polyvinylidene fluoride membrane (PVDF, Millipore, Bedford, MA, USA, Cat.no. IPVH00005). Blocking with 5% non-fat dried milk dissolved in 1× TBST (Tris-buffered saline with 0.1% v/v Tween-20) for 1 h at room temperature. Blots were then incubated at 4 °C overnight with primary antibodies diluted as recommended in the manufacturer’s instructions. The following primary antibodies were used: anti-PGC1α (1:200; Santa Cruz, CA, USA, Cat.no. sc-517,380), p-CREB (1:1000; Cell signaling, Beverly, MA, USA, Cat.no. 3895S), p-ERK (1:1000; Cell signaling, Beverly, MA, USA, Cat.no. 4693116001). The following primary antibodies were used: anti-PGC1α (1:200; Santa Cruz, CA, USA, Cat.no. sc-517,380), p-CREB (1:1000; Cell signaling, Beverly, MA, USA, Cat.no. 4693116001).
USA, Cat.no. ab18723), and anti-Cat.no. MAP5374), DCX antibody (1:1000; abcam, Cambridge, MA, peroxidase-conjugated secondary anti-mouse or anti-rabbit antibodies Santa Cruz, CA, USA, Cat.no. sc-7382), Caspase3 (1:1000; Cell signaling, anti-Bax (1:200; Santa Cruz, CA, USA, Cat.no. sc-7480), BCL-2 (1:200; M. Lee et al.

to manufacturer
WST-1 (Roche, Mannheim, Germany, Cat.no. CELLPRO-RO) according plates and incubated with young and old serum-exo 200 WelGene, Daegu, Korea) using a triple (Invitrogen, Carlsbad, CA, USA, Cat.no. 556463) staining was used. Neuronal cytometry using annexin V-FITC (BD Biosciences Pharmingen, San Jose, CA, Cat.no. 556420) and propidium iodide (PI) (BD Biosciences Pharmingen, Westwood, MA, Cat.no. 556463) staining was used. Neuronal stem cells were washed and harvested in PBS (phosphate buffered saline, WelGene, Daegu, Korea) using a triple (Invitrogen, Carlsbad, CA, USA, Cat.no. 23604013). Cells were counted and 1 × 106 cells were sus- pended in 1 ml cold binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl2). Cells were aliquoted into 1.5 ml tube at 1 × 105 cells per tube, and were incubated with 10 μl of annexin V-FITC at room temperature for 30 min and 2 μg/ml of PI at room temperature for 10 min. After incubation, 400 μl of binding buffer was added and flow cytometric analysis was performed (FACS Calibur, BD Bioscience, CA, USA). FITC and PI fluorescences were passed through 520 and 630 nm bandpass filters, respectively, and the data were analyzed using Flowing Software (www.flowingsoftware.com).

2.12. Flow cytometry

To analyze the apoptosis population of neuronal stem cells, flow cytometry using annexin V-FITC (BD Biosciences Pharmingen, San Jose, CA, Cat.no.CELLPRO-RO) according to manufacturer’s instruction. Briefly, cells were seeded in 96-well plates and incubated with young and old serum-exo 200 μg/ml for 72 h. After 72 h, WST-1 reagent was added to each well, and cells were incubated at 37 °C and 5% CO2 for 2 h. Absorbance was measured using a plate reader at 450 nm (reference 650 nm) and the result shown represent the averages of four independent experiments.

2.11. Cell survival assay

Cell survival rate was measured by a colorimetric assay using the WST-1 (Roche, Mannheim, Germany, Cat.no. CELLPRO-RO) according to manufacturer’s instruction. Briefly, cells were seeded in 96-well plates and incubated with young and old serum-exo 200 μg/ml for 72 h. After 72 h, WST-1 reagent was added to each well, and cells were incubated at 37 °C and 5% CO2 for 2 h. Absorbance was measured using a plate reader at 450 nm (reference 650 nm) and the result shown represent the averages of four independent experiments.

