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엑소좀 수득 조건 최적화 및 퇴행성 신경 질환 치료에의 응용

OPTIMIZATION OF HANDLING CONDITIONS FOR THE EXOSOME AND APPLICATION TO THE NEURODEGENERATIVE DISEASES

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ABSTRACT

Optimization of Exosome Handling Condition and Application to the Neurodegenerative Diseases

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Exosomes are small extracellular vesicles with 30–100 nm diameter that originate from many type of cells. Exosomes serve as a role of intercellular messenger unit by inward budding and scission of vesicles from the limiting endosomal membranes and released from the MVB lumen into the extracellular environment during exocytosis. Secreted exosomes can be isolated and characterized in blood, urine, saliva in vivo and conditioned medium in vitro. They include various molecular constituents of their cell origin, including proteins, mRNA, and microRNA. Therefore, exosomes have been widely studied as a therapeutic agent, biomarker, and drug delivery vehicle.

Despite of their importance in normal physiology and disease progression, the standard criteria of storage conditions, which are generally the pH and the temperature, are indefinite and controversial. Therefore, in this study, we isolated the exosomes after incubation in various pH conditions and analyzed the recovery yield of exosomes, and we examined the stability of exosomes at various storage temperatures. We have

discovered that acidic condition was favorable for the high yield for exosome isolation, and the temperature below -70 °C is crucial for the preservation of exosomes in fresh condition for clinical and basic applications.

After optimizing the isolation and storage conditions for the exosome, we examined that the human adipose stem cell derived exosome (ASC-exo) has a therapeutic potential for treating Alzheimer's disease (AD), Huntington's disease (HD), Amyotrophic lateral sclerosis (ALS) by modulating representative cellular phenotypes. Adipose stem cells (ASCs) are regarded as the potential source of regenerative medicine, since ASCs express multiple factors for the paracrine effects and have a high proliferation rate with a lower senescence than other tissue-derived stem cells. Although exosomes derived from stem cells has therapeutic potential on many illnesses, precise mechanism of how ASC-exo affect the progression of HD, AD, and ALS has not been studied.

We examined the effects of ASC-exo on key phenotypes of HD including cell death, and mitochondrial dysfunction in R6/2-derived neuronal cells, and mHtt aggregates levels. Also, we examined the effects of ASC-exo on key hallmarks of ALS including SOD1 aggregation, elevated SOD1 level and abnormal mitochondrial protein levels in G93A neuronal cells. Furthermore, we developed an in vitro AD model using neuronal stem cells (NSCs) from the transgenic mice line, TG2576. The differentiated NSCs of AD mice, which showed major phenotypes of AD including the Aβ pathology and increased apoptosis, were treated by ASC-exo. In addition, changes in neurite outgrowth were examined, as neurites are involved in the synapse formation of the central nervous system. We proved that ASC-exo ameliorate the progression of Aβ-induced neuronal death in AD, and ASC-exo modulates SOD-1 aggregation and mitochondrial dysfunction, making it a therapeutic candidate for ALS.

In HD, we confirmed that the ASC-exo up-regulates PGC1, phospho-CREB and amelio rates abnormal apoptotic protein level, and reduces mHtt aggregates level. In addition, MitoSOX Red, JC-1 and cell viability assay showed that ASC-exo reduces mitochondrial dysfunction and cell apoptosis. These findings suggest that ASC-exo has a therapeutic potential for treating neurodegenerative diseases by modulating representative cellular phenotypes.

Next focus is ameliroration of HD by heterochronic parabiosis (HP) which have been reported by a few paper in AD, presenting that exposure to a young blood circulation through HP reverses cognitive deficits observed with normal aging. To determine the effect of young circulatory factors on HD-like disease in R6/2 mice, we used HP, in which we joined the circulatory systems of young, wildtype animals together with R6/2 mice which express exon1 of the human HD gene with around 150CAG repeats. We analyzed the changes in protein expression in the brains of these mice with established disease after heterochronic parabiosis, and we examined the effects of young serum exosome on key phenotypes of HD including p-CREB-PGC-1 pathway, apoptotic proteins in R6/2-derived neuronal cells. mutant Huntingtin aggregation protein mice with established disease showed a restoration in levels of HD pathology. Moreover, young blood associated with improved mitochondria dysfunction and cognition, decreased cell death after exposure to young blood in heterochronic parabiosis. In addition, in vitro HD model cells treated with young blood serum and serum exosome showed decreased mHtt aggregation, mitochondrial dysfunction, cell death and cell proliferation.

This study provides useful information for understanding the optimum conditions for the isolation and storage of exosomes, and by identifying the exosomes as a

messenger unit that can transport the positive factors for the diseases, this study opens the possibilities for adopting the exosomes to various neurodegenerative diseases.

Keywords: Neurodegenerative disease, Human adipose stem cells, Exosomes, storage condition, temperature, Acidic microenvironment, parabiosis

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two different isolation methods. Two different protocols were used to isolate exosomes. One was ultracentrifugation method and the other was exosome precipitation reagent ExoQuick-TC. Exosomal proteins were extracted from each exosomes from pH4 and pH7 and measured by BCA protein assay kit. As a result, total exosomal protein concentration in pH4 is higher than that of pH7. This result indicated that ultracentrifugation-based method and commercial reagent ExoQuick-TC showed similar result. Thus, representative exosome components were increased by acidic environment.

Figure 4.1 Isolation of conditioned medium derived exosomes. Exosome from exosome-free medium and HEK293 derived conditioned medium were isolated using Exo-Quick method. Extracted proteins were immunoblotted with antibodies for representative exosome markers, HSP70, CD63 and CD9.

Figure 4.2 Effects of short-term storage condition on exosome recovery. Isolated exosomes from HEK293 cells were suspended with protein extraction buffer and stored at from 4 to 90°C for 30 min. After incubation, exosomes were isolated and exosomal protein markers were detected using western blot (A). Bar graph shows relative quantification of band intensity for HSP70, CD63 and CD9 (B). $n = 3$; $*p < 0.05$, $**p < 0.01$ vs. 4°C.

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Figure 7.5 ASC-Exo reduces Ab levels and the Aβ 42/40 ratio. Neuronal stem cells (NSCs) from TG2576 or wild-type littermate mice were cultured and differentiated for 2 day and Aβ levels were measured (A). NSCs were cultured in the presence of ADSC-Exo for 12, 24, 48, 72 h and Aβ levels of the conditioned medium were analyzed using ELISA. Aβ 40 (B) and Aβ 42 (C) levels of AD cells, and ADSC-Exo treated AD cells are represented as a bar graph. (D) Aβ 42/40 ratio of AD NSCs treated with or without ADSC-Exo. * $p < 0.05$, ** $p < 0.01$ vs AD.

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caspase-3 were examined using western blot (A). Relative protein band densities were normalized using b-actin, and represented as a bar graph (B). * $p < 0.05$, ** $p < 0.01$ vs AD.

Figure 7.7 ADSC-Exo ameliorates the apoptosis of AD cells. Cells isolated from WT or AD mice were cultured and differentiated. ADSC-Exo treatment was conducted for 2 days and cells were then harvested and incubated with annexin V-FITC for 5 min at room temperature. Propidium iodide was added to all samples just before the analysis. The viable (lower left), early apoptotic (lower right), late apoptotic (upper right), necrotic (upper left) cell populations were analyzed using flow cytometry (A). The cell percentage was analyzed according to annexin V and propidium iodide staining and presented as a bar graph (B). This result used ADSC-Exo from three different volunteers and similar result was obtained. ** $p < 0.01$.

Figure 7.8 ADSC-Exo increases neurite number and the length of AD neuronal cells. NSCs from the subventricular zone of AD transgenic mice were cultured and differentiated using a differentiation medium. NSCs from AD mice were cultured in the presence of ADSC-Exo (200 lg/mL) on day 1 after differentiation and incubated for 2 days. (A) Cells were photographed using the light microscope. The numbers (B) and lengths (C) of neurites were analyzed and represented as bar graphs (n = 8 per group per experiment) (B) The small box displays an enlarged image of the dotted line. This result used ADSC-Exo from three different

volunteers and similar result was obtained. Bar = 100 μm, * $p < 0.05$, ** $p < 0.01$.

Figure 8.1 Schematic depicting the 4 different parabiotic pairing: isotype wildtype (WT), heterotype HD, and isotype HD. Isotype pairs are age-matched and the HD mouse from the heterotype pair, which is connected to a young WT mouse (6 weeks old) or old WT mouse (18-22 months old). All pairs were surgically connected for 6 weeks.

Figure 8.2 Actual photo for the parabiosis of mice.

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Figure 8.4 BrdU was injected into one mice. The formation of shared blood circulation between the parabiotic animals was tested by injection of BrdU (150 mg/kg) to the intraperitoneal administration of one of the mice in parabiosis after 2weeks postsurgically and the pair was

killed after 4 weeks. brain from the injected mice and the attached mice in all groups of parabionts showed similar BrdU signals, indicating a joined circulatory system.

Figure 8.5 Parabiotic pairs between the WT (Young or Old) and HD mice were established as described in Materials and Methods. Survival of the pairs was monitored for 6 weeks; HD young heterotype (n=6), HD old heterotype (n=8). In contrast with old parabiosis R6/2 mice, young parabiosis R6/2 mice showed increased survival. Measures of each pair or each partner in a pair are plotted separately in different colors.

Figure 8.6 Body weight of each pair. Absolute weights of the pairs (parabionts) measured at various times during recovery; WT isotype (n=5), HD heterotype (n=7), HD isotype (n=6). Immediately following the surgery, both groups appeared to slight lose weight, which was followed by steady normalisation of the body weight. Heterotyep HD mouse group mitigated weight loss in isotype HD mouse group at 5 and 6 weeks after parabiosis surgery. **$p<0.01$. Values are mean ± SEM. Measures of each pair or each partner in a pair are plotted separately in different colors.

Figure 8.7 Body weight of each pair. Absolute weights of the pairs (parabionts) measured at various times during recovery; HD old heterotype (n=5), HD young heterotype (n=5), HD isotype (n=5). Immediately following the surgery, both groups appeared to slight lose weight, which was followed by steady normalisation of the body weight. HD young heterotype mitigated weight loss in HD

Figure 8.13 As shown by western blotting, Heterotyep HD mouse group brain increased the levels of Bcl-2 compared with isotype HD mouse group and decreased the levels of p53, Bax, and cleaved caspase-3. ***p<0.001 (n=3).

Figure 8.14 As shown by western blotting, HD young heterotype brain increased the levels of Bcl-2 compared with HD old heterotype and decreased the levels of p53, Bax, and cleaved caspase-3. *p<0.05, *p<0.01, ***p<0.001 (n=3).

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Figure 8.16 Double labeling of BrdU-positive cells (red) and DCX-positive cells (green) in parabiosis model. BrdU labeling is widely distributed in the dentate gyrus of the hippocampus, as well as DCX-positive cells, which are spread out over of the brain. DCX and BRDU double staining is heterotype HD whereas almost no DCX and BRDU positive cells are isotype HD. The number of double positive cells is higher in the heterotype HD.

Figure 8.17 DCX-positive cells (green) in parabiosis model. DCX-

positive labeling cells is widely distributed in the dentate gyrus of the hippocampus which are spread out over of the brain. DCX is HD young heterotype whereas almost no DCX positive cells are HD old heterotype. The number of DCX positive cells is higher in the HD young heterotype.

Figure 8.18 Isolation of blood serum derived exosomes. Exosomes from blood serum were isolated using Exo-Quick and ultracentrifuge method. Extracted exosomal proteins were immunoblotted with antibodies for representative exosome markers, HSP70, CD63 and CD9. We thus confirmed the presence of exosomes.

Figure 8.19 Identification of markers confirm exosomes isolation from serum and High concentraction in Exosomal fractions isolated from serum of young blood versus old blood. To investigate exosomal protein and RNA concentration in young blood and old blood, proteins and total RNA were extracted by protein extraction buffer and total RNA isolation kit respectively after isolating exosomes from young blood and old blood. Exosomes from blood serum were isolated using Exo-Quick method. Extracted exosomal proteins were immunoblotted with antibodies for representative exosome markers, HSP70, CD63 and CD9. Interestingly, somewhat higher exosomal protein and RNA revels were observed in young blood. Two representative methods for exosome isolation are serial ultracentrifugation and Exo-Quick reagent. We identified increased yield of exosomal protein by young blood using ultracentrifugation method. These results suggest that exosomal protein and RNA from

Figure 8.28 As shown by western blotting, Treatment with young blood serum and serum exosome decreased the levels of p53, Bax, and cleaved caspase-3 compared with HD cells and increased the levels of Bcl-2. *P < 0.05, **P < 0.01, ***P < 0.001 (n = 3).

Figure 8.29 Young blood serum and serum exosome ameliorates the apoptosis of HD cells. NSCs from the subventricular zone of WT and HD transgenic mice were cultured and differentiated using a differentiation medium. NSCs from HD mice were cultured in the presence of young blood serum and serum exosome (200 lg/mL) on day 2 after differentiation. young blood serum and serum exosome treatment was conducted for 3 days and cell were then harvested and incubated with annexin V-FITC for 30 min at room temperature. Propidium iodide was added to all samples just before the analysis. The viable (lower-left), early apoptotic (lower-right), late apoptotic (upper-right), necrotic (upper-left) cell populations were analyzed using flowcytometry. The cell percentage was analyzed according to annexin V and propidium iodide staining and presented as a bar graph. This result used young serum and serum exosome from three different volunteers and similar result was obtained. *** p < 0.001.

Figure 8.30 As shown by western blotting, Treatment with Old blood serum and serum exosome no decreased the levels of p53, Bax, and cleaved caspase-3 compared with HD cells and no increased the levels of Bcl-2. (n = 3).

CHAPTER 1

Introduction

1.1. Neurodegeneration process

Neurodegeneration is the progressive loss of structure or function of neurons, including death of neurons. Many neurodegenerative diseases – including amyotrophic lateral sclerosis, Parkinson's disease (PD), Alzheimer's disease (AD), and Huntington's disease (HD) – occur as a result of neurodegenerative processes. Such diseases are incurable, resulting in progressive degeneration and/or death of neuron cells. [1] As research progresses, many similarities appear that relate these diseases to one another on a sub-cellular level. Discovering these similarities offers hope for therapeutic advances .that could ameliorate many diseases simultaneously. There are many parallels between different neurodegenerative disorders including atypical protein assemblies as well as induced cell death. [2,3] Neurodegeneration can be found in many different levels of neuronal circuitry ranging from molecular to systemic.

The greatest risk factor for neurodegenerative diseases is aging. Mitochondrial DNA mutations as well as oxidative stress both contribute to aging. [4] Many of these

diseases are late-onset, meaning there is some factor that changes as a person ages for each disease.[2] One constant factor is that in each disease, neurons gradually lose function as the disease progresses with age. It has been proposed that DNA damage accumulation provides the underlying causative link between aging and neurodegenerative disease. [5, 6]. About 20-40% of healthy people between 60 and 78 years old experience discernable decrements in cognitive performace in several domains including working, spatial, and episodic memory, and processing speed. [7]

Several neurodegenerative diseases are classified as proteopathies as they are associated with the aggregation of misfolded proteins. Alpha-synuclein can aggregate to form insoluble fibrils in pathological conditions characterized by Lewy bodies, such as Parkinson's disease, dementia with Lewy bodies, and multiple system atrophy. Alpha-synuclein is the primary structural component of Lewy body fibrils. In addition, an alpha-synuclein fragment, known as the non-Abeta component (NAC), is found in amyloid plaques in Alzheimer's disease. Hyperphosphorylated tau protein is the main component of neurofibrillary tangles in Alzheimer's disease. Beta amyloid is the major component of senile plaques in Alzheimer's disease. Prion is the main component of prion diseases and transmissible spongiform encephalopathies. There are various causes for these diseases.

Damage to the membranes of organelles by monomeric or oligomeric proteins could also contribute to these diseases. Alpha-synuclein can damage membranes by inducing membrane curvature, [8] and cause extensive tubulation and vesiculation when incubated with artificial phospholipid vesicles. [8] The tubes formed from these lipid vesicles consist of both micellar as well as bilayer tubes. Extensive induction of membrane curvature is deleterious to the cell and would eventually lead to cell death.

Apart from tubular structures, alpha-synuclein can also form lipoprotein nanoparticles similar to apolipoproteins.

The most common form of cell death in neurodegeneration is through the intrinsic mitochondrial apoptotic pathway. This pathway controls the activation of caspase-9 by regulating the release of cytochrome c from the mitochondrial intermembrane space (IMS). Reactive oxygen species (ROS) are normal byproducts of mitochondrial respiratory chain activity. ROS concentration is mediated by mitochondrial antioxidants such as manganese superoxide dismutase (SOD2) and glutathione peroxidase. Over production of ROS (oxidative stress) is a central feature of all neurodegenerative disorders. In addition to the generation of ROS, mitochondria are also involved with life-sustaining functions including calcium homeostasis, PCD, mitochondrial fission and fusion, lipid concentration of the mitochondrial membranes, and the mitochondrial permeability transition. Mitochondrial disease leading to neurodegeneration is likely, at least on some level, to involve all of these functions. [9] There is strong evidence that mitochondrial dysfunction and oxidative stress play a causal role in neurodegenerative disease pathogenesis, including in four of the more well known diseases Alzheimer's, Parkinson's, Huntington's, and Amyotrophic lateral sclerosis. [4] Neurons are particularly vulnerable to oxidative damage due to their strong metabolic activity associated with high transcription levels, high oxygen consumption, and weak antioxidant defense. [10, 11]

The brain metabolizes as much as a fifth of consumed oxygen, and reactive oxygen species produced by oxidative metabolism are a major source of DNA damage in the brain. Damage to a cell’s DNA is particularly harmful because DNA is the blueprint for protein production and unlike other molecules it cannot simply be replaced by re-synthesis. The vulnerability of post-mitotic neurons to DNA damage (such as

oxidative lesions or certain types of DNA strand breaks), coupled with a gradual decline in the activities of repair mechanisms, could lead to accumulation of DNA damage with age and contribute to brain aging and neurodegeneration.[12] DNA single-strand breaks are common and are associated with the neurodegenerative disease ataxia-oculomotor apraxia.[11, 13] Increased oxidative DNA damage in the brain is associated with Alzheimer's disease and Parkinson's disease. [13] Defective DNA repair has been linked to neurodegenerative disorders such as Alzheimer's disease, amyotrophic lateral sclerosis, ataxia telangiectasia, Cockayne syndrome, Parkinson's disease and xeroderma pigmentosum. [12, 13]

Axonal swelling and spheroids have been observed in many different neurodegenerative diseases. This suggests that defective axons are not only present in diseased neurons, but also that they may cause certain pathological insult due to accumulation of organelles. Axonal transport can be disrupted by a variety of mechanisms including damage to: kinesin and cytoplasmic dynein, microtubules, cargoes, and mitochondria. [14] When axonal transport is severely disrupted a degenerative pathway known as Wallerian-like degeneration is often triggered.[15] Programmed cell death (PCD) is death of a cell in any form, mediated by an intracellular program.[16] This process can be activated in neurodegenerative diseases including Parkinson's disease, amytrophic lateral sclerosis, Alzheimer's disease and Huntington's disease.[17] There are, however, situations in which these mediated pathways are artificially stimulated due to injury or disease. [3]

Apoptosis is a form of programmed cell death in multicellular organisms. It is one of the main types of programmed cell death (PCD) and involves a series of biochemical events leading to a characteristic cell morphology and death. Extrinsic apoptotic pathways occur when factors outside the cell activate cell surface death

receptors (e.g., Fas) that result in the activation of caspases-8 or -10. [3] Intrinsic apoptotic pathways result from mitochondrial release of cytochrome c or endoplasmic reticulum malfunctions, each leading to the activation of caspase-9. The nucleus and Golgi apparatus are other organelles that have damage sensors, which can lead the cells down apoptotic pathways. [3, 18]

Caspases (cysteine-aspartic acid proteases) cleave at very specific amino acid residues. There are two types of caspases: initiators and effectors. Initiator caspases cleave inactive forms of effector caspases. This activates the effectors that in turn cleave other proteins resulting in apoptotic initiation. [3]

Transglutaminases are human enzymes ubiquitously present in the human body and in the brain in particular.[19] The main function of transglutaminases is bind proteins and peptides intra- and intermolecularly, by a type of covalent bonds termed isopeptide bonds, in a reaction termed transamidation or crosslinking. [19] Transglutaminase binding of these proteins and peptides make them clump together. The resulting structures are turned extremely resistant to chemical and mechanical disruption.[19] Most relevant human neurodegenerative diseases share the property of having abnormal structures made up of proteins and peptides. [19] Each of these neurodegenerative disesases have one (or several) specific main protein or peptide. In Alzheimer's disease, these are amyloid-beta and tau. In Parkinson's disease, it is alpha-synuclein. In Huntington's disease, it is huntingtin. [19] Amyloid-beta, tau, alpha-synuclein and huntingtin have been proved to be substrates of transglutaminases in vitro or in vivo, that is, they can be bonded by trasglutaminases by covalent bonds to each other and potentially to any other transglutaminase substrate in the brain. [19] It has been proved that in these neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, and Huntington's disease) the expression of the transglutaminase

enzyme is increased. [19] The presence of isopeptide bonds (the result of the transglutaminase reaction) have been detected in the abnormal structures that are characteristic of these neurodegenerative diseases.[19] Co-localization of transglutaminase mediated isopeptide bonds with these abnormal structures has been detected in the autopsy of brains of patients with these diseases. [19]

The process of neurodegeneration is not well understood, so the diseases that stem from it have, as yet, no cures. In the search for effective treatments (as opposed to palliative care), investigators employ animal models of disease to test potential therapeutic agents. Model organisms provide an inexpensive and relatively quick means to perform two main functions: target identification and target validation. Together, these help show the value of any specific therapeutic strategies and drugs when attempting to ameliorate disease severity.

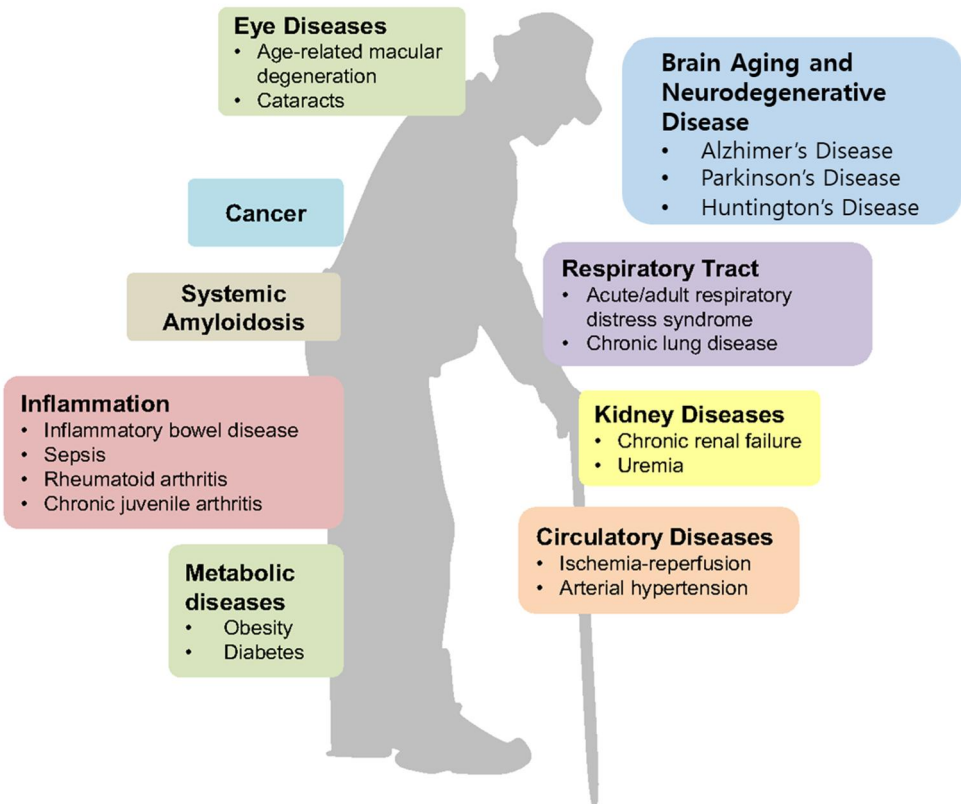


Figure 1.1 Protein oxidation in age-related diseases; it shows the numerous diseases in which protein oxidation has been demonstrated so far.

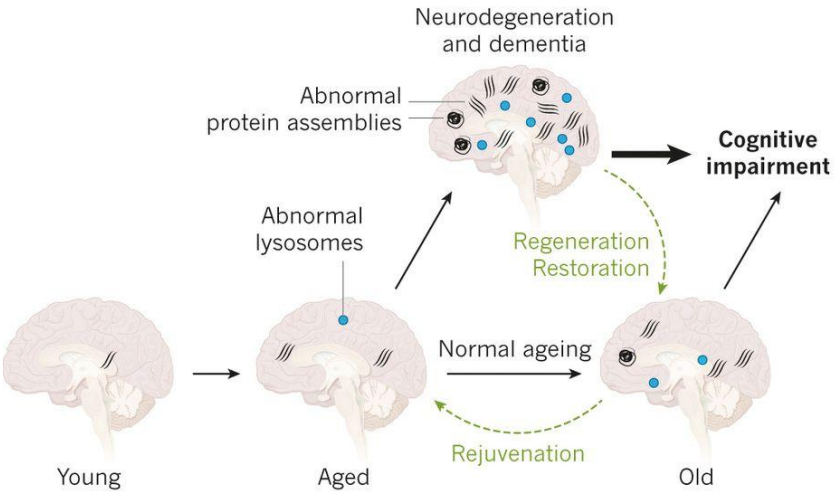


Figure 1.2 The process for the aging and the rejuvenation process of the brain.

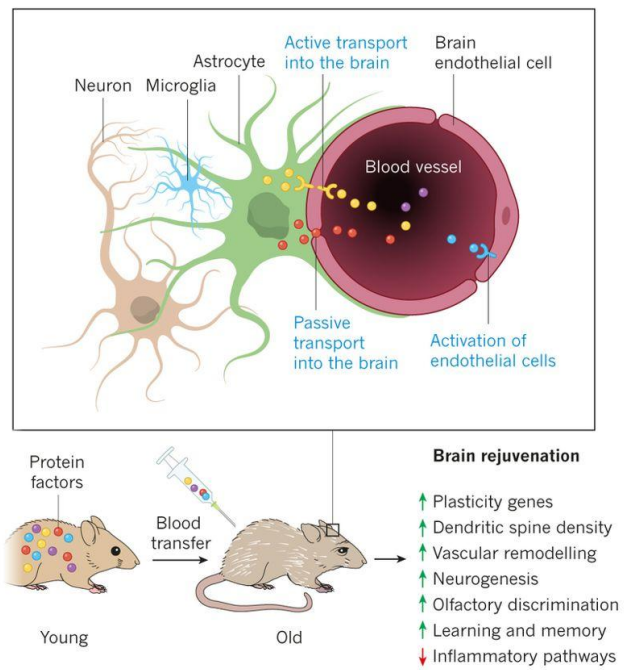


Figure 1.3 Protein factors and other molecules that circulate in the blood of a young mouse exert rejuvenating effects on the brain of an old mouse.

1.2. Exosomes

1.2.1. Role of exosomes as an intercellular cargo unit

Exosomes are extracellular vesicles (EVs) that are produced in the endosomal compartment of most eukaryotic cells. [20-22] The multivesicular body (MVB) is an endosome defined by intraluminal vesicles (ILVs) that bud inward into the endosomal lumen. If the MVB fuses with the cell surface (the serum membrane), these ILVs are released as exosomes. In multicellular organisms, exosomes and other EVs are present in tissues and can also be found in biological fluids including blood, urine, and cerebrospinal fluid. They are also released in vitro by cultured cells into the culture-conditioned medium. [23-25] Since the size of exosomes is limited by that of the parent MVB, exosomes are generally thought to be smaller than most other EVs, from about 30 to several hundred nm in diameter: around the same size as many lipoproteins but much smaller than cells. [23] EVs including exosomes carry markers of cells of origin and have specialized functions in physiological processes, from coagulation and intercellular signalling to waste management.[23] Consequently, there is a growing interest in clinical applications as biomarkers and therapies alike.[26] Compared with EVs in general, it is unclear whether exosomes have unique characteristics or functions or can be distinguished effectively from other vesicles.[20]

Exosomes contain various molecular constituents of their cell of origin, including proteins and RNA. Although the exosomal protein composition varies with the cell and tissue of origin, most exosomes contain an evolutionarily-conserved common set of protein molecules. The protein content of a single exosome, given certain assumptions of protein size and configuration, and packing parameters, can be about 20,000 molecules. [27] The cargo of mRNA and miRNA in exosomes was first

discovered at the University of Gothenburg in Sweden. [28] In that study, the differences in cellular and exosomal mRNA and miRNA content was described, as well as the functionality of the exosomal mRNA cargo. Exosomes have also been shown to carry double-stranded DNA. [29]

Exosomes can transfer molecules from one cell to another via membrane vesicle trafficking, thereby influencing the immune system, such as dendritic cells and B cells, and may play a functional role in mediating adaptive immune responses to pathogens and tumors. [30, 31] Therefore, scientists that are actively researching the role that exosomes may play in cell-to-cell signaling, often hypothesize that delivery of their cargo RNA molecules can explain biological effects. For example, mRNA in exosomes has been suggested to affect protein production in the recipient cell. [28, 32, 33] However, another study has suggested that miRNAs in exosomes secreted by mesenchymal stem cells (MSC) are predominantly pre- and not mature miRNAs. [34] Because the authors of this study did not find RNA-induced silencing complex-associated proteins in these exosomes, they suggested that only the pre-miRNAs but not the mature miRNAs in MSC exosomes have the potential to be biologically active in the recipient cells. Multiple mechanisms have been reported to be involved in loading miRNAs into exosomes, including specific motifs in the miRNA sequences, interactions with lncRNAs localized to the exosomes, interactions with RBPs, and post-translational modifications of Ago [35].

Conversely, exosome production and content may be influenced by molecular signals received by the cell of origin. As evidence for this hypothesis, tumor cells exposed to hypoxia secrete exosomes with enhanced angiogenic and metastatic potential, suggesting that tumor cells adapt to a hypoxic microenvironment by secreting exosomes to stimulate angiogenesis or facilitate metastasis to more favorable

environment. [36]

Scientists are actively researching the role that exosomes may play in cell-to-cell signaling, hypothesizing that because exosomes can merge with and release their contents into cells that are distant from their cell of origin (see membrane vesicle trafficking), they may influence processes in the recipient cell [53]. For example, RNA that is shuttled from one cell to another, known as "exosomal shuttle RNA," could potentially affect protein production in the recipient cell. [32, 54] By transferring molecules from one cell to another, exosomes from certain cells of the immune system, such as dendritic cells and B cells, may play a functional role in mediating adaptive immune responses to pathogens and tumors. [30, 44]

Conversely, exosome production and content may be influenced by molecular signals received by the cell of origin. As evidence for this hypothesis, tumor cells exposed to hypoxia secrete exosomes with enhanced angiogenic and metastatic potential, suggesting that tumor cells adapt to a hypoxic microenvironment by secreting exosomes to stimulate angiogenesis or facilitate metastasis to more favorable environment. [36] It has recently been shown that exosomal protein content may change during the progression of chronic lymphocytic leukemia. [55]

A study hypothesized that intercellular communication of tumor exosomes could mediate further regions of metastasis for cancer. Hypothetically, exosomes can plant tumor information, such as tainted RNA, into new cells to prepare for cancer to travel to that organ for metastasis. The study found that tumor exosomal communication has the ability to mediate metastasis to different organs. Furthermore, even when tumor cells have a disadvantage for replicating, the information planted at these new regions, organs, can aid in the expansion of organ specific metastasis. [56]

Exosomes carry cargo, which can augment innate immune responses. For example,

exosomes derived from Salmonella enterica-infected macrophages but not exosomes from uninfected cells stimulate naive macrophages and dendritic cells to secrete pro-inflammatory cytokines such as TNF-α, RANTES, IL-1ra, MIP-2, CXCL1, MCP-1, sICAM-1, GM-CSF, and G-CSF. Proinflammatory effects of exosomes are partially attributed to lipopolysaccharide, which is encapsulated within exosomes [57].

1.2.2. Application of exosoems for diseases

Exosomes from red blood cells contain the transferrin receptor which is absent in mature erythrocytes. Dendritic cell-derived exosomes express MHC I, MHC II, and costimulatory molecules and have been proven to be able to induce and enhance antigen-specific T cell responses in vivo. In addition, the first exosome-based cancer vaccination platforms are being explored in early clinical trials. [37] Exosomes can also be released into urine by the kidneys, and their detection might serve as a diagnostic tool. [38-40] Urinary exosomes may be useful as treatment response markers in prostate cancer. [41, 42] Exosomes secreted from tumour cells can deliver signals to surrounding cells and have been shown to regulate myofibroblast differentiation. [43] In melanoma, tumor-derived vesicles can enter lymphatics and interact with subcapsular sinus macrophages and B cells in lymph nodes. [44] A recent investigation showed that exosome release positively correlates with the invasiveness of ovarian cancer. [45] Exosomes released from tumors into the blood may also have diagnostic potential. Exosomes are remarkably stable in bodily fluids strengthening their utility as reservoirs for disease biomarkers. [46, 47] Patient blood samples stored in biorepositories can be used for biomarker analysis as colorectal cancer cell-derived exosomes spiked into blood could be recovered after 90 days of storage at various

temperatures. [48]

In malignancies such as cancer, the regulatory circuit which guards exosome homeostasis is co-opted to promote cancer cell survival and metastasis.[33, 49] Urinary exosomes have also proven to be useful in the detection of many pathologies, such as genitourinary cancers and mineralocorticoid hypertension, through their protein and miRNA cargo."[50, 51]

With neurodegenerative disorders, exosomes appear to play a role in the spread of alpha-synuclein, and are being actively investigated as a tool to both monitor disease progression as well as a potential vehicle for delivery of drug and stem cell based therapy. [52] An online open access database containing genomic information for exosome content has been developed to catalyze research development within the field. [52]

Increasingly, exosomes are being recognized as potential therapeutics as they have the ability to elicit potent cellular responses in vitro and in vivo.[58-60] Exosomes mediate regenerative outcomes in injury and disease that recapitulate observed bioactivity of stem cell populations. [61] Mesenchymal stem cell exosomes were found to activate several signaling pathways important in wound healing (Akt, ERK, and STAT3) and bone fracture repair. [62, 63] They induce the expression of a number of growth factors (hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF1), nerve growth factor (NGF), and stromal-derived growth factor-1 (SDF1)). [64] Exosomes secreted by human circulating fibrocytes, a population of mesenchymal progenitors involved in normal wound healing via paracrine signaling, exhibited in-vitro proangiogenic properties, activated diabetic dermal fibroblasts, induced the migration and proliferation of diabetic keratinocytes, and accelerated wound closure in diabetic mice in vivo. Important components of the exosomal cargo were heat

shock protein-90α, total and activated signal transducer and activator of transcription 3, proangiogenic (miR-126, miR-130a, miR-132) and anti-inflammatory (miR124a, miR-125b) microRNAs, and a microRNA regulating collagen deposition (miR-21). [65] Researchers have also found that exosomes released from oral keratinocytes can accelerate wound healing, even when human exosomes were applied to rat wounds. [66] Exosomes can be considered a promising carrier for effective delivery of small interfering RNA due to their existence in body's endogenous system and high tolerance. [67, 68] Patient-derived exosomes have been employed as a novel cancer immunotherapy in several clinical trials. [69]

Exosomes offer distinct advantages that uniquely position them as highly effective drug carriers. Composed of cellular membranes with multiple adhesive proteins on their surface, exosomes are known to specialize in cell–cell communications and provide an exclusive approach for the delivery of various therapeutic agents to target cells. [70] For example, researchers used exosomes as a vehicle for the delivery of cancer drug paclitaxel. They placed the drug inside exosomes derived from white blood cells, which were then injected into mice with drug-resistant lung cancer. Importantly, incorporation of paclitaxel into exosomes increased cytotoxicity more than 50 times as a result of nearly complete co-localization of airway-delivered exosomes with lung cancer cells. [71]

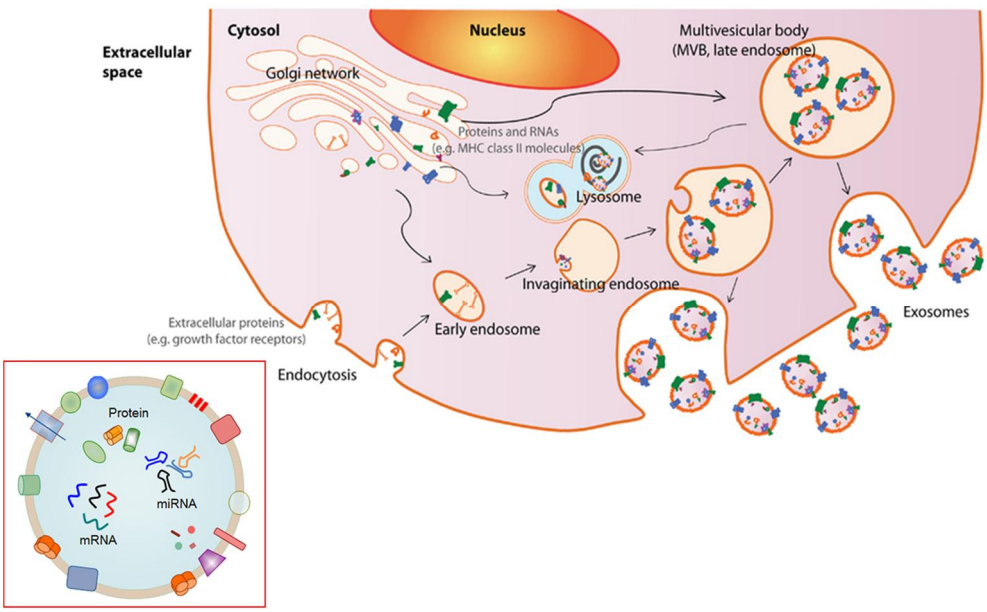


Figure 1.4 Biogenesis and secretion of exosome. Exosomes are released by cells when intracellular organelles called multivesicular bodies (MVBs) fuse with the plasma membrane.

