

Identifying Exosomes as a Messenger Unit During Heterochronic Parabiosis for Amelioration of Huntington's Disease

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Abstract

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. We demonstrated the blood sharing effect by heterochronic parabiosis in HD and explored the underlying mechanism for transferring positive factors in the young blood serum. A shared blood circulation by heterochronic parabiosis has improved behavioral performance and HD pathology through mediation of mitochondria dysfunction and cell death. Furthermore, the messenger unit for the effective components in young blood is identified for the first time to the best of our knowledge.

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 200 µg/ml 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, and 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<0.5) showed a near complete restoration. In addition, treatment of exosomes from young blood serum to the *in vitro* cellular model of HD 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 is improved by the shared blood circulation through heterochronic parabiosis, furthermore, we demonstrated that the exosomes are messengers for transferring positive factors, showing the potential of exosomes from young blood for the amelioration of HD.

Background

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.[1-3] HD is caused by an expanded CAG repeat in the HD gene on chromosome 4.[1, 3, 4] This mutation produced an extended N-terminal polyglutamin stretch in the huntingtin (Htt) protein leading to intracellular accumulation and aggregation of mutant huntingtin (mHtt).[1] Accumulation of mHtt aggregates causes striatal cell death through transcriptional dysregulation, activation of intrinsic apoptosis pathways, mitochondrial dysfunction, and alters protein-protein interactions.[5, 6] To present date, there is no promising cure or beneficial treatment for the disease and developing therapies are in high priority.[7, 8]

Several studies in other neurodegenerative diseases like AD have shown that exposure to a young blood circulation through heterochronic parabiosis, which is a surgical union of 2 organisms of different phenotype that leading to the formation of a vascular anastomosis and a shared circulatory system between two mice,[9] reverses cognitive deficits that is observed with normal aging. Although the idea of adopting heterochronic parabiosis to the HD seems pretty straightforward, however, it has not been reported so far in R6/2 mice. It is because the mouse model for HD, are highly vulnerable to stress and at 3 to 4 months of age, they develop tremoring and cause death by muscle loss.[10, 11]

Also, a thorough understanding for the transportation mechanism of positive factors from the young blood to the old during heterochronic parabiosis is necessary in order to adopt the heterochronic parabiosis as the cure for HD. However, previous studies have only focused on which factors of 'young blood' have a positive effect on neurodegenerative disease.[9, 12-14] There was 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 (40-100nm) that has cargo ability for intercellular matter exchange.[15-17] 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.[18, 19] 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.[20, 21]

By minimizing the damage of parabiosis surgery, we were able to make R6/2 mice survive over 12 month in the case of control group, and over 17 month in the case of heterochronic parabiosis group, successively producing the HD R6/2 animal model for the heterochronic parabiosis for the first time. By the heterochronic parabiosis of R6/2 mice, we found that young blood has positive factors in improving the HD pathology, and the identical results could be obtained by processing the exosomes extracted from the young serum into *in vitro* model. We discovered that the exosomes serve 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 therapeutic benefits for HD.

Methods

Experimental model of HD

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. Transgenic R6/2 (B6CBA-Tg(HDexon1)62Gpb/1J, 111 CAGs) and their WT littermates used

in this study were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice used in this study were zQ175 KI heterozygous (CHDI-81003003) or WT littermates on a C57BL/6J background strain obtained from the CHDI colony at Jackson Laboratories (Bar Harbor, ME) or bred in-house at PsychoGenics, Inc. (Tarrytown, NY). ZQ175 mice, originating from the CAG 140 mice (from germline CAG expansion) were heterozygous and wild-type mice were generated by crossing heterozygous ZQ175 mice on a C57BL/6J background. The R6/2 transgenic mice model expresses exon 1 of a human mHtt and is the most widely used animal model for studying HD. These mice were obtained by crossing ovarian transplant hemizygote females with B6CBAF1/J males. R6/2 Mice and ZQ175 were bred at the Seoul National University Hospital under specific pathogen-free conditions and mice homozygous offspring of heterozygous matings were identified by polymerase chain reaction (PCR) typing of tail-tip genomic DNA. The mice were housed in groups with ad libitum access to food and water and a 12 hours light / 12 hours dark cycle. Mouse body weight was recorded weekly.

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/1J}, 111 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. Pairs of 8- to 10-week-old and 28- to 30-week-old, sex-matched ZQ175 (C57BL/6J B6.129S1-Htt^{tm1Mfc}/190JChdi) mice were housed together in a single cage for 16 weeks.