3. Results

3.1. Construction of parabiosis animal model for HD and modulation of HD pathology

The R6/2 mice were surgically joined either with another R6/2 mice or with their corresponding wild-type littermate (Fig. 1A). WT-WT is composed of 5- to 6-week-old wild type mice, HD-HD is composed of 5- to 6-week-old transgenic mice, WT(Young)-HD is composed of 5- to 6-week-old mice of wild type and transgenic, and WT(Old)-HD is composed of 28- to 30-week-old wild type mice and 5- to 6-week-old transgenic mice. The composition of females and males is equal in all experimental groups. To successively perform the parabiosis of R6/2 mice, minimizing the damage and the stress of parabiosis were essential due to the high vulnerability of R6/2 mice. The binding site and method had a critical influence on the survival of R6/2 parabionts, and we were able to obtain stable R6/2 parabionts for the first time to the best of our knowledge. Suturing the abdomen and the back of the mice was per- formed through clamping and tying their forelimbs with threads. More details about the methods of parabiosis are demonstrated in the sup- plement (Figs. S1 and S2, Videos S1 and S2). The mice were observed periodically as described in materials and methods. No obvious signs of stress were noted during observations after 6 weeks from the surgery, and no parabionts displayed parabiotic syndrome among the whole experimental groups (n = 32).

To demonstrate a connected circulation between parabionts, BrdU was injected into Het-WT. Brain from the injected mice and the attached mice in all groups of parabions showed similar BrdU signals, indicating a joined circulatory system (Fig. 1B). The shared circulation was also demonstrated by examining the genomic DNA of blood cells in paired mice at four weeks after parabiotic surgery. By comparing the genotype of DNA from the Het-WT and the Iso-HD, the two bands that can be seen in Iso-HD were also observed in Het-WT (Fig. 1C).

To evaluate the functional consequences of heterochronic parabiosis, we measured weight loss and lifespan. Phenotypes were examined from the parabions paired for six weeks. In order to analyze the effect of age of wild type during parabiosis, survival of WT(Young)-HD and WT(Old)-HD was compared (Fig. 1D). The survival curves for the other experimental groups are demonstrated in the supplement (Fig. S3). WT-WT and WT(Young)-HD showed no death during 6 weeks of observation after the parabiosis surgery, while WT(Old)-HD and HD-HD showed a survival rate less than 50% (P < 0.05). It also reduced tremor and improved the movement (Video S3 – S5). Also, WT(Young)-HD showed delayed progression of weight loss at 12 weeks old (p < 0.01) while HD-HD showed gradual weight loss from 10 to 12 weeks of age (Fig. 1E), while R6/2 mice (with CAG repeats of between 154 and 200) die typically at around 3–4 months of age. In addition, WT-WT serve as controls to ensure that the surgical procedures did not cause ectopic mineralization. Also, to extend the universality of parabiosis and to reproduce the results reported by Rieux et al., the improvement on the behavior (Video S6 – S8) and the weight loss (Fig. S4) were also shown in zQ175 mice.

The R6/2 mice showed mHtt aggregation in striatum and cortex during disease progression. To examine histological changes of the brain, mHtt aggregation were evaluated at 12 weeks of age. To evaluate the effect of parabiosis on mHtt aggregation, brain was sectioned and sliced tissues were stained with an EM48 antibody which detects ag- gregation of mHtt (Fig. 1F). Quantification of the image demonstrated that Het-HD shows reduced mHtt aggregation in the striatum and cortex compared to Iso-HD (Fig. S5). Also, we extracted proteins from R6/2 mice brain and mHtt aggregation were measured by western blot analysis (Fig. 1G), and WT(Young)-HD showed reduced mHtt aggrega- tion in the brain.

3.2. Modulation of pathological phenotypes of HD by parabiosis

Dysfunction of CREB-PGC-1a pathway has been regarded as the key molecule for HD progression. To examine effects of heterochronic parabiosis on this pathway, western blot analysis was performed for parabionts after six weeks from the surgery. Compared to Iso-HD, Het- HD showed increased expression of p-CREB and PGC-1A (p < 0.05 vs. R6/2 control), which are from the striatum of mice (Fig. 2A). To investigate whether heterochronic parabiosis protects against apoptosis, we examined the levels of apoptosis-related proteins by western blotting. p53, Bax, and cleaved caspase-3 levels were lower in the Het-HD than Iso-HD (Fig. 2B).