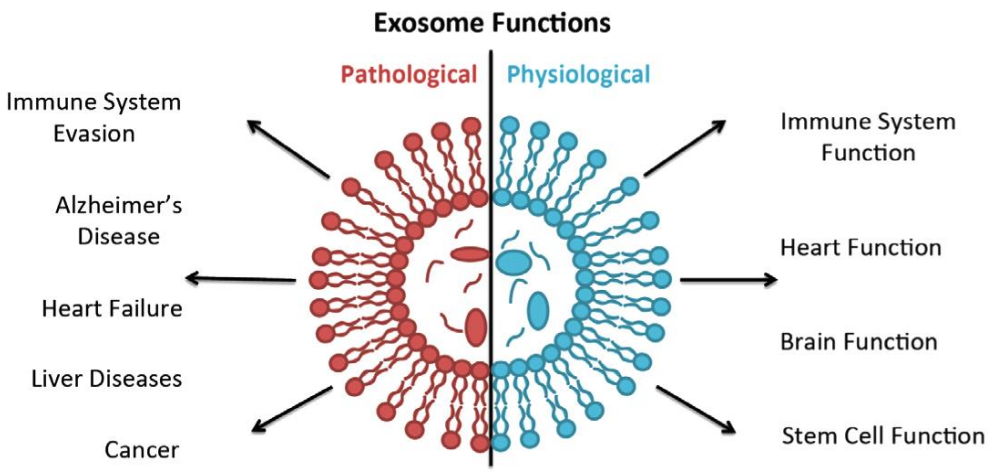


Figure 1.5 Role of the exosome.

1.3. Objective of the thesis

Exosomes are small extracellular vesicles with 30–100 nm diameter that originate from many type of cells. Exosomes serve as a role of intercellular messenger unit by inward budding and scission of vesicles from the limiting endosomal membranes and released from the MVB lumen into the extracellular environment during exocytosis.

To obtain high quality exosome in high yield and use them in fresh condition by optimizing the storage condition, we isolated the exosomes after incubation in various pH conditions and analyzed the recovery yield of exosomes, and we examined the stability of exosomes at various storage temperatures. We have discovered that acidic condition was favorable for the high yield for exosome isolation, and the temperature below -70 °C is crucial for the preservation of exosomes in fresh condition for clinical and basic applications.

After optimizing the isolation and storage conditions for the exosome, we examined that the human adipose stem cell derived exosome (ASC-exo) has a therapeutic potential for treating Alzheimer's disease (AD), Huntington's disease (HD), Amyotrophic lateral sclerosis (ALS) by modulating representative cellular phenotypes.

We proved that ASC-exo ameliorate the progression of Aβ-induced neuronal death in AD, and ASC-exo modulates SOD-1 aggregation and mitochondrial dysfunction, making it a therapeutic candidate for ALS. In HD, we confirmed that the ASC-exo up-regulates PGC1, phospho-CREB and ameliorates abnormal apoptotic protein level, and reduces mHtt aggregates level. In addition, MitoSOX Red, JC-1 and cell viability assay showed that ASC-exo reduces mitochondrial dysfunction and cell apoptosis. These findings suggest that ASC-exo has a therapeutic potential for treating neurodegenerative diseases by modulating representative cellular phenotypes.

Next focus is ameliroration of HD by heterochronic parabiosis (HP) which have been reported by a few paper in AD, presenting that exposure to a young blood circulation through HP reverses cognitive deficits observed with normal aging. To determine the effect of young circulatory factors on HD-like disease in R6/2 mice, we used HP, in which we joined the circulatory systems of young, wildtype animals together with R6/2 mice which express exon1 of the human HD gene with around 150CAG repeats. We analyzed the changes in protein expression in the brains of these mice with established disease after heterochronic parabiosis, and we examined the effects of young serum exosome on key phenotypes of HD including p-CREB-PGC-1 pathway, apoptotic proteins, cell death and mitocondria dysfunction in R6/2-derived neuronal cells.

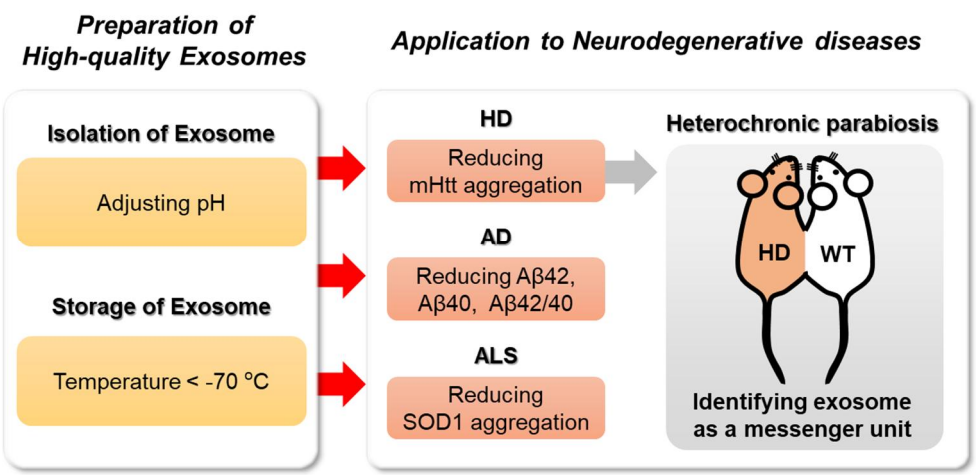


Figure 1.6 Schematics for the objective of the thesis

1.4. Organization of the thesis

This thesis consists of nine chapters. The principles of neurodegenerative diseases, process about parabiosis, and the analysis techniques are reviewed in Chapter 2. In Chapter 3, effect of pH on the yield of exosome isolation is discussed. The yield of total exosomal protein and RNA after incubation in acidic, neutral, and alkaline conditioned medium are studied. As a result, the concentrations of exosomal protein and nucleic acid were increased after incubation in the acidic medium. Chapter 4 discusses the influence of storage condition on exosome recovery. The temperature dependence of both the short-term stability and long-term stability is studied. The termperature below -70 ^{o}C was favorable condition for preservation of exosomes at fresh condition while incubation at 4 ^{o}C and RT resulted in major loss of CD63. Chapter 5 discusses the amelioration of HD phenotype in vitro model by ASC-exo. ASC-exo decreased mHtt aggregates in R6/2 mice-derived neuronal cells, and it up-regulates PGC-1, phospho-CREB, and abnormal apoptotic protein level. Chapter 6 discusses alleviation of ALS pathology by ASC-exo. ASC-exo was treated to the neuronal stem cells isolated from G93A ALS mice model. ASC-exo alleviated aggregation of SOD1 and decreased the phospho-CREB and PGC-1α. Chapter 7 discusses about reducing β-amyloid pathology and apoptosis of neuronal cells derived from the transgenic mouse model of AD by ASC-exo. ASC-exo was treated to the neuronal stem cells from the brains of TG2576 AD mice. ASC-exo treatment resulted in reduced Aβ42 levels, Aβ40 levels, and the Aβ42/40 ratio of AD cells. Also, apoptotic molecules, p53, Bax, pro-caspase-3 and cleaved-caspase-3, and Bcl-2 protein level were normalized by ASC-exo treatment. Chapter 8 discusses the

alleviation of Huntington's disease by heterochronic parabiosis (HP). Parabiosis between the young wild type mouse and HD mouse was performed, and the alleviation of HD pathology was observed after the parabiosis. Furthermore, the exosomes extracted from the young wild type mouse showed the alleviation of HD pathology in vitro model, indicating that the exosome is a messenger unit for the positive factors during the heterochronic parabiosis. Chapter 9 summarizes this study and opens the possibilities for adopting the exosomes to curing various neurodegenerative diseases.

CHAPTER 2

Theoretical background

2.1. Neurodegenerative diseases

2.1.1. Huntington's disease (HD)

Huntington's disease (HD), also known as Huntington's chorea, is an inherited disorder that results in the death of brain cells. [1] The earliest symptoms are often subtle problems with mood or mental abilities. [2] A general lack of coordination and an unsteady gait often follow. [3] As the disease advances, uncoordinated, jerky body movements become more apparent. [2] Physical abilities gradually worsen until coordinated movement becomes difficult and the person is unable to talk. [2, 3] Mental abilities generally decline into dementia. [4] The specific symptoms vary somewhat between people. [2] Symptoms usually begin between 30 and 50 years of age, but can start at any age. [4-5] The disease may develop earlier in life in each successive generation. [2] About eight percent of cases start before the age of 20 years and typically present with symptoms more similar to Parkinson's disease.[4] People with HD often underestimate the degree of their problems. [2]

HD is typically inherited, although up to 10% of cases are due to a new mutation. [2] The disease is caused by an autosomal dominant mutation in either of an individual's

two copies of a gene called Huntingtin. [5] This means a child of an affected person typically has a 50% chance of inheriting the disease. [5] The Huntingtin gene provides the genetic information for a protein that is also called "huntingtin". [2] Expansion of CAG (cytosine-adenine-guanine) triplet repeats in the gene coding for the Huntingtin protein results in an abnormal protein, which gradually damages cells in the brain, through mechanisms that are not fully understood. [5] Diagnosis is by genetic testing, which can be carried out at any time, regardless of whether or not symptoms are present. [6] This fact raises several ethical debates: the age at which an individual is considered mature enough to choose testing; whether parents have the right to have their children tested; and managing confidentiality and disclosure of test results. [3]

There is no cure for HD. [5] Full-time care is required in the later stages of the disease. [3] Treatments can relieve some symptoms and in some improve quality of life. [4] The best evidence for treatment of the movement problems is with tetrabenazine. [4] HD affects about 4 to 15 in 100,000 people of European descent. [2, 4] It is rare among Japanese, while the occurrence rate in Africa is unknown. [4] The disease affects men and women equally. [4] Complications such as pneumonia, heart disease, and physical injury from falls reduce life expectancy. [4] Suicide is the cause of death in about 9% of cases. [4] Death typically occurs fifteen to twenty years from when the disease was first detected. [5]

The first likely description of the disease was in 1841 by Charles Oscar Waters. [6] The condition was described in further detail in 1872 by the physician George Huntington, after whom it is named. [6] The genetic basis was discovered in 1993 by an international collaborative effort led by the Hereditary Disease Foundation. [7, 8] Research and support organizations began forming in the late 1960s to increase public awareness, to provide support for individuals and their families, and to promote

research. [8] Current research directions include determining the exact mechanism of the disease, improving animal models to aid with research, testing of medications to treat symptoms or slow the progression of the disease, and studying procedures such as stem cell therapy with the goal of repairing damage caused by the disease. [7]

Symptoms of HD most commonly become noticeable between the ages of 35 and 44 years, but they can begin at any age from infancy to old age. [10, 11] In the early stages, there are subtle changes in personality, cognition, and physical skills. [10] The physical symptoms are usually the first to be noticed, as cognitive and behavioral symptoms [12] are generally not severe enough to be recognized on their own at the earlier stages. [10] Almost everyone with HD eventually exhibits similar physical symptoms, but the onset, progression and extent of cognitive and behavioral symptoms vary significantly between individuals. [13, 14]

The most characteristic initial physical symptoms are jerky, random, and uncontrollable movements called chorea. [10] Chorea may be initially exhibited as general restlessness, small unintentionally initiated or uncompleted motions, lack of coordination, or slowed saccadic eye movements. [10] These minor motor abnormalities usually precede more obvious signs of motor dysfunction by at least three years. [13] The clear appearance of symptoms such as rigidity, writhing motions or abnormal posturing appear as the disorder progresses. [10] These are signs that the system in the brain that is responsible for movement has been affected. [15] Psychomotorfunctions become increasingly impaired, such that any action that requires muscle control is affected. Common consequences are physical instability, abnormal facial expression, and difficulties chewing, swallowing, and speaking. [10] Eating difficulties commonly cause weight loss and may lead to malnutrition. [16, 17] Sleep disturbances are also associated symptoms. [18] Juvenile HD differs from these

symptoms in that it generally progresses faster and chorea is exhibited briefly, if at all, with rigidity being the dominant symptom. Seizures are also a common symptom of this form of HD. [10]

Cognitive abilities are progressively impaired. [15] Especially affected are executive functions, which include planning, cognitive flexibility, abstract thinking, rule acquisition, initiation of appropriate actions, and inhibition of inappropriate actions. [15] As the disease progresses, memory deficits tend to appear. Reported impairments range from short-term memory deficits to long-term memory difficulties, including deficits in episodic (memory of one's life), procedural (memory of the body of how to perform an activity) and working memory.[15] Cognitive problems tend to worsen over time, ultimately leading to dementia.[15] This pattern of deficits has been called a subcortical dementia syndrome to distinguish it from the typical effects of cortical dementias e.g. Alzheimer's disease.[15]

Reported neuropsychiatric manifestations are anxiety, depression, a reduced display of emotions (blunted affect), egocentrism, aggression, and compulsive behavior, the latter of which can cause or worsen addictions, including alcoholism, gambling, and hypersexuality.[9] Difficulties in recognizing other people's negative expressions have also been observed.[15] The prevalence of these symptoms is highly variable between studies, with estimated rates for lifetime prevalence of psychiatric disorders between 33% and 76%.[9] For many sufferers and their families, these symptoms are among the most distressing aspects of the disease, often affecting daily functioning and constituting reason for institutionalization.[9] Suicidal thoughts and suicide attempts are more common than in the general population.[10] Often individuals have reduced awareness of chorea, cognitive and emotional impairments.[19]

Mutant Huntingtin is expressed throughout the body and associated with abnormalities in peripheral tissues that are directly caused by such expression outside the brain. These abnormalities include muscle atrophy, cardiac failure, impaired glucose tolerance, weight loss, osteoporosis, and testicular atrophy.[20]

All humans have two copies of the Huntingtin gene (HTT), which codes for the protein Huntingtin (HTT). The gene is also called HD and IT15, which stands for 'interesting transcript 15'. Part of this gene is a repeated section called a trinucleotide repeat, which varies in length between individuals and may change length between generations. If the repeat is present in a healthy gene, a dynamic mutation may increase the repeat count and result in a defective gene. When the length of this repeated section reaches a certain threshold, it produces an altered form of the protein, called mutant Huntingtin protein (mHTT). The differing functions of these proteins are the cause of pathological changes which in turn cause the disease symptoms. The Huntington's disease mutation is genetically dominant and almost fully penetrant: mutation of either of a person's HTT alleles causes the disease. It is not inherited according to sex, but the length of the repeated section of the gene and hence its severity can be influenced by the sex of the affected parent.[10]

Generally, people have fewer than 36 repeated glutamines in the polyQ region which results in production of the cytoplasmic protein Huntingtin.[10] However, a sequence of 36 or more glutamines results in the production of a protein which has different characteristics.[10] This altered form, called mutant huntingtin (mHTT), increases the decay rate of certain types of neurons. Regions of the brain have differing amounts and reliance on these types of neurons, and are affected accordingly.[10] Generally, the number of CAG repeats is related to how much this process is affected, and accounts for about 60% of the variation of the age of the onset

of symptoms. The remaining variation is attributed to environment and other genes that modify the mechanism of HD.[10] 36–39 repeats result in a reduced-penetrance form of the disease, with a much later onset and slower progression of symptoms. In some cases the onset may be so late that symptoms are never noticed.[10] With very large repeat counts, HD has full penetrance and can occur under the age of 20, when it is then referred to as juvenile HD, akinetic-rigid, or Westphal variant HD. This accounts for about 7% of HD carriers.[21]

HD affects the whole brain, but certain areas are more vulnerable than others. The most prominent early effects are in a part of the basal ganglia called the neostriatum, which is composed of the caudate nucleus and putamen.[10] Other areas affected include the substantia nigra, layers 3, 5 and 6 of the cerebral cortex, the hippocampus, purkinje cells in the cerebellum, lateral tuberal nuclei of the hypothalamus and parts of the thalamus.[10] These areas are affected according to their structure and the types of neurons they contain, reducing in size as they lose cells. [10] Striatal spiny neurons are the most vulnerable, particularly ones with projections towards the external globus pallidus, with interneurons and spiny cells projecting to the internal pallidum being less affected.[10, 22] HD also causes an abnormal increase in astrocytes and activation of the brain's immune cells, microglia.[23]

The basal ganglia—the part of the brain most prominently affected in early HD—play a key role in movement and behavior control. Their functions are not fully understood, but current theories propose that they are part of the cognitive executive system[15] and the motor circuit.[24] The basal ganglia ordinarily inhibit a large number of circuits that generate specific movements. To initiate a particular movement, the cerebral cortex sends a signal to the basal ganglia that causes the inhibition to be released. Damage to the basal ganglia can cause the release or

reinstatement of the inhibitions to be erratic and uncontrolled, which results in an awkward start to motion or motions to be unintentionally initiated, or a motion to be halted before, or beyond, its intended completion. The accumulating damage to this area causes the characteristic erratic movements associated with HD.[24] The spontaneous and erratic physical movements associated with HD are classified as a type of hyperkinetic dysarthria. Because of the basal ganglia's inability to inhibit movements, individuals affected by it will inevitably experience a reduced ability to produce speech and swallow foods and liquids (dysphagia).[25]

Medical diagnosis of the onset of HD can be made following the appearance of physical symptoms specific to the disease.[10] Genetic testing can be used to confirm a physical diagnosis if there is no family history of HD. Even before the onset of symptoms, genetic testing can confirm if an individual or embryo carries an expanded copy of the trinucleotide repeat in the HTT gene that causes the disease. Genetic counseling is available to provide advice and guidance throughout the testing procedure, and on the implications of a confirmed diagnosis. These implications include the impact on an individual's psychology, career, family planning decisions, relatives and relationships. Despite the availability of pre-symptomatic testing, only 5% of those at risk of inheriting HD choose to do so.[10]

There is no cure for HD, but there are treatments available to reduce the severity of some of its symptoms.[26] For many of these treatments, evidence to confirm their effectiveness in treating symptoms of HD specifically are incomplete.[10, 27] As the disease progresses the ability to care for oneself declines, and carefully managed multidisciplinary caregiving becomes increasingly necessary.[10] Although there have been relatively few studies of exercises and therapies that help rehabilitate cognitive symptoms of HD, there is some evidence for the usefulness of physical therapy,

occupational therapy, and speech therapy.[10] An association between caffeine intake and earlier age of onset in Huntington's disease has been found,[28, 29] but, since this finding was based on retrospective questionnaire data rather than a blinded, randomized trial or case-control study, this work is a poor basis for guiding lifestyle decisions.[30]

The length of the trinucleotide repeat accounts for 60% of the variation in the age symptoms appear and the rate they progress. A longer repeat results in an earlier age of onset and a faster progression of symptoms.[10, 31] Individuals with more than sixty repeats often develop the disease before age 20, while those with fewer than 40 repeats may not ever develop noticeable symptoms.[32] The remaining variation is due to environmental factors and other genes that influence the mechanism of the disease.[10]

Life expectancy in HD is generally around 20 years following the onset of visible symptoms.[10] Most life-threatening complications result from muscle coordination and, to a lesser extent, behavioral changes induced by declining cognitive function. The largest risk is pneumonia, which causes death in one third of those with HD. As the ability to synchronize movements deteriorates, difficulty clearing the lungs and an increased risk of aspirating food or drink both increase the risk of contracting pneumonia. The second greatest risk is heart disease, which causes almost a quarter of fatalities of those with HD.[10] Suicide is the third greatest cause of fatalities, with 7.3% of those with HD taking their own lives and up to 27% attempting to do so. It is unclear to what extent suicidal thoughts are influenced by behavioral symptoms, as they signify sufferers' desires to avoid the later stages of the disease.[33 - 35] Other associated risks include choking, physical injury from falls, and malnutrition.[10]

2.1.2. Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS), also known as motor neurone disease (MND) or Lou Gehrig's disease, is a specific disease that causes the death of neurons controlling voluntary muscles.[36][37] Some also use the term motor neuron disease for a group of conditions of which ALS is the most common.[38] ALS is characterized by stiff muscles, muscle twitching, and gradually worsening weakness due to muscles decreasing in size.[38] It may begin with weakness in the arms or legs, or with difficulty speaking or swallowing.[39] About half of the people affected develop at least mild difficulties with thinking and behavior and most people experience pain.[40][41] Most eventually lose the ability to walk, use their hands, speak, swallow, and breathe.[42] The cause is not known in 90% to 95% of cases,[36] but is believed to involve both genetic and environmental factors.[43] The remaining 5–10% of cases are inherited from a person's parents.[44] About half of these genetic cases are due to one of two specific genes.[36] The underlying mechanism involves damage to both upper and lower motor neurons.[38] The diagnosis is based on a person's signs and symptoms, with testing done to rule out other potential causes.[36] No cure for ALS is known.[36] The goal of treatment is to improve symptoms.[40] A medication called riluzole may extend life by about two to three months.[45] Non-invasive ventilation may result in both improved quality and length of life.[42] Mechanical ventilation can prolong survival but does not stop disease progression.[46] A feeding tube may

help.[47] The disease can affect people of any age, but usually starts around the age of 60 and in inherited cases around the age of 50.[44] The average survival from onset to death is two to four years, though this can vary.[42][47] About 10% survive longer than 10 years.[36]Most die from respiratory failure.[44] In Europe, the disease affects about two to three people per 100,000 per year.[48] Rates in much of the world are unclear.[49] In the United States, it is more common in white people than black people.[50] ALS is a motor neuron disease, also spelled "motor neurone disease", which is a group of neurological disorders that selectively affect motor neurons, the cells that control voluntary muscles of the body.[38] Motor neuron diseases include amyotrophic lateral sclerosis (ALS), primary lateral sclerosis (PLS), progressive muscular atrophy (PMA), progressive bulbar palsy, pseudobulbar palsy, and monomelic amyotrophy (MMA).[51] ALS itself can be classified a few different ways: by how fast the disease progresses (slow vs fast progressors), by whether it is inherited or sporadic, and by where it starts.[36] In about 25% of cases, muscles in the face, mouth, and throat are affected first, because motor neurons in the part of the brain stem called the medulla oblongata (formerly called the "bulb") start to die first along with lower motor neurons. This form is called "bulbar onset". In about 5% of cases muscles in the trunk of the body are affected first.[44] In most cases the disease spreads and affects other spinal cord regions. A few people with ALS have symptoms that are limited to one spinal cord region for at least 12 to 24 months before spreading to a second region; these regional variants of ALS are associated with a better prognosis.[52] The disorder causes muscle weakness, atrophy, and muscle spasms throughout the body due to the degeneration of the upper motor and lower motor neurons. Individuals affected by the disorder may ultimately lose the ability to initiate and control all voluntary movement,[42] although bladder and bowel function and the

extraocular muscles (the muscles responsible for eye movement) are usually spared[53] until the final stages of the disease.[54] Cognitive or behavioral dysfunction is present in 30–50% of individuals with ALS.[55] Around half of people with ALS will experience mild changes in cognition and behavior, and 10–15% will show signs of frontotemporal dementia.[42] Repeating phrases or gestures, apathy, and loss of inhibition are frequently reported behavioral features of ALS.[56] Language dysfunction, executive dysfunction, and troubles with social cognition and verbal memory are the most commonly reported cognitive symptoms in ALS; a meta-analysis found no relationship between dysfunction and disease severity.[57] However, cognitive and behavioral dysfunctions have been found to correlate with reduced survival in people with ALS and increased caregiver burden; this may be due in part to deficits in social cognition.[57] About half the people who have ALS experience emotional lability, in which they cry or laugh for no reason; it is more common in those with bulbar-onset ALS.[42] Pain is a symptom experienced by most people with ALS and can take the form of neuropathic pain (pain caused by nerve damage), spasticity, muscle cramps, and nociceptive pain caused by reduced mobility and muscle weakness; examples of nociceptive pain in ALS include contractures (permanent shortening of a muscle or joint), neck pain, back pain, shoulder pain, and pressure ulcers.[41] Sensory nerves and the autonomic nervous system are generally unaffected, meaning the majority of people with ALS maintain hearing, sight, touch, smell, and taste.[36] Though the exact cause of ALS is unknown, genetic factors and environmental factors are thought to be of roughly equal importance.[43] The genetic factors are better understood than the environmental factors; no specific environmental factor has been definitively shown to cause ALS. A liability threshold model for ALS proposes that cellular damage accumulates over time due to genetic

factors present at birth and exposure to environmental risks throughout life.[49] The defining feature of ALS is the death of both upper motor neurons (located in the motor cortex of the brain) and lower motor neurons (located in the brainstem and spinal cord).[58] In ALS with frontotemporal dementia, neurons throughout the frontal and temporal lobes of the brain die as well.[54] The pathological hallmark of ALS is the presence of inclusion bodies (abnormal aggregations of protein) in the cytoplasm of motor neurons. In about 97% of people with ALS, the main component of the inclusion bodies is TDP-43 protein;[39] however, in those with SOD1 or FUS mutations, the main component is SOD1 protein or FUS protein, respectively.[59] The gross pathology of ALS, which are features of the disease that can be seen with the naked eye, include skeletal muscle atrophy, motor cortex atrophy, sclerosis of the corticospinal and corticobulbar tracts, thinning of the hypoglossal nerves (which control the tongue), and thinning of the anterior roots of the spinal cord.[39] Besides for the death of motor neurons, two other characteristics common to most ALS variants are focal initial pathology, meaning that symptoms start in a single spinal cord region, and progressive continuous spread, meaning that symptoms spread to additional regions over time. Prion-like propagation of misfolded proteins from cell to cell may explain why ALS starts in one area and spreads to others.[59] No test can provide a definite diagnosis of ALS, although the presence of upper and lower motor neuron signs in a single limb is strongly suggestive.[55] Instead, the diagnosis of ALS is primarily based on the symptoms and signs the physician observes in the person and a series of tests to rule out other diseases.[55] Physicians obtain the person's full medical history and usually conduct a neurologic examination at regular intervals to assess whether symptoms such as muscle weakness, atrophy of muscles, hyperreflexia,

and spasticity are worsening.[55] A number of biomarkers are being studied for the condition, but so far are not in general medical use.[60][61]

2.1.3. Alzheimer's disease (AD)

Alzheimer's disease (AD), also referred to simply as Alzheimer's, is a chronic neurodegenerative disease that usually starts slowly and gradually worsens over time.[62][63] It is the cause of 60–70% of cases of dementia.[62][63] The most common early symptom is difficulty in remembering recent events.[62] As the disease advances, symptoms can include problems with language, disorientation (including easily getting lost), mood swings, loss of motivation, not managing self care, and behavioural issues.[62][63] As a person's condition declines, they often withdraw from family and society.[62] Gradually, bodily functions are lost, ultimately leading to death.[64] Although the speed of progression can vary, the typical life expectancy following diagnosis is three to nine years.[65][66] The cause of Alzheimer's disease is poorly understood.[62] About 70% of the risk is believed to be inherited from a person's parents with many genes usually involved.[67] Other risk factors include a history of head injuries, depression, and hypertension.[62] The disease process is associated with plaques and neurofibrillary tangles in the brain.[67] A probable diagnosis is based on the history of the illness and cognitive testing with medical imaging and blood tests to rule out other possible causes.[68] Initial symptoms are often mistaken for normal ageing.[62] Examination of brain tissue is needed for a definite diagnosis.[67]Mental and physical exercise, and avoiding obesity may decrease the risk of AD; however, evidence to support these recommendations is weak.[67][69] There are no medications or supplements that have been shown to

decrease risk.[70] No treatments stop or reverse its progression, though some may temporarily improve symptoms.[63] Affected people increasingly rely on others for assistance, often placing a burden on the caregiver.[71] The pressures can include social, psychological, physical, and economic elements.[71] Exercise programs may be beneficial with respect to activities of daily living and can potentially improve outcomes.[72] Behavioural problems or psychosis due to dementia are often treated with antipsychotics, but this is not usually recommended, as there is little benefit with an increased risk of early death.[73][74] In 2015, there were approximately 29.8 million people worldwide with AD.[63][75] It most often begins in people over 65 years of age, although 4–5% of cases are early-onset Alzheimer's. It affects about 6% of people 65 years and older.[62] In 2015, dementia resulted in about 1.9 million deaths.[76] It was first described by, and later named after, German psychiatrist and pathologist Alois Alzheimer in 1906.[77] In developed countries, AD is one of the most financially costly diseases.[78][79] The cause for most Alzheimer's cases is still mostly unknown except for 1% to 5% of cases where genetic differences have been identified.[80][81] for example, In 1991, the amyloid hypothesis postulated that extracellular amyloid beta (Aβ) deposits are the fundamental cause of the disease.[82][83] Support for this postulate comes from the location of the gene for the amyloid precursor protein (APP) on chromosome 21, together with the fact that people with trisomy 21 (Down Syndrome) who have an extra gene copy almost universally exhibit at least the earliest symptoms of AD by 40 years of age.[84][85] Also, a specific isoform of apolipoprotein, APOE4, is a major genetic risk factor for AD. While apolipoproteins enhance the breakdown of beta amyloid, some isoforms are not very effective at this task (such as APOE4), leading to excess amyloid buildup in the brain.[86] Further evidence comes from the finding that transgenic mice that

express a mutant form of the human APP gene develop fibrillar amyloid plaques and Alzheimer's-like brain pathology with spatial learning deficits.[87] An experimental vaccine was found to clear the amyloid plaques in early human trials, but it did not have any significant effect on dementia.[88] Researchers have been led to suspect non-plaque Aβ oligomers (aggregates of many monomers) as the primary pathogenic form of Aβ. These toxic oligomers, also referred to as amyloid-derived diffusible ligands (ADDLs), bind to a surface receptor on neurons and change the structure of the synapse, thereby disrupting neuronal communication.[89] One receptor for Aβoligomers may be the prion protein, the same protein that has been linked to mad cow disease and the related human condition, Creutzfeldt–Jakob disease, thus potentially linking the underlying mechanism of these neurodegenerative disorders with that of Alzheimer's disease.[90] In 2009, this theory was updated, suggesting that a close relative of the beta-amyloid protein, and not necessarily the beta-amyloid itself, may be a major culprit in the disease. The theory holds that an amyloid-related mechanism that prunes neuronal connections in the brain in the fast-growth phase of early life may be triggered by ageing-related processes in later life to cause the neuronal withering of Alzheimer's diseases.[91] N-APP, a fragment of APP from the peptide's N-terminus, is adjacent to beta-amyloid and is cleaved from APP by one of the same enzymes. N-APP triggers the self-destruct pathway by binding to a neuronal receptor called death receptor 6 (DR6, also known as TNFRSF21).[91] DR6 is highly expressed in the human brain regions most affected by Alzheimer's, so it is possible that the N-APP/DR6 pathway might be hijacked in the ageing brain to cause damage. In this model, beta-amyloid plays a complementary role, by depressing synaptic function. In early 2017, a trial of verubecestat, which inhibits the beta-secretase protein responsible for creating beta-amyloid protein was discontinued as an

independent panel found "virtually no chance of finding a positive clinical effect".[92] In 2018 and 2019, more trials, including aducanumab which reduce amyloid beta concentrations, failed leading some to question the validity of the amyloid hypothesis.[93][94] The tau hypothesis proposes that tau protein abnormalities initiate the disease cascade.[83] In this model, hyperphosphorylated tau begins to pair with other threads of tau. Eventually, they form neurofibrillary tangles inside nerve cell bodies.[95] When this occurs, the microtubules disintegrate, destroying the structure of the cell's cytoskeleton which collapses the neuron's transport system.[96] This may result first in malfunctions in biochemical communication between neurons and later in the death of the cells.[97] Alzheimer's disease is characterised by loss of neurons and synapses in the cerebral cortex and certain subcortical regions. This loss results in gross atrophy of the affected regions, including degeneration in the temporal lobe and parietal lobe, and parts of the frontal cortex and cingulate gyrus.[98] Degeneration is also present in brainstem nuclei like the locus coeruleus.[99] Studies using MRI and PET have documented reductions in the size of specific brain regions in people with AD as they progressed from mild cognitive impairment to Alzheimer's disease, and in comparison with similar images from healthy older adults.[100][101] Both amyloid plaques and neurofibrillary tangles are clearly visible by microscopy in brains of those afflicted by AD.[102] Plaques are dense, mostly insoluble deposits of beta amyloid peptide and cellular material outside and around neurons. Tangles (neurofibrillary tangles) are aggregates of the microtubule-associated protein tau which has become hyperphosphorylated and accumulate inside the cells themselves. Although many older individuals develop some plaques and tangles as a consequence of ageing, the brains of people with AD have a greater number of them in specific brain regions such as the temporal lobe.[103] Lewy bodies are not rare in the brains of

people with AD.[104] Alzheimer's disease is usually diagnosed based on the person's medical history, history from relatives, and behavioural observations. The presence of characteristic neurological and neuropsychological features and the absence of alternative conditions is supportive.[105][106] Advanced medical imaging with computed tomography (CT) or magnetic resonance imaging (MRI), and with single-photon emission computed tomography (SPECT) or positron emission tomography (PET) can be used to help exclude other cerebral pathology or subtypes of dementia.[107] Moreover, it may predict conversion from prodromal stages (mild cognitive impairment) to Alzheimer's disease.[108] Assessment of intellectual functioning including memory testing can further characterise the state of the disease.[109] Medical organisations have created diagnostic criteria to ease and standardise the diagnostic process for practising physicians. The diagnosis can be confirmed with very high accuracy post-mortem when brain material is available and can be examined histologically.[110] There is no definitive evidence to support that any particular measure is effective in preventing AD.[70] Global studies of measures to prevent or delay the onset of AD have often produced inconsistent results. Epidemiological studies have proposed relationships between certain modifiable factors, such as diet, cardiovascular risk, pharmaceutical products, or intellectual activities among others, and a population's likelihood of developing AD. Only further research, including clinical trials, will reveal whether these factors can help to prevent AD.[70] There is no cure for Alzheimer's disease; available treatments offer relatively small symptomatic benefit but remain palliative in nature. Current treatments can be divided into pharmaceutical, psychosocial and caregiving. The early stages of Alzheimer's disease are difficult to diagnose. A definitive diagnosis is usually made once cognitive impairment compromises daily living activities, although the person

may still be living independently. The symptoms will progress from mild cognitive problems, such as memory loss through increasing stages of cognitive and non-cognitive disturbances, eliminating any possibility of independent living, especially in the late stages of the disease.[111] Life expectancy of people with AD is reduced.[112] Following diagnosis it typically ranges from three to ten years.[112] Fewer than 3% of people live more than fourteen years.[113] Disease features significantly associated with reduced survival are an increased severity of cognitive impairment, decreased functional level, history of falls, and disturbances in the neurological examination. Other coincident diseases such as heart problems, diabetes or history of alcohol abuse are also related with shortened survival.[114-116] While the earlier the age at onset the higher the total survival years, life expectancy is particularly reduced when compared to the healthy population among those who are younger.[117] Men have a less favourable survival prognosis than women.[113][118] Pneumonia and dehydration are the most frequent immediate causes of death brought by AD, while cancer is a less frequent cause of death than in the general population.[118]

2.2. Parabiosis

2.2.1. Surgical process of parabiosis

Parabiosis, meaning "living beside", is a technical term in various contexts in fields of study related to ecology and physiology. It accordingly has been defined independently in at least three disciplines, namely experimental or medical physiology, the ecology of inactive physiological states, and the ecology of certain classes of social species that share nests. In the field of experimental physiology, parabiosis is a class of techniques in which two living organisms are joined together surgically and develop single, shared physiological systems, such as a shared circulatory system. [119][120] Through surgically connecting two animals, researchers can prove that the feedback system in one animal is circulated and affects the second animal via blood and serum exchange. Total blood volume is exchanged approximately ten times per day in rat experiments using parabiosis. One limitation of the experiments is that outbred rats cannot be used because it can lead to a significant loss of pairs due to intoxication of the blood supply from a dissimilar rat. [121] In the mid-1800s, parabiotic experiments were pioneered by Paul Bert. He postulated that surgically connected animals could share a circulatory system. Bert was awarded the Prize of Experimental Physiology of the French Academy of Science in 1866 for his discoveries. Parabiotic experiments were scarcely revisited until the 20th century. [122] Many of the parabiotic experiments since 1950 involve research regarding metabolism. One of these experiments was published in 1959 by G. R. Hervey in the Journal of Physiology. This experiment was to support the theory that damage to the hypothalamus, particularly the ventromedial hypothalamus, leads to obesity caused by the overconsumption of food. This results from the ventromedial hypothalamus failing

to respond to physiological signals that suppress appetite. The result is attributed to the feedback control system in the brain. Rats in the study were from the same litter, which had been a closed colony for multiple years. The two rats in each pair had no more than 3% difference in weight. Rats were paired at four weeks old. Unpaired rats were used as controls. The rats were conjoined in three ways. In early experiments, the peritoneal cavities were opened and connected between the two rats. In later experiments, to avoid the risk of tangling the two rats' intestines together, smaller cuts were made. After further refinement of the experimental procedure, the abdominal cavities were not opened and the rats were conjoined at the hip bone with minimal cutting. In order to prove that the two animals were sharing blood, researchers injected dye into the veins of one rat and the pigment would show up in the conjoined rat. The scientists refined the lesion placement by practicing the procedure on other rats. The rats were killed with ether and weighed at the conclusion of the experiment and the amount of fat in each animal was quantified. Many of the parabiotic pairs died throughout the experiment before its conclusion. In each pair, one rat became obese and exhibited hyperphagia. The weight of the rat with the surgical lesion rose rapidly for a few months, then reached a plateau as a direct result of the surgical procedure. After the procedure, the rat with the impaired hypothalamus voraciously ate and the paired rat decreased their appetite. The paired rat became obviously thin throughout the experiment, even rejecting food when it was offered. Some of the paired rats starved to death. Lesions subsequently made in the hypothalamus of two paired rats resulted in hyperphagia and obesity. That result verifies that the paired rat decreased its eating in a direct response to the signals in the blood from the rat with the lesion. If its brain was similarly altered, it also ate voraciously and became obese. The control rats who were given surgical lesions in the hypothalamus became similarly obese to

the parabiotic counterparts. [123] Later studies linked the effects of the previous parabiotic experiments about metabolism to the discovery of leptin. Many hormones and metabolites were proven to not be the satiety factor that caused one rat to starve in the experiments. Leptin seemed like a viable candidate. Starting in 1977, Ruth B.S. Harris, a graduate student under Hervey, repeated previous studies about parabiosis in rats and mice. Due to the discovery of leptin, she analyzed leptin concentrations of the mice in the parabiotic experiments. After injecting leptin into the obese mouse of each pair, she found that leptin circulated between the conjoined animals, but the circulation of leptin took some time to reach equilibrium. As a result of the injections, almost immediate weight loss resulted in the parabiotic pairs due to increased inhibition. Approximately 50–70% of fat was lost in the pairs. The obese mouse lost only fat. The lean mouse lost muscle mass and fat. Harris concluded that leptin levels are increased in the obese animal, but other factors could also affect the animals. Also, leptin was determined to decrease fat storage in both the obese and thin animals. [121] Parabiotic experiments have also been used to study diabetes. Douglas Coleman did further parabiotic experiments to determine diabetic chromosomal relationships. A major gene that causes obesity in mice was identified on chromosome 6. The obese gene (ob/ob) was determined from one mutant mouse, discovered in 1950. Coleman and researchers further identified a gene on chromosome 4 that led to hyperphagia and obesity in mice. The gene also correlated to the severe onset of diabetes (db/db gene). The experimenters used parabiosis to conjoin a db/db mouse to a normal mouse, and the normal mouse would starve to death after one week. The db/db mouse would still have a high blood sugar and food in its system. The db/db mouse was determined to have a satiety factor so potent that the other mouse would starve to death. A db/db mouse was conjoined with an ob/ob mouse and the result was the same as the first

experiment. The obese mouse starved in 20–30 days, similar to the thin counterpart in the first experiment. The db/db mutant mouse overproduced a satiety factor but could not respond to it, perhaps due to a defective receptor, whereas the ob/ob mutant recognized and responded to the factor but could not reproduce it. Further studies with db/db mice with lesions in the arcuate nucleus of the hypothalamus suggested that the receptor for the satiety factor was found in these brain receptors. [124] Early parabiotic experiments also included cancer research. One study, published in 1966 by Friedell, studied the effects of radiation with X-rays on ovarian tumors. To study the tumors, two adult female rats were conjoined. The left rat was shielded and the right rat was exposed to high levels of radiation. The rats were given a controlled amount of food and water for the remainder of their natural lives. After death, the rats were autopsied and 149 of 328 pairs showed the presence of possible ovarian tumors in one or both of the two animals. This result matched previous studies of single rats. Since Friedell's experiment, other parabiotic experiments have been useful in researching many types of cancer. [125] Chronic diseases of age have been saluted as prime candidates for parabiotic research because of the potential to conjoin an older animal with a younger animal. This process could be used to research cardiovascular disease, diabetes, osteoarthritis, and Alzheimer's disease. As animals age, their oligodendrocytes reduce in efficiency, resulting in decreased myelination, causing negative effects on the central nervous system (CNS). Julia Ruckh and fellow researchers have used parabiosis to study remyelinationfrom adult stem cells to see if conjoining young with older mice could reverse or delay this process. In the experiment, the two mice were conjoined and demyelination was induced via injection into the older mice. The experiment determined that factors from the younger mice reversed CNS demyelination in older mice by revitalizing the oligodendrocytes. The

monocytes from the younger mice also enhanced the ability of the older mice to clear myelin debris because the young monocytes can clear lipids from myelin sheaths more effectively than older monocytes. The conjoining of the two animals reversed the effects of age on the myelination cells. The ability of the young mouse's cells was unaffected. Enhanced immunity from the younger mouse also promoted the general health of the older mouse in each pair. The results of this experiment could lead to therapy processes for people with demyelinating diseases like multiple sclerosis. [126] Parabiotic research can be controversial. Due to accusations of animal cruelty, the practice is now shunned in many countries. Compared to other research techniques, there are relatively few parabiotic experiments. Some scientists argue for the benefits of parabiotic research. Eggel and Wyss-Coray argue that conjoining animals mimics naturally occurring parabiosis in nature due to the shared blood supply of conjoined twins. The experiments give insight into the way that bodily systems circulate, which has led to many advances in the study of a variety of diseases. Parabiotic experiments have been used to study obesity, chronic diseases of age, stem cell research, tissue regeneration, diabetes, transplants, tumor biology, and endocrinology. [122] Wyss-Coray has also conducted research into young blood transfusion, the donation of blood from a young donor to an older recipient.