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 parabiotic animals was tested by injection of BrdU (150mg/kg, Sigma-Aldrich) to the intraperitoneal administration of one of the parabionts after 2 weeks post-surgically and the pair was killed after 4 weeks. Mice were anesthetized and perfused through the heart with 10ml 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 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 BrdU antibody (1:300, Abcam, Cambridge, MA, USA). 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) for 2 hours. BrdU (red) or DAPI (blue)-stained cells were identified using an inverted microscope (BX61, Olympus Corporation, Tokyo, Japan).

Weight measurement

Mice from the R6/2×TG2 KO line and from the zQ175×TG2 KO line were weighed every week for 6 weeks and for 16 weeks, respectively.

Preparation of *in vitro* cellular model for HD

A colony was maintained by breeding ovarian transplant R6/2 females (B6CBATg(HDexon1)62Gpb/1J) obtained from the Jackson Laboratories with B6CBAF1/J males, based on a C57BL/6 background. F1 offspring of the first mating generated mice with either the wild-type or R6/2 genotype. 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. All animal experiments were studied with the approval of the Institutional Animal Care and Use Committee (IACUC, Approval number: 13-0058-C2A1) of Seoul National University Hospital. We developed an *in vitro* HD model by culturing neural stem cells from R6/2 mice. In brief, after sacrificed by CO₂ gas, brain tissues of 9-week-old mice were used for primary culture and neural stem cells were isolated by dissection and trypsin treatment. Cells were incubated in culture medium consisting of DMEM/F12 (Invitrogen, Carlsbad, CA, USA), 1% P/S (penicillin-streptomycin), 2% B27 Supplement (Gibco BRL, Carlsbad, CA, USA), 10 ng/mL EGF (Invitrogen, Carlsbad, CA, USA) and 10 ng/mL bFGF (Invitrogen, Carlsbad, CA, USA) at 37°C in a 95% O₂, 5% CO₂ humidified atmosphere. neural stem cells were differentiated in the differentiation medium, which was composed of DMEM/F12, 1% PSA, 2% B27, and 5% FBS.

Isolation serum exosome and treatment of young and old serum-exo

To deplete exosomes from the mice serum, all centrifugation steps were performed at 4 °C. Exosomes were isolated by Exo-quick exosome precipitation solution (System Biosciences, Mountain View, CA), according to manufacturer's specifications. Briefly, the serum 250µl was mixed thoroughly with 63µl of Exo-Quick exosome precipitation solution and centrifuged at 1500g for 30 min. The supernatant was then removed and centrifuged at 1500g for 5 min after adding buffer. The remaining exosome pellets were then suspended in 100µl PBS. Exosome concentration was measured using BCA protein assay kit for treatment. HD cells were treated with 200 µg/ml of young or old serum-exo and young or old serum at 2 days of differentiation and incubated for 3 days. Control groups were treated with same volume of PBS.

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) after fixing with 4% paraformaldehyde. The cells were then counterstained with DAPI (1:300, Sigma, Deisenhofen, Germany). For the fluorescence staining analysis, we performed three independent experiments and over 500 cells are counted in each group.^[22] Em48 (red) or DAPI (blue)-stained cells were counted using an inverted microscope (BX61, Olympus Corporation, Tokyo, Japan).

Tissue preparation and Fluorescent Immunohistochemistry

For immunohistochemistry, mice were anesthetized and perfused through the heart with 10ml 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). 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) for 2 hours. EM48 (red) or DAPI (blue)-stained cells were identified using an upright microscope (Ni-E, Nikon Corporation, Tokyo, Japan). [23, 24]

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 (1:200; Santa Cruz, CA, USA) and BrdU antibody (1:300, Abcam, Cambridge, MA, USA). 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) for 2 hours. DCX (fitc) and BrdU (red) or DAPI (blue)-stained cells were identified using an inverted microscope (BX61, Olympus Corporation, Tokyo, Japan).