IHC and western blot were performed to show the possibility of improving the neurogenesis (Fig. 2C, D). It was found that DCX and BrdU, which are representative signals for neurogenesis, were increased
Fig. 1. Construction of animal model of heterochronic parabiosis for HD and modulation of HD pathology. A, Schematics of parabiotic paring. WT-WT indicates the isochronic paring of wild-types \((n = 5)\), HD-HD indicates the isochronic paring of HD mouse \((n = 6)\), and each parabions are indicated as Iso-WT and Iso-HD, respectively. WT (Young)-HD indicates the heterochronic paring of young wild-type and HD mouse \((n = 6)\), of which parabions are indicated as Het-WT and Het-HD, respectively, and WT (Old)-HD indicates the heterochronic paring of old wild-type and HD mouse \((n = 8)\). B, Analysis of BrdU signal for WT(Young)-HD with the injection of BrdU to the abdomen of Het-WT. C, Comparison of the genotype of DNA from the Het-WT with that of the WT and the HD mice. D, Effect of the age of wild-type on survival of HD mice paired with the wild-type. E, Analysis of body weight of parabions. Inset is relative comparison of the body weight of WT(Young)-HD and HD-HD. F, mHtt aggregation in striatum and cortex for Iso-HD and Het-HD (Red: EM48). G, Evaluation of mHtt aggregation from the proteins in the brain of parabions by western blot.
in Het-HD, and the same result was confirmed by western blot. Also, BDNF expression is evaluated to support the neurogenesis by parabiosis, showing the increased BDNF expression in Het-HD compared to Iso-HD (Fig. S6). It shows the possibility that young wild-type blood could be effective in improving neurogenesis. In addition, as hippocampal neurogenesis is increased by parabiosis, reduction of cell death in SVZ could be observed (Fig. S7). Also in the case of zQ175 mice, pathological phenotypes of HD were modulated by parabiosis (Figs. S8 and S9).

3.3. Amelioration of mHtt aggregation by exosome treatment

To construct the in vitro cellular model for HD, neural stem cells were
separated from the SVZ which was isolated from the brain of the R6/2 mice. Neural stem cells showed a spherical shape after the separation, and the expression of mHtt aggregation protein was not observed. The mHtt aggregation protein can be seen from 5 to 7 days after the differentiation of the cell, which is expressed as red in the cell nucleus (Fig. S10). To harvest the exosomes from the blood serum, there are two representative methods for exosome isolation: serial ultracentrifugation and Exo-Quick reagent. Western bolt analyses were performed to determine the expression of exosome-specific markers which are CD9, CD63 (tetraspanin proteins) and HSP70. The isolated products expressed all the markers, thus confirming the presence of exosomes (Fig. S11).

As shown earlier in Fig. 1D, paring the HD mice with the young wild-type increased survival, whereas paring with old wild-type did not. To investigate the underlying mechanism of age-dependent effect of the parabiosis with WT, the exosomal protein and RNA concentration in young blood and old blood were analyzed. Young blood indicates the blood from WT(Young) mice which are 5- to 6-week-old, and Old blood indicates the blood from WT(Old) mice which are 28- to 30-week-old.

**Fig. 3.** Amelioration of mHtt aggregation by exosome treatment. In vitro cellular model for HD was developed by culturing neural stem cells (NSCs) from the SVZ of R6/2 mice. A, Comparison of the amount of exosomes, exosomal proteins, and RNA levels of blood serum from young and old wild-type. Young serum-exosome and Old serum-exosome indicates the exosomes derived from the blood serum from the young mice and old mice, respectively. B, C, Immunohistochemistry and western blot of cells from demonstrate mHtt aggregates in cells. CTL indicates the cells from the wild-type mice, and HD indicates the cells from the HD mice. HD + Young and HD + Young-exo indicates the HD cells treated by blood serum and exosomes derived from the young wild-type mice, respectively. HD + Old and HD + Old-exo indicates the HD cells treated by blood serum and exosomes derived from the old wild-type mice, respectively. All data are produced from three separate experiments.
Proteins and total RNA were extracted by protein extraction buffer and total RNA isolation kit, respectively, after isolating exosomes. Higher amount of exosomes, exosomal protein, and RNA levels were observed in young blood (Fig. 3A).

To investigate whether young serum-exosomes has a protective role in HD, we treated an in vitro cellular model for HD, which showed mHtt aggregations in nucleus after day 7 of induction, with young serum-exosomes. Young serum-exosomes (200 μg/ml) was applied to the cells for 3 days after inducing mHtt aggregation. At day 7, the control and young serum-exosomes groups were fixed with 4% formaldehyde and processed for immunofluorescence for analysis (Fig. 4).