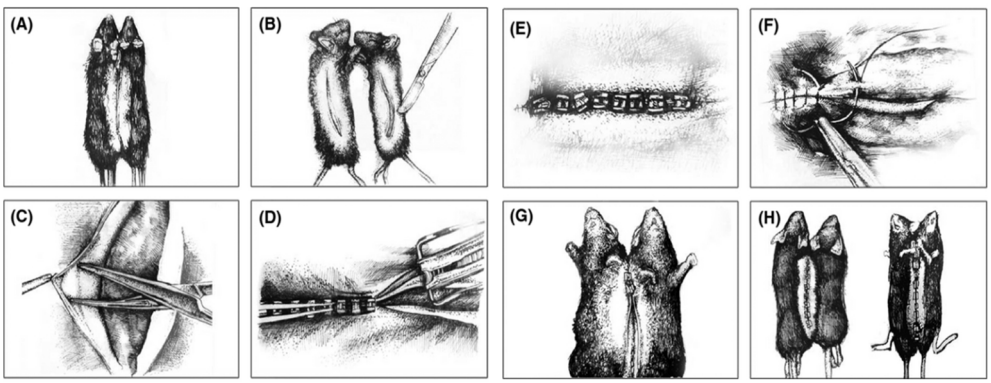


Figure 2.1 Illustrations of stages of the parabiosis surgery. Following all the requisite surgical preparations and shaving of the skin along the flanks of mice to be joined (A), a skin incision is made along the opposing flanks of each mouse (B). The skin is freed from the underlying peritoneal lining of each skin flap (C), being careful not to damage the peritoneum. With the mice side-by-side in a prone position, the dorsal skin flaps from the two mice are pinched together and stapled in a rostralto-caudal fashion (D), until the dorsal flaps are stably joined (E). The connection is secured by suturing the corresponding joints (elbows and knees), using a suture passed through the soft tissues of each joint while avoiding passing through the joints themselvess (F). With the pair flipped over to reveal the ventral skin flaps (G), the process of stapling the skin and suturing the joints is repeated, resulting in the generation of stable parabionts (H).

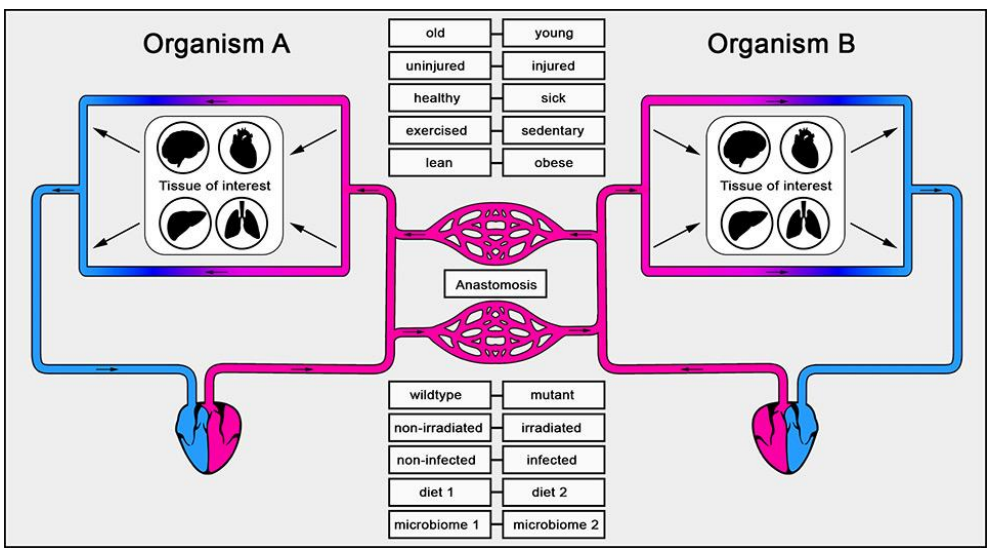


Figure 2.2 Circulatory system in parabiosis. Organism A and B share a common blood supply, which spontaneously develops through anastomosis after surgery. Organisms with different physiological conditions might be used for parabiosis in order to assess the systemic effect of one organism on a particular tissue of interest in its attached partner.

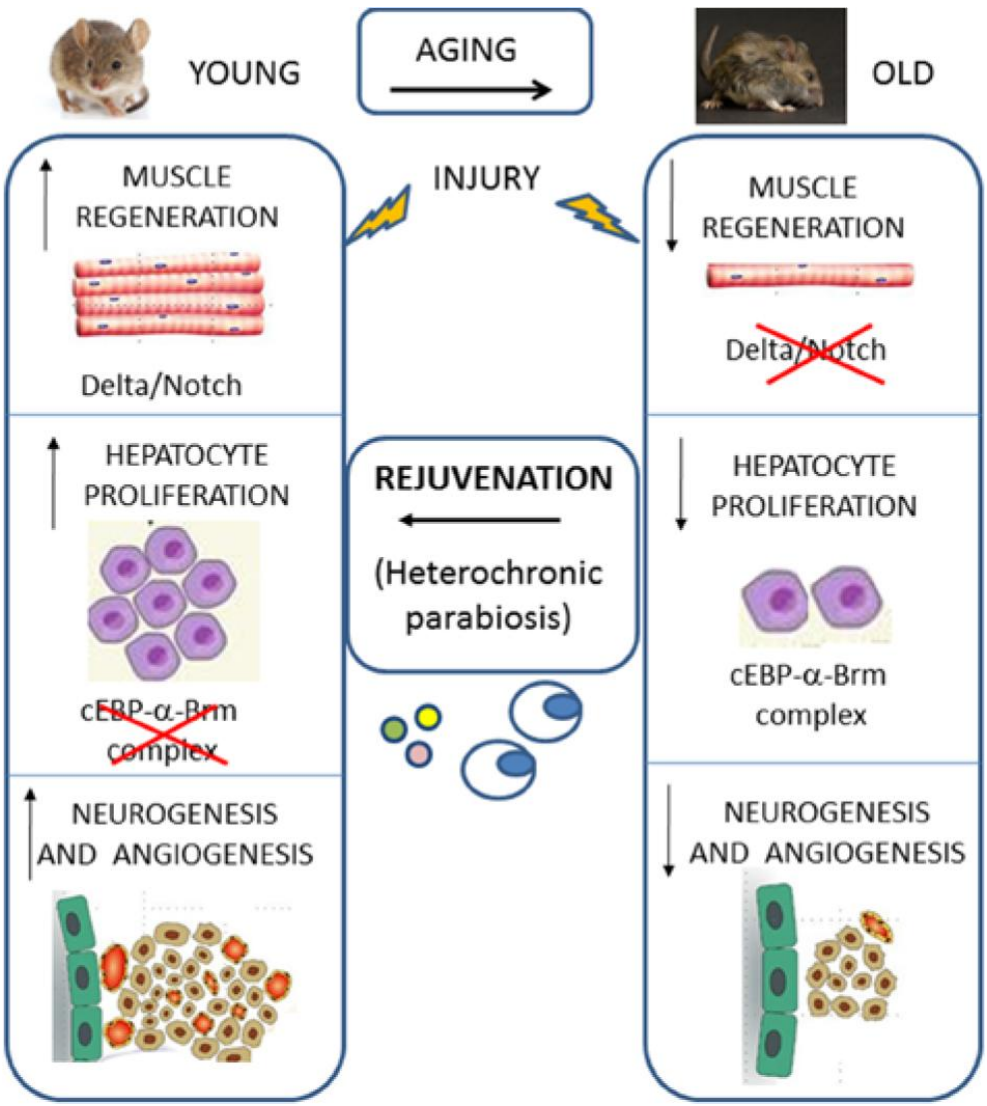

YOUNG
AGING
OLD
MUSCLE REGENERATION
Delta/Notch
INJURY
MUSCLE REGENERATION
Delta/Notch
HEPATOCYTE PROLIFERATION
cEBP-α-Brm complex
REJUVENATION
(Heterochronic parabiosis)
HEPATOCYTE PROLIFERATION
cEBP-α-Brm complex
NEUROGENESIS AND ANGIOGENESIS
NEUROGENESIS AND ANGIOGENESIS

Figure 2.3 Process for the rejuvenation by heterochronic parabiosis.

2.2.2. Application of parabiosis for diseases

Different studies have shown that the regenerative capacity of tissues and organs are dependent on the proliferative activity of progenitor cells derived from tissue resident stem cells [127–131]. A major hallmark of aging is that the regenerative properties significantly decline in most tissues. This has partially been attributed to impaired stem cell function [132–134]. However, whether these age-related effects were due to cell intrinsic changes or alterations in the microenvironment of stem cells required further investigation. In 2005 Conboy et al. used heterochronic parabiosis experiments to address this question. They showed that factors derived from the young systemic environment are able to activate molecular signaling pathways in hepatic or muscle stem cells of the old parabiont leading to increased proliferation and tissue regeneration. These in vivo results were furthermore confirmed ex vivo by culturing muscle stem cells in medium containing serum from young animals [135]. Their findings clearly suggest that the age-associated impairment of stem cell function is induced to a significant extent by the molecular composition of the surrounding niche rather than by cell intrinsic changes alone. In 2011 our group published a similar finding suggesting an old systemic environment can be detrimental for stem cell function and negatively regulate adult neurogenesis in brains of young heterochronic parabionts. This led to the discovery that factors in old blood are sufficient to decrease synaptic plasticity and impair contextual fear conditioning and spatial memory. Using a systematic proteomic approach we were able to identify soluble factors that were significantly increased in blood serum of old mice and humans. One of these factors was the chemokine CCL11 (eotaxin), known to chemotactically attract eosinophils to tissues. Indeed, application of CCL11 was sufficient to induce impaired adult

neurogenesis [136]. Again, these findings provide evidence that the age-related decline in stem cell function can be attributed to changes in the systemic environment. Three more recent publications using heterochronic parabiosis further support this conclusion. Ruckh et al. reported that recovery from experimentally induced demyelination in the CNS is enhanced in old mice that were exposed to a young systemic environment [137]. Salpeter and colleagues showed that the decline in pancreatic β-cell proliferation in old mice can be reversed in old parabionts paired with young mice [138]. And most recently, Loffredo et al. demonstrated that age-related loss of normal cardiac function leading to diastolic heart failure is partially due to the lack of certain circulating factors in old mice. They reported that this hypertrophy is reversible upon exposure of an aged animal to a youthful systemic environment through heterochronic parabiosis. They identified growth differentiation factor 11 (GDF11), which is significantly reduced in the blood serum of old mice, as a crucial factor to prevent cardiac hypertrophy. The value of parabiosis as an experimental model is most evident for physiological or pathophysiological studies that affect the organism as a whole or that induce changes in the circulatory system. Naturally, such (patho)physiological studies are most relevant to understanding the complexity of higher organisms and disease processes, but they are also the most challenging to conduct and they cannot be replaced by in vitro experiments. Indeed, it becomes increasingly evident that many diseases and biological processes, including aging, result in organism-wide, systemic changes contributing to local tissue alterations. Thus, studying an individual organ or cell type in isolation may not lead to a holistic understanding of events. This shift in thinking has been particularly striking with respect to the brain, where decades of neuron-centric research has started to give way to include studies on other brain cell types as critical regulators of cognition and

disease, and where a growing number of studies document effects of factors outside the brain including gut microbiota, diet, and other systemic changes on CNS function [139–143]. We think parabiosis is an ideal tool to ask whether alterations occurring in an organism as a consequence of disease, aging, genetic background, infection, diet, exercise etc. might result in circulatory changes altering the status of a healthy, young, uninfected or sedentary organism. Thus, parabiosis may help assessing the effects of any number of functional states of one organism on a partner organism through a shared circulatory system. This is, of course, only a first step in linking particular factors or cells to a newly discovered transmissible effect. But as the above cited reports show, it has indeed been possible to identify, for example, cells that regenerate an injured brain [137] or proteins that induce satiety [144], regenerate an aging heart [145], or accelerate aspects of brain aging [136]. A generalized approach to reveal such factors or cells using heterochronic parabiosis is to analyze systemic changes and correlate them with local alterations in a particular tissue of interest. Whether the identified candidates are necessary or sufficient to induce pathophysiology may subsequently be assessed by exogenous application or neutralization as well as endogenous overexpression or ablation experiments in suitable animal models. As many of the major untreatable diseases of our time are chiefly dependent on aging, understanding them will require more insight into the systemic changes and the resulting molecular alterations occurring with age. Animal models can replicate many aspects of chronic diseases including heart disease, stroke, or neurodegeneration, yet we know very little about the contribution of the systemic environment and aging to these conditions. Parabiosis and heterochronic parabiosis in particular could help answering some of the fundamental questions in this regard: are circulatory factors or cells in a young organism protecting against age-related disease, and vice versa, are

factors or cells in the old organism predisposing or promoting disease in a younger organism? Parabiosis between mutant mice genetically manipulated to develop disease and age-matched or heterochronic wildtype littermates or between other genetically engineered mice can help address the importance of systemic factors in the disease process. Variations of this paradigm can help elucidate pathways and mediators in many other conditions.

2.3. In vivo analysis technique

2.3.1. Animal model (R6/2 and ZQ175)

Transgenic R6/2 mice exhibit a progressive neurological phenotype that mimics many of the features of HD. Onset of phenotype is apparent from approximately 8 weeks of age based on home cage behavior. Some functional tests indicate the presence of a motor impairment from 5-6 weeks and cognitive impairment from 3 weeks. Epileptic seizures are seen in a small percentage of transgenic mice. A failure to gain weight is more pronounced in males than females. Immunohistochemistry with antibodies raised against the N-terminus of huntingtin reveals aggregates in the form of intranuclear inclusions and neuropil aggregates. Transgenic mice on a background that involves C57BL/6 and CBA display a progressive neurological phenotype that mimics many of the features of Huntington Disease in humans, including choreiform-like movements, involuntary stereotypic movements, tremor, and epileptic seizures, as well as nonmovement disorder components, including unusual vocalization. Frequent urination, loss of body weight and muscle bulk occurs through the course of the disease. Neurological developments include Neuronal Intranuclear Inclusions (NII), which contain both the huntingtin and ubiquitin proteins (NII have subsequently been identified in human HD patients); the onset of HD symptoms occurs between 9 and 11 weeks. A human HD fragment containing a polyglutamine-repeat expansion was isolated from a clone derived from a patient with Huntington's disease. The transgene contained approximately 1 kb of 5' UTR region, exon 1 which initially contained 142 CAG repeats, and 262 bp of intron 1. Subsequent analysis showed that the number of CAG repeats was prone to increase when inherited through the male line due to instability in the germline. A range of 141 to

157 was observed. On a background that involves C57BL/6 and CBA, transgenic mice have been observed to carry >(CAG)200 repeat expansions. The insertion site has been localized to a position on mouse chromosome 4 in an intron of predicted gene GM12695. The transgene is ubiquitously expressed.

The zQ175 knock-in (zQ175 KI) allele has the mouse Htt exon 1 replaced by the human HTT exon 1 sequence with a ~190 CAG repeat tract. Huntington's disease (HD) is an autosomally dominant, fatal neurodegenerative disorder characterized by uncontrolled movements, psychiatric disturbances and cognitive impairment. HD is caused by an unstable trinucleotide (CAG) repeat expansion in the huntingtin gene (HTT; HD or Hdh). The zQ175 knock-in (zQ175 KI) allele replaces mouse Htt exon 1 with the human HTT exon 1 sequence with ~190 repeats of a pure CAG tract [(CAG)nCAACAG, encoding polyglutamine]. The CAG repeat number is subject to germline and somatic instability, and may expand or contract. zQ175 mice have mutant mouse/human chimeric protein expression in brain at similar levels of normal endogenous huntingtin protein. Homozygous and heterozygous mice are viable and fertile with some characteristics that phenocopy Huntington's disease. Homozygous mice exhibit weakened grip strength (~4 weeks of age), motor deficit (~8 weeks of age), impaired rotarod and climbing activity (~30 weeks of age), circardian rhythm disruption (~9 months of age), cognitive deficits (~1 year of age), operant learning deficits (~1 year of age) and significantly reduced survival (median ~90 weeks of age). In addition, homozygous mice have huntingtin inclusions/aggregates (~2-4 months of age), early and significant decreased striatal gene markers (from ~12 weeks of age) and decreased neuronal cell counts. Heterozygous mice show behavioral deficits from around 4.5 months of age, especially in the dark phase of the diurnal cycle. Heterozygous mice also show motivational deficits (~30 weeks of age) and operant

learning deficits (~1 year of age). Decreased expression of striatal gene markers are detected in heterozygous mice from ~18 weeks of age. Both heterozygotes and homozygotes have decreased body weight (from ~8 weeks of age). This zQ175 KI allele is the same design as the zQ175 neo-deleted knock-in allele, with the exception that this zQ175 KI retains the floxed neo cassette. It is not specifically characterized if deletion of the neo cassette alters the zQ175DN KI phenotype compared to the zQ175 KI phenotype.

Name	Period	Symptoms	Behavioral test	Possession
R6/2 (CAG 150) 	6-12 week	Striatal atrophy mHtt aggregation Hypokinesia Jerk	Rotarod test Clasping test Body weight	2006 Year~ Mating group: 5 pairs
YAC128 (CAG 128) 	6-18 month	Loss of Neurons Hyperactivity Hypokinesia	Rotarod test	2008 Year~ Mating group: 5 pairs Experiment : 30 mice (In 1 year : 20 mice)
zQ175 (CAG175) 	5-15 month	Loss of Neurons Cognitive deficits Hypokinesia	Rotarod test Grip strength Climbing Body weight	2014 Year~ Mating group: 10 pairs Experiment : 70 mice

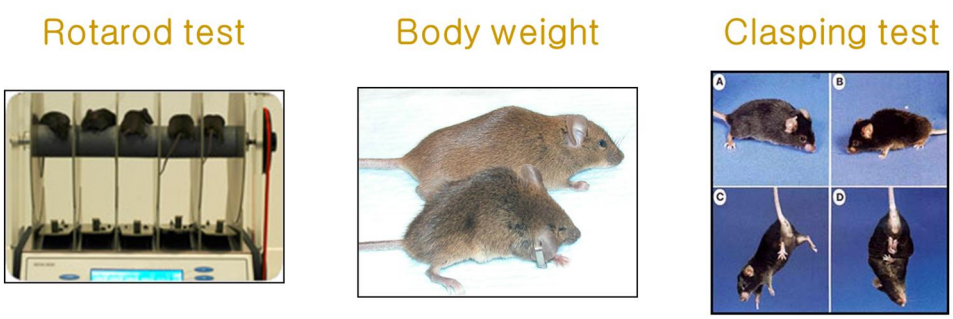


Figure 2.4 Animal models of Huntington's disease and behavior test.

2.3.2. Animal modeling for parabiosis

Parabiosis is a surgical union of two organisms allowing sharing of the blood circulation. Attaching the skin of two animals promotes formation of microvasculature at the site of inflammation. Parabiotic partners share their circulating antigens and thus are free of adverse immune reaction. First described by Paul Bert in 1864, the parabiosis surgery was refined by Bunster and Meyer in 1933 to improve animal survival. In the current protocol, two mice are surgically joined following a modification of the Bunster and Meyer technique. Animals are connected through the elbow and knee joints followed by attachment of the skin allowing firm support that prevents strain on the sutured skin. The blood chimerism allows one to examine the contribution of the circulating cells from one animal in the other.

2.3.3. BrdU injection

Bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU, BUdR, BrdUrd, broxuridine) is a synthetic nucleoside that is an analog of thymidine. BrdU is commonly used in the detection of proliferating cells in living tissues. 5-Bromodeoxycytidine is deaminated to form BrdU. BrdU can be incorporated into the newly synthesized DNA of replicating cells (during the S phase of the cell cyclеduring which DNA is replicated), substituting for thymidine during DNA replication. Antibodies specific for BrdU can then be used to detect the incorporated chemical (see immunohistochemistry), thus indicating cells that were actively replicating their DNA. Binding of the antibody requires denaturation of the DNA, usually by exposing the cells to acid or heat. BrdU

can be passed to daughter cells upon replication. BrdU has been demonstrated to be detectable over two years post-infusion. Because BrdU can replace thymidine during DNA replication, it can cause mutations, and its use is therefore potentially a health hazard. However, because it is neither radioactive nor myelotoxic at labeling concentrations, it is widely preferred for in vivo studies of cancer cell proliferation. However, at radiosensitizing concentrations, BrdU becomes myelosuppressive, thus limiting its use for radiosensitizing. BrdU differs from thymidine in that BrdU substitutes a bromine atom for thymidine's CH3 group. The Br substitution can be used in X-ray diffraction experiments in crystals containing either DNA or RNA. The Br atom acts as an anomalous scatterer and its larger size will affect the crystal's X-ray diffraction enough to detect isomorphous differences as well. Bromodeoxyuridine releases gene silenced by association with the histone caused by DNA methylation. BrdU can also be used to identify microorganisms that respond to specific carbon substrates in aquatic and soil environments. A carbon substrate added to incubations of environmental samples will cause the growth of microorganisms that can utilize that substrate. These microorganisms will then incorporate BrdU into their DNA as they grow. Community DNA can then be isolated and BrdU-labeled DNA purified using an immunocapture technique. Subsequent sequencing of the labeled DNA can then be used to identify the microbial taxa that participated in the degradation of the added carbon source. However, it is not certain whether all microbes present in an environmental sample can incorporate BrdU into their biomass during de novo DNA synthesis. Therefore, a group of microorganisms may respond to a carbon source but go undetected using this technique. Additionally, this technique is biased towards identifying microorganisms with A- and T-rich genomes.

2.4. In vitro analysis technique

2.4.1. Neural stem cell

Neural stem cells (NSCs) are self-renewing, multipotent cells that firstly generate the radial glial progenitor cells that generate the neurons and glia of the nervous system of all animals during embryonic development. Some neural progenitor stem cells persist in highly restricted regions in the adult vertebrate brain and continue to produce neurons throughout life. Stem cells are characterized by their capacity to differentiate into multiple cell types. They undergo symmetric or asymmetric cell division into two daughter cells. In symmetric cell division, both daughter cells are also stem cells. In asymmetric division, a stem cell produces one stem cell and one specialized cell. NSCs primarily differentiate into neurons, astrocytes, and oligodendrocytes. Epidermal growth factor (EGF) and fibroblast growth factor (FGF) are mitogens that promote neural progenitor and stem cell growth in vitro, though other factors synthesized by the neural progenitor and stem cell populations are also required for optimal growth. It is hypothesized that neurogenesis in the adult brain originates from NSCs. The origin and identity of NSCs in the adult brain remain to be defined.

2.4.2. Exosome isolation

In order to facilitate the study and application of these unique extracellular vesicles, it is crucial that exosomes are specifically isolated from a wide spectrum of cellular debris and interfering components. The techniques employed in the isolation of exosomes should exhibit high efficiency and are capable of isolating exosomes from

various sample matrices. To examine the quality of isolated exosomes, several optical and non-optical techniques have been developed to gauge their size, size distribution, morphology, quantity, and biochemical composition.43 With the fast advances in science and technology, many techniques have been developed for the isolation of exosomes in appreciable quantity and purity. Each technique exploits a particular trait of exosomes, such as their density, shape, size, and surface proteins to aid their isolation. Variants within each group also bring about a unique set of advantages and disadvantages to exosome isolation.

2.4.3. Immunohistochemistry

Immunohistochemistry (IHC) is the most common application of immunostaining. It involves the process of selectively identifying antigens (proteins) in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues. IHC takes its name from the roots "immuno", in reference to antibodies used in the procedure, and "histo", meaning tissue (compare to immunocytochemistry). Albert Coons conceptualized and first implemented the procedure in 1941. Immunohistochemical staining is widely used in the diagnosis of abnormal cells such as those found in cancerous tumors. Specific molecular markers are characteristic of particular cellular events such as proliferation or cell death (apoptosis). Immunohistochemistry is also widely used in basic research to understand the distribution and localization of biomarkersand differentially expressed proteins in different parts of a biological tissue. Visualising an antibody-antigen interaction can be accomplished in a number of ways. In the most common instance, an antibody is conjugated to an enzyme, such as peroxidase, that can catalyse a colour-producing

reaction (see immunoperoxidase staining). Alternatively, the antibody can also be tagged to a fluorophore, such as fluorescein or rhodamine (see immunofluorescence).

2.4.4. Immunocytochemistry

Immunocytochemistry (ICC) is a common laboratory technique that is used to anatomically visualize the localization of a specific protein or antigen in cells by use of a specific primary antibody that binds to it. The primary antibody allows visualization of the protein under a fluorescence microscope when it is bound by a secondary antibody that has a conjugated fluorophore. ICC allows researchers to evaluate whether or not cells in a particular sample express the antigen in question. In cases where an immunopositive signal is found, ICC also allows researchers to determine which sub-cellular compartments are expressing the antigen. Immunocytochemistry differs from immunohistochemistry in that the former is performed on samples of intact cells that have had most, if not all, of their surrounding extracellular matrix removed. This includes individual cells that have been isolated from a block of solid tissue, cells grown within a culture, cells deposited from suspension, or cells taken from a smear. In contrast, immunohistochemical samples are sections of biological tissue, where each cell is surrounded by tissue architecture and other cells normally found in the intact tissue. Immunocytochemistry is a technique used to assess the presence of a specific protein or antigen in cells (cultured cells, cell suspensions) by use of a specific antibody, which binds to it, thereby allowing visualization and examination under a microscope. It is a valuable tool for the determination of cellular contents from individual cells. Samples that can be analyzed include blood smears, aspirates, swabs, cultured cells, and cell suspensions.

There are many ways to prepare cell samples for immunocytochemical analysis. Each method has its own strengths and unique characteristics so the right method can be chosen for the desired sample and outcome. Cells to be stained can be attached to a solid support to allow easy handling in subsequent procedures. This can be achieved by several methods: adherent cells may be grown on microscope slides, coverslips, or an optically suitable plastic support. Suspension cells can be centrifuged onto glass slides (cytospin), bound to solid support using chemical linkers, or in some cases handled in suspension. Concentrated cellular suspensions that exist in a low-viscosity medium make good candidates for smear preparations. Dilute cell suspensions existing in a dilute medium are best suited for the preparation of cytospins through cytocentrifugation. Cell suspensions that exist in a high-viscosity medium, are best suited to be tested as swab preparations. The constant among these preparations is that the whole cell is present on the slide surface. For any intercellular reaction to take place, immunoglobulin must first traverse the cell membrane that is intact in these preparations. Reactions taking place in the nucleus can be more difficult, and the extracellular fluids can create unique obstacles in the performance of immunocytochemistry. In this situation, permeabilizing cells using detergent (Triton X-100 or Tween-20) or choosing organic fixatives (acetone, methanol, or ethanol) becomes necessary. Antibodies are an important tool for demonstrating both the presence and the subcellular localization of an antigen. Cell staining is a very versatile technique and, if the antigen is highly localized, can detect as few as a thousand antigen molecules in a cell. In some circumstances, cell staining may also be used to determine the approximate concentration of an antigen, especially by an image analyzer.

2.4.5. Western blot

The western blot (sometimes called the protein immunoblot) is a widely used analytical technique in molecular biology, immunogenetics and other molecular biology disciplines to detect specific proteins in a sample of tissue homogenate or extract. In brief, the sample undergoes protein denaturation, followed by gel electrophoresis. A synthetic or animal-derivedantibody (known as the primary antibody) is created that recognises and binds to a specific target protein. The electrophoresis membrane is washed in a solution containing the primary antibody, before excess antibody is washed off. A secondary antibody is added which recognises and binds to the primary antibody. The secondary antibody is visualised through various methods such as staining, immunofluorescence, and radioactivity, allowing indirect detection of the specific target protein. Other related techniques include dot blot analysis, quantitative dot blot, immunohistochemistry, and immunocytochemistrywhere antibodies are used to detect proteins in tissues and cells by immunostaining, and enzyme-linked immunosorbent assay (ELISA). The name western blot is a play on the eponymously-named Southern blot, a technique for DNA detection. Similarly, detection of RNA is termed northern blot.[1] The term "western blot" was given by W. Neal Burnette, although the method itself originated in the laboratory of Harry Towbin at the Friedrich Miescher Institute. The western blot is extensively used in biochemistry for the qualitative detection of single proteins and protein-modifications (such as post-translational modifications). It is used as a general method to identify the presence of a specific single protein within a complex mixture

of proteins. A semi-quantitative estimation of a protein can be derived from the size and color intensity of a protein band on the blot membrane. In addition, applying a dilution series of a purified protein of known concentrations can be used to allow a more precise estimate of protein concentration. The western blot is routinely used for verification of protein production after cloning. It is also used in medical diagnostics, e.g., in the HIV test or BSE-Test. The confirmatory HIV test employs a western blot to detect anti-HIV antibody in a human serum sample. Proteins from known HIV-infected cells are separated and blotted on a membrane as above. Then, the serum to be tested is applied in the primary antibody incubation step; free antibody is washed away, and a secondary anti-human antibody linked to an enzyme signal is added. The stained bands then indicate the proteins to which the patient's serum contains antibody. A western blot is also used as the definitive test for variant Creutzfeldt-Jakob Disease, a type of prion disease linked to the consumption of contaminated beef from cattle with Bovine spongiform encephalopathy (BSE, commonly referred to as 'mad cow disease'). Another application is in the diagnosis of tularemia. An evaluation of the western blot's ability to detect antibodies against F. tularensis revealed that its sensitivity is almost 100% and the specificity is 99.6%. Some forms of Lyme disease testing employ western blotting.[6] A western blot can also be used as a confirmatory test for Hepatitis B infection and HSV-2 (Herpes Type 2) infection. In veterinary medicine, a western blot is sometimes used to confirm FIV+ status in cats. Further applications of the western blot technique include its use by the World Anti-Doping Agency (WADA). Blood doping is the misuse of certain techniques and/or substances to increase one's red blood cell mass, which allows the body to transport more oxygen to muscles and therefore increase stamina and performance. There are three widely known substances or methods used for blood doping, namely, erythropoietin (EPO),

synthetic oxygen carriers and blood transfusions. Each is prohibited under WADA's List of Prohibited Substances and Methods. The western blot technique was used during the 2014 FIFA World Cup in the anti-doping campaign for that event. In total, over 1000 samples were collected and analyzed by Reichel, et al. in the WADA accredited Laboratory of Lausanne, Switzerland. Recent research utilizing the western blot technique showed an improved detection of EPO in blood and urine based on novel Velum SAR precast horizontal gels optimized for routine analysis. With the adoption of the horizontal SAR-PAGE in combination with the precast film-supported Velum SAR gels the discriminatory capacity of micro-dose application of rEPO was significantly enhanced.

2.4.6. Fluorescence Activated Cell Sorting (FACS)

Originally developed in the late 1960s, flow cytometry is a popular analytical cell-biology technique that utilizes light to count and profile cells in a heterogenous fluid mixture. Flow cytometry is a particularly powerful method because it allows a researcher to rapidly, accurately, and simply collect data related to many parameters from a heterogeneous fluid mixture containing live cells. Flow cytometry is used extensively throughout the life and biomedical sciences, and can be applied in any scenario where a researcher needs to rapidly profile a large population of loose cells in a liquid media. For example, in immunology flow cytometry is used to identify, separate, and characterize various immune cell subtypes by virtue of their size and morphology. When additional information is required, antibodies tagged with fluorescent dyes, and raised against highly specific cell surface antigens (e.g. clusters

of differentiation or CD markers) can be used to better identify and segregate specific sub-populations within a larger group.

2.4.7. Enzyme Linked Immunosorbent Assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) is a commonly used analytical biochemistryassay, first described by Engvall and Perlmann in 1972. The assay uses a solid-phase enzyme immunoassay(EIA) to detect the presence of a ligand (commonly a protein) in a liquid sample using antibodies directed against the protein to be measured. ELISA has been used as a diagnostic tool in medicine, plant pathology, and biotechnology, as well as a quality control check in various industries. In the most simple form of an ELISA, antigens from the sample are attached to a surface. Then, a matching antibody is applied over the surface so it can bind to the antigen. This antibody is linked to an enzyme, and in the final step, a substance containing the enzyme's substrate is added. The subsequent reaction produces a detectable signal, most commonly a color change. Performing an ELISA involves at least one antibody with specificity for a particular antigen. The sample with an unknown amount of antigen is immobilized on a solid support (usually a polystyrene microtiter plate) either non-specifically (via adsorption to the surface) or specifically (via capture by another antibody specific to the same antigen, in a "sandwich" ELISA). After the antigen is immobilized, the detection antibody is added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme or can itself be detected by a secondary antibody that is linked to an enzyme through bio-conjugation. Between each step, the plate is typically washed with a mild detergent solution to

remove any proteins or antibodies that are non-specifically bound. After the final wash step, the plate is developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of antigen in the sample. Of note, ELISA can perform other forms of ligand binding assays instead of strictly "immuno" assays, though the name carried the original "immuno" because of the common use and history of development of this method. The technique essentially requires any ligating reagent that can be immobilized on the solid phase along with a detection reagent that will bind specifically and use an enzyme to generate a signal that can be properly quantified. In between the washes, only the ligand and its specific binding counterparts remain specifically bound or "immunosorbed" by antigen-antibody interactions to the solid phase, while the nonspecific or unbound components are washed away. Unlike other spectrophotometric wet lab assay formats where the same reaction well (e.g., a cuvette) can be reused after washing, the ELISA plates have the reaction products immunosorbed on the solid phase, which is part of the plate, and so are not easily reusable.

CHAPTER 3

Effect of pH on the Yield of Exosome Isolation

3.1. Introduction

Exosomes are small extracellular vesicles with 30 - 100 nm diameter that originate from many type of cells [1]. Exosomes are formed as part of the multivesicular body pathway in which intraluminal vesicles progressively accumulate during endosome maturation. Exosomes are formed by inward budding and scission of vesicles from the limiting endosomal membranes and released from the MVB lumen into the extracellular environment during exocytosis [1,2]. Secreted exosomes can be isolated and characterized in blood [3,4], urine [5], saliva [6] in vivo and conditioned medium [2] in vitro. They include various molecular constituents of their cell origin, including proteins, mRNA, and microRNA [7-9]. Because of their importance in normal physiology and disease progression, exosomes have been widely studied as a therapeutic agent [10-13], biomarker [3,6] and drug delivery vehicle [14].