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) using a cell scraper. Protein extracts were prepared using RIPA buffer (Radio immunoprecipitation assay buffer, Thermo-Scientific, Waltham, MA, USA) containing freshly added protease inhibitor and phosphatase inhibitor (Roche, NJ, USA). The protein content was determined using a BCA (Bicinchoninic acid assay) protein assay kit (Pierce, Rockford, IL, USA). 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). 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), p-CREB (1:1000; Cell-signaling), CREB (1:1000; Cell-signaling), anti-p53 (1:1000; Cell-signaling, MA, USA), anti-Bax (1:200; Santa Cruz), BCL-2 (1:200; Santa Cruz), Caspase3 (1:1000; Cell-signaling), Em48 (1:500, Millipore), DCX antibody (1:500, abcam), and anti-β-actin (1:200, Santa Cruz). Blots were then incubated with horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit antibodies (1:3000, GE Healthcare, NJ, USA), and developed using ECL solution (Enhanced chemiluminescence solution, Advanta, CA, USA). Band intensities were measured using ImageJ software from three independent results normalized by β-actin (Park, 2015). All western blot figures show the representative one from three separate experiments.

Cell survival assay

Cell survival rate was measured by a colorimetric assay using the WST-1 (Roche, Mannheim, Germany) 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 hours. After 72 hours, 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.

Flow cytometry

To analyze the apoptosis population of neuronal stem cells, flow cytometry using annexin V-FITC and propidium iodide (PI) staining was used. Neuronal stem cells were washed and harvested in PBS (phosphate buffered saline, WelGene, Daegu, Korea) using a cell scraper. Cells were counted and 1×10^6 cells were suspended in 1ml 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 1×10^5 cells per tube, and were incubated with 10 µl of annexin V-FITC at room temperature for 30min 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).

Statistical analysis

All values indicated in the figures are presented as mean ± standard error. Results of western blot were analyzed using Student's t-test. A 2-tailed probability value below 0.05 was considered statistically significant. Data were analyzed by SPSS version 17.0 (SPSS Inc., USA).

Results

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. In addition, WT-WT serve as controls to ensure that the surgical procedures did not cause ectopic mineralization (Fig. 1a). To successively perform the parabiosis of R6/2 mice, minimizing the damage of parabiosis and the stress 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. Suturing the abdomen and the back of the mice was performed through clamping and tying their forelimbs with threads. More details about the methods of parabiosis are demonstrated in the Supplement (Additional file 1: Figure S1, Additional file 2: Figure S2, Additional file 3: Video 1, and Additional file 4: Video 2). The mice were observed periodically as described

in materials and methods. No obvious signs of stress were noted during observations, after 6 weeks after surgery.

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 parabionts 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 parabionts paired for six weeks. We also recorded survival, weight loss and lifespan of each group among the same composition of females and males. In contrast to the WT(Old)-HD, WT(Young)-HD showed increased survival (Fig. 1d). Heterochronic parabiosis with the young mice delayed the onset of mortality from 84 to 100 days, the average survival from 102 to 123 days, and the maximum survival from 118 to 145 days ($P < 0.05$). It also reduced tremor (data not shown), improved movement measured (Additional file 5 - 7: Video3 - 5), and improved clasping test (Additional file 8 - 10: Video6 - 8) at 9 and 12 weeks of age. 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. The improvement on the behavior (Additional file 11 – 13: Video9 - 11) and the weight loss (Additional file 14: Figure S3) 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 effect on mHtt aggregation, brain was sectioned and sliced tissues were stained with an EM48 antibody which detects aggregation of mHtt, and Het-HD showed reduced mHtt aggregation in the striatum and cortex (Fig. 1f). 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 aggregation in the brain.

Modulation of Pathological Phenotypes of HD by Parabiosis

Dysfunction of CREB-PGC-1 α pathway has been regarded as the key molecule for HD progression. To examine effects of Heterochronic parabiosis on this pathway, 12 weeks old R6/2 mice were paired and western blot analysis was performed after six weeks from the surgery. Het-HD showed increased expression of p-CREB and PGC-1 α ($p < 0.05$ vs. R6/2 control) compared to Iso-HD (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).

Also, IHC and western blot were performed to examine the improvement of cognitive function. It was found that DCX and BrdU, which are the representative signals for neurogenesis, were increased in Het-HD (Fig. 2c), and the same result was confirmed by western blot (Fig. 2d). It proves that young wild-type blood is also effective in improving cognition. Also, the same result was obtained for the modulation of pathological phenotypes of HD by parabiosis in the case of ZQ175 mice (Additional file 15 – 16: FigureS4 -S5).

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 R6/2 mice brain. 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 (Additional file 17: FigureS6). To harvest the exosomes from the blood serum, there are two representative methods for exosome isolation: serial ultracentrifugation and Exo-Quick reagent. Western blot 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 (Additional file 18: FigureS7).