Fig. 4. Modulation of molecular pathology of HD by exosome treatment. A, Improvement of mitochondrial dysfunction and modulation cell death, respectively. B, Analysis for cell death and cell proliferation. C, Evaluation of cell viability with exosome treatment. All data are produced from three separate experiments.
paraformaldehyde and stained with the Em48 antibody to detect mHtt aggregates, with DAPI as a counter stain (Fig. 3B). We counted DAPI(+) and Em48(+) cells in the HD and HD + Young-exo. The ratios of double-positive cells to DAPI(+) cells were 20.8 ± 2.3% for HD and 12.1 ± 0.3% for HD + Young-exo. To confirm the reduction of mHtt aggregates, aggregates were also quantified by western blot (Fig. 3C). In HD + Young-exo, levels of mHtt aggregates in cells were significantly decreased. To further confirm that the exosome is responsible for the amelioration of HD, We treated the in vitro model cell for HD with the exosome-depleted blood serum (Fig. S12). The exosome-depleted serum had no effect on improving the mHtt aggregation, which indicates that the positive factors that can modulate the HD are transported through exosomes.

3.4. Modulation of molecular pathology of HD by exosome treatment

To examine the effects of young serum-exosomes on the p-CREB-PGC1α pathway, cells were treated with control medium or young serum-exosomes for 3 days after 2 days of differentiation. Treatment with young serum-exosomes promoted expression of p-CREB and PGC1α (Fig. 4A). To examine the protection against apoptosis, the levels of apoptosis-related proteins were evaluated by western blotting. p53, Bax, and cleaved caspase-3 levels were lower in the HD + Young-exo than in the HD (Fig. 4B). To definitely demonstrate the effect of exosomes, we evaluated the effect of exosome-depleted blood serum on molecular pathology of HD (Fig. S13). The exosome-depleted serum had no effect on improving the mitochondrial dysfunction and cell death, which support that exosomes are the messenger unit for transferring positive factors. Also, to confirm the anti-apoptotic effect of young serum-exosomes, neural stem cells were differentiated and treated by the young serum-exosomes for 3 days, and flow cytometry analysis was performed using annexin-V and propidium iodide (Fig. 4C). Cell population was analyzed as viable/early-apoptotic/late-apoptotic/necrosis, and this calculation was conducted using the annexin-V and propidium iodide positive cell count. More necrotic population and less viable population were shown in HD, however, HD + Young-exo showed significant reduction of the apoptotic/necrotic cell population and an increase in the viable cell population. Taken together, young serum-exo treatment resulted in more cell survival and less cell death, accompanied by a reduction of mHtt aggregation protein, and apoptotic signaling. To examine the cell survival effects, we investigated the WST-1. The result showed that treatment of young serum-exosomes significantly increases cell survival (Fig. 4D). On the contrary, old serum-exosomes did not improve mitochondrial activation and cell survival (Fig. S14).

4. Discussion

In this study, we demonstrated that heterochronic parabiosis of HD mice with the young wild-type could modulate the body weight loss, mHtt aggregation, mitochondrial dysfunction, cell death, and cognitive impairment (Khakh and Sofroniew, 2014; Menalled et al., 2014). These are the representative pathologies of HD, thus extending the survival of HD mice. It is worth noting that the R6/2 mice die typically at around 4 months of age accompanied by trimming and muscle loss (Li et al., 2005; Menalled et al., 2014) and highly vulnerable to stress. Therefore minimizing the damage of parabiosis surgery were indispensable to risk procedure already offered as a therapy with limited complications (Colao et al., 2018; Kamerkar et al., 2017), and a group of soluble factors in exosome targeting several pathways may help therapeutic benefits. Therefore, it is feasible to test the efficacy of young serum exosome in patients with HD and possibly other forms of mitochondrial dysfunction and neurodegeneration.

5. Conclusion

In summary, our results show that the overall pathology of HD is improved by the shared blood circulation through parabiosis. Furthermore, by the in vitro cellular model for HD, we demonstrated that the exosome might have the potential for messenger units that transfer positive factors inside the blood. Thus, the therapeutic potential of the exosomes from young serum has been confirmed through our study, and young serum-exo can be a valuable tool for treating HD.

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Ethics approval

All experimental animal procedures performed were approved by the Institutional Animal Care and Use Committee (IACUC, Approval number: 16-0043-C2A1) of Seoul National University Hospital, which was accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

ML designed and conceptualized study, analyzed the data, and drafted the manuscript for intellectual content. WI and MK interpreted the data, and revised the manuscript for intellectual content. All authors read and approved the final manuscript.
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