Extracellular pH is various dependent on the tissues microenvironment and pathologic conditions, affecting the amount and characteristic of exosome. For example, the pH of gastric juice is severely low and there are RNAs in the stomach's

acidic condition. These gastric juice-derived RNAs might be associated with gastric juice exosomes [15]. Abnormally proliferated cancer cells change their microenvironmental pH into acidic, resulting in cancer progression and metastasis [16-18]. Recent research of cancer exosomes revealed that acidic microenvironment promotes exosome traffic and uptake of cancer cells, contributing tumor favorable environment [16,19,20]. Exosomes seem to have a crucial role in propagation of Prion protein in CreutzfeldteJakob disease and low pH triggers Prion protein misfolding [21,22]. Considering these studies of exosome and pH on diseases, it is plausible that pH have some crucial role in exosome physiology.

Despite of this importance of pH condition in exosome and related diseases, most in vitro experiments of exosome isolation and characterization have been performed without regard to this situation. Thus, the effects of pH on the exosome isolation and characterization should be thoroughly studied for future exosome researches. In this study, we isolated the exosomes after incubation in various pH conditions and investigated the yield of exosomes by measuring exosomal total protein and RNA concentration. Representative exosome markers were also investigated to confirm the yield of exosome isolation.

3.2. Methods

3.2.1. HEK 293 cell culture

To deplete bovine exosomes in the culture medium, Dulbecco's modified eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum(FBS; Gibco, Carlsbad, CA, USA) and 1% penicillin-streptomycinwas centrifuged at 100,000 g for 15 h at 4 ^{o}C and supernatantwas used for cell culture. HEK 293 cellswere seeded at a density of 1×10^5 cells in 15ml of exosome free medium into T-75 culture flask and cultured for 5 days at 37 ^{o}C in a 5% CO_2 incubator.

3.2.2. Adjustment of pH in conditioned medium

Cells and cellular debris were eliminated from conditioned medium (CM) by centrifugation at 3000 g for 10 min at room temperature. pH in CM was adjusted with 1 M hydrogen chloride (HCl) or 1 M sodium hydroxide (NaOH) solution and measured by a pH meter (Thermo Fisher Scientific, MA, USA) for making pH 4, 7 and 11 CM. Exosomes were isolated from pH4, 7 or 11 CM after incubating for 30 min at room temperature.

3.2.3. Exosomes isolation

Exosome isolation using Exo-quick exosome precipitation kit (System Biosciences, CA, USA) was performed according to manufacturer's specifications. Briefly, the 3ml CM was mixed thoroughly with 0.6 ml of Exo-Quick exosome precipitation solution

and incubated for 24 h at 4 ^{o}C. CM complex were centrifuged at 1500 g for 30 min, and then the supernatant was removed and centrifuged at 1500 g for 5 min again. The remaining exosome pellet was used for protein or RNA isolation.

3.2.4. Protein isolation and western blot

Proteins in exosomes were extracted by 100 ml of Radio Immuno Precipitation Assay buffer (Pierce, IL, USA) with freshly added protease inhibitor (Roche, USA) and exosomal proteins were measured by BCA protein assay kit (Pierce, IL, USA). Proteins were loaded on sodium dodecyl sulfateopolyacrylamide gel and electrotransferred to a polyvinylidene fluoride membrane (Millipore, MA, USA). The blots were probed with primary antibodies: CD9 (1:200; Santa Cruz, CA, USA), CD63 (1:200; Santa Cruz, CA, USA), HSP70 (1:200; Santa Cruz, CA, USA), followed by horseradish peroxidase conjugated secondary anti-mouse or rabbit antibody (1:3000; GE Healthcare, USA). The relative protein levels were analyzed by ImageJ software.

3.2.5. RNA isolation

Isolation of RNA from exosomes was carried out by the mirVana isolation kit (Ambion, TX, USA) according to the manufacturer's protocol. Briefly, exosomes pellet was lysed with lysis buffer and exosomal RNA was extracted by miRNA homogenate solution and chloroform. The exosomal RNAwas suspended with 100 ml of DEPC water after purifying by the filter cartridge and the concentration of RNA

was quantified using the Nano Drop ND-1000 Spectrophotometer (Nano-Drop Technologies, DE, USA).

3.2.6. Statistical analysis

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

3.3. Isolation and characterization of exosomes

HEK 293 cells were cultured for 3 days and exosomes were isolated by Exoquick isolation kit. After isolation, proteins were extracted by protein extraction buffer and western blot was performed. As a result, representative exosome markers including CD9, CD63 and HSP70 were examined in the conditioned medium derived exosomal protein (Figure 3.1). Exosome-free media was examined as a negative control and didn't show any exosome markers. The result of this experiment indicates that our exosome-free media and isolation method is suitable for cell line-derived exosome research.

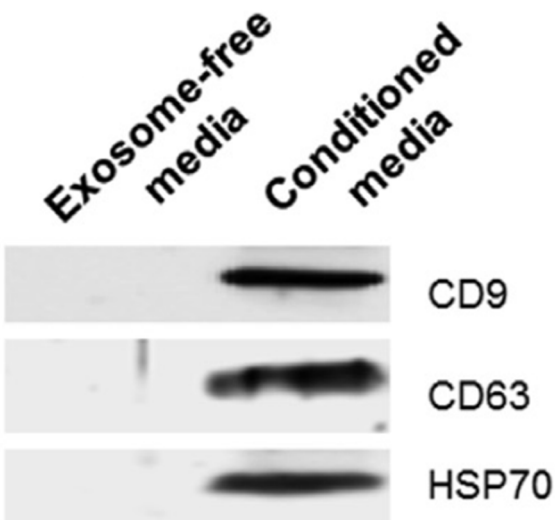


Figure 3.1 Western blot analysis with exosome markers. Exosome isolated from exosome-free media or conditioned media were immunoblotted with CD9, CD63 and HSP70

3.4. Effect of pH on the representative exosome markers

To investigate the level of representative exosome markers in exosomes of CM in different pH conditions, the pH of CM was adjusted to pH4, 7 and 11 by HCl or NaOH, and exosomal proteins of pH4, pH7 or pH11 CM were immunoblotted with CD9, CD63 and HSP70 antibodies. As a result, pH4 CM shows higher level of exosome markers than pH7 CM. On the contrary, the sample that had been incubated in pH11 showed no exosomal markers (Figure 3.2). The effect of pH on exosomal HSP70 level was measured in a range from pH3 to 10, and result showed inverse correlation of pH and protein level (Figure 3.3)

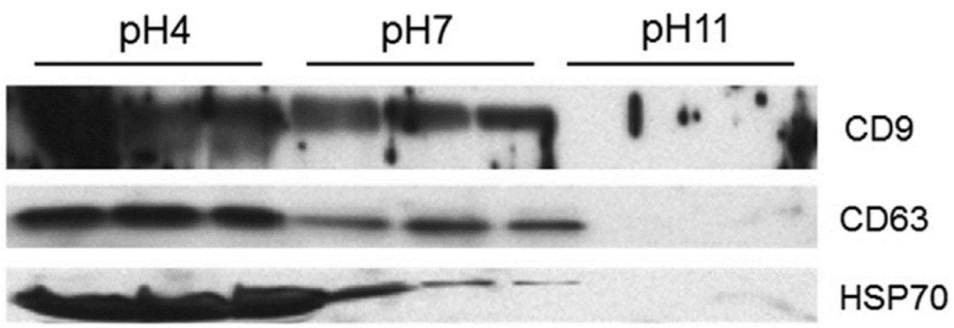


Figure 3.2 Western blot analysis with exosome markers. Exosomes were incubated with pH4, pH7 or pH11 medium and tested with western blot for analyzing the level of CD9, CD63 and HSP70

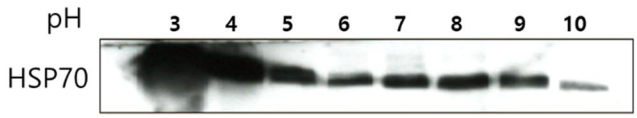


Figure 3.3 Comparison of exosomal HSP70 protein level by pH. After removing cell debris from CM, The pH was adjusted from pH3 to 10. After 30 min incubation at 37 °C, exosomes were isolated and exosomal HSP70 level was examined. Western blot result of HSP70 showed that low pH leads to higher HSP70 protein level and high pH showed vice versa.

3.5.
Effect of pH on the exosomal protein and RNA concentration

To investigate exosomal protein and RNA concentration in different pH conditions, proteins and total RNA were extracted by protein extraction buffer and total RNA isolation kit respectively after isolating exosomes from each pH4, pH7 or pH11 CM. As a result, total exosomal protein concentration in pH4 CM is fivefold higher than that of pH7 CM (Figure 3.4) and RNA concentration of pH4 CM also showed five times increased level compared to pH7 CM (Figure 3.5). Two representative methods for exosome isolation are serial ultracentrifugation and Exoquick reagent. We identified increased yield of exosomal protein by low pH using ultracentrifugation method (Figure 3.6). These results suggest that the yield of isolation of representative exosome components, such as proteins and nucleic acids, could be increased by acidic environment.

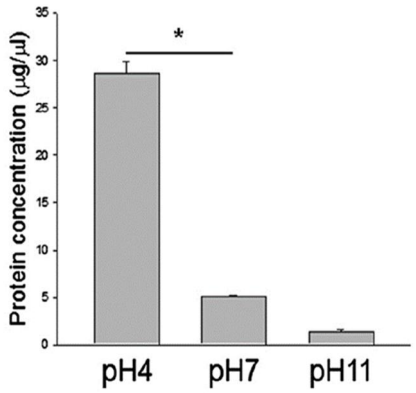


Figure 3.4 Measurement of exosomal protein and RNA concentration. Exosomal protein and RNA were extracted from each exosomes incubated with pH4, pH7 or pH11 and protein concentration was measured by BCA protein quantification. *$p < 0.01$.

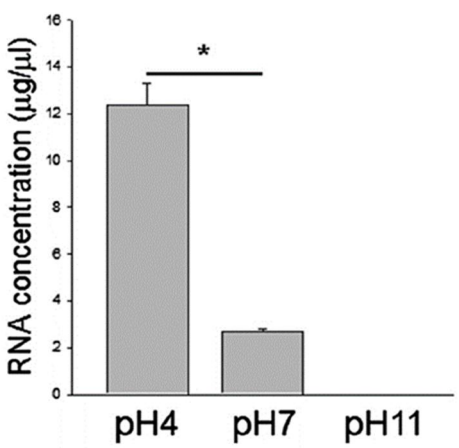


Figure 3.5 Measurement of exosomal protein and RNA concentration. Exosomal protein and RNA were extracted from each exosomes incubated with pH4, pH7 or pH11 and protein concentration was measured by Nano

Drop for RNA concentration. *p < 0.01.

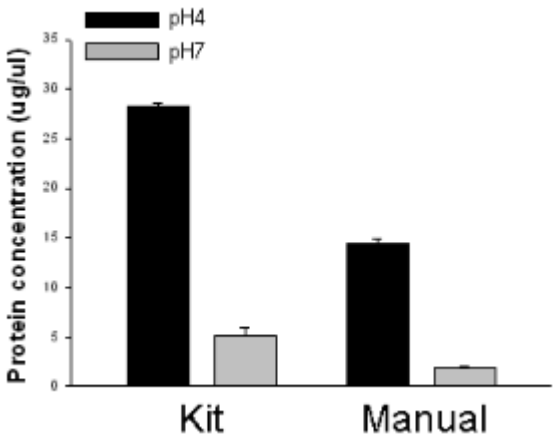


Figure 3.6 Comparing the effect of pH on the exosomal protein yield using two different isolation methods. Two different protocols were used to isolate exosomes. One was ultracentrifugation method and the other was exosome precipitation reagent ExoQuick-TC. Exosomal proteins were extracted from each exosomes from pH4 and pH7 and measured by BCA protein assay kit. As a result, total exosomal protein concentration in pH4 is higher than that of pH7. This result indicated that ultracentrifugation-based method and commercial reagent ExoQuick-TC showed similar result. Thus, representative exosome components were increased by acidic environment.

3.6. Summary

In this study, we demonstrated that acidic pH could increase the stability of exosomes in vitro, resulting in higher yield of exosome isolation. Incubation in acidic medium showed increased exosomal protein and RNA concentration. Immunoblot of representative exosome markers also showed increased isolation of intact exosomes by acidic condition. On the contrary, exosome isolation failed after incubation in an alkaline medium.

Cancer cell derived exosomes seem to induce cancer favorable microenvironment and tumor core is relatively acidic [16-18]. Recent study shows that precipitation of exosomes from tumor cells has improved after treatment of acetate [23]. Parolini et al. found that intercellular acidic pH could increase cancer exosome secretion and uptake, leading to tumor progression and metastasis [19]. Prion is propagated by extracellular exosomes in Creutz-feldte-Jakob disease and misfolding of Prion protein is related with low pH [21,22]. Based on these exosome researches, it is plausible that pH could affect stability of exosomes. Our study showed that acidic condition could increase the amount of isolation of cellderived exosomes, which enables to extract exosomal proteins and nucleic acids from the small amount of the specimen. Proteins and nucleic acids in exosomes are protected against low pH condition of gastric fluid by showing that they are present in high concentration in gastric fluid while there are almost no exosomes after incubation in alkaline condition.

Exosome mediates genesis and progression of diseases including cancer, Creutz-feldte-Jakob disease and Alzhermer's disease [20,21,24]. Despite of their promising potential in medical field, the exact mechanism of biogenesis, secretion and uptake of exosome is still unknown. In addition to this, clear protocol for handling, storage and

isolation method for exosome experiment need to be established. In summary, our results show that low pH increases the yield of exosome isolation and high pH degrades the exosome, suggesting that environmental pH of exosome needs to be carefully considered for exosome researches.

CHAPTER 4

Influence of Storage Condition on Exosome Recovery

4.1. Introduction

Most of eukaryotic cells release extracellular vesicle (EV) to maintain or change their environmental cells. Extracellular membrane vesicles called "microparticles" have been regarded as cell fragments shed from cell membrane [1]. However, it was not until the 1980s when vesicles which are derived from cell embosoms followed by fusion with the cell membrane were first described by Johnstone et al. while culturing sheep reticulocyte [2]. Exosomes are small extracellular vesicles with 30 ~ 100 nm diameters that are secreted from most of cell types [3]. Exosomes are formed by inward budding and scission of vesicles from the limiting endosomal membranes and released from the multivesicular body lumen into the extracellular environment during exocytosis [3,4]. Exosomes can be isolated and characterized in blood [5,6], urine [7], saliva [8] in vivo and cell culture conditioned medium (CM) in vitro [4]. They contain various molecular constituents of their cell origin, such as proteins [9], mRNAs [10],

and microRNAs [10,11].

Many exosome studies reported their significance in biology and the medical field. Tumor derived exosomes can promote angiogenesis, metastasis and immune suppression [12,14]. Also, exosome mediates pathogenic propagation of amyloid-β and tau proteins in Alzheimer's disease [15,16]. Exosomes seem to have a crucial role in propagation of Prion protein in Creutzfeldt–Jakob disease [17,18]. Because of their importance in normal physiology and disease progression, exosomes have been widely studied as a therapeutic agent [19,22], disease biomarker [5,8] and drug delivery vehicle [23].

Thus, standard preservation protocol needs to be used for clinical application and basic researches of exosomes. Due to their small size, exosomes cannot be easily isolated and detected by conventional method such as low g-force centrifugation and light microscopy. Although deep freezer at -70°C is widely used for specimen preservation for exosome researches, proper storage condition of exosomes for short-term or long-term period need to be evaluated.

Despite the significance of the exosomes in biological field, optimal storage conditions are insufficiently studied and more researches need to be done for stringent criteria of preservation of homogeneous exosome populations. In this study, we evaluated the recovery yield of cell derived exosomes which were stored in various storage temperature and period.

4.2. Methods

4.2.1. HEK 293 cell culture

We used HEK 293 cell, since this cell line was used in many exosome researches [24,27] and secreted consistent and abundant exosomes. HEK 293 cell was cultured in Dulbecco's modified eagle's medium (DMEM; Gibco, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA) and 1% penicillin-streptomycin. To deplete bovine exosomes from the culture medium, DMEM containing 10% FBS was centrifuged at 100,000 g for 15 h at 4°C and supernatant was used for cell culture. HEK 293 cells were seeded at a density of 1×10^5 cells in 15 mL of exosome free medium into T-75 culture flask and cultured for 5 days at 37 °C in a 5% CO_2 incubator.

4.2.2. Isolation of exosomes

Exosome isolation using Exo-Quick exosome precipitation kit (System Biosciences, CA, USA) was performed according to manufacturer's specifications. Briefly, the 3 mL CM was mixed thoroughly with 0.6 mL of Exo-Quick exosome precipitation solution and incubated for 24 h at 4°C. CM complex were centrifuged at 1,500 g for 30 min, and then the supernatant was removed and centrifuged at 1,500 g for 5 min again. The remaining exosome pellet was used for protein or RNA isolation. Exosomes were resuspended with 100 μL of Radio Immuno Precipitation Assay (RIPA) buffer (Pierce, IL, USA).

4.2.3. Storage of exosomes

Isolated exosomes were aliquoted in cooled 1.5 mL microcentrifuge tubes and stored at ice before keeping at various temperatures. For storage at short-term period, aliquoted exosomes were kept at temperatures ranging from 4 to 90°C for 30 min using heatblock. For long-term period storage experiments, aliquoted exosomes were stored at various temperatures at from -70°C to room temperature (RT) for 10 days. Samples were mixed with 4× sample buffer, boiled for 5 min, and resolved by SDS-PAGE.

4.2.4. Protein concentration measurement and western blot

Proteins in exosomes were extracted by 100 μL of RIPA buffer (Pierce, IL, USA) with freshly added protease inhibitor (Roche, USA) and exosomal proteins were measured by BCA protein assay kit (Pierce, IL, USA). After incubation at indicated temperature, protein samples were loaded on SDS-PAGE and electrotransferred to a polyvinylidene fluoride membrane (Millipore, MA, USA). The blots were probed with primary antibodies: CD9 (1:200; Santa Cruz, CA, USA), CD63 (1:200; Santa Cruz, CA, USA), HSP70 (1:200; Santa Cruz, CA, USA), followed by horseradish peroxidase conjugated secondary anti-mouse or rabbit antibody (1:3,000; GE Healthcare, USA). The intensities of band were quantified by ImageJ [28].

4.2.5. Flow cytometry

Exosomes were fixed with 2% paraformaldehyde and investigated by FACScalibur (BD bioscience, San Jose, CA, USA) after storage. Nano-sized exosome population below 200 nm was calibrated by Submicron Particle Size reference (Thermo Fisher, Carlsbad, CA, USA) and data was analyzed with Winmdi 2.9 [29].

4.2.6. Statistical analysis

All values shown in the figures are presented as mean +/- standard deviation. Western blot results were measured by ImageJ software and all data were analyzed by one-way analysis of variance followed by post hoc t-test using SPSS (Statistical Package for the Social Sciences) version 17.0 (SPSS Inc., USA). A 2-tailed probability value below 0.05 was considered statistically significant.

4.3. Exosome isolation from conditioned medium

HEK 293 cells were cultured for 3 days in exosome-depleted medium and exosomes were isolated by Exo-Quick isolation kit according to the manufacturer's protocol. After isolation, proteins were extracted by protein extraction buffer and protein markers of exosomes were measured using western blot. HSP70, CD63 and CD9 have been used as representative exosome markers in many studies [26,30,31]. The representative exosome markers were detected in the CM derived exosomal protein, whereas exosome-free control medium did not contain any exosome marker proteins (Figure 4.1). These data indicates that our culture condition and exosome isolation protocol were appropriate for in vitro exosome researches.

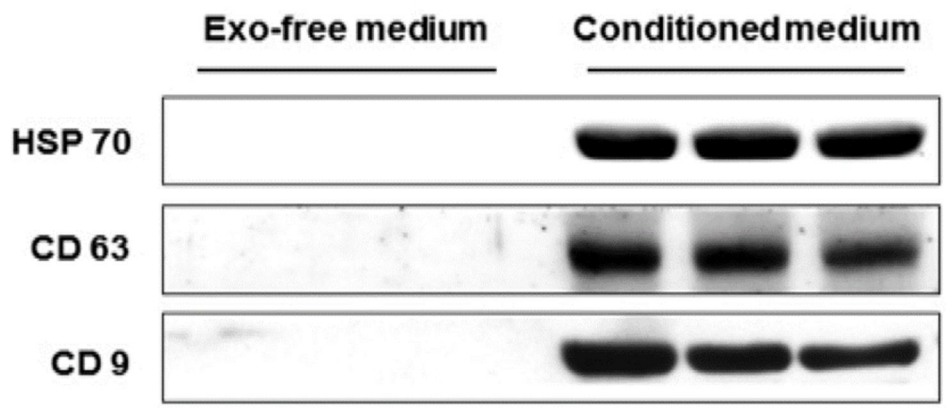


Figure 4.1 Isolation of conditioned medium derived exosomes. Exosome from exosome-free medium and HEK293 derived conditioned medium were isolated using Exo-Quick method. Extracted proteins were immunoblotted with antibodies for representative exosome markers, HSP70, CD63 and CD9.

4.4. Temperature dependence on short-term storage of exosomes

For clinical usage or basic research of exosomes, short-term storage of exosomes may be needed. We incubated the exosomes isolated by Exo-Quick method at ranging from 4 to 90°C for 30 min. And then HSP70, CD63 and CD9 level were measured by immunoblot assay. As shown in Figure 4.2, all representative exosome markers were detected at 4°C, 37°C and RT. The exosomes incubated at 60°C for 30 min showed slight loss of HSP70 compared with samples below 37°C. Furthermore, 90°C incubation resulted in degradation of all exosomal proteins. This result indicated that differential vulnerability of exosome markers to temperature and the temperature higher than 37°C is not optimal for short-term storage of exosomes.

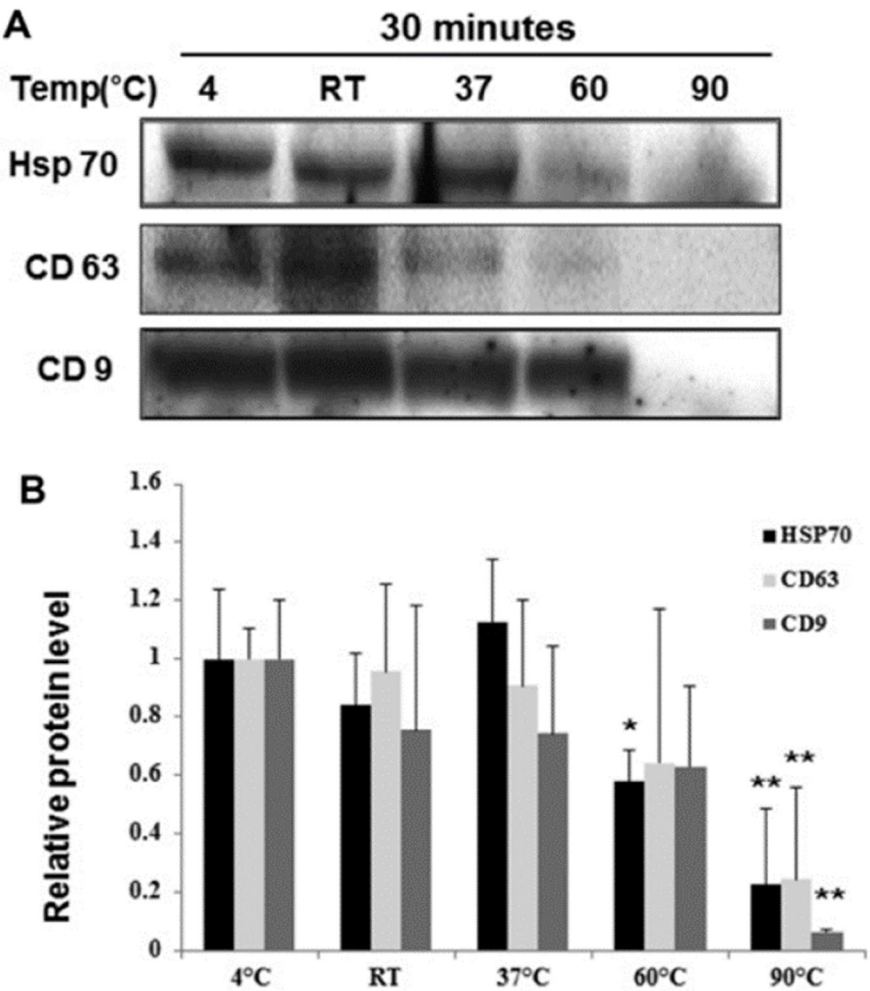


Figure 4.2 Effects of short-term storage condition on exosome recovery. Isolated exosomes from HEK293 cells were suspended with protein extraction buffer and stored at from 4 to 90°C for 30 min. After incubation, exosomes were isolated and exosomal protein markers were detected using western blot (A). Bar graph shows relative quantification of band intensity for HSP70, CD63 and CD9 (B). n = 3; *p < 0.05, **p < 0.01 vs. 4°C.

4.5. Temperature dependence on short-term storage of exosomes

To investigate exosomal protein level in different long-term storage conditions, proteins were extracted by protein extraction buffer and stored under a wide range of temperatures for 10 days. Exosome-associated protein levels were examined using same amount of exosomes from different storage condition. Immunoblot assay result showed that intact exosome markers were shown in -20 and -70 °C samples. Conversely, complete loss of CD63 was examined by incubation at 4°C and RT, and exosomes incubated at RT showed partial decrease of HSP70 level (Figure 4.3). Exosomes were isolated from same amount of medium after storage at various temperature conditions for 10 days, and concentrations of proteins and RNA were measured. Compared with samples from 4 °C, -70 °C and freshly isolated exosomes, sample stored at RT showed higher reduction of protein and RNA amount (Figure 4.4). In addition, more dispersed exosome population was examined in RT storage sample using flow cytometry (Figure 4.5). These results indicated that freezing temperature below -20 °C is ideal for long-term storage of exosomes, and CD63 and/or HSP70 was more susceptible to high temperature damage than CD9 marker.

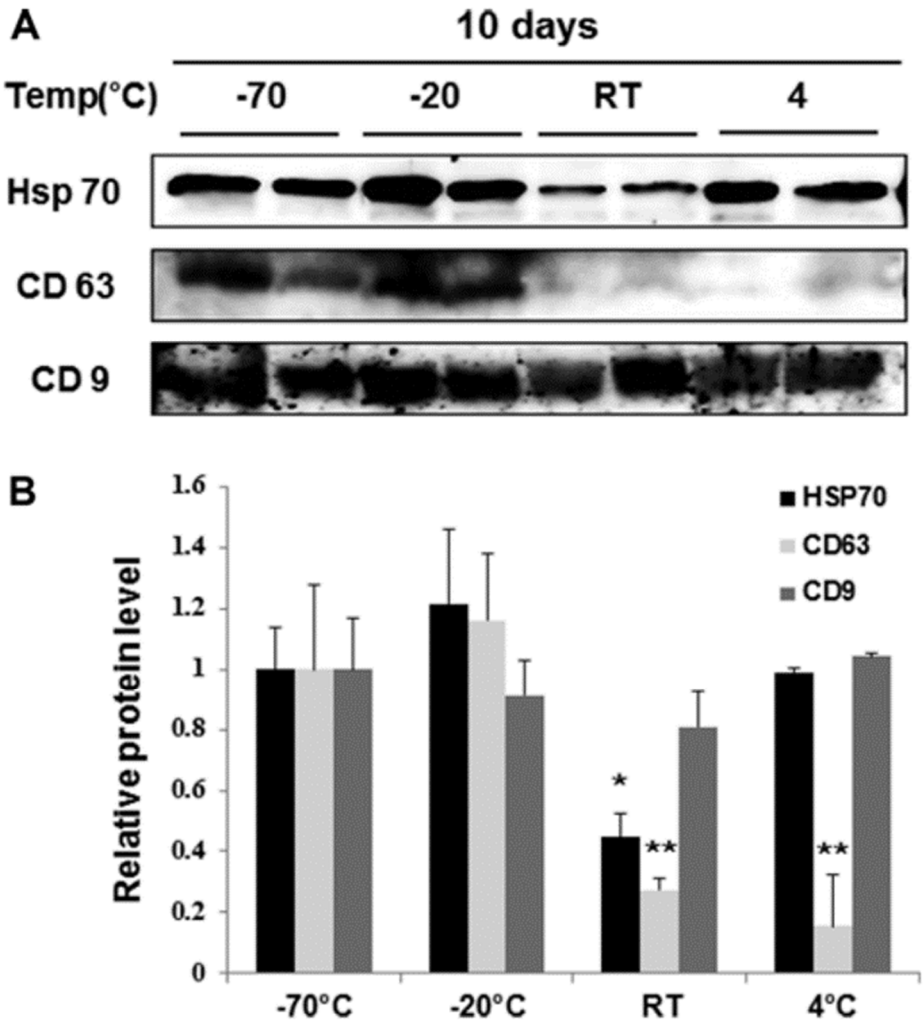


Figure 4.3 Effects of long-term storage condition on exosome recovery. Isolated exosomes from HEK293 cells-derived conditioned medium were stored at from -70°C to RT for 10 days and exosomal proteins from isolated exosomes were examined using western blot (A), and bar graph represents relative band intensity for HSP70, CD63 and CD9 (B). n = 3; *p < 0.05, **p < 0.01 vs. -70°C.

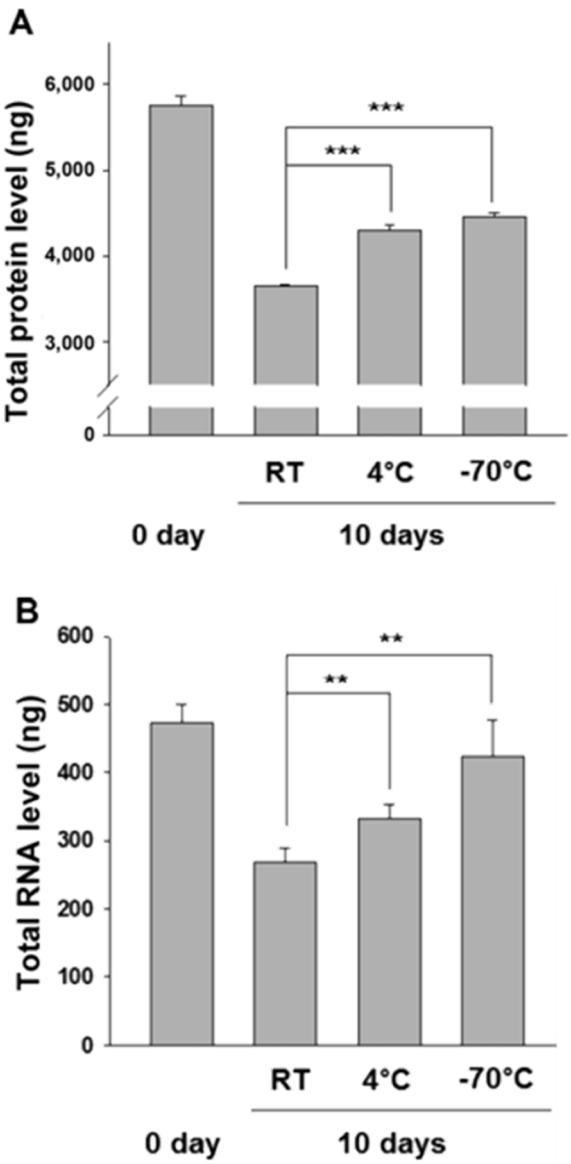


Figure 4.4 Exosomal protein and RNA concentration measurement. Exosomal protein and RNA were extracted from exosomes stored at various temperatures and protein concentration was measured by BCA protein quantification (A) and

NanoDrop for RNA concentration (B). n = 4; **p < 0.01, ***p < 0.001.

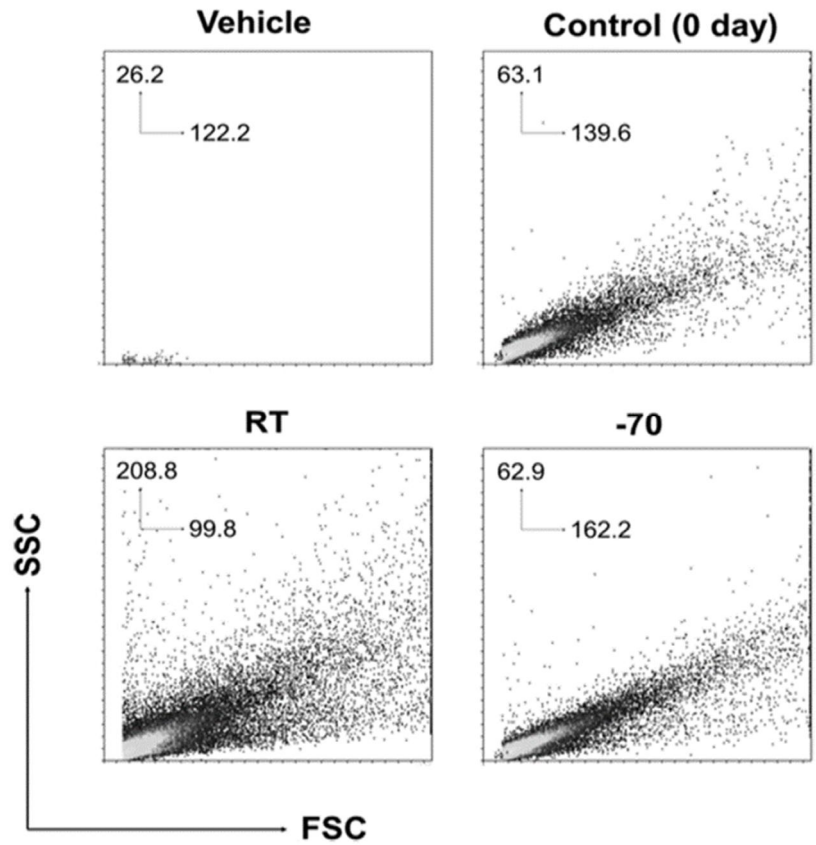


Figure 4.5 Flow cytometric analysis of exosome population. Flow cytometry buffer (Vehicle), freshly isolated exosomes (Control) or exosomes stored at RT or -70 °C for 10 days were analyzed using flow cytometry. Numbers indicate mean of side scatter (SSC) or forward scatter (FSC) of total exosome population.

4.6. Summary

Despite recent advances in our understanding of exosomes, most of information from many researches has been obtained from impure population of exosomes because of the absence of stringent criteria for short-term or long-term preservation of exosomes. For consistency of the results from different researchers, proper protocol for storage of exosomes needs to be established. In this study, we demonstrated that incubation at high temperature degraded exosomal protein, and freezing storage conditions were more proper for longterm storage.

Exosomes were regarded as a critical mediator of pathogenesis. Cancer cell derived exosomes seem to induce cancer favorable microenvironment, leading to cancer progression [12,13]. Amyloid beta protein, major component of Alzheimer's disease, is associated with neuronal cell derived exosomes, resulting in propagation of disease protein [15,16]. Furthermore, cells release pathogenic prion protein of CJD, contributing progression of the disease [17,18]. Exosome associated microRNA from dendritic cells represses target mRNA of recipient cells, modulating inflammatory response to endotoxin [32]. In addition to these pathogenic roles, protein and microRNA profile of exosomes have been regarded as a promising biomarker of diseases [5,6,8,33,35]. Since exosomes partially share their characteristics with other extracellular vesicles including MVs and apoptotic blebs [36], partial contamination of other extracellular vesicles is inevitable. In order to study homogeneous exosomes population from samples, influence of difference of storage condition on exosome population needs to be thoroughly studied.

Although -70 ℃ is the most used condition of exosome preservation, storage of higher temperature of exosome is necessary during procedure of the experiment.

Therefore, our results showed that higher temperature than -20 °C is not suitable for preservation of intact exosomes. Researchers must be aware of experiment's limitation in short-term and/or long-term storage of exosomes which can cause partial change of samples. Concentration, protein and nucleic acids profiles, functions of exosomes could be changed by wide range of factors. For example, pH influences isolation yield of exosomal protein and nucleic acids [31]. Based on our results, temperature is definitely a critical factor in exosome preservation and isolation.

In summary, our results show that storage temperature and period differentially affects exosome stability, and exosomes should be kept at freezing temperature, which can preserve all exosome populations, for clinical application and basic research of exosomes.

CHAPTER 5

In vitro Modeling of Exosomes from ASCs for Huntington's disease

5.1. Introduction

Huntington's disease (HD) is a progressive autosomal dominant neurodegenerative disorder caused by abnormal CAG repeats in the huntingtin gene, resulting in the production of mutant huntingtin (mHtt). The accumulation of mHtt aggregates causes striatal cell death through transcriptional dysregulation, activation of intrinsic apoptosis pathways, mitochondrial dysfunction, and altered protein– protein interactions (Davies et al., 1997; Panov et al., 2002; Browne, 2008; Oliveira, 2010). Dysregulated mitochondrial activation has been detected in the brains of both HD patients and transgenic HD mice (Panov et al., 2002), and mitochondrial dysfunction is a major causative factor in neurodegenerative disorders (Filosto et al., 2011; Johri & Beal, 2012). Recent studies have shown that activation and protection of mitochondria using stem cells delays the progression of in vitro and in vivo HD (Lee et al., 2009; Im et al., 2013). Downregulation of the p-CREB-PGC1a pathway is associated with mitochondrial dysfunction in HD; therefore, this pathway is a potential therapeutic target (McGill & Beal, 2006; St-Pierre et al., 2006). Human adipose-derived stem

cells (hASCs) are a feasible source for stem cell-based therapy, owing to their abundance, multipotency, and paracrine effects, as well as from the standpoint of ethical considerations (Schaffler & Buchler, 2007; Mizuno, 2010). Stem cells release various factors, including neurotrophic growth factors, which can improve the hostile microenvironment associated with disease (Gnecchi et al., 2008; Lee et al., 2011). The beneficial paracrine effects of hASCs on the progression of HD have been studied in vitro and in vivo (Lee et al., 2009; Im et al., 2013).