As shown earlier in Fig. 1d, pairing the HD mice with the young wild-type increased survival, whereas pairing 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. 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 (200µg/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% 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 $20.8 \pm 2.3\%$ for HD and $12.1 \pm 0.3\%$ 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.

Modulation of Molecular Pathology of HD by Exosome Treatment

To examine the effects of young serum-exosomes on the p-CREB-PGC1 α pathway, cells were treated with control medium or young serum-exosomes for three days after days 2 of differentiation. Treatment with

young serum-exosomes promoted expression of p-CREB and PGC1 α (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). 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). Young serum-exosomes improves mitochondrial activation and cell survival while old serum-exosomes does not (Additional file 19: Figure S8).

Discussion

In this study, we demonstrated that heterochronic parabiosis of HD mice with the young wild-type modulates the body weight loss, mHtt aggregation, mitochondrial dysfunction, cell death, and cognitive impairment.[1, 10] These are the representative pathologies of HD, thus extending the survival of HD mice. The positive effect of heterochronic parabiosis originates from the shared blood circulation,[25, 26] just like the other neurodegenerative diseases ameliorated by heterochronic parabiosis.[9, 27, 28] It is worth noting that the R6/2 mice die typically at around 3–4 months of age accompanied by trimming and muscle loss[10, 11] and highly vulnerable to stress. Therefore minimizing the damage of parabiosis surgery were indispensable to make stable parabiotic pairs. We were able to obtain stable R6/2 parabionts for the first time by optimizing the binding site and method, which had a critical influence on the survival of R6/2 parabionts.

As we have already demonstrated that the positive factors exist in the young blood affecting the transgenic ones,[14] it should also exist in the messenger unit in the shared circulatory system. We identified that the exosomes are messenger units for transferring positive factors inside the blood by the *in vitro* cellular model for HD. Young blood serum as well as serum exosomes also modulated mHtt aggregation, mitochondrial dysfunction, cell death, and cell viability.

Although there are several limitations that further study is required to identify the positive factor itself that are responsible for elucidating the mechanisms of exosome treatment.[16, 29] However, we demonstrated that exosome showed more than equivalent effect compared to serum, which could lead to the development of the intravenous administration of serum exosome in humans, which is a low risk procedure already offered as a therapy with limited complications,[30, 31] 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 mitochondria dysfunction and neurodegeneration.

Conclusion

In summary, our results show that the overall pathology of HD is improved by the shared blood circulation through parabiosis, furthermore, we demonstrated that the exosomes are messenger units for transferring positive factors inside the blood by the *in vitro* cellular model for HD. 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.

Abbreviations

HD: Huntington's disease, mHtt: Mutant Huntingtin, NSC: Neural stem cell, SVZ: Subventricular zone

Declarations

- 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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Figures