Exosomes are small extracellular vesicles of 30–100 nm diameter, containing nucleic acids and proteins, that act as messengers, delivering factors and signaling molecules to close and distant cells (Mathivanan et al., 2010; Vlassov et al., 2012; Bernal et al., 2014). Exosomes are present in most biological fluids, including blood and urine, and in the cell culture medium (Keller et al., 2006; van der Pol et al., 2012). They have been actively studied as a therapeutic agent, biomarker, and drug delivery vehicle (Michael et al., 2010; Arslan et al., 2013; Ohno et al., 2013). Thus, exosomes may be potential vehicles for delivering paracrine factors.

Although paracrine factors of stem cells has therapeutic potential on many neurodegenerative diseases including HD, precise effect of ASC-exo on HD have not been investigated. In this study, we examined the effects of ASC-exo on key phenotypes of HD including p-CREB-PGC1 pathway, apoptotic proteins, cell death, and mitochondrial dysfunction in R6/2-derived neuronal cells.

5.2. Methods

5.2.1. Preparation of human ASCs

Human adipose stem cell harvesting and culture Samples of human subcutaneous abdominal adipose tissue were taken from three consenting patients undergoing reconstructive surgery at Seoul National University Hospital, Korea. The study using human samples was performed with approval from the Institutional Review Board (IRB) in Seoul National University Hospital. hASCs were cultured as previously described (Im et al., 2013). Briefly, adipose tissues derived from subcutaneous were digested in collagenase type I solution (Invitrogen, CA, USA) for 1 h at 37 °C. The stromal fraction (a pellet) was treated with red blood cell lysis buffer for 5 min at room temperature after removing fat fractions by centrifugation, filtered through a 100 lm nylon mesh, and centrifuged at 1200 g for 10 min. The pellet was resuspended with EGM-2MV (Clontecs, MD, USA), and cultured in the same medium.

5.2.2. Preparation of in vitro HD model

A colony was maintained by breeding ovarian transplant R6/2 females (B6CBA-Tg(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 to 20:00 hours) 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 (NSCs) from R6/2 mice. In brief, after killed by CO2 gas, brain tissues of 9-week-old mice were used for primary culture and NSCs were isolated by dissection and trypsin treatment. Cells were incubated in culture medium consisting of DMEM/F12, 1% PSA (penicillin-streptomycin-amphotericin; Invitrogen, Carlsbad, CA, USA), 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% O_2, 5% CO_2 humidified atmosphere. NSCs were differentiated in the differentiation medium, which was composed of DMEM/F12, 1% PSA, 2% B27, and 5% FBS.

5.2.3. Isolation and treatment of ASC derived exosome

To deplete exosomes from the culture medium, EGM-2MV was centrifuged at 100 000 g for 15 h at 4 °C and supernatant was used for culture of ASC. ASC culture medium was centrifuged at 1200 g for 20 min to eliminate cells and cellular debris (Lee et al., 2015). All centrifugation steps were performed at 4 °C. Exosomes were isolated by Exo-quick exosome precipitation kit (System Biosciences, Mountain View, CA), according to manufacturer's specifications. Briefly, the supernatant (10 mL) was mixed thoroughly with 1 mL of Exo-Quick exosome precipitation solution and centrifuged at 1500 g for 30 min. The supernatant was then removed and centrifuged at 1500 g for 5 min after adding buffer. The remaining exosome pellets were then suspended in 1 mL PBS. Exosome concentration was measured using microBCA protein assay kit (Thermo Scientific/Pierce, Rockford, IL, USA) for treatment. HD cells were treated with 200 lg/mL of ASC-exo at 2 days of differentiation and

incubated for 5 days. Control groups were treated with same volume of PBS.

5.2.4. 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 300 cells are counted in each group (Im et al., 2015). Em48 (red) or DAPI (blue)-stained cells were counted using an inverted microscope (BX61, Olympus Corporation, Tokyo, Japan).

5.2.5. Protein extraction and western blot analysis

Cultured HD cells were washed and harvested in PBS (phosphatebuffered 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 of HD cells 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, 4–12% Novex NuPage Bis-Tris gel, Invitrogen, Mount Waverley, Australia) and transferred to a polyvinylidene fluoride membrane (PVDF, Millipore, Bedford, MA, USA) in transfer buffer after blocking with 5% non-fat-dried milk dissolved in 19 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-PGC1a (1:200; Santa Cruz, CA, USA), p-CREB (1:1000; Cell signaling, Beverly, MA, USA), anti-p53 (1:1000; Cell signaling, MA, USA), anti-Bax (1:200; Santa Cruz, CA, USA), Em48 (1:500, Millipore, CA, USA), and anti-b-actin (1:200, Santa Cruz, CA, USA). Blots were then incubated with horseradish peroxidase-conjugated secondary anti-mouse or antirabbit antibodies (1:3000, GE Healthcare, NJ, USA), and developed using ECL solution (Enhanced chemiluminescence solution, Advansta, 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.

5.2.6. Mitochondrial Dysfunction analysis

Cells in a plate were washed with PBS and stained with MitoSOX Red (Invitrogen, CA, USA) for 15 min. Cells were harvested and washed once with buffer after staining. Cells were measured by flow cytometry and the fluorescence was analyzed by Winmdi 2.9 (Kim et al., 2015).

5.2.7. 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 ASC-exo 200 μg/mL for 48 h. After 48 h,

WST-1 reagent was added to each well, and cells were incubated at 37 °C and 5% CO_2 for 2 h. Absorbance was measured using a plate reader at 450 nm (reference 650 nm) and the result shown represent the averages of three independent experiments.

5.2.8. Statistical analysis

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

5.3 Reduction of accumulation of mHtt aggregates by ASC-exo

To investigate whether ASC-exo has a protective role in HD, we treated an in vitro HD model, which showed mHtt aggregations in nucleus after day 7 of induction, with ASC-exo. ASC-exo (200 lg/mL) was applied to the cells for 5 days after inducing mHtt aggregation. At day 7, the control and ASC-exo groups were fixed with 4% paraformaldehyde and stained with the Em48 antibody to detect mHtt aggregates, with DAPI as a counter stain (Figure 5.1A). We counted DAPI (+) and Em48 (+) cells in the control (HD control) and ASC-exo treated groups (HD ASC-exo). The ratios of double-positive cells to DAPI (+) cells were 20.8 ± 3.3% for HD control and 10.1 ± 0.3% for HD ASC-exo (Figure 5.1B). To confirm the reduction of mHtt aggregates in cells treated with ASC-exo, aggregates were also quantified by western blot (Figure

5.2A). In HD ASC-exo groups, levels of mHtt aggregates in cells were significantly decreased (Figure 5.2B).

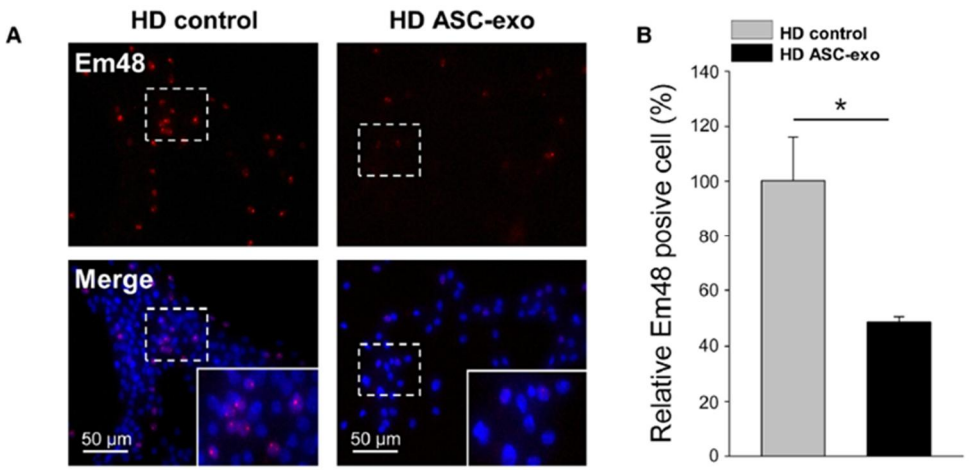


Figure 5.1 Treatment of ASC-exo decreases the accumulation of mHtt aggregates. mHtt aggregates were analyzed by immunocytochemistry. For immunocytochemistry, cells were stained with Em48 (red) and DAPI (blue) and the ratios of Em48 (+) to DAPI (+) cells were calculated (A). The ASCexo group showed less mHtt aggregates than the control group (B). Scale bar = 50 μm. *P < 0.05, **P < 0.001 (n = 3).

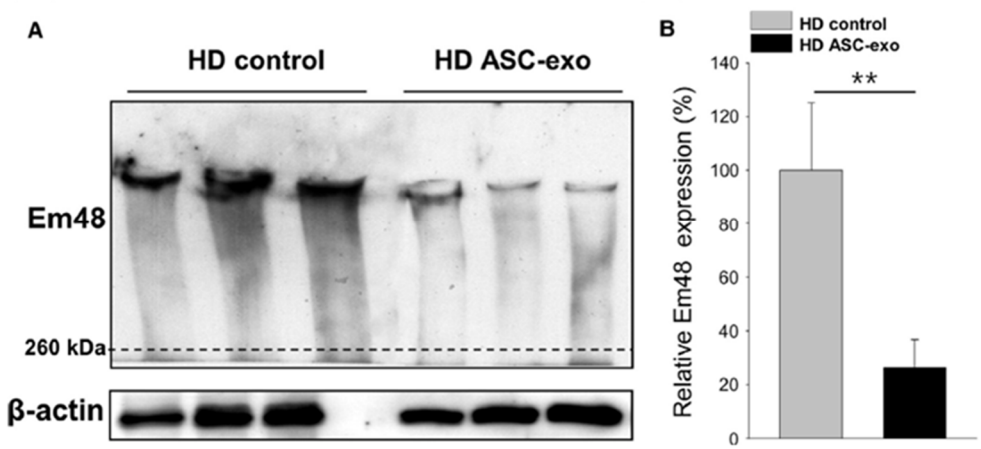


Figure 5.2 Statistical Treatment of ASC-exo decreases the accumulation of mHtt aggregates. mHtt aggregates were analyzed by western blotting. Western blotting confirmed that treatment with ASC-exo decreased the accumulation of mHtt aggregates (A). Normalized mutant huntingtin level was represented as bar graph (B). Scale bar = 50 μm. *P < 0.05, **P < 0.001 (n = 3).

5.4 Activation of mitochondria and apoptotic proteins by ASC-exo

To examine the effects of ASC-exo on the p-CREB-PGC1a pathway, cells were treated with control medium or ASC-exo for 2 days after day 2 of differentiation. Treatment with ASC-exo promoted expression of p-CREB and PGC1a (Figure 5.3). To investigate whether ASC-exo protect against apoptosis in vitro HD model, we determined the levels of apoptosis-related proteins by western blotting. We found that p53, Bax, and cleaved caspase-3 levels were lower in the HD ASC-exo groups than in the HD control groups (Figure 5.4).

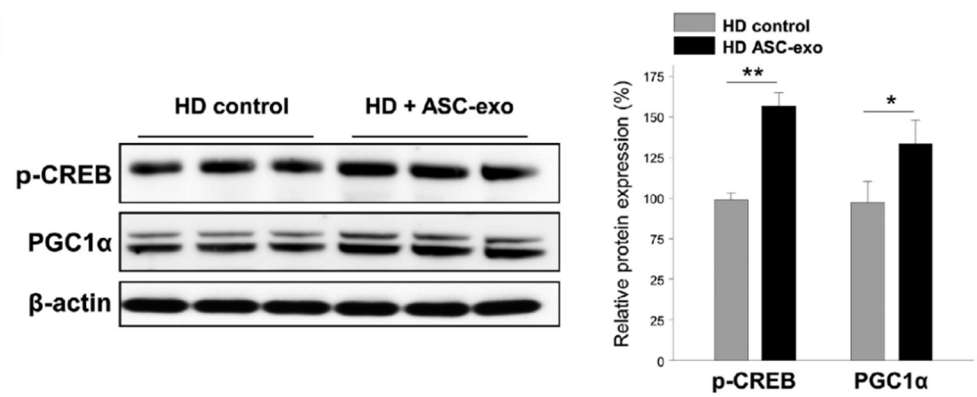


Figure 5.3 Treatment of ASC-exo improves mitochondrial function and reduces apoptosis. As shown by western blotting, treatment with ASCexo increased the levels of PGC1a and p-CREB. *P < 0.05, **P < 0.001 (n = 3).

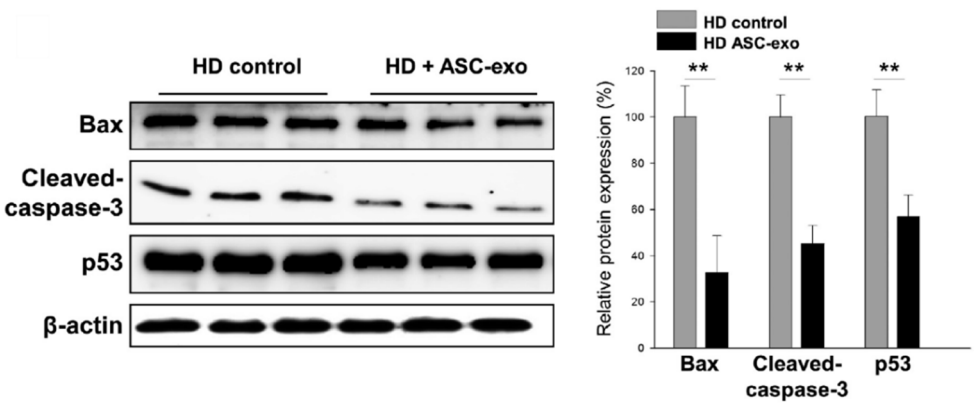


Figure 5.4 Treatment Treatment of ASC-exo improves mitochondrial function and reduces apoptosis. As shown by western blotting, treatment with ASCexo decreased the levels of p53, Bax, and cleaved caspase-3. *P < 0.05, **P < 0.001 (n = 3).

5.5 Mitochondrial protection and cell survival roles of ASC-exo

To test whether ASC-exo has mitochondrial protection and cell survival roles, we investigated the mitochondrial superoxides and cell survival using the MitoSOX Red and WST-1. The result showed that the fluorescent intensity (red) is reduced in cells treated with ASC-exo compared to HD control (Figure 5.5A) and treatment of

ASCexo significantly increases cell survival (Figure 5.5B). As the results, ASC-exo improves mitochondrial dysfunction and cell survival in vitro HD model.

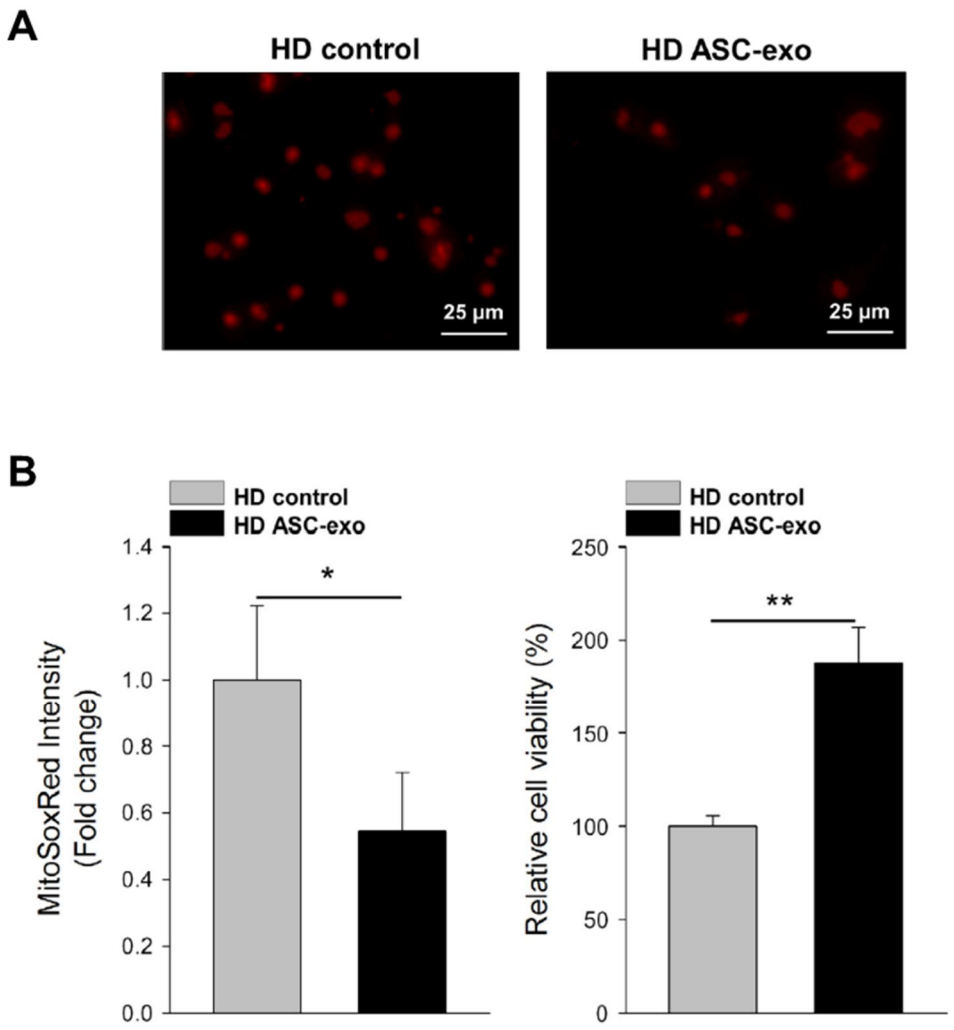


Figure 5.5 The effects of ASC-exo on cell protection. HD control and HD ASC-exo cells were subjected to MitoSOX Red, which was observed by

fluorescence microscopy. MitoSOX Red intensity was reduced in cells treated with ASC-exo

(A). Cell survival was investigated using the WST-1. ASC-exo significantly increased cell survival (B). Scale bar = 25 lm. *P < 0.05, **P < 0.01 (n = 3).

5.6 Summary

In this study, we investigated the neuroprotective effects of ASCexo in vitro HD model by examining the activation of mitochondrial function and modulation of apoptosis. Our results suggest that ASCexo decreases accumulation of mHtt aggregates, activates the p-CREB-PGC1a pathway, and modulates the expression of apoptotic proteins. Cell therapy using hASC has been suggested as a feasible strategy due to the multipotency and ease of culture of ASCs, the variety of factors by them, and the minimal ethical considerations associated with their use (Rehman et al., 2004; Schaffler & Buchler, 2007; Lee et al., 2009; Mizuno, 2010). However, although ASC transplantation into in vivo models has shown beneficial effects, it is not clear whether transplanted ASCs can replace damaged cells (Atsma et al., 2007; Lee et al., 2009; Prockop, 2009). We have previously demonstrated that injection of human adipose-derived stem cell extracts (hASC-extracts) slows progression in the R6/2 model of HD through activation of mitochondrial function (Im et al., 2013). Further study has demonstrated hASC-extracts protect against mitochondrial dysfunction and apoptosis through downregulation of p53 (Liu et al., 2014; Jeon et al., 2016). These findings indicate that the paracrine effects of ASCs may be beneficial to the treatment of neurodegenerative disorders, although further studies are required to elucidate the specific factors mediating these paracrine effects.

Exosomes may be ideal carriers for delivery of therapeutic agents to tissues that are difficult to access, such as those in the brain. Methods of isolating exosomes from blood, urine, or saliva in vivo and conditioned medium in vitro have been developed. However, the levels of exosome cargos, such as proteins or non-coding RNAs, are too low for accurate quantification or analysis. We aim to further investigate which factors derived from ASC-exo delay progression of HD.

Exosomes are small extracellular vesicle and difficult to isolate for analysis and experiments. As normal cell culture medium contains FBS, pure isolation of ASC-exo is not easy (Lv et al., 2013). We deplete cell culture medium for ASC-exo experiment and compare exosome-depleted culture medium with conditioned medium using both ultracentrifuge and Exo-Quick kit. Immunoblot result showed that exosome-depleted medium has no exosome. Conversely, abundant exosomes markers were confirmed in conditioned medium using both ultracentrifuge and Exo-Quick kit samples (Figure 5.6).

Exosomes isolated using Exo-Quick kit have heterogeneous vesicles including exosomes. Otherwise, ultracentrifuge method can isolate pure exosomes, but isolation yield of ultracentrifugation is relatively low. To compare the effects of exosomes isolated using Exo-Quick kit and ultracentrifugation method, we treated both exosomes to HD cells and examined mHtt level. As a result, we confirmed similar result of reduction in mHtt (Figure 5.7), and chose Exo-Quick kit considering its high isolation yield.

We used JC-1 and MitoSOX Red for analyzing mitochondrial dysfunctions as mitochondrial oxidative stress and mitochondrial membrane potential (MMP) are general indicators of mitochondrial health. ASC-exo has significantly protective roles in mitochondrial function (Figure 5.5 and Figure 5.8); however, it is still unclear

whether any factors affected mitochondria though we confirmed ASC-exo to delivery various growth factors and cytokines (data not shown). Therefore, we will further investigate which specific molecules of ASC-exo exert therapeutic effects.

In summary, the therapeutic potential of ASC-exo was confirmed in an in vitro HD model. Considering its easiness of storage and safety, ASC-exo could be an effective and safe strategy than stem cell transplantation for treatment of HD.

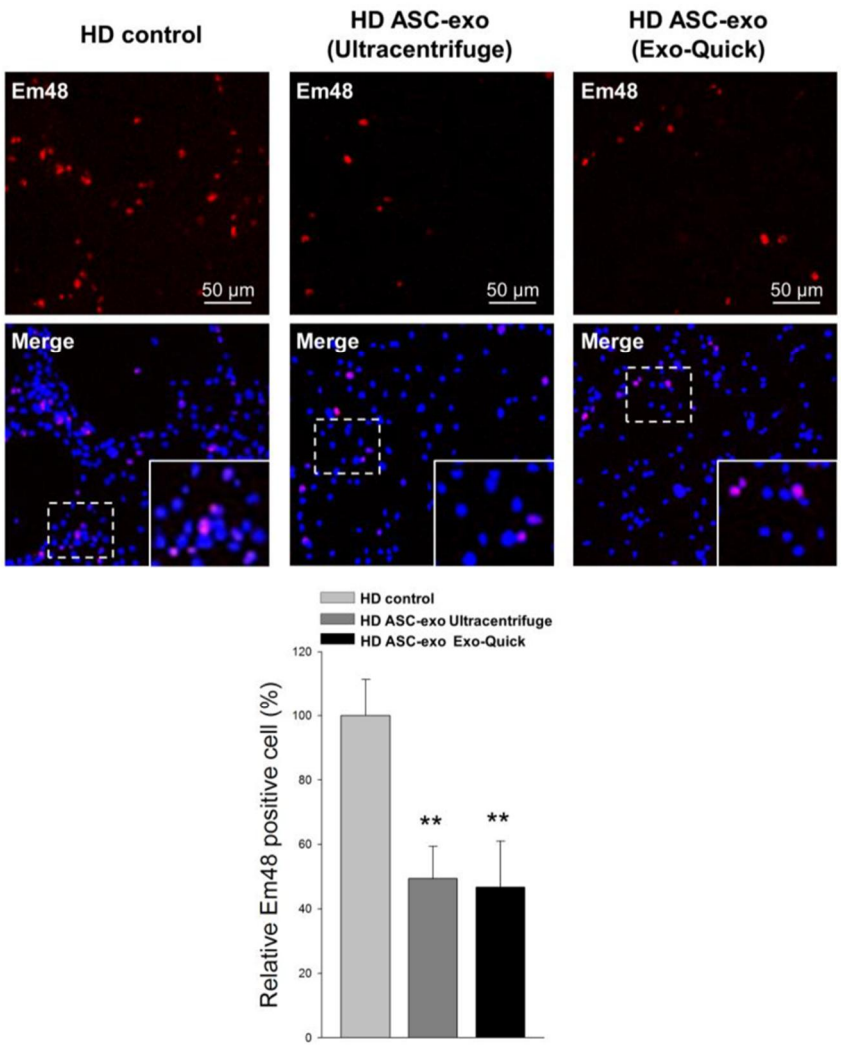


Figure 5.6 ASC-exo extracted by both ultracentrufuge and Exo-Quick kit methods decrease accumulation of mHtt aggregation.Cells were treated with ASC-exo isolated using ultracentrifuge or Exo-Quick method and stained with Em48 (red) and DAPI (blue), and the ratios of Em48 positive cells were calculated. Normalized mHtt level was represented as bar graph. Scale bar = 50μm. **p<0.01 (n=3).

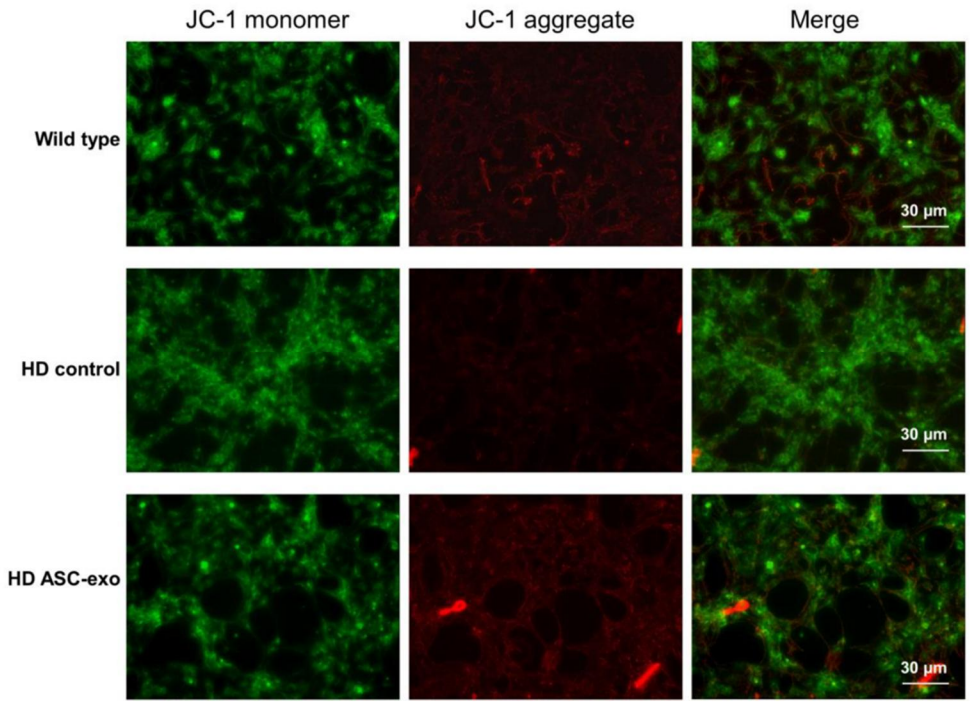


Figure 5.7 ASC-exo improves mitochondrial dysfunction in vitro HD model. *In vitro* HD cells were treated with ASC-exo for 48 hand stained with JC-1, which showed red and green fluorescence to represent aggregated and monomeric JC-1. The fluorescence observed by fluorescence microscopy shows that the JC-1 aggregate was significantly increased in cells treated with ASC-exo (HD ASC-exo) compared to HD control. Scale bar = 25 μm.

CHAPTER 6

Alleviation of Amyotrophic Lateral Sclerosis by ASC-exo

6.1. Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating neurologic disorder that is caused by selective death of motor neurons of the brain, brainstemand spinal cord [1,2]. However, precise mechanism of ALS remains unclear and there is no cure for ALS. A pathologic hallmark of familial ALS is mutant superoxide dismutase 1 (SOD1) aggregation [3]. Aggregation of mutant SOD1 is often found in the motor neurons of mouse model and some percentage of patients of ALS [4-7], and most widely used animal model of this disease is G93A mice model, which have multiple copies of human mutant form of the SOD1 [8,9]. Mitochondrial abnormalities in the neuronal cells are phenotypes of many neurodegenerative diseases including ALS, and normalization of mitochondrial function can be the novel therapeutic strategy of ALS [10].

Adipose-derived stem cells (ASCs) are regarded as potential source of regenerative medicine, since their multipotency, abundance and minimal ethical consideration [11-14]. In addition to direct replacement of damaged cells using stem cells, recent studies

have reported that stem cells secrete various beneficial factors and modulate a hostile environment during illness. In many reports, this is called a bystander or paracrine effect [15-17]. ASCs express and secrete multiple factors for the paracrine effects and have a high proliferation rate with a lower senescence than other tissuederived stem cells [12,15]. Thus, ASCs are reliable source for paracrine function based stem cell therapy. Exosomes are extracellular vesicles that are released by cells and mediate intercellular communication via microRNA [18,19], mRNA [19] and proteins [20]. Exosomes can be isolated from most biological fluids, such as blood [21,22], saliva [23], urine [24] and cerebrospinal fluid [25]. Recent exosome researches have revealed that exosomes mediates many diseases such as Alzheimer's disease [26,27], Creutzfeldt-Jacob disease [28,29], cancer [30,31] and ALS [32]. Since their significant role in biology, exosomes have been widely studies as a biomarker and therapeutic agent [21-23].

Although exosomes derived from stem cells has therapeutic potential on many illnesses, precise mechanism of how ASC derived exosomes (ASC-exo) affect ALS progression has not been investigated. In this study, we examined the effects of ASC-exo on key hallmarks of ALS including SOD1 aggregation, elevated SOD1 level and abnormal mitochondrial protein levels in G93A neuronal cells.

6.2. Methods

6.2.1. Human ASC culture

Subcutaneous adipose samples were obtained from normal humans who provided written informed consent to participate in the experiment. Adipose samples obtained from the patients were kept in phosphate buffered saline (PBS) containing antibiotics/ antimycotics (Invitrogen, USA) and transported to our laboratory within a day. The adipose samples were chopped with sterile scissors, then digested in 0.075% collagenase type I solution (Invitrogen, USA) with gentle shaking for 1 h at 37 ^{o}C. Mature adipocyte fractions were removed from stromal fractions by centrifugation at 1200g for 10 min. The remaining stromal fractions were treated with red blood cell lysis buffer, for 10 min at room temperature, filtered through a 100 μm-nylon mesh, and centrifuged at 1200g for 10 min. The remaining stromal fractions of the samples were resuspended and cultured in endothelial growth mediume2 MV (EGM-2MV; Clonetics, MD, USA), which contained vascular endothelial growth factor, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), insulin-like growth factor-1, hydrocortisone, and ascorbic acid with 5% fetal bovine serum (FBS). The ADSCs used for the generation of ADSC-exo were at passage 3-4 [33].

6.2.2. G93A primary neuronal cell culture

We modified a previously published method [34]. To culture primary neurosphere cells, neural stem cells (NSCs) were isolated from the subventricular zone (SVZ) of 9 weeks G93A mouse brain. Brain tissue was dissociated in Tryple-Express (Invitrogen,

CA, USA) by gentle pipetting. Dissociated cells were obtained by centrifugation at 200g for 10 min. Cell pellet was resuspended with PBS and filtered through 40 mm nylon mesh cell filters (Falcon, Suffolk, UK), and then washed three times. Cells were seeded at a density of 1×10^5 cells/ml in 25 cm^2 flasks in DMEM/F12 with B27 (Invitrogen, USA),1% antibiotics/antimycotics, 20 ng/ml bFGF and 20 ng/ ml EGF (R&D Systems, USA). NSCs were differentiated in the differentiation medium composed of DMEM/F12 with 2% B27 and 5% FBS for in vitro ALS model.

6.2.3. Isolation of ASC-exo

To deplete bovine exosomes from the medium, EGM-2MV was centrifuged at 100,000g for 15 h at 4 ^oC and supernatant was used for ADSC culture [35]. For the preparation of ADSC-exo, the cultured ADSCs were cultured for 5 days at 37 ^oC in a 5 % CO_2 incubator. Exosome isolation using Exo-quick exosome precipitation kit (System Biosciences, USA) was performed according to manufacturer's specifications. Briefly, culture medium was centrifuged at 1200g for 20 min to remove cells and debris, and the 3 ml CM was mixed thoroughly with 0.6 ml of Exo-Quick exosome precipitation solution and incubated for 24 h at 4 ^oC. CM complex were centrifuged at 1,500g for 30 min, and then the supernatant was removed and centrifuged at 1,500g for 5 min again. The remaining exosome pellet was resuspended in 1 ml PBS. Exosome concentration was measured using BCA protein assay kit (Pierce, IL, USA). ALS cells were treated with 200 μg/ml of ADSC-exo at day 2 and day 6 of differentiation. Control groups were treated with same volume of PBS.

6.2.4. Immunocytochemistry

Cells fixed on coverslips were treated with blocking solution and permeabilized for 60 min in 4% normal goat serum and 0.2% Triton X-100 in PBS [14]. Primary antibodies for SOD1 (1:1000, Abcam, USA), microtubule-associated protein-2 (MAP2) (1:200, Millipore, MA, USA), glial fibrillary acidic protein (GFAP) (1:200, Sigma- Aldrich, USA) were added, coverslips were incubated overnight at 4 ^{o}C. After a wash in PBS, the coverslips were incubated with secondary antibodies (1:500, phycoerythrin (PE)-conjugated antimouse IgG) for 2 h at room temperature. DAPI (Invitrogen, USA) counter staining was performed. Fluorescent images were obtained using the Leica DM5500 (Leica Microsystem, Switzerland). Images were processed and merged by Leica Application Suite software.

6.2.5. Protein extraction and western blot

Protein extracts and exosomal proteins were prepared using RIPA buffer (Radio immunoprecipitation assay buffer, Thermo Scientific, USA) containing freshly added protease inhibitor and phosphatase inhibitor (Roche, USA). The concentrations of total cell lysates and isolated exosomes were measured by BCA protein assay kit. Samples were loaded on sodium dodecyl sulfatepolyacrylamidem gels (SDS-PAGE) and electrotrasnferred to a polyvinylidene fluoride membrane (Millipore, USA). The blots were probed with primary antibodies: CD9 (1:200; Santa Cruz, USA), CD63 (1:200; Santa Cruz, USA), heat shock protein-70 (HSP70) (1:200; Santa Cruz, USA), SOD1 (1:1000, Abcam, USA), PGC-1a (1:1000; Santa Cruz, USA), p-CREB (1:1000;

Santa Cruz, USA), CREB (1:1000; Santa Cruz, USA), Sirt-1 (1:1000; Santa Cruz, USA), Actin (1:1000; Santa Cruz, USA) followed by horseradish peroxidase conjugated secondary anti-mouse or rabbit antibody (1:3000; GE Healthcare, USA). The experiment was repeated three times for analysis and the data shown are representative. The relative band intensities and protein levels were analyzed by ImageJ software [36].

6.2.6. Preparation of cytoplasmic extracts

Cytoplasmic extracts was prepared as previously described [37]. Briefly, cytoplasmic extracts were prepared using NE-PER nuclear and cytosolic extraction reagents (Pierce, USA). Briefly, cells were harvested and then gently lysed for 15 min in 100 µl cold PRO-PREP lysis buffer. Lysates were centrifuged at 14,000g for 10 min. Supernatants were collected and protein concentrations determined using BCA protein assay kit. The proteins were separated on SDSPAGE and transferred to PVDF membranes.

6.2.7. Dot blot assay

The Cultured ALS cell extracts were prepared using RIPA buffer containing freshly added protease inhibitor and phosphatase inhibitor (Roche, USA). Five micrograms of protein samples were loaded by PVDF membrane (Millipore, USA). The membrane was dried for half-hour at room temperature and blocked with 5% nonfat dried milk for 1 h in a rotational shaker at room temperature. Blots were then incubated at 4 _C overnight with primary antibodies diluted. The following primary antibodies were

used: SOD1 (1:3000, Abcam, USA). Blot was then incubated with horseradish peroxidase-conjugated secondary anti-rabbit antibodies (1:3000; GE Healthcare, USA), and developed. Band intensities were measured using ImageJ software.

6.2.8. Statistical analysis

All values shown in the figures are presented as the mean ± standard deviation. Results were analyzed by analysis of variance (ANOVA) followed by post-hoc test or student's t-test. A 2- tailed probability value below 0.05 was considered statistically significant. Data were analyzed using SPSS version 17.0 (SPSS Inc., USA).

6.3. Primary cell culture and exosome isolation

Human ADSC was isolated and cultured as the previously described [38]. Exosomes from ADSC was isolated and western blot result showed representative exosome markers, suggesting that our exosome isolation method is optimal for exosome research (Figure 6.1). NSCs were cultured from SVZ-derived single cell population using neurosphere method and differentiated using differentiation media, DMEM/F12 with 2% B27 and 5% FBS, resulting in expressions of neuronal cell markers including GFAP and MAP2 (Figure 6.2). Differentiated NSCs from G93A ALS mice model showed gradual increase of mutant SOD1 in the cytosol and we used this NSC for in vitro model of ALS (Figure 6.3).

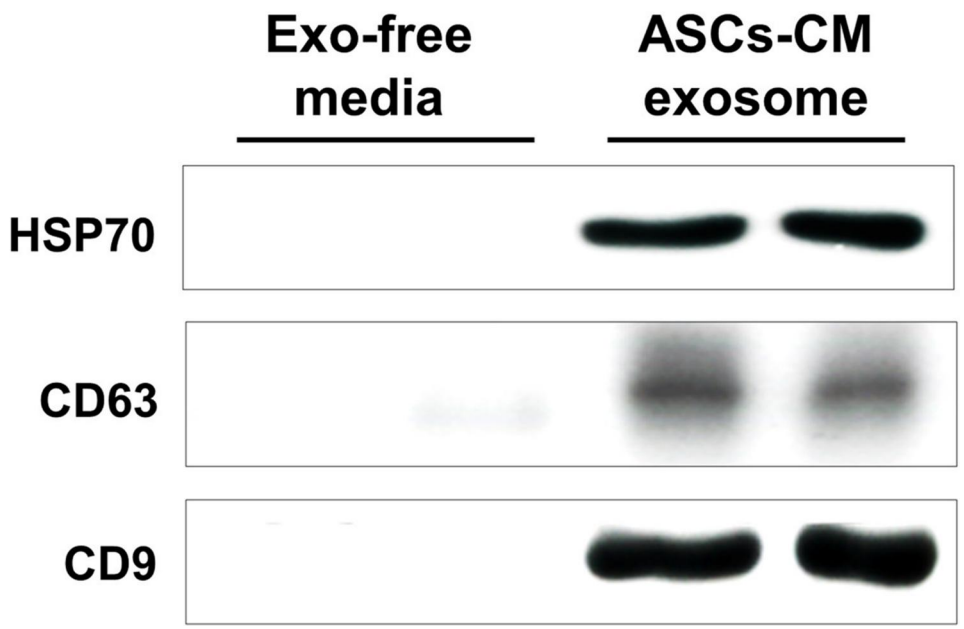


Figure 6.1 Exosome characterization. ADSC was culture with exosome depleted media for 5 days and exosomes in conditioned medium were isolated using Exo-quick method. Isolated exosomes were lysed with RIPA buffer and western blot was performed using representative exosome markers, HSP70, CD63 and CD9.

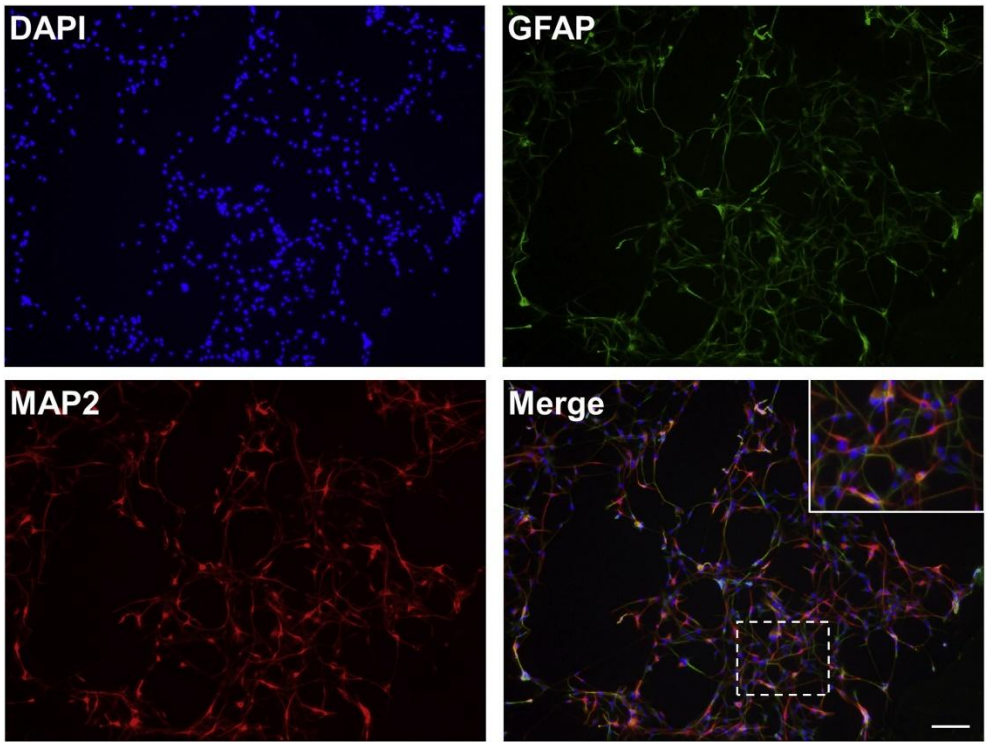


Figure 6.2 Neuronal stem cell culture. Primary dissociated SVZ-derived NSCs were maintained in DMEM/F12/B27 with EGF and bFGF. Cells were maintained using neurosphere culture method and were transferred into growth factor free differentiation medium containing FBS. After 10 days of differentiation, microscopic images showed that representative neuronal cell markers, GFAP and MAP2. Scale bar = 50 μm.

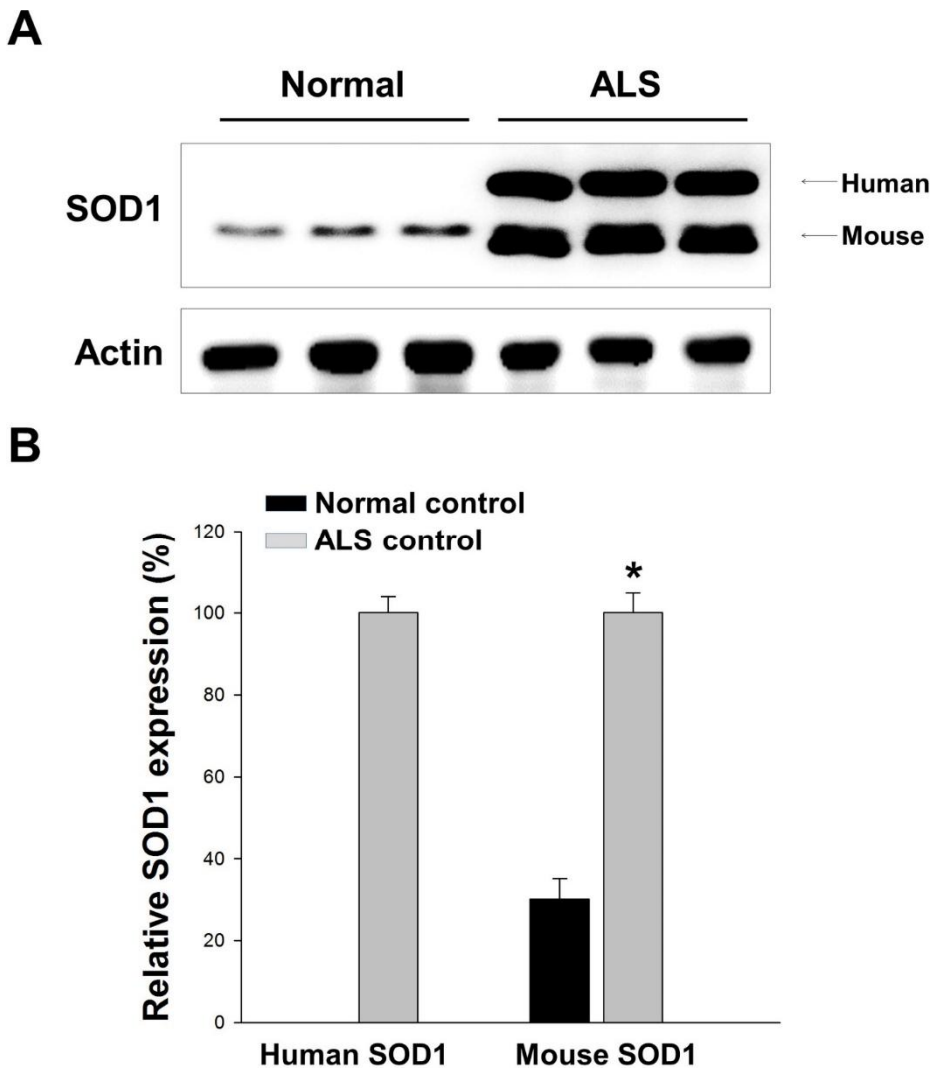


Figure 6.3 Elevated SOD1 level in G93A neuronal cells. Wild type or G93A mouse brain SVZ-derived NSCs were differentiated for 10 days and SOD1 antibody was probed for western blot analysis. Western blot result showed that human SOD1 was only expressed in G93A derived cell, not in wild type derived cells (A). Bar graph showed mean intensity of SOD1 from three independent results. *$p < 0.001$.

6.4. Reduction of mutant SOD1 aggregation in G93A by ASC-exo

To investigate whether ADSC-exo affects accumulation of mutant SOD1 aggregation in G93A neuronal cells, we used an in vitro ALS model using NSCs, which showed SOD1 aggregation in cytoplasm after day 10 of differentiation. After differentiation induction, ADSC-exo (200 μg/ml) were treated two times at day 2 and day 6. The control, ALS and ADSC-exo cells were fixed and stained with mutant SOD1 antibody and DAPI counter stain (Figure 6.4 and Figure 6.5). As shown in Figure 6.4, control cells showed no SOD1 staining and G93A primary neuronal cells showed SOD1 aggregation in the cytoplasm at day 10 of differentiation. Dot-blot assay also showed decrease of SOD1 protein in the ADSC-exo treated cells (Figure 6.5). To confirm the cytosolic SOD1 level in the ADSC-exo treated cells, cells were lysed and SOD1 level was examined using western blot. Western blot result showed that significant decrease of human mutant SOD1 level (Figure 6.6) in ADSC-exo group. There results showed that ADSCexo ameliorates SOD1 protein level and aggregates in the neuronal cells derived from G93A ALS mice.

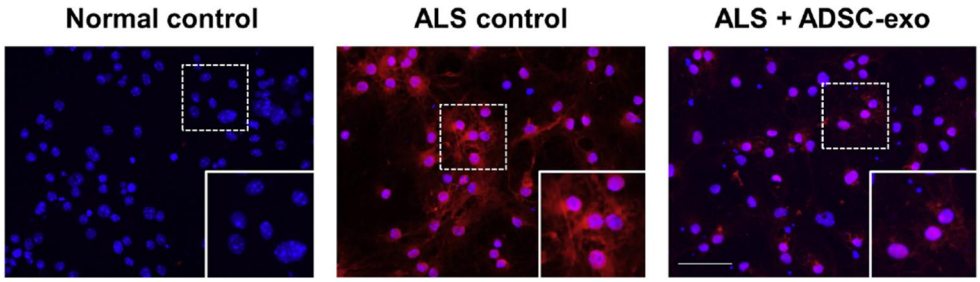


Figure 6.4 ADSC-exo decreases SOD1 aggregation of G93A neuronal cells. To culture NSCs from G93A mice, primary dissociated SVZ-derived NSCs were maintained in DMEM/F12/B2 with EGF and bFGF. After neurosphere cell expansion, these cells were transferred into growth factor free differentiation medium containing FBS. At day 2 and day 6 of differentiation, medium was changed and ADSC-exo was treated. Microscopic images showed that mutant SOD1 aggregation in the cytoplasm (red) and DAPI staining (blue). Scale Bar = 50 μm.

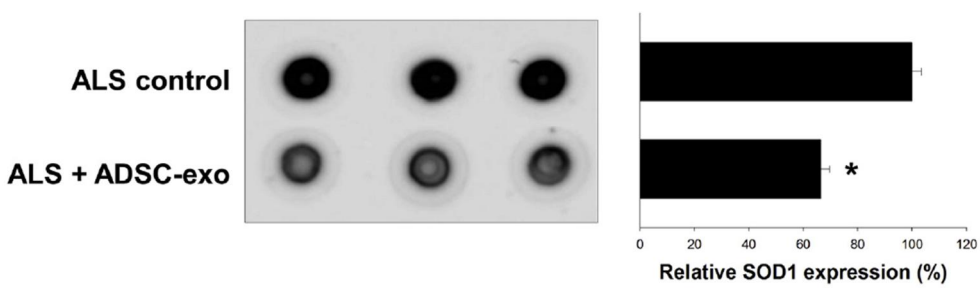


Figure 6.5 Dot blot assay using G93A cell lysates with or without ADSC-exo treatment. Bar graph shows mean intensity of dot blot. *$p < 0.001$.

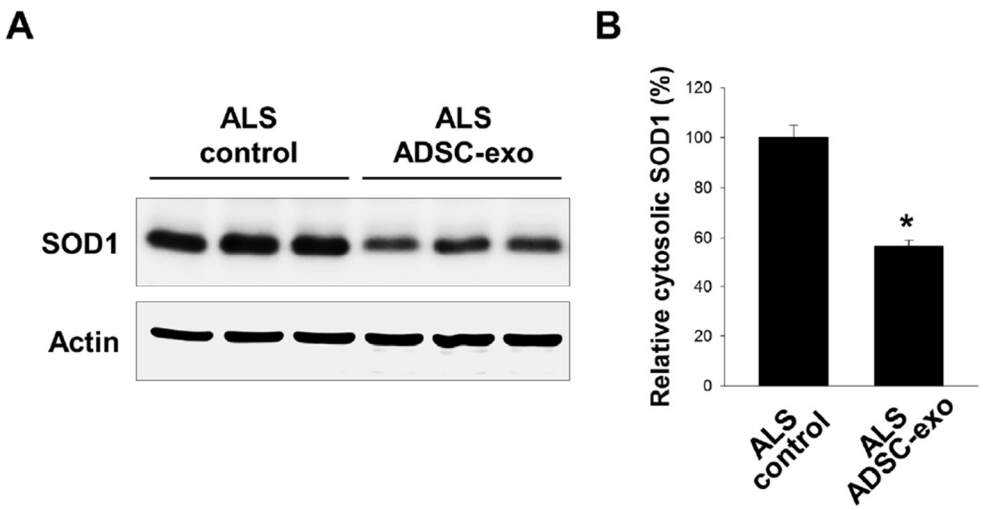


Figure 6.6 ASC-exo reduces cellular level of SOD1 in G93A neuronal cells. NSCs were differentiated for 10 days with or without ASC-exo at day 2 and day 6 of induction, and cells were lysed for western blot analysis. (A) SOD1 level was significantly decreased by two times treatment of ADSC-exo. (B) Bar graph shows the level of human SOD1 normalized to bactin. *$p < 0.001$ vs ALS control.

6.5. Normalization of p-CREB-PGC1α pathway in G93A by ASC-exo

Mitochondrial defects were major phenotypes of neurodegenerative diseases including ALS [10]. Our G93A cells showed abnormal expression of mitochondrial proteins, such as downregulation of p-CREB/CREB ratio, PGC-1a and Sirt-1 level at day 10 of differentiation. To examine the effects of ADSC-exo on these mitochondrial function proteins, cells were treated with ADSC-exo at day 2 and day 6 of differentiation, and cells were lysed at day 10 for western blot analysis. As a result, ADSC-exo treatment normalized decreased levels of p-CREB and PGC-1a of the G93A cells without alteration of decreased Sirt-1 level (Figure 6.7). Normalization of PGC-1a by ADSC-exo was also confirmed in mRNA level (Figure 6.8). This result suggests that abnormal expression of mitochondrial function proteins of ALS cells can be rescued by application of ADSC-exo.

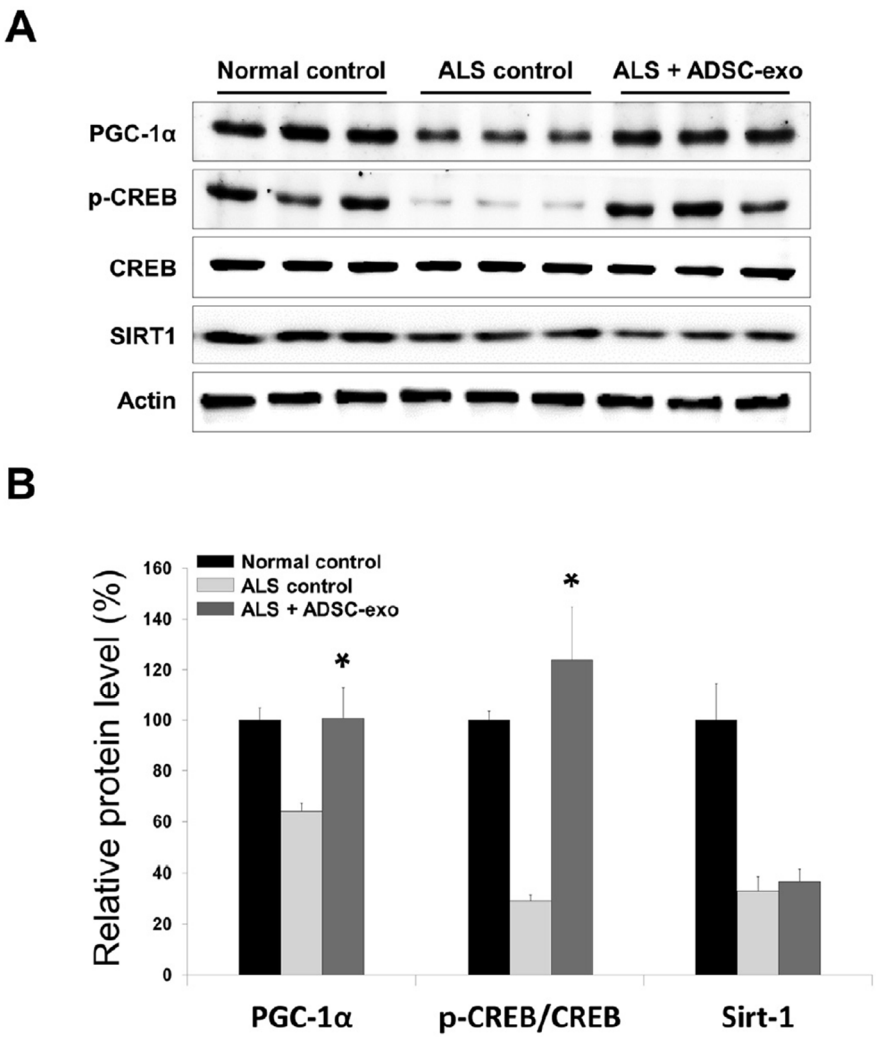


Figure 6.7 ASC-exo rescues abnormal expressions of mitochondrial proteins of G93 cells. NSCs were differentiated for 10 days and ADSC-exo was applied at day 2 and da 6 of induction. (A) Total cell lysates were prepared and western blot analysis was performed using antibodies for p-CREB, CREB, PGC-1a and Sirt-1. (B) Bar graph shows the relative levels of expressions normalized to b-actin. *$p < 0.01$ vs ALS control.

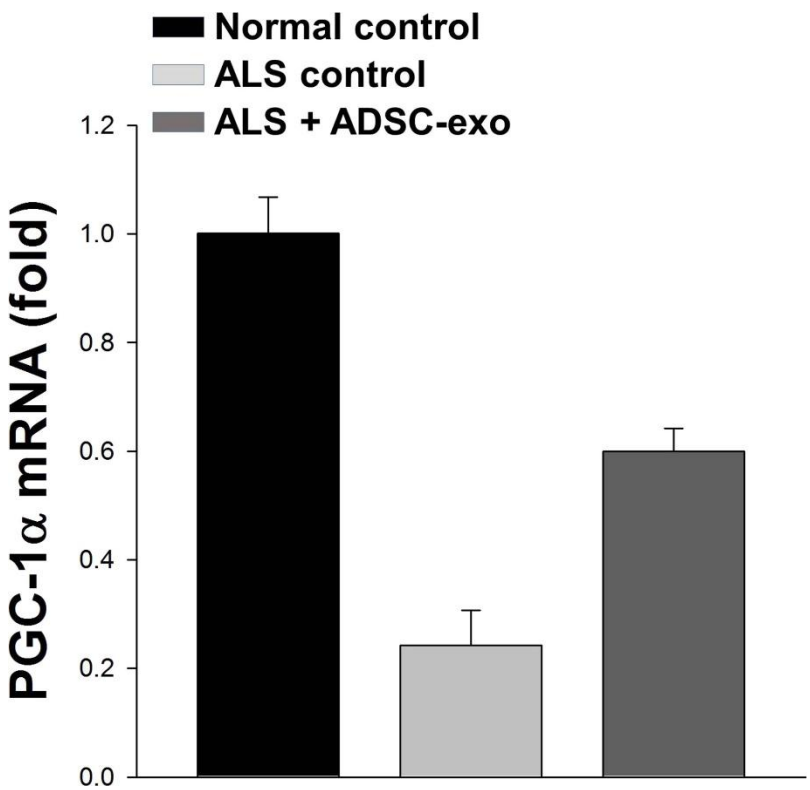


Figure 6.8 Restoration of PGC-1α mRNA level. G93A mouse-derived NSCs were differentiated for 10 days with ADSC-exo or vehicle and total RNA was isolated. qRT-PCR result showed that significant decrease of PGC-1α in G93A cells. Treatment of ADSC-exo resulted in restoration of PGC-1α mRNA level of G93A neuronal cells.

6.6. Summary

In this study,we examined ADSC-exo can reduce increased SOD-1 aggregation in the G93A ALS in vitro model. In addition, G93A primary neuronal cells showed abnormal decrease of mitochondrial function proteins including p-CREB and PGC-1a. These cellular phenotypes of ALS can be normalized by ADSC-exo treatment, suggesting that therapeutic potential of ADSC-exo on ALS.

Stem cells have been widely studies as a novel candidate for regenerative medicine, and ADSC can be regarded as an ideal source of adult stem cells [11]. In addition to multipotency of stem cells, many stem cells showed paracrine function which can be treat hostile pathological microenvironment [12,15,17]. ADSC also secrete various paracrine factors, and ADSC-derived exosomes could be the potential paracrine factor considering their roles in the organism. Our result showed promising effects of ADSC-derived extracellular vesicles. ADSC-exo could be the safer source than stem cell transplantation because it can have similar effects of stem cell without invasive methods and side effects of cell injection.

Although there are many in vitro models for drug screening and mechanism studies, immortalized cell lines cannot reflect normal cells in vivo. Our in vitro system used primary NSCs to examine the kinetics of aggregation and phenotypes of normal neuronal cells from ALS mice model. Immunocytochemistry and western blot result showed that differentiated NSCs showed gradual increase of mutant SOD1 aggregation and ADSC-exo treatment resulted in slowed progression of aggregation formation and protein increase of SOD1.

The mitochondrion is an important organelle on the onset and progression of many illnesses, and CREB-PGC-1a pathway, activator of mitochondrial biogenesis, have

been shown to be dysregulated in many neurological disorders such as Alzheimer's disease [39], Parkinson's disease [40] and Huntington's disease [41e43]. Mitochondria-mediated apoptosis of neurons was found in ALS patients and mice models, and mutant SOD1 proteins have been found in the intermembrane space, matrix and ourter membrane of mitochondria [44e46]. Thus, strategies of prevention of mitochondrial dysfunctions can be effective in ALS therapy. In this research, we found that ADSC-exo activates CREB and PGC-1a in G93A ALS cells, indicating that beneficial effects of ADSC-exo may be derived from restoration of mitochondrial functions.

In this study, we isolated primary NSCs from the G93A ALS model, and these neuronal cells showed mutant SOD1 aggregation and abnormal expression of mitochondrial proteins. We examined the ameliorative effects of ADSC-exo on the major cellular phenotypes of ALS. Finding from our research suggest that ADSC-exo can be a potential source of ALS treatment strategy, and application of ADSC-exo may be possible after confirmation of its effects and safety has been proven.

CHAPTER 7

Amelioration of pathology of Alzheimer's disease by ASC-exo

7.1. Introduction

Alzheimer's disease (AD) is the most common type of dementia with cognitive deficits, resulting from progressive neuronal death in the hippocampus and the cerebral cortex. It is well known that the accumulation of amyloid beta (Ab) is responsible for the progression of AD, which includes widespread neuronal dysfunction and ultimately, cell death (Hardy, 1997; Hardy and Selkoe, 2002). Pathologically, the brain of AD patients is characterized by an accumulation of senile plaques, atrophy of neurites, and neurofibrillary tangles. The plaques contain large amounts of Ab peptide derived from cleavage of the amyloid precursor protein (APP). In familial AD, mutations in APP have been shown to increase the production of Ab42 leading to disease, and this association has led to the hypothesis that Ab is the key to the pathogenesis of AD (Selkoe, 2001). Mitochondria are known to have revevance in AD and Ab accumulates in the mitochondrial membrane and impairs mitochondrial functions. This results in the induction of pro-apoptotic factors, whose activation induces apoptosis (Cha et al., 2012). p53, known as a tumor suppressor, has a critical

role in determining cell fate, and there is abundant evidence that p53-dependent cell death is increased by oxidative damage of cells during AD (Gabbita et al., 1998; Lyras et al., 1997; Miller et al., 2000). p53 induces apoptosis by regulating apoptotic proteins such as Bcl-2-associated X (Bax) and caspase-3 (Eckert et al., 2003; Mattson, 2000).

For quite some time, stem cells have been in the spotlight as a promising candidate for regenerative medicine and tissue engineering. Amongst the many adult stem cell types, adiposederived stem cells (ADSCs) isolated from fat tissues are a feasible variety for experimentation purposes, owing to their multipotency, abundance, and minimal ethical considerations (Dicker et al., 2005; Gimble et al., 2007; Katz et al., 2005). Stem cells secrete various molecules such as growth factors and extracellular vesicles. Recent studies have also reported that in addition to the direct replacement of damaged cells, stem cells secrete various beneficial factors that can ameliorate certain pathological changes in diseases. In many reports, stem cell secretory factors have shown antiapoptotic (Bruno et al., 2012), angiogenic (Deregibus et al., 2007; Sahoo et al., 2011), anti-wrinkling (Kim et al., 2009), and wound healing effects (Hu et al., 2016).

Exosomes are small extracellular vesicles with 30–100 nm diameter. They are secreted from most cell types, and contain various molecular components of their cell of origin, including proteins, mRNAs, and microRNAs (Valadi et al., 2007). Exosomes can be found and isolated in most biological fluids, including saliva (Michael et al., 2010), blood (Gupta et al., 2010; Tsujiura et al., 2010), urine (Zhou et al., 2006), and cerebrospinal fluid (Street et al., 2012). In various diseases, including cancer (Park et al., 2010; Webber et al., 2010), Creutzfeldt-Jakob disease (Fevrier et al., 2004; Vella et al., 2008), and AD (Rajendran et al., 2006; Saman et al., 2012),

exosomes play critical roles in disease progression. Due to their significance in the biological and medical fields, they have been widely studied as a therapeutic agent (Bruno et al., 2012; Hu et al., 2016), biomarker (Gupta et al., 2010; Michael et al., 2010; Povero et al., 2014; Taylor and Gercel-Taylor, 2008; Tsujiura et al., 2010), and a drug delivery vehicle (Ohno et al., 2013).

Although the paracrine function of stem cells is reported to have a therapeutic potential in many neurological disorders, the effects of ADSC-derived exosomes (ADSC-Exo) on the cellular phenotypes of AD have not yet been investigated. In this study, we hypothesized that ADSC-Exo may have an influence on AD phenotypes. Hence, we used an in vitro AD model using neuronal stem cells (NSCs) from the transgenic mice line, TG2576, for AD. NSCs were isolated from the subventricular zone of the brain in diseased mice, and differentiated in a differentiation medium. These neuronal cells derived from the diseased brain showed major phenotypes of AD, including the Ab pathology and increased apoptosis. Therefore, differentiated NSCs of AD mice were treated with ADSC-Exo; subsequently, AD related phenotypes were analyzed. In addition, changes in neurite outgrowth were examined, as neuritis are involved in the synapse formation of the central nervous system.

7.2. Methods

7.2.1. In vitro AD model

We used a previously published method for the in vitro AD model (Liu et al., 2017). To culture primary neurosphere cells, TG2576 mice or wild-type littermate (B6;SJL) were killed by decapitation. Dissections were performed in phosphate-buffered saline (PBS) at 4 ^{o}C with 0.6% glucose and a pH of 7.4; sterile scissor and forceps were used. In order to dissect the subventricular zone, each brain was sliced into 2 mm coronal sections. These tissues were washed in PBS and digested in papain–dispase–DNase solution (Lorne Laboratories, Twyford, UK) with 0.1% dispase (Roche Diagnostics, Hertfordshire, UK) for 4 min, filtered through 40 mm nylon mesh cell filters (Falcon, Suffolk, UK), and then washed again three times. Live cells were calculated using the trypan blue dye (Sigma) exclusion method and seeded at a density of 1×10^5 cells/mL in 25 cm^2 flasks containing Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) with 2% B27 (Invitrogen, Eugene, Oregon, USA), 1% penicillin/ streptomycin/ fungizone (Invitrogen, Eugene, Oregon, USA), 20 ng/mL basic fibroblast growth factor (bFGF), and 20 ng/mL epidermal growth factor (EGF) (R&D Systems, MN, USA).

7.2.2. Culture of ASCs

Previously published method were used for ADSC culture (Im et al., 2013; Lee et al., 2016). Subcutaneous adipose tissues samples were obtained from cognitively normal human subjects who provided written, informed consent to participate in the experiment. Adipose tissues obtained from the patients were maintained in PBS containing antibiotics (Invitrogen, CA, USA), and transported to our laboratory within a day. Adipose samples were digested in 0.075% collagenase type I solution (Invitrogen, CA, USA) with gentle shaking for 1 h at 37 ^{o}C. Mature adipocyte fractions were removed from stromal fractions by centrifugation at 1200×g for 10 min. The remaining stromal fractions were treated with red blood cell lysis buffer (Sigma) for 10 min at room temperature, filtered through a 100 μm nylon mesh, and centrifuged at 1200×g for 10 min. Further remaining stromal fractions of the samples were resuspended and cultured in endothelial growth medium-2 MV (EGM-2 MV; Clonetics, MD, USA), which contained vascular endothelial growth factor, bFGF, EGF, insulin-like growth factor-1, hydrocortisone, and ascorbic acid with 5% fetal bovine serum (FBS). The cells were used for the generation of ADSC-Exo after 3 or 4 passages.

7.2.3. Isolation and treatment of ASC-exo

To deplete exosomes from the culture medium, EGM-2MV was centrifuged at 100,000×g for 15 h at 4 °C, and the supernatant was used for the ADSC culture. Exosomes were isolated using the ExoQuick-TC exosome precipitation kit (System Biosciences, Mountain View, CA), according to the manufacturer's specifications. The ADSC culture medium was centrifuged at 3000×g for 15 min to eliminate cells and cellular debris, and all centrifugation steps were performed at 4 °C. The supernatant (10 ml) was mixed thoroughly with 2 ml of Exo-Quick exosome precipitation solution, and stored at 4 °C overnight. The supernatant mixed with ExoQuick solution was centrifuged at 1500×g for 30 min. The supernatant was then removed and centrifuged at 1500×g for 5 min, and all remaining fluid was removed. The remaining exosome pellets were then suspended in 100 μl PBS. The exosome concentration was measured using the bicinchoninic acid assay (BCA) protein assay kit for treatment. Exosomal markers, HSP70, CD63, and CD9 were not detected in the exosome-free media used for cell culture. On the contrary, in the case of exosomes obtained from the conditioned medium using the exoquick or ultracentrifuge method, exosomal markers were identified (Figure 7.1). AD cells were treated with 200 μg/mL of ADSC-Exo at 2 days of differentiation and incubated for 24 or 48 h. Control groups were treated with an equivalent volume of PBS.

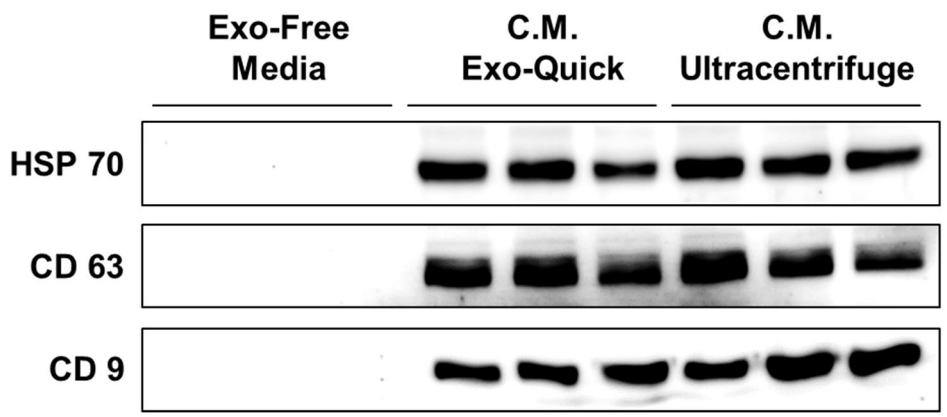


Figure 7.1 Characterization of ADSC-Exo. The representative exosome markers, HSP70, CD63, and CD9, were not detected in the exosome-free media in which the bovine exosome was removed by ultracentrifugation. Representative exosome markers were detected in both the ExoQuick and ultracentrifuge methods in the conditioned medium of adipose-derived stem cells

7.2.4. Aβ ELISA

Concentrations of Ab40 and 42 were analyzed using Aβ ELISA kit according to the manufacturer's instruction (Thermo Scientific, USA). Briefly, conditioned medium of NSCs was centrifuged 3000×g for 15 min to eliminate cells and cellular debris. Aβ Standard was reconstituted to 1 μg/ml with Standard Diluent Buffer (55 mM sodium bicarbonate, pH 9.0) and serially diluted using Standard Diluent Buffer. Standards and diluted conditioned medium were incubated with antibody coated plate, and 50 μl of Aβ antibody was added to each well. After incubation for 3 h at room temperature, 100 μl of Anti-Rabbit IgG HRP solution was added to each well, and incubated for 30 min. After washing 4 times with 1X Wash Buffer, 100 μl of Stabilized Chromogen was added and incubated 30 min in the dark. After adding Stop Solution, 450 nm of absorbance was measured using a microplate reader (VersaMax Tunable Microplate Reader Molecular Devices, USA).

7.2.5. Western blot analysis

Cultured AD cells were washed and harvested in PBS (WelGene, Daegu, Korea) using a cell scraper and collected. Protein extracts were prepared using radioimmunoprecipitation assay buffer (RIPA buffer, Thermo Scientific, USA) containing a protease inhibitor (cOmpleteTM Protease Inhibitor Cocktail, Sigma, USA) and a phosphatase inhibitor (PhosSTOPTM, Sigma, USA), added just before experimentation (1 tablet per 10 ml of RIPA buffer). The protein content of AD cells was determined using a BCA protein assay kit (Thermo Scientific, 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 transferred to a polyvinylidene fluoride membrane (PVDF, Millipore, Bedford, MA, USA) in transfer buffer, after 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 ^{o}C overnight with primary antibodies diluted as recommended in the manufacturer's instructions. The following primary antibodies were used: anti-p53 (1:200;Santa Cruz, CA, USA), anti-Bax (1:200; Santa Cruz, CA, USA), anti-Cleaved Caspase-3 (1:1000; Cell signaling, MA, USA), and anti-β-Actin (1:200, Santa Cruz, CA, USA). Blots were then incubated with horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit antibodies (1:3000, GE Healthcare, NJ, USA), and developed using an ECL solution (enhanced chemiluminescence solution, Advansta, CA, USA). Band intensities were measured using ImageJ software (version 1.48) from three independent results normalized by β-actin.

7.2.6. Flow cytometry

To analyze the apoptosis population of neuronal cells, flow cytometry using annexin V-FITC and propidium iodide (PI) staining was used. Neuronal cells were trypsinized, collected, and washed with PBS. Cells were counted and 1×10^6 cells were suspended 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 1×10^5 cells per tube, and were incubated with 5 μl of annexin V-FITC and 2 μg/ml of PI at room temperature for 15 min. After incubation, 400 μl of binding buffer was added and flow cytometric analysis was performed. 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).

7.2.7. Statistical analysis

All values shown in the figures are presented as the mean ± the standard error of the mean. Intergroup comparison of ELISA and western blot results was performed by one-way analysis of variance followed by Tukey's post hoc test. A probability value below 0.05 was considered statistically significant. Data were analyzed using SPSS version 17.0 (SPSS Inc., USA)

7.3. Reduction of Aβ levels and Aβ 42/40 by ASC-exo

To examine whether ADSC-Exo modulates Ab expression levels and the Aβ42/40 ratio in AD neuronal cells, we used an in vitro AD model using NSCs isolated and cultured from Tg2575 mice brains, using a previously described method. We cultured NSCs in a neurosphere-containing medium of EGF and FGF, and aided their differentiated into neuronal cells under growth factor deprivation conditions (Figure 7.2 and Figure 7.3). NSCs derived from TG2576 mice showed a positive result in terms of Aβ staining (Figure 7.4). Compared to control NSCs, our in vitro AD model showed an increase in Aβ42 levels, Aβ40 levels, and the Aβ42/40 ratio (Figure 7.5A). Treatment with ADSC-Exo (200 μg/mL) was conducted on the first day after differentiation, and the amount of Aβ was measured at 12, 24, 48, and 72 h after ADSC-exo treatment. Increased Aβ42 levels, Aβ40 levels, and an increased Aβ42/40 ratio in AD neuronal cells were reduced from 24 h after ADSC-Exo treatment (Figure 7.5 B-D). These results confirm that the ADSC-Exo treatment alleviates an increase in Aβ levels and the ratio of Aβ42/40, which is the typical pathology of AD.

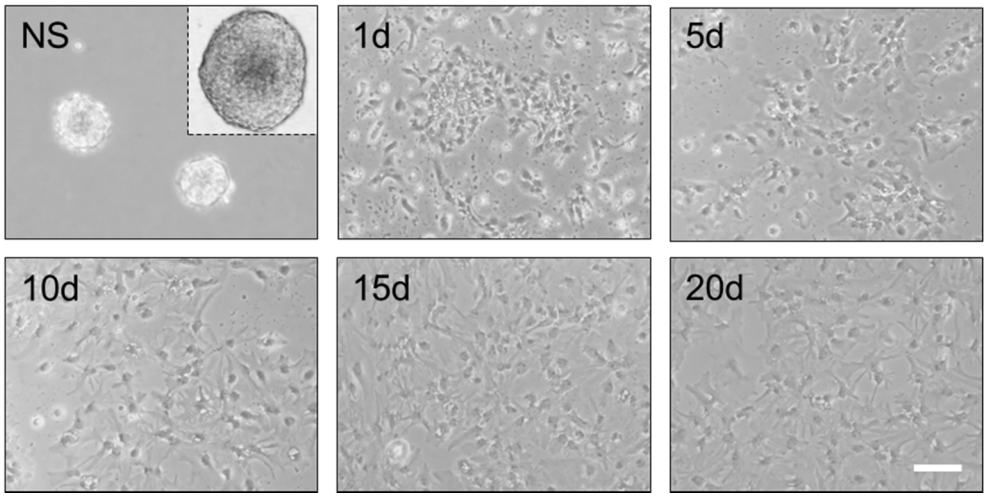


Figure 7.2 Culture of neuronal stem cells derived from mouse brain. Neural stem cells derived from the subventricular zone, which is an in vitro model, could be cultured in the form of spheres in the presence of FGF and EGF. When cultured in growth factor deprivation media, the neurosphere is dissocia7ted and attached to a Poly-L-lysine coated dish. Scale bar = 100 μm.