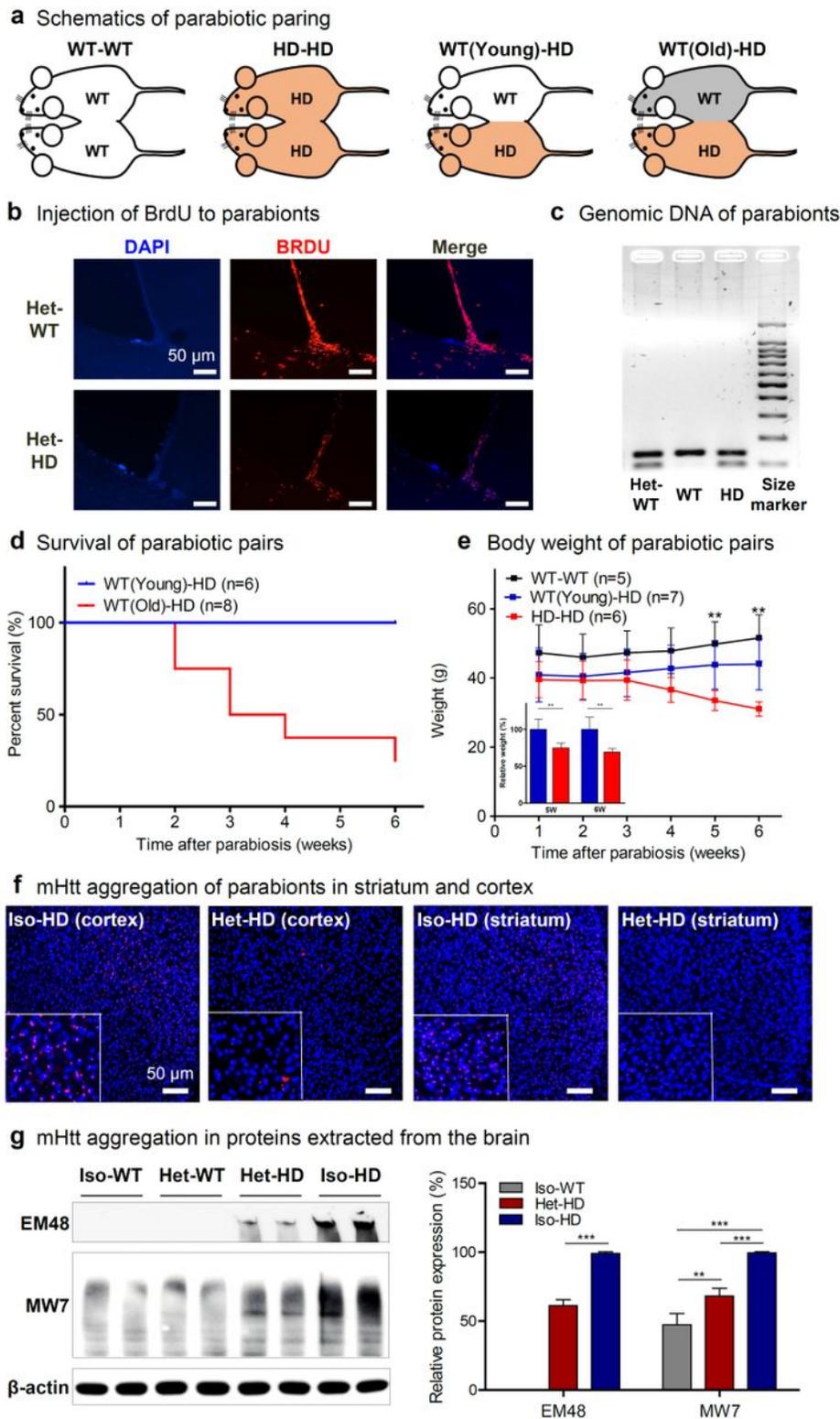
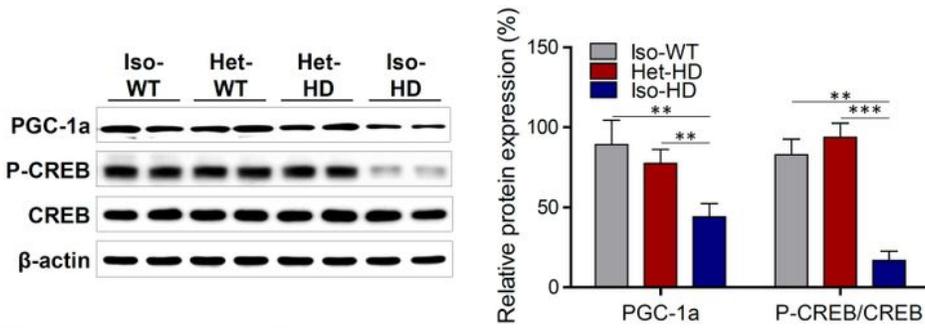


Figure 1

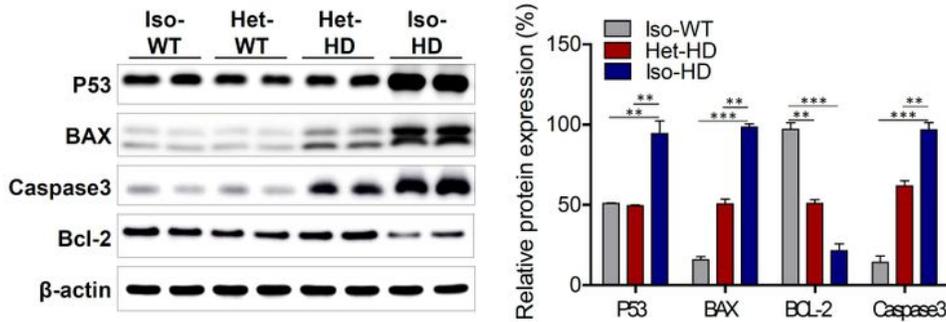
Construction of animal model of heterochronic parabiosis for HD and modulation of HD pathology. **a** Schematics of parabiotic pairing. WT-WT indicates the isochronic pairing of wild-types, HD-HD indicates the isochronic pairing of HD mouse, and each parabionts are indicated as Iso-WT and Iso-HD, respectively. WT(Young)-HD indicates the heterochronic pairing of young wild-type and HD mouse, which are indicated as Het WT and Het HD, respectively, and WT(Old)-HD indicates the heterochronic pairing of old wild-type

and HD mouse. 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 parabionts. 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. g Evaluation of mHtt aggregation from the proteins in the brain of parabionts by western blot.

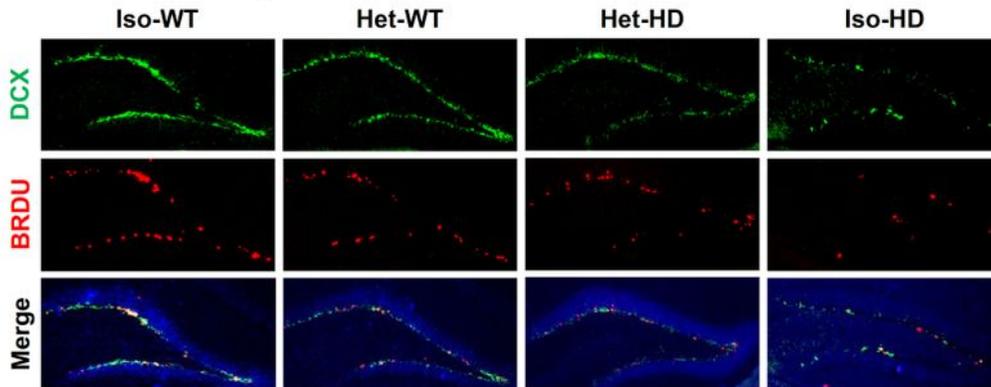
a Improvement of mitochondrial dysfunction



b Modulations of cell death



c Immunohistochemistry of parabiotic individuals



d Western blot of parabiotic individuals

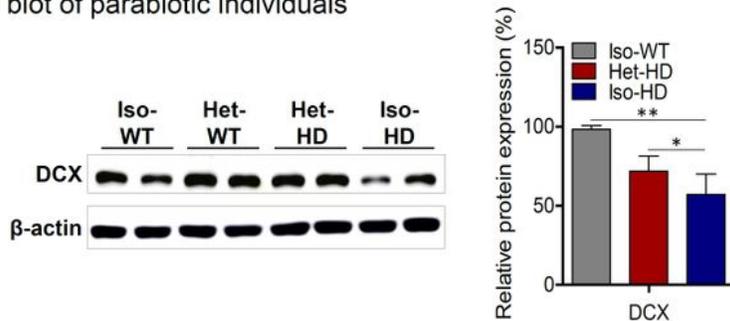
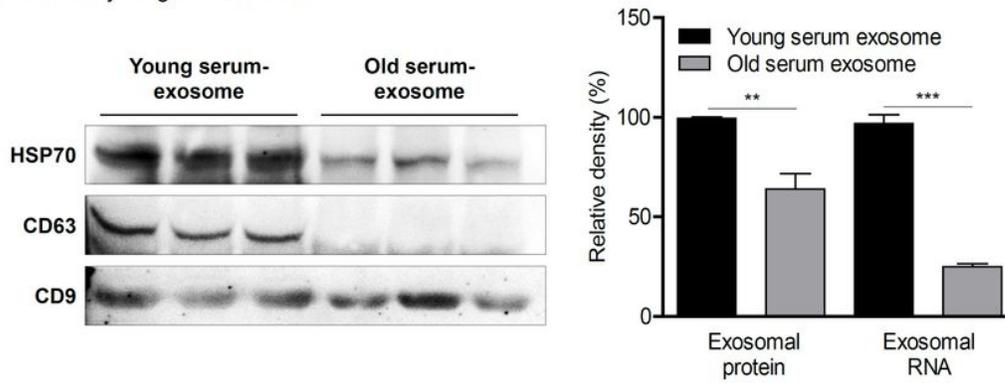