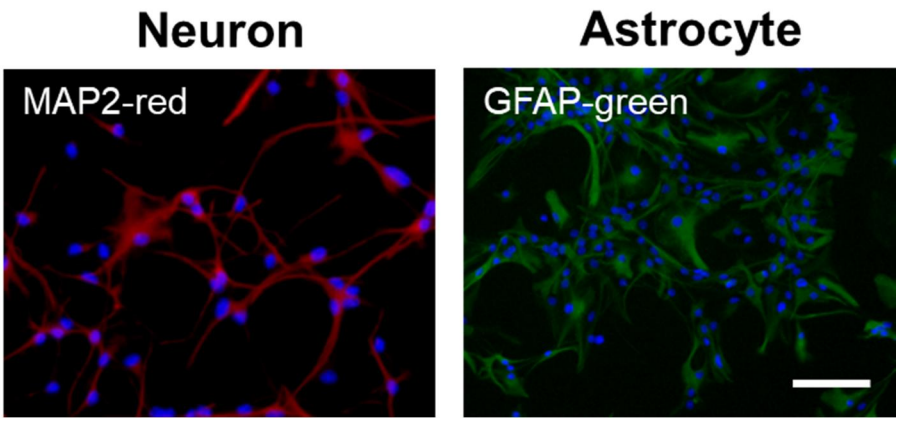


Figure 7.3 Culture of neuronal stem cells derived from mouse brain. When differentiated cells were stained with MAP2 (neuron) and GFAP (astrocyte), positive cells were observed, confirming that neuronal cell population. Scale bar = 100 μm.

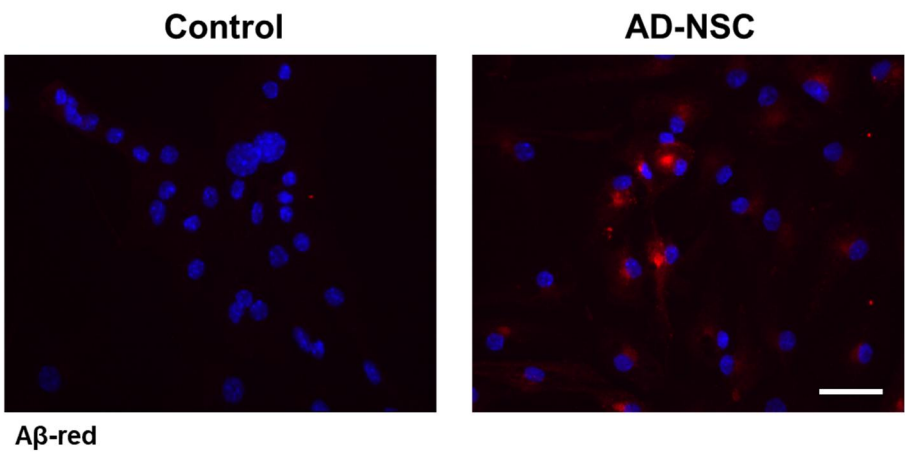


Figure 7.4 Aβ accumulation in TG2576-derived neuronal cells. Neuronal stem cells (NSCs) from TG2576 or wild-type littermate mice were cultured and differentiated for 3 days. Aβ staining result showed that accumulation of Aβ in the cell body of TG2576-derived neuronal cells. Scale bar = 50 μm

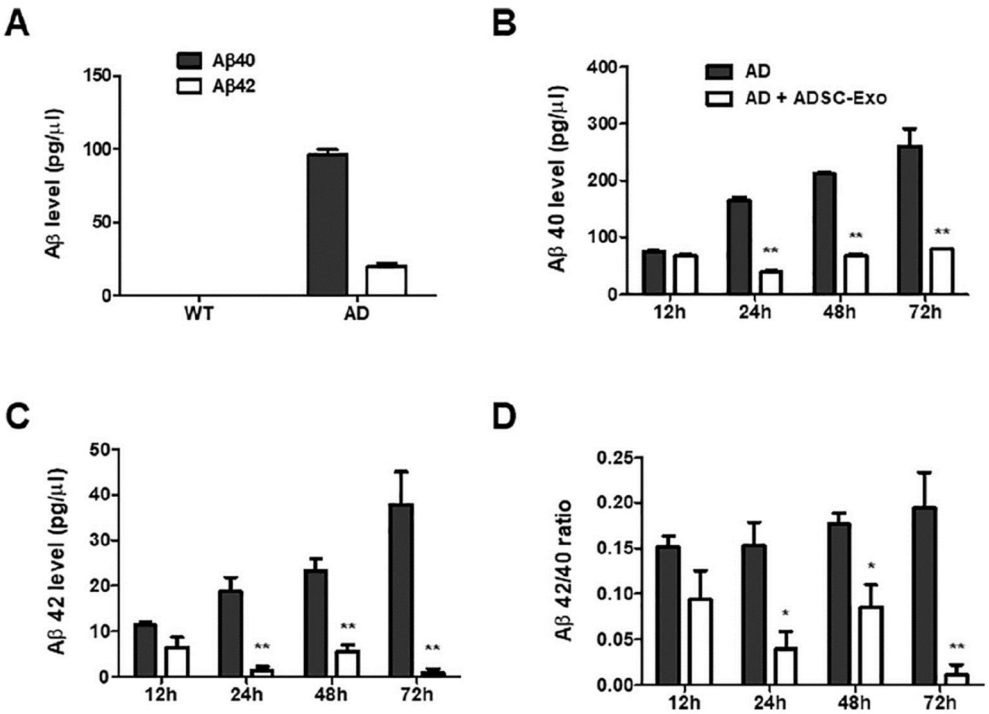


Figure 7.5 ASC-Exo reduces Ab levels and the Aβ 42/40 ratio. Neuronal stem cells (NSCs) from TG2576 or wild-type littermate mice were cultured and differentiated for 2 day and Aβ levels were measured (A). NSCs were cultured in the presence of ADSC-Exo for 12, 24, 48, 72 h and Aβ levels of the conditioned medium were analyzed using ELISA. Aβ 40 (B) and Aβ 42 (C) levels of AD cells, and ADSC-Exo treated AD cells are represented as a bar graph. (D) Aβ 42/40 ratio of AD NSCs treated with or without ADSC-Exo. * $p < 0.05$, ** $p < 0.01$ vs AD.

7.4. Increase in apoptotic molecules by ASC-exo

Many lines of evidence have shown that p53 levels increase in AD brains. Similarly, our in vitro AD model also showed an upregulation of p53, compared to wild-type cells. Apoptotic proteins, such as p53, Bax, pro-caspase-3 and cleaved-caspase-3, were upregulated and anti-apoptotic protein, Bcl-2, was down-regulated in our in vitro AD model. ADSC-Exo treatment was performed on the first day of differentiation and the total protein was extracted 2 days after ADSC-Exo treatment. As we expected, ADSC-Exo treatment normalized the increased p53, Bax, and caspase-3 levels and decreased Bcl-2 level in AD model cells (Figure 7.6). This result shows that ADSC-Exo attenuates an increase in apoptosis signaling of neuronal cells in AD mice.

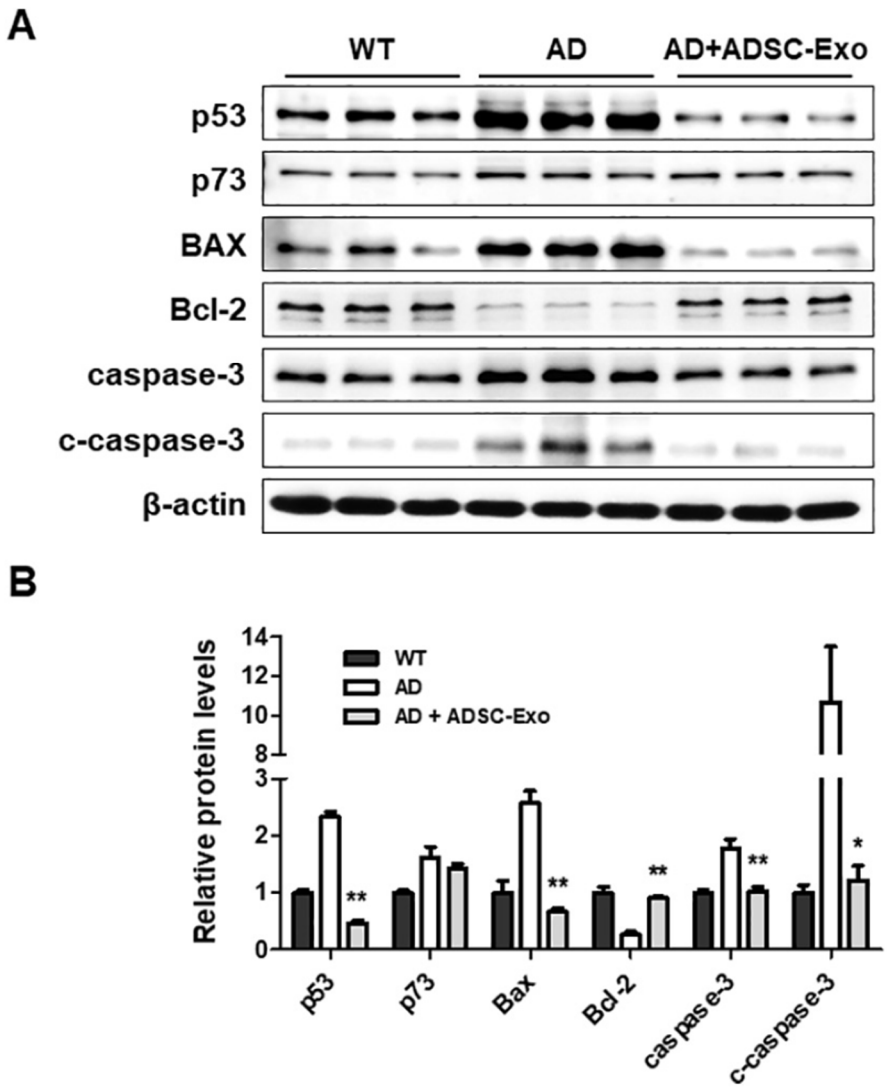


Figure 7.6 Normalization of apoptosis-related proteins by ADSC-Exo. Wild-type or AD neuronal cells were treated with ADSC-Exo for 2 days and the total cellular proteins were prepared. Apoptosis-related proteins including p53, p73, Bax, total caspase-3 and cleaved-caspase-3 were examined using western blot (A). Relative protein band densities were normalized using b-actin, and represented as a bar graph (B). * $p < 0.05$, ** $p < 0.01$ vs AD.

7.5. Attenuation of the apoptosis of AD model cells by ASC-exo

To confirm the anti-apoptotic effect of ADSC-Exo on the AD in vitro model, NSCs derived from the WT or AD model were differentiated and treated with ADSC-Exo for 2 days, and flow cytometry analysis was performed using annexin-V and propidium iodide (Figure 7.7A). Cell populations were analyzed as viable/early apoptotic/ late apoptotic/necrotic cells, and this calculation was conducted using the annexin-V and propidium iodide positive cell count. AD neuronal cells were found to have a more necrotic population and less viable population compared to wild-type cells. However, ADSC-Exo treated AD cells showed significant reduction of the necrotic cell population and an increase in the viable cell population. In addition, WT cells treated with ADSC-Exo showed that increased viable cells and decreased apoptotic/necrotic cells, indicating that naturally occurring cell death can be also attenuated by ADSC-Exo (Figure 7.7B). Taken together, ADSC-Exo treatment results in more cell survival and less cell death, accompanied by a reduction of Aβ levels, the Aβ42/40 ratio, and apoptotic signaling.

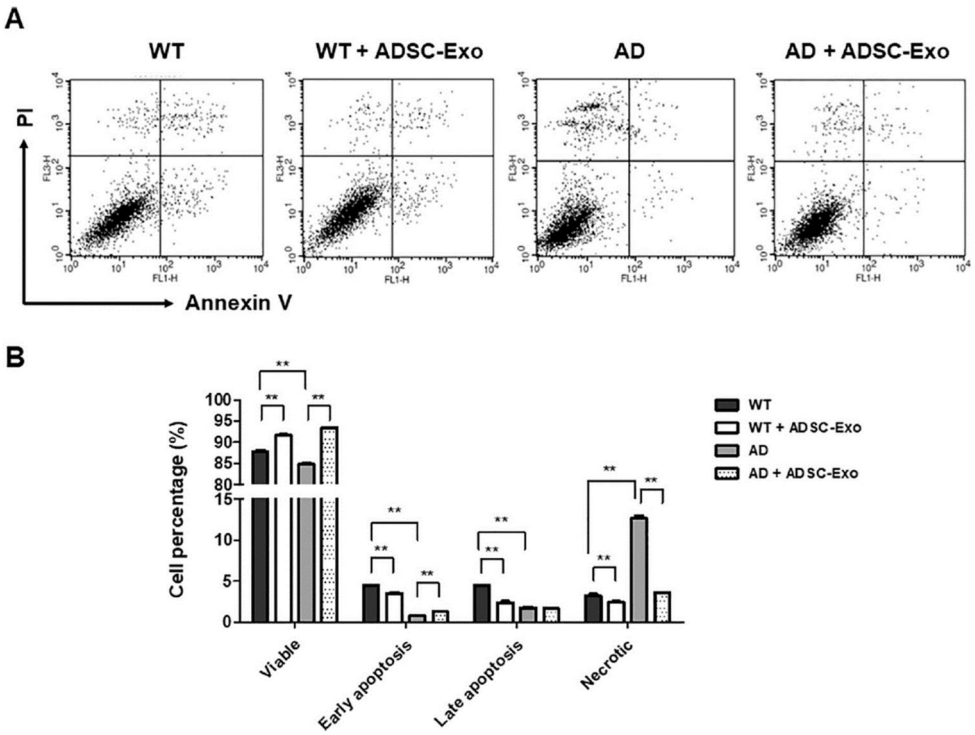


Figure 7.7 ADSC-Exo ameliorates the apoptosis of AD cells. Cells isolated from WT or AD mice were cultured and differentiated. ADSC-Exo treatment was conducted for 2 days and cells were then harvested and incubated with annexin V-FITC for 5 min at room temperature. Propidium iodide was added to all samples just before the analysis. The viable (lower left), early apoptotic (lower right), late apoptotic (upper right), necrotic (upper left) cell populations were analyzed using flow cytometry (A). The cell percentage was analyzed according to annexin V and propidium iodide staining and presented as a bar graph (B). This result used ADSC-Exo from three different volunteers and similar result was obtained. ** $p < 0.01$.

7.6. Neurite outgrowth of Neuronal Stem Cells by ASC-exo

Neurite growth and synaptogenesis are precisely controlled in neuronal development (Read and Gorman, 2009; Skaper, 2005), regeneration (Schiwy et al., 2009), and plasticity (Hrvoj-Mihic et al., 2013). The function of NSCs seems to be impaired in many neurodegenerative diseases and aging. Differentiated NSCs of AD were treated with ADSC-Exo for 2 days and light microscope images were captured. We investigated the changes in neurite genesis of NSCs caused by a treatment with ADSC-Exo (Figure 7.8A). The neurtite of AD neuronal cells showed shorter length than WT. AD neuronal cells treated with ADSC-Exo for 48 h showed higher neurite numbers and length, compared to vehicle treated AD cells (Figure 7.8B). In addition, lower number of neurites per cell in AD cells were restored to WT level by treatment with ADSC-Exo for 48 h (Figure 7.8C). This examination indicates that ADSC-Exo contains components that promote neurite growth associated with neuronal synapse formation.

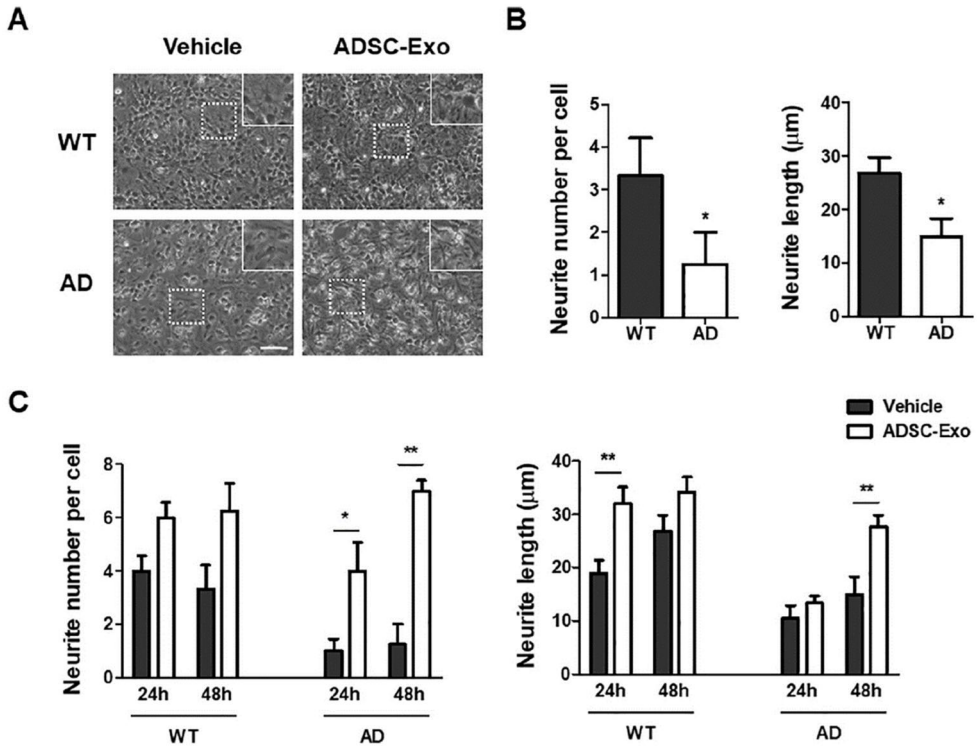


Figure 7.8 ADSC-Exo increases neurite number and the length of AD neuronal cells. NSCs from the subventricular zone of AD transgenic mice were cultured and differentiated using a differentiation medium. NSCs from AD mice were cultured in the presence of ADSC-Exo (200 lg/mL) on day 1 after differentiation and incubated for 2 days. (A) Cells were photographed using the light microscope. The numbers (B) and lengths (C) of neurites were analyzed and represented as bar graphs (n = 8 per group per experiment) (B) The small box displays an enlarged image of the dotted line. This result used ADSC-Exo from three different volunteers and similar result was obtained. Bar = 100 μm, * $p < 0.05$, ** $p < 0.01$.

7.7. Summary

In this study, we investigated the preventive role of ADSC-Exo in the AD related phenotypes within the in vitro AD model. NSCs were isolated from TG2576 mice and differentiated into neuronal cells. Differentiated AD neuronal cells showed increased Aβ42 and Aβ40 levels, and an Aβ42/40 ratio. The cells also showed an increase of apoptotic molecules and decrease of anti-apoptotic protein accompanied with cellular apoptosis, which are the major phenotypes of AD in vivo. Our results showed that increased Aβ levels and the Aβ42/40 ratio of AD cells were decreased by ADSC-Exo treatment. The increase of apoptotic signaling and the apoptotic/necrotic cell population of AD cells were normalized by ADSC-Exo treatment.

Exosomes contain various molecular components of its cell of origin, such as proteins, mRNAs, and microRNAs to maintain or change environmental conditions (Valadi et al., 2007). Many studies have reported the biological and medical significance of exosomes. Cancer cell exosomes mediate cancer progression by inducing a cancer favorable microenvironment (Park et al., 2010; Webber et al., 2010). Aβ protein seems to be associated with the exosomes of AD neurons, resulting in the propagation of the amyloid beta protein (Rajendran et al., 2006). Furthermore, prion protein of exosomes in Creutzfeldt-Jakob disease contributes to the progression of the disease (Fevrier et al., 2004; Vella et al., 2008). In addition to its pathological roles, exosomes have been regarded as a biomarker of various diseases (Gupta et al., 2010; Michael et al., 2010; Povero et al., 2014; Tsujiura et al., 2010).

ADSCs have been regarded as a feasible cell model in the medical field due to ease of isolation, multipotency, and minimal ethical problems. Although stem cells have shown many beneficial effects for various disorders, it is not clear whether

transplanted cells move to the damaged area and differentiate into wanted cell types. Several reports have suggested that stem cells, including ADSCs, secrete various cytokines and vesicles, which can modulate a hostile pathological environment. Our results prove once again that the secretory vesicles alone, apart from the cell itself, contain some of the beneficial components of ADSCs.

An exosome is an extracellular vesicle released from most cell types. Exosomes from stem cells have beneficial effects on wound healing, ischemic injury, fibrosis, and tissue regeneration. Recent evidence shows that ADSC transplantation results in improving cognitive function and neuropathology through different underlying mechanisms (Blurton-Jones et al., 2009; Lee et al., 2010). Furthermore, ADSC secretory factors also ameliorate AD phenotypes by Aβ degradation (Katsuda et al., 2013) and an anti-depressive effect (Yamazaki et al., 2015). In addition to these previous reports of the therapeutic potential of ADSCs on AD, our results further demonstrate that ADSC-Exo is effective in modulating AD through the reduction of cellular apoptosis and the Ab pathology.

Although our results show that ADSC-Exo improves the in vitro AD phenotype, stem cell therapy may also vary in efficacy by cell donor. Typically, the age or gender of the donor may influence the efficiency, characteristics, and differentiation potential of stem cells (Choudhery et al., 2014; Lo Sardo et al., 2017; Siegel et al., 2013). For future clinical application, thorough investigation of ADSC-Exo according to the donor characteristics will be required. We confirmed anti-apoptotic effect and neurite genesis through adipose tissue obtained from three different volunteers, and same results were obtained in three independent experiments. Thus, although there may be slight difference in efficiency, it has been confirmed that ADSC-Exo has anti-apoptotic and neurite genesis effect in common.

Aβ accumulation and mitochondrial dysfunction are considered as a major biomarker and key step of AD (Moreira et al., 2006; Moreira et al., 2009; Reddy and Beal, 2008; Reddy et al., 2010; Su et al., 2008). In addition, reducing Aβ deposition and mitochondrial oxidative stress have been studied to date as a beneficial strategy for AD (Chow et al., 2010; Manczak et al., 2010; Yuede et al., 2009). Our primary neuronal stem cells from TG2576 mice exhibit increased level of Aβ and mitochondrial superoxide as representative phenotypes with a typical Aβ cascade. Our study demonstrated that these in vitro phenotypes were attenuated by ADSC-Exo treatment, therefore, therapeutic potential of ADSC-Exo on AD phenotypes was confirmed by in vitro. In order to verify the effect of ADSC-Exo on sporadic AD which accounts for the majority of human dementia, preclinical and clinical trials will be essential. In summary, our results show that ADSC-Exo have modulating effects on the in vitro model of AD. Neuronal cells from AD transgenic mice show an increase in Aβ levels and the Aβ 42/40 ratio, apoptotic molecules, and cellular apoptosis. ADSC-Exo reduces these pathological phenotypes of the AD in vitro model and augment neurite outgrowth of AD cells. Thus, the therapeutic potential of the exosomes from ADSC has been confirmed through our study, and ADSC-Exo can be a valuable tool for treating AD.

CHAPTER 8

Alleviation of Huntington's disease by Heterochronic Parabiosis

8.1. 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 impairments. [1] HD is caused by an expanded CAG repeat in the HD gene on chromosome 4. [2,3] This mutation produced an extended N-terminal polyglutamin stretch in the huntingtin (Htt) protein leading to intracellular accumulation and aggregation of mutant huntingtin (mHtt). [3] The accumulation of mHtt aggregates causes striatal cell death through transcriptional dysregulation, activation of intrinsic apoptosis pathways, mitochondrial dysfunction, and altered protein-protein interactions. [4,5] To date, there exists no cure or beneficial treatment for the disease and developing therapies are a high priority. [6] Dysregulated mitochondrial activation has been detected in the brains of both HD patients and transgenic HD mice and mitochondrial dysfunction is a major causative factor in neurodegenerative disorders.

Several studies in other neurodegenerative diseases like AD have shown that exposure to a young blood circulation through heterochronic parabiosis (HP) reverses cognitive deficits observed with normal aging, which is a surgical union of 2 organisms of different phenotype that leading to the formation of a vascular anastomosis and a shared curculatory system between, e.g., 2 mice. Although the idea of adopting HP to the HD seems straightforward because HD is also caused by the neurodegeneration, however, it has not been reported so far to the best of our knowledge.

Although a thorough understanding for the transportation mechanism of positive factors from the young blood to the old during HP is necessary in order to adopt HP to the HD, however, previous studies on parabiosis have focused on which factors of young blood have a positive effect on neurodegenerative disease. 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 (40-100nm) that has cargo ability for intercellular matter exchange thus could be the potential vehicles for deivering paracrine factors during HP. These nanovesicles are secreted by diverse cell types (e.g., neurons, tumor cells, and kidney cells) and are found in various body fluids such as urine, amniotic fluid, breast milk, saliva, blood, and cerebrospinal fluid. intracellularly, exosomes atr generated via the inward budding of endosomes to form multivesicular bodies (MVBs) that fuse with the plasma membranes to release exosomes into the surrounding environment. [7-9] exosomes, depending on their parental origin, contain a variety of proteins, lipids, non-coding RNAs, mRNA, AND miRNA, collectively termed as "cargo" contains, and are

delivered ti the surrounding cells or carried to the distal cells. [4-6] Due to their cargo ability, exosomes represent a novel form of intercellaul 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. [7.8]

Here, through in vivo R6/2 mice HP, we proved that young blood has positive factors to improve the HD pathology, and by extracting exosomes from young serum and processing them into in vitro model cell, we discovered that the exosomes serve as a cargo unit for the positive factor of young serum during the HP. It is worth noting that we have succeeded in producing the HD R6/2 animal model for the HP for the first time. Producing the HP animal model for the HD have been impossible because unlike other neurodegenerative diseases, R6/2 mice are highly vulnerable to stress and at 3 to 4 months of age, the develop trimming and die of muscle loss. However, by minimizing the stress and 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 HP group.

To determine the effect of young circulatory factors on HD-like disease in R6/2 mice, we used HP, in which we joined the circulatory systems of young, wildtype animals together with R6/2 mice which express exon1 of the human HD gene with around 150CAG repeats. First, we analyzed the changes in protein expression in the brains of these mice with established disease after heterochronic parabiosis, and second, we examined the effects of young serum exosome on key phenotypes of HD including p-CREB-PGC-1 pathway, apoptotic proteins, cell death and mitocondria dysfunction in R6/2-derived neuronal cells.

By identifying the exosomes as a messenger unit that can transport the positive factors in the young blood to the old during the HP, this study opens the possibilities for adopting the exosomes to various neurodegenerative diseases.

8.2. Methods

8.2.1. Animal modeling

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. 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. 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). 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. ZQ175 and R6/2 Mice 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 house 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.

8.2.2. 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). 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. 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 (B6CBA-Tg(HDexon1)62Gpb/1J, 111 CAGs) mice were housed together in a single cage for 6 weeks. Mice were housed under standard conditions (12 h light cycle from 08:00 h to 20:00 h) with *adlibitum* access to food and water. 100ul blood was collected from eyes to document joint circulation by polymerase chain reaction (PCR) of genomic DNA. At the time of killing, parabiotic mice were anesthetized with Zoletil/Rompun intraperitoneal (i.p.) and separated through transection at the anastomosis site. Before separation, 0.5~0.8mL blood was obtained by hearts. Also, other organs was obtained by mice.

8.2.3. Administration of BrdU and immunohistochemistry

To demonstrate a connected circulation between prabionts, BrdU was injected into one mice. The formation of shared blood circulation between the parabiotic animals was tested by injection of BrdU (150 mg/kg, Sigma□Aldrich, St. Louis, MO, USA) to the intraperitoneal administration of one of the mice in parabiosis after 2weeks postsurgically 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 12weeks 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 2hours. BrdU (red) or DAPI (blue)-stained cells were identified using an inverted microscope (BX61, Olympus Corporation, Tokyo, Japan).

8.2.4. Preparation of in vitro Huntington's disease model

A colony was maintained by breeding ovarian transplant R6/2 females (B6CBATg(HDexon1)62Gpb/1J) obtained from the Jack-son 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 *adlibitum* 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 (NSCs) from R6/2 mice. In brief, after sacrificed by CO2 gas, brain tissues of 9-week-old mice were used for primary culture and NSCs 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. NSCs were differentiated in the differentiation medium, which was composed of DMEM/F12, 1% PSA, 2% B27, and 5% FBS.

8.2.5. Isolation of exosomes from serum and treatment

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 1500*g* for 30 min. The supernatant was then removed and centrifuged at 1500*g* 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.

8.2.6. 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 (Im *et al.*, 2015). Em48 (red) or DAPI (blue)-stained cells were counted using an inverted microscope (BX61, Olympus Corporation, Tokyo, Japan).

8.2.7. Preparation of tissue 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 12weeks 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 2hours. EM48 (red) or DAPI (blue)-stained cells were identified using an upright microscope (Ni-E, Nikon Corporation, Tokyo, Japan).

8.2.8. DAB Immunohistochemistry

Immunocytochemistry was used to visualize DCX. Prior to staining, sections were mounted on glass (MUTO Pure Chemicals Co., Ltd., Tokyo, Japan) and antigen retrieval was performed by heating the sections in 0.1M citrate buffer (pH6) in a microwave (Samsung RE-C20DV) to a temperature of ±95 °C for 15 min. Sections were incubated with 0.3% H2O2 for 15 min to block endogenous peroxidase activity, and were next incubated for 30 min in blocking buffer (1% BSA, 0.3% Triton X-100 in 0.05 M TBS). Primary antibody DCX neurogenesis antibody (1:200; Santa Cruz, CA, USA) was incubated for 2 h at room temperature and overnight at 4 °C. Sections were incubated with biotinylated secondary antibody(1:200, biotinylated anti-goat, Vector Labs, Burlingame, CA, USA) for 2 h at room temperature followed by a 90 min incubation with avidin-biotin complex (1:50; Vectastain Elite ABC-HRP kit, PK-6100, Vector Laboratories, Burlingame, CA, USA). Subsequent chromogen development was performed with diaminobenzidine (DAB; 20 mg/100 mL 0.05 M Tris, 0.01% H2O2, Agilent, Santa Clara, CA, USA).

8.2.9. Protein extraction and western blot analysis

Brains of ZQ175 and R6/2 mice 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 1h 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), p-CREB (1:1000; Cell signaling, Beverly, MA, USA), CREB (1:1000; Cell signaling, Beverly, MA, USA), anti-p53 (1:1000; Cell signaling, MA, USA), anti-Bax (1:200; Santa Cruz, CA, USA), BCL-2 (1:200; Santa Cruz, CA, USA), Caspase3 (1:1000; Cell signaling, Beverly, MA, USA), Em48 (1:500, Millipore, CA, USA), and anti-β-actin (1:200, Santa Cruz, CA, USA). 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 chemiluminescenece solution, Advansta, 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.

8.2.10. 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.

8.2.11. 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 $CaCl_2$). 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).

8.2.12. 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).

8.3. HP modeling and reduction of mHtt aggregation

The R6/2 mice were surgically joined either with another R6/2 mice or with their correspnding wile-type littermate to form KOKO (KO-KO isochronic), KO wild-type (KO wild-type heterochronic) pairs respectively. In addition, two-wild-type mice were joined to form wild-type pairs (WT-WT isochronic), to serve as controls to ensure that the surgical procedures will not cause ectopic mineralization (Figure 8.1). Mice were surgically joined by the muscles in the abdominal wall and the skin for a period of 6 weeks and then killed (Figure 8.2). 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. The shared circulation was demonstrated by examination of the genomic DNA of blood cells in paired mice at 4 weeks after parabiotic surgery. At 4 weeks of parabiosis, the right mice (originally wild-type) showed the presence of the mutuant allele in their circulations (Figure 8.3). Also, to demonstrate a connected circulation between parabionts, BrdU was injected into one mice. Brain from the injected mice and the attached mice in all groups of parabionts showed similar BrdU signals, indicating a joined circulatory system (Figure 8.4). It was confirmed that blood sharing was achieved through parabiosis. R6/2 mice, a representative mouse model of Huntington's disease, exhibit loss of body weight and muscle bulk through the course of the disease. To evaluate the functional consequences of Heterochronic parabiosis, we measured lifespan and weight loss in HD Transgenic R6/2 mice. Heterochronic parabiosis had been paired with young mice for 6 weeks and phenotypes were examined. we also recorded survival. R6/2 mice (with CAG repeats of between 154 and 200) death typically at around 3–4 months of age (Stack et al., 2005). Notably, survival also increased dramatically, from around 3-4 months. In

contrast with Old mice heterochronic parabiosis R6/2 mice, young mice heterochronic parabiosis R6/2 mice showed survival increased. Young mice heterochronic parabiosis delayed the onset of mortality in HD transgenic R6/2 mice from 84 to 100 days, the average survival from 102 to 110 days, and the maximum survival from 140 to 145 days (Figure 8.5). Isochronic parabiosis mice showed gradual weight loss from 10 to 12 weeks of age. In contrast with isochronic parabiosis R6/2 mice, heterochronic parabiosis R6/2 mice showed delayed progression of weight loss at 11 and 12 weeks old (**$p<0.01$) (Figure 8.6). ZQ175 old heterotype (n=5), ZQ175 young heterotype (n=5), ZQ175 isotype (n=5). ZQ175 young heterotype mitigated weight loss in ZQ175 old heterotype and ZQ175 isotype at 16 weeks after parabiosis surgery (Figure 8.7). Young mice heterochronic parabiosis also reduced tremor (data not shown), and improved movement measured at 9 and 12 weeks of age. Lifespan and weight loss of each group had the same composition of females and males. R6/2 mice show mHtt aggregation in cortex and striatum during disease progression. To examine histological changes of the brain, mHtt aggregation were evaluated at 12 weeks of age. To evaluate effect on mHtt aggegation brains were sectioned and sliced tissues were stained with an EM48 antibody which detects aggregation of mutant huntingtin. As shown in Figure 8.8, Heterochronic parabiosis had been paired with young mice for 6 weeks reduced mHtt aggregation in the cortex and striatum. We extracted proteins from R6/2 mice brain and mHtt aggegation was measured by western blot. As shown in Figure 8.9, Heterochronic parabiosis group showed decreased mHtt aggregation in the brain compared with isochronic parabiosis (***$P<0.001$). Dot blots of ZQ175 old heterotype and ZQ175 young heterotype brain extracts. ZQ175 young heterotype decrease the levels of EM48 compared with ZQ175 old heterotype. (Figure 8.10).

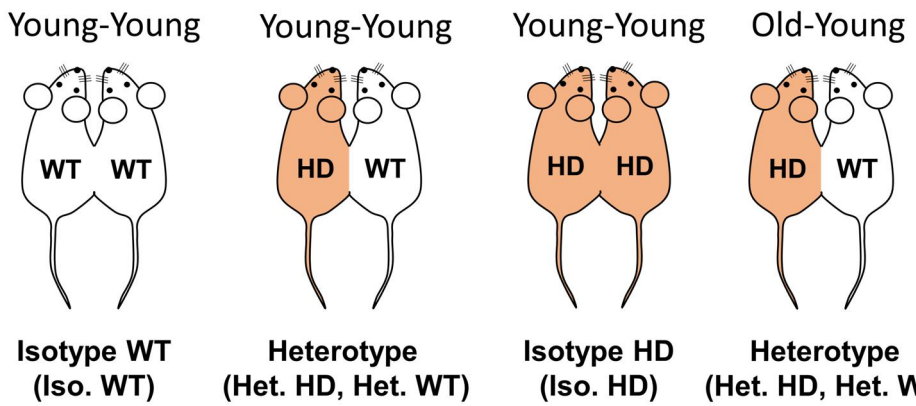


Figure 8.1 Schematic depicting the 4 different parabiotic pairing: isotype wildtype (WT), heterotype HD, and isotype HD. Isotype pairs are age-matched and the HD mouse from the heterotype pair, which is connected to a young WT mouse (6 weeks old) or old WT mouse (18-22 months old). All pairs were surgically connected for 6 weeks.

Figure 8.2 Actual photo for the parabiosis of mice.

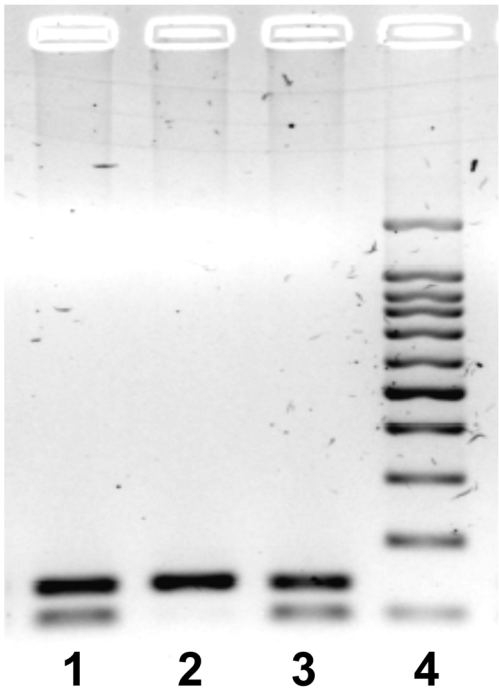


Figure 8.3 DNA was isolated from mouse blood. Each mouse was examined by two separate PCR reactions with two sets of primers designed for Hdh and Neo, respectively. In Hdh amplification, the 140-bp band represents the wild-type allele, whereas the 100-bp PCR product is derived from amplification of the knock-in allele. For the Neo gene, the knock-in allele (100-bp), wild-type allele (140-bp), or both alleles were amplified in the PCR reaction before surgical pairing, respectively. Line 4, molecular weight markers. 1, genotype of parabiotic WT mouse pairs is shown. Quantitative PCR for genotyping of the Neo gene was performed with blood samples collected at 4 weeks postoperatively. The 140-bp and 100-bp bands represent the parabiotic WT allele after surgery.

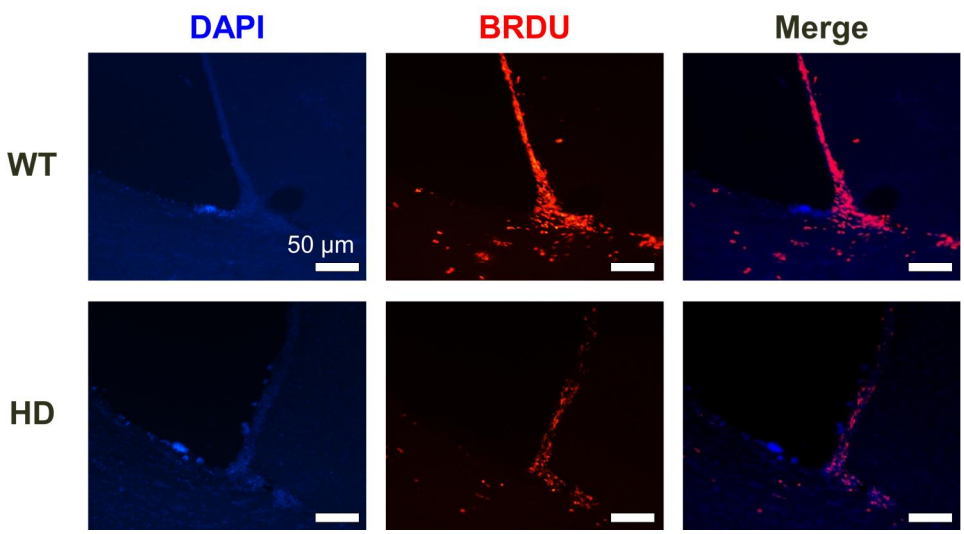


Figure 8.4 BrdU was injected into one mice. The formation of shared blood circulation between the parabiotic animals was tested by injection of BrdU (150 mg/kg) to the intraperitoneal administration of one of the mice in parabiosis after 2weeks postsurgically and the pair was killed after 4 weeks. brain from the injected mice and the attached mice in all groups of parabionts showed similar BrdU signals, indicating a joined circulatory system.