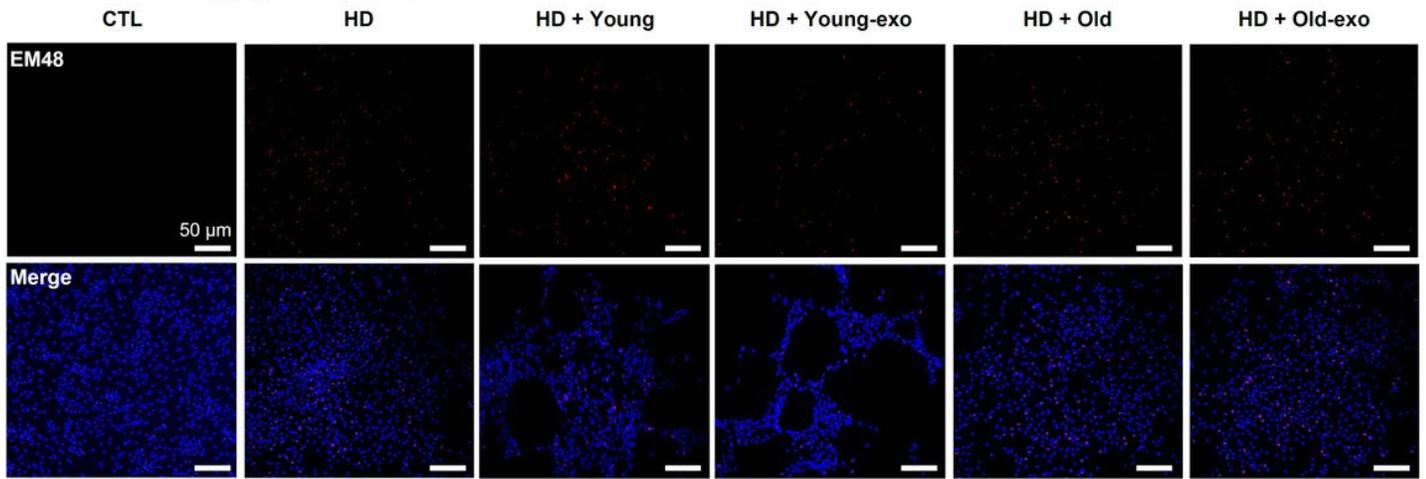
Figure 2

Modulation of pathological phenotypes of HD by parabiosis. a Improvement of mitochondrial dysfunction. b Modulation of cell death. c, d Immunohistochemistry and western blot of parabionts to demonstrate cognitive function of parabionts, respectively.

a Exosomes derived from young and old serum



b Amelioration of mHtt aggregation by exosome treatment



c Western blot analysis for exosome treatment

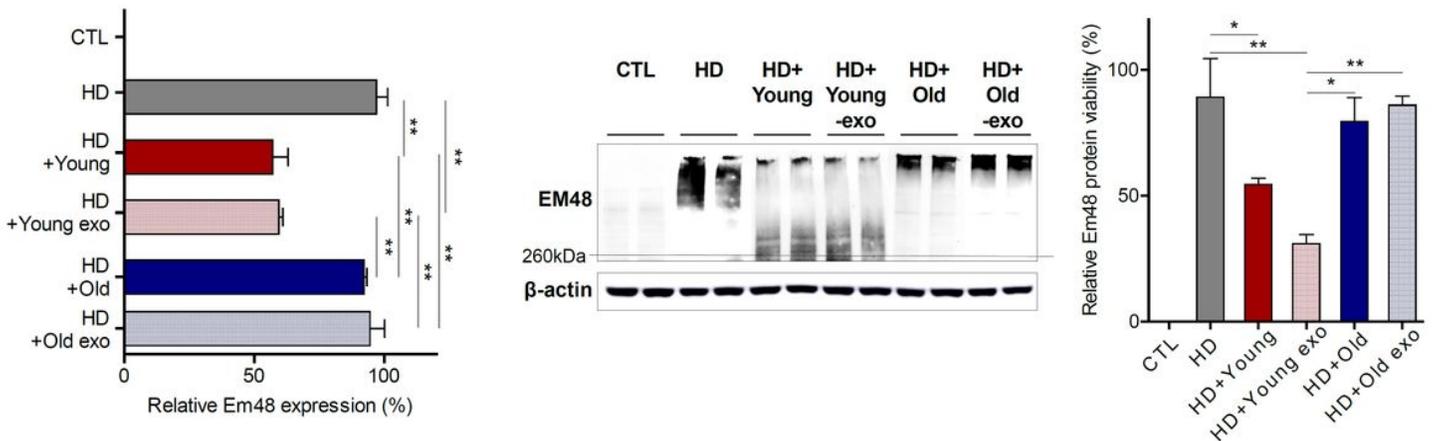
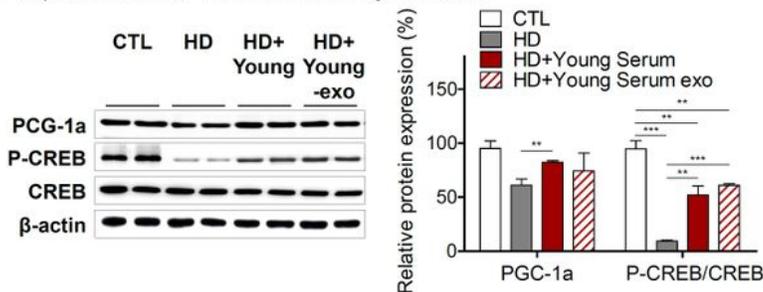


Figure 3

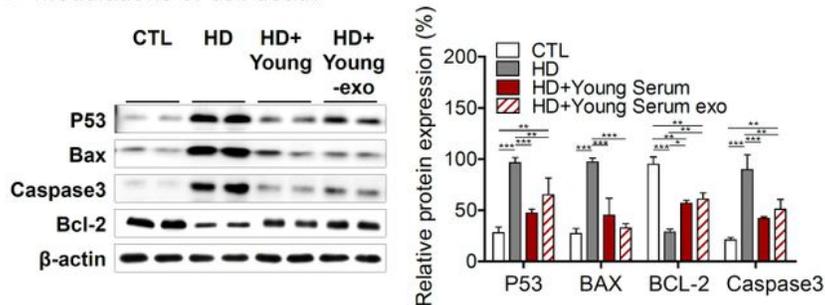
Amelioration of mHtt aggregation by exosome treatment. a Comparison of the amount of exosomes, exosomal proteins, and RNA levels of blood serum from young and old wild-type. Young serum-exosome and Old serum-exosome indicates the exosomes derived from the blood serum from the young mice and old mice, respectively. b, Immunohistochemistry and western blot of cells from demonstrated 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 by blood serum from the young wild-type mice, respectively. HD+Old and HD+Old-exo indicates the HD cells treated by blood serum and exosomes derived by blood serum from the old wild-type mice, respectively.

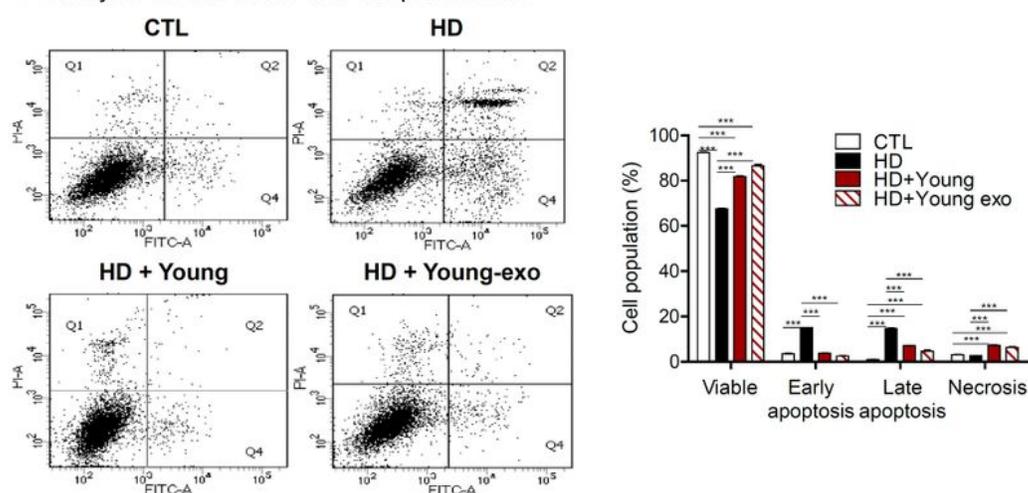
a Improvement of mitochondrial dysfunction



b Modulations of cell death



c Analysis for cell death and cell proliferation



d Evaluation of cell viability with exosome treatment

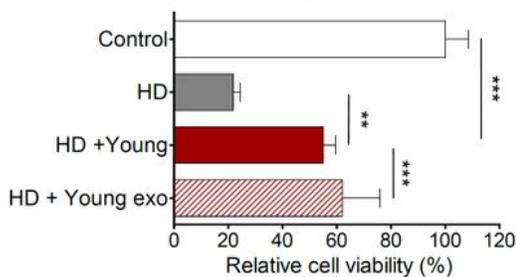


Figure 4

Modulation of molecular pathology of HD by exosome treatment.a, b Improvement of mitochondrial dysfunction and modulation cell death, respectively. c Analysis for cell death. d Evaluation of cell viability with exosome treatment.

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