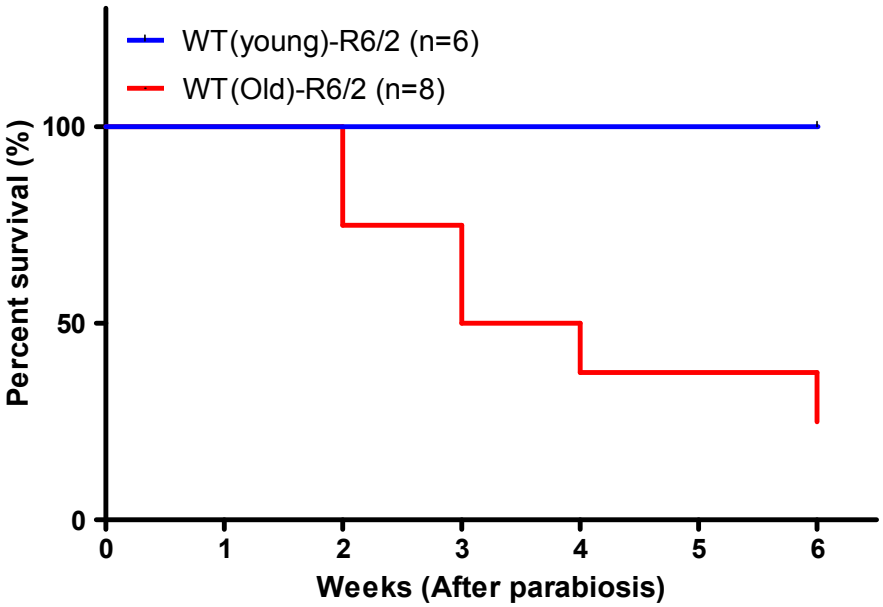


Figure 8.5 Parabiotic pairs between the WT (Young or Old) and HD mice were established as described in Materials and Methods. Survival of the pairs was monitored for 6 weeks; HD young heterotype (n=6), HD old heterotype (n=8). In contrast with old parabiosis R6/2 mice, young parabiosis R6/2 mice showed increased survival. Measures of each pair or each partner in a pair are plotted separately in different colors.

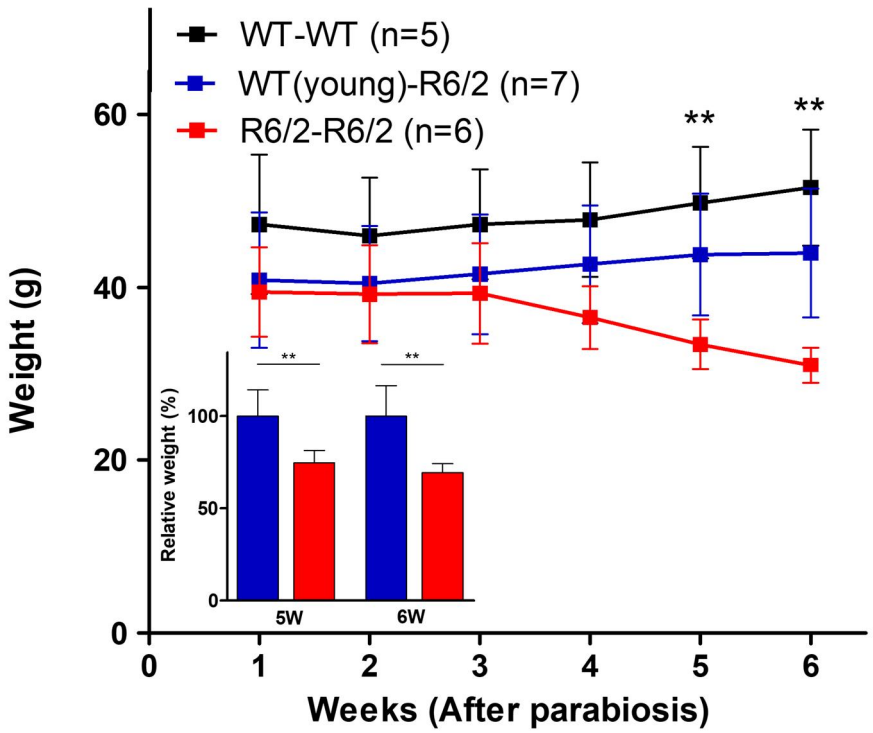


Figure 8.6 Body weight of each pair. Absolute weights of the pairs (parabionts) measured at various times during recovery; WT isotype (n=5), HD heterotype (n=7), HD isotype (n=6). Immediately following the surgery, both groups appeared to slight lose weight, which was followed by steady normalisation of the body weight. Heterotyep HD mouse group mitigated weight loss in isotype HD mouse group at 5 and 6 weeks after parabiosis surgery. **$p<0.01$. Values are mean ± SEM. Measures of each pair or each partner in a pair are plotted separately in different colors.

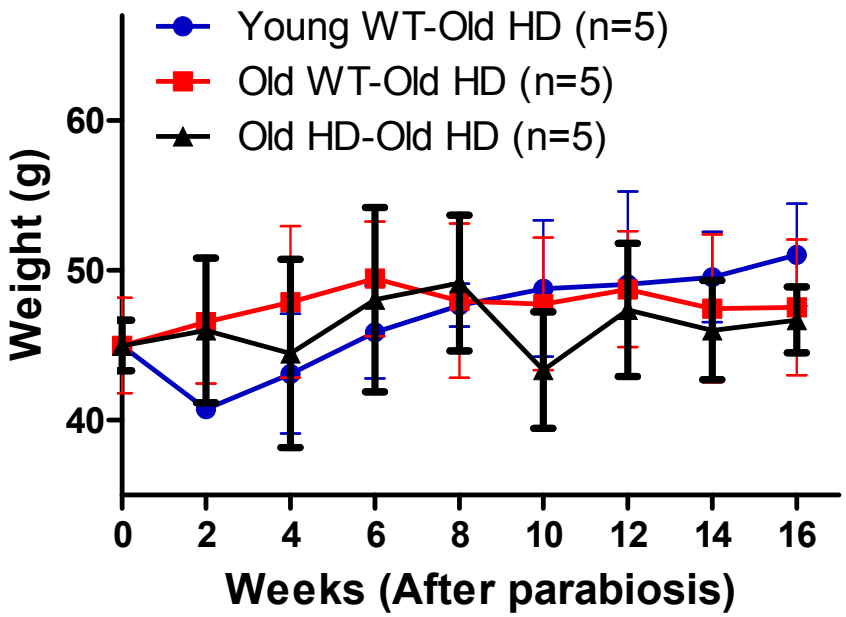


Figure 8.7 Body weight of each pair. Absolute weights of the pairs (parabionts) measured at various times during recovery; HD old heterotype (n=5), HD young heterotype (n=5), HD isotype (n=5). Immediately following the surgery, both groups appeared to slight lose weight, which was followed by steady normalisation of the body weight. HD young heterotype mitigated weight loss in HD old heterotype and HD isotype at 16 weeks after parabiosis surgery. Measures of each pair or each partner in a pair are plotted separately in different colors.

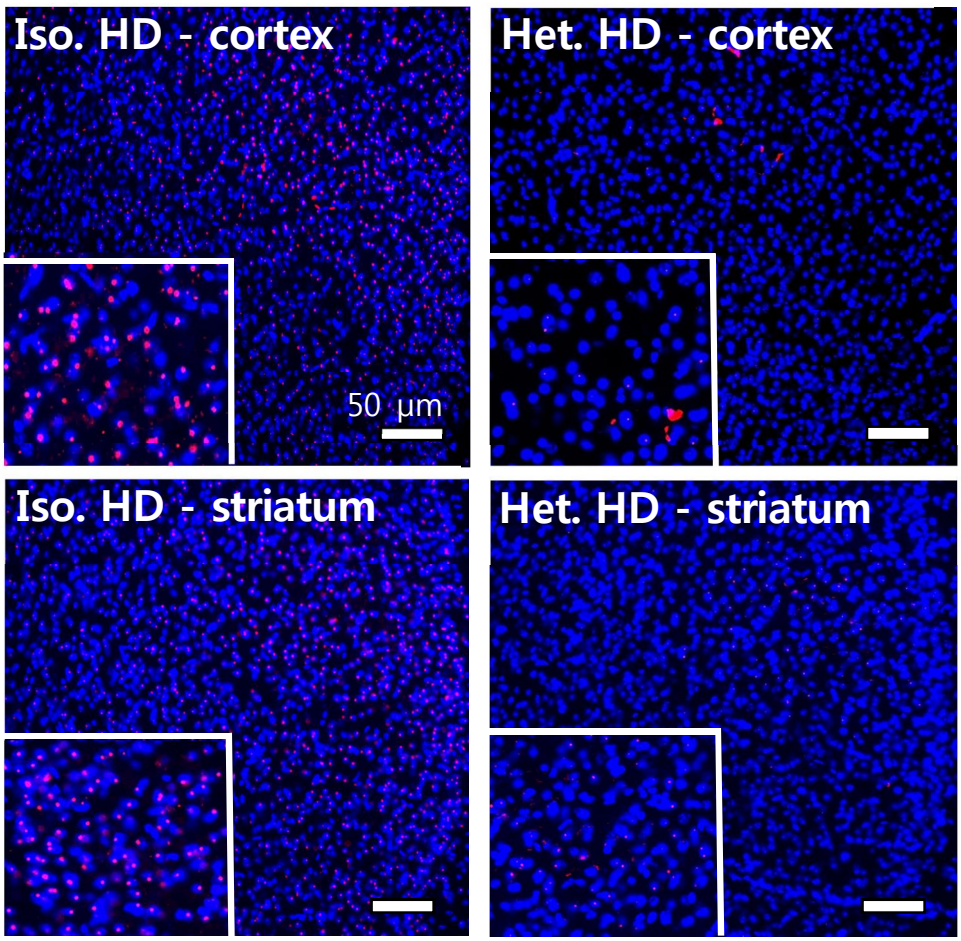


Figure 8.8 Cortex and Striatum was sectioned and stained with EM48 in Heterotyep HD mouse or isotype HD mouse. Cortex and Striatal mHtt aggregation was mitigated in Heterotyep HD mouse group. Bar = 50 μm

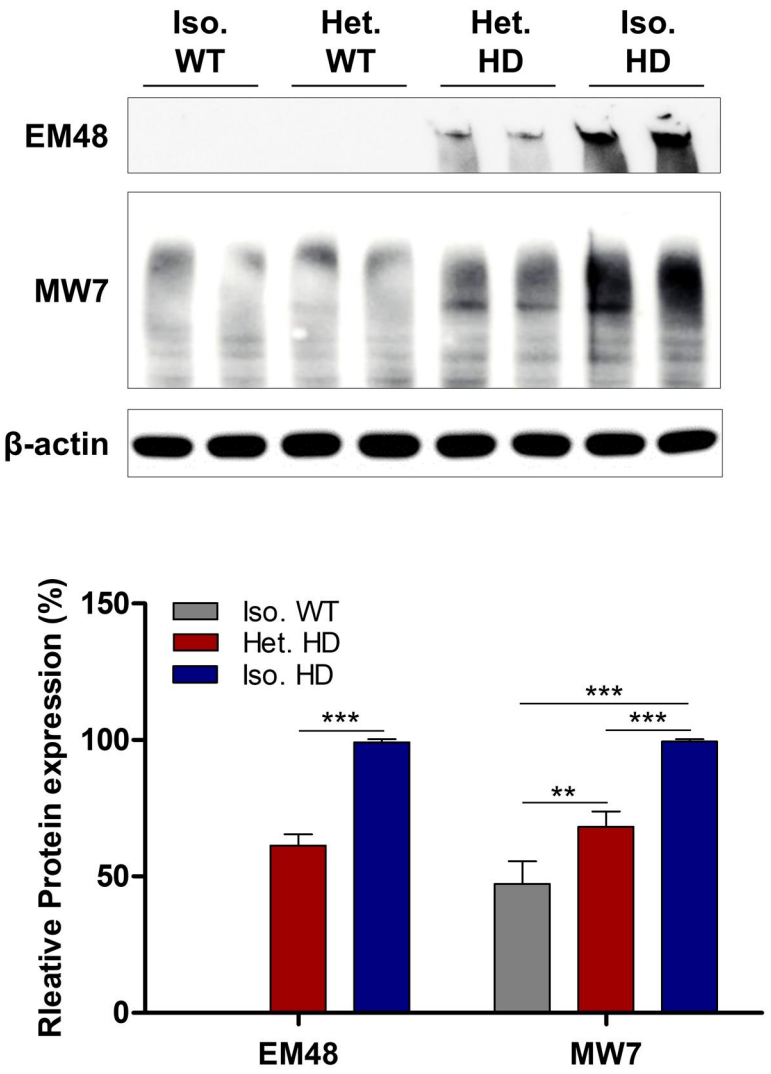


Figure 8.9 Western blots of Heterotyep HD mouse and isotype HD mouse brain extracts. Em48 and MW7 were used to identify mutant Htt. MHtt aggregation was visualized by western blot. MHtt aggregation level was ameliorate in

Heterotyep HD mouse group brain compared with isotype HD mouse group. **P<0.01, ***p<0.001.

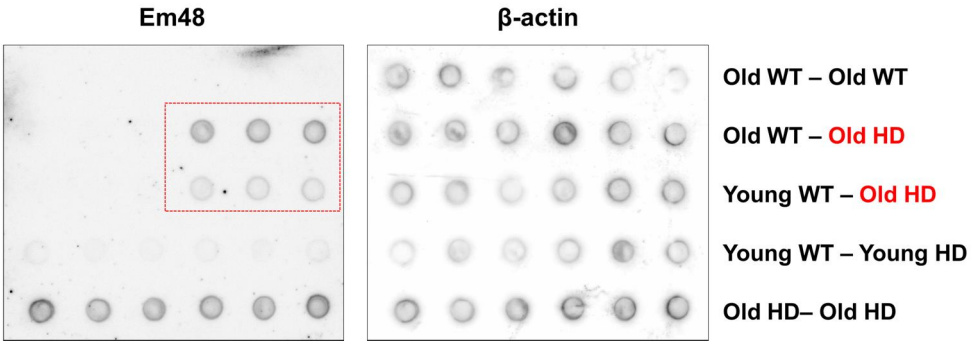


Figure 8.10 Dot blot assay was performed using parabiosis model. Dot blots of HD old heterotype and HD young heterotype brain extracts. HD young heterotype decrease the levels of EM48 compared with HD old heterotype. (n=3).

8.4. Activation of mitochondria and modulation of apoptotic proteins

Dysfunction of PGC1α-CREB pathway has been regarded as the key molecules for HD progression. To examine effects of Heterochronic parabiosis on this pathway, 12 weeks old R6/2 mice heterochronic parabiosis and isochronic parabiosis for 6 weeks were sacrificed and western blot analysis was performed. Heterochronic parabiosis groups promoted expression of PGC1α and p-CREB (*$p<0.05$, **$p<0.01$ vs. isochronic HD) (Figure 8.11). Western blots of ZQ175 old heterotype and ZQ175 young heterotype brain extracts. ZQ175 young heterotype brain increased the levels of PGC1α and p-CREB compared with ZQ175 old heterotype (Figure 8.12). To investigate whether heterochronic parabiosis protect against apoptosis, we determined the levels of apoptosis-related proteins by western blotting. We found that p53, Bax, and cleaved caspase-3 levels were lower in the HD heterochronic parabiosis groups than in the HD isochronic parabiosis groups and increased BCL-2 (***$p<0.001$) (Figure 8.13). ZQ175 young heterotype increased the levels of Bcl-2 compared with ZQ175 old heterotype and decreased the levels of p53, Bax, and cleaved caspase-3 (Figure 8.14). Likewise, exposure to this young environment completely restored the loss of hippocampal DCX observed in isochronic HD mice. This deficit was significantly reversed in HD heteroronic parabionts that had been paired with young mice for 6weeks (*$p<0.05$) (Figure 8.15). Double labeling of BrdU-positive cells (red) and DCX-positive cells (green) in parabiosis model. BrdU labeling is widely distributed in the dentate gyrus of the hippocampus, as well as DCX-positive cells, which are spread out over of the brain. DCX and BRDU double staining is heterotype HD whereas almost no DCX and BRDU positive cells are isotype HD. The number of

double positive cells is higher in the heterotype HD (Figure 8.16). DCX-positive labeling cells is widely distributed in the dentate gyrus of the hippocampus which are spread out over of the ZQ175 mice brain. DCX is ZQ175 young heterotype whereas almost no DCX positive cells are ZQ175 old heterotype. The number of DCX positive cells is higher in the ZQ175 young heterotype (Figure 8.17).

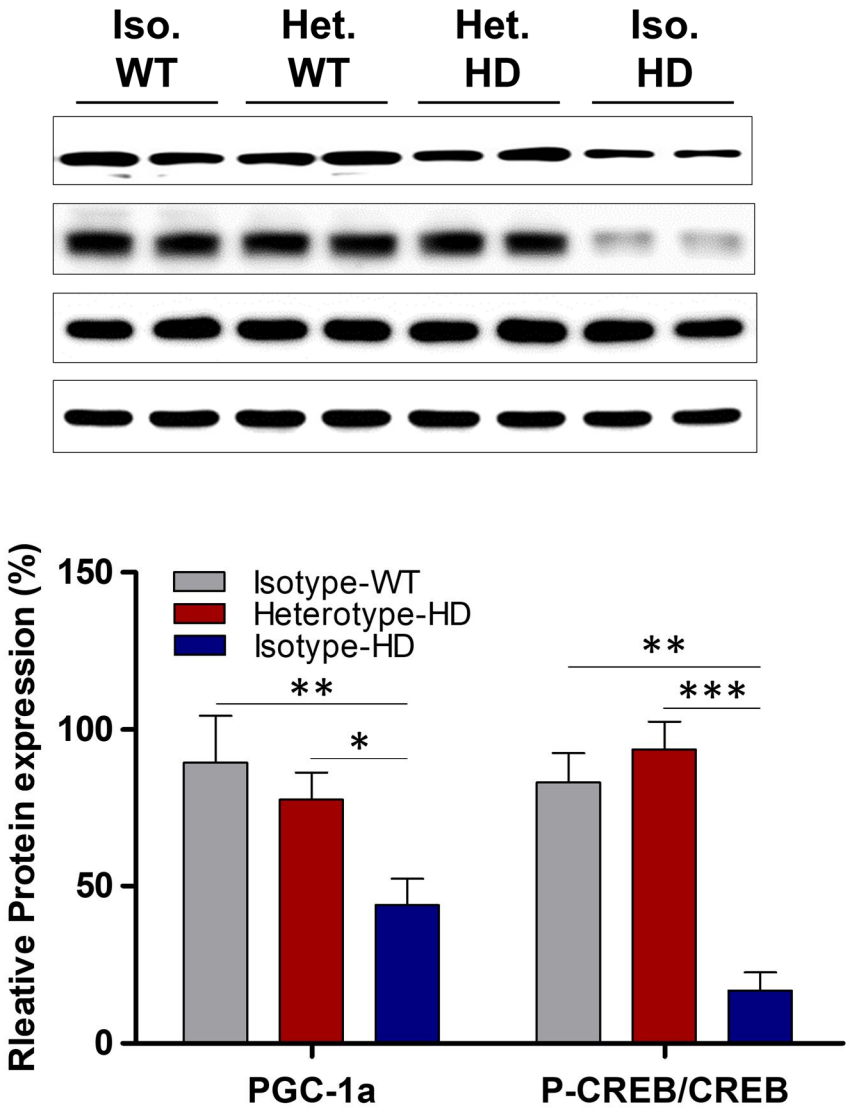


Figure 8.11 Western blots of Heterotyep HD mouse and isotype HD mouse brain extracts. Heterotyep HD mouse group brain increased the levels of PGC1a and p-CREB compared with isotype HD mouse group. *p<0.05, **P<0.01, ***p<0.001 (n=3).

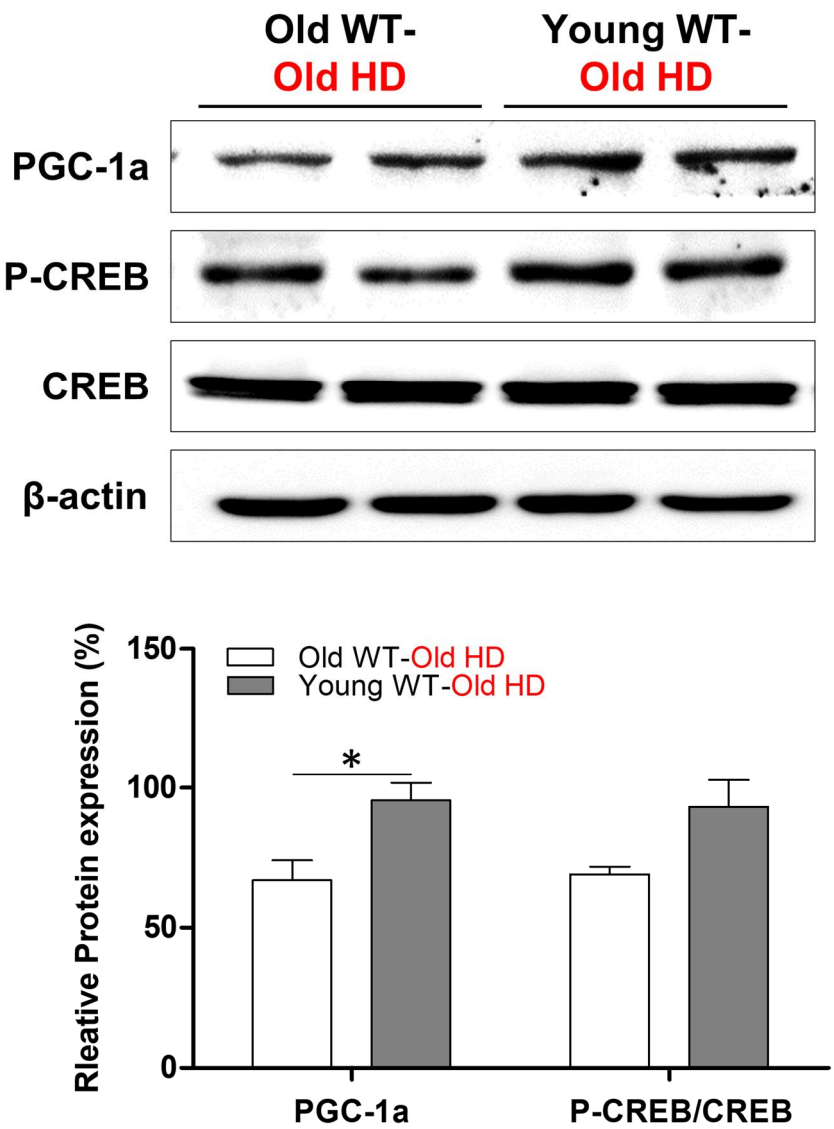


Figure 8.12 Western blots of HD old heterotype and HD young heterotype brain extracts. HD young heterotype brain increased the levels of PGC1a (*p<0.05) and p-CREB compared with HD old heterotype. (n=3).

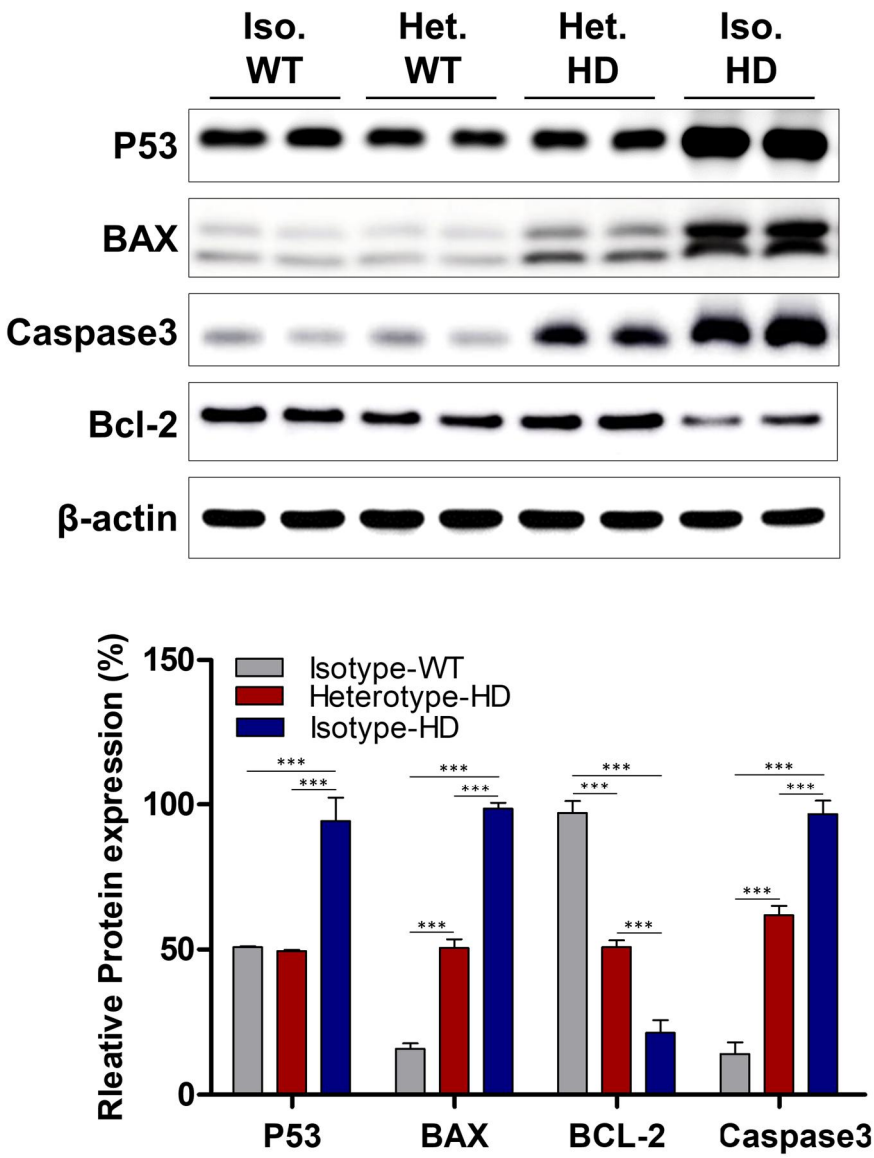


Figure 8.13 As shown by western blotting, Heterotyep HD mouse group brain increased the levels of Bcl-2 compared with isotype HD mouse group and decreased the levels of p53, Bax, and cleaved caspase-3. ***p<0.001 (n=3).

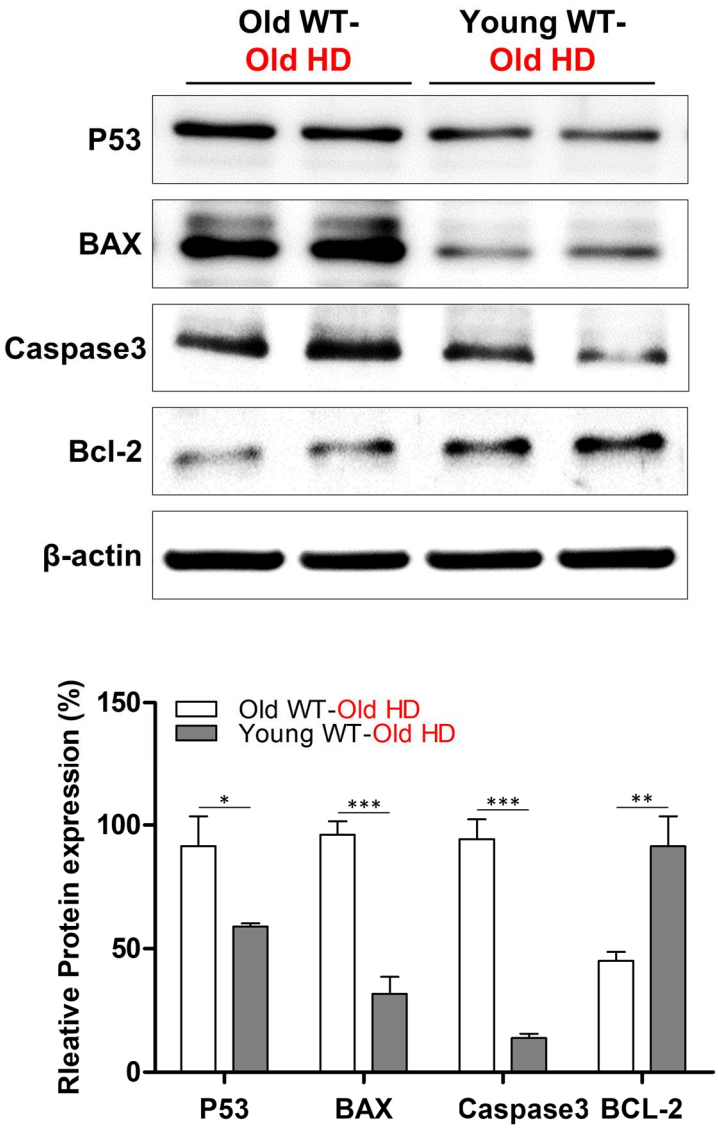


Figure 8.14 As shown by western blotting, HD young heterotype brain increased the levels of Bcl-2 compared with HD old heterotype and decreased the levels of p53, Bax, and cleaved caspase-3. *p<0.05, *p<0.01, ***p<0.001 (n=3).

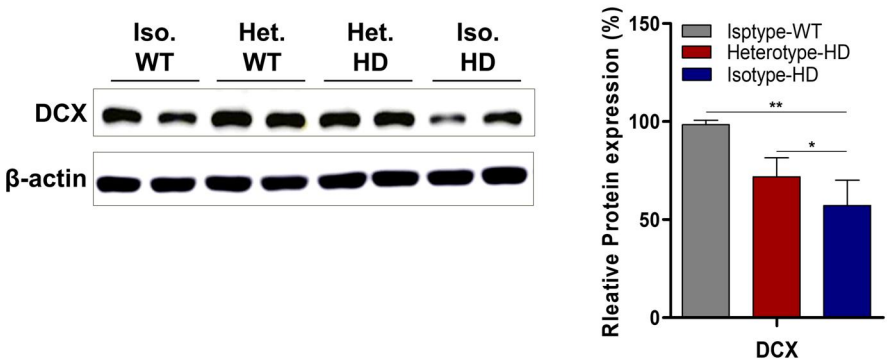


Figure 8.15 DCX immunoreactivity in the DG of WT isochronic, HD isochronic, and HD heterochronic parabionts. Heterotyep HD mouse group brain increased the levels of DCX compared with isotype HD mouse group; WT isochronic (n=5), HD isochronic (n=6), and HD heterochronic (n=7). As shown by western blotting, Heterotyep HD mouse group brain increased the levels of DCX compared with isotype HD mouse group. *p<0.05, **P<0.01 (n=3).

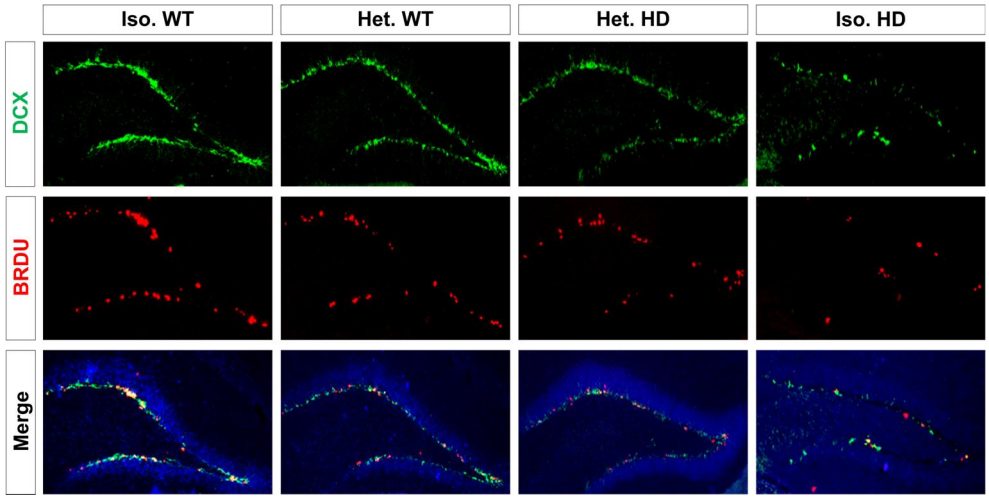


Figure 8.16 Double labeling of BrdU-positive cells (red) and DCX-positive cells (green) in parabiosis model. BrdU labeling is widely distributed in the dentate gyrus of the hippocampus, as well as DCX-positive cells, which are spread out over of the brain. DCX and BRDU double staining is heterotype HD whereas almost no DCX and BRDU positive cells are isotype HD. The number of double positive cells is higher in the heterotype HD.

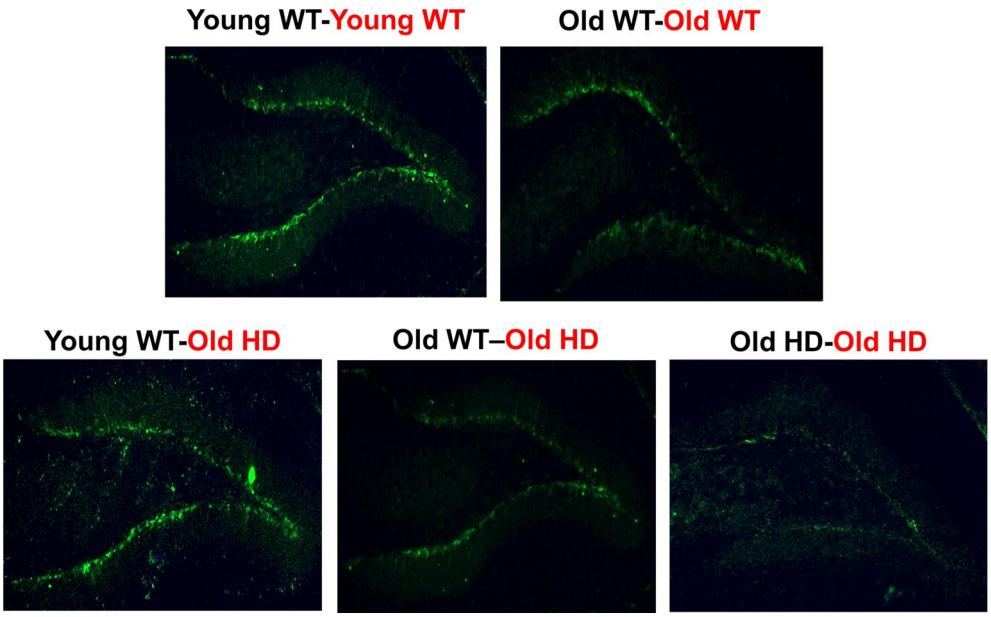


Figure 8.17 DCX-positive cells (green) in parabiosis model. DCX-positive labeling cells is widely distributed in the dentate gyrus of the hippocampus which are spread out over of the brain. DCX is HD young heterotype whereas almost no DCX positive cells are HD old heterotype. The number of DCX positive cells is higher in the HD young heterotype.

8.5. Reduction of mHtt aggregation by serum exosomes

Isolation of blood serum derived exosomes. Exosomes from blood serum were isolated using Exo-Quick and ultracentrifuge method. Extracted exosomal proteins were immunoblotted with antibodies for representative exosome markers, HSP70, CD63 and CD9. We thus confirmed the presence of exosomes (Figure 8.18). To investigate exosomal protein and RNA concentration in young blood and old blood, proteins and total RNA were extracted by protein extraction buffer and total RNA isolation kit respectively after isolating exosomes from young blood and old blood. Exosomes from blood serum were isolated using Exo-Quick method. Extracted exosomal proteins were immunoblotted with antibodies for representative exosome markers, HSP70, CD63 and CD9. Interestingly, somewhat higher exosomal protein and RNA revels were observed in young blood. Two representative methods for exosome isolation are serial ultracentrifugation and Exo-Quick reagent. We identified increased yield of exosomal protein by young blood using ultracentrifugation method. These results suggest that exosomal protein and RNA from serum was significantly greater in young blood than old blood (Figure 8.19). To investigate whether young serum and serum exosomes has a protective role in HD, we treated an in vitro HD model cells, which showed mHtt aggregations in nucleus after day 7 of induction, with young serum-exo. Young serum-exo (200 μg/mL) was applied to the cells for 5 days after inducing mHtt aggregation. At 7 days, the control and young serum and serum-exo groups were fixed with 4% paraformaldehyde and stained with the Em48 antibody to detect mHtt aggregates, with DAPI as a counter stain (Figure 8.20). We counted DAPI (+) and Em48 (+) cells in the control (HD cells) and young serum and serum-exo treated groups (HD serum and serum-exo). The ratios of double-positive

cells to DAPI (+) cells were HD control and HD young serum and serum-exo. Young serum and serum-exo decreased expression of EM48/DAPI (**p<0.01 vs. no treatment HD cells). Treatment with Old blood serum and serum-exo no decreased the levels of EM48/DAPI. (Figure 8.21). To confirm the reduction of mHtt aggregates in cells treated with young serum and serum-exo, aggregates were also quantified by western blot (Figure 8.22). In HD young serum and serum-exo groups, levels of mHtt aggregates in cells were significantly decreased (*P<0.05, **p<0.01 vs. no treatment HD cells). Treatment with Old blood serum and serum-exo no decreased the levels of EM48 (Figure 8.23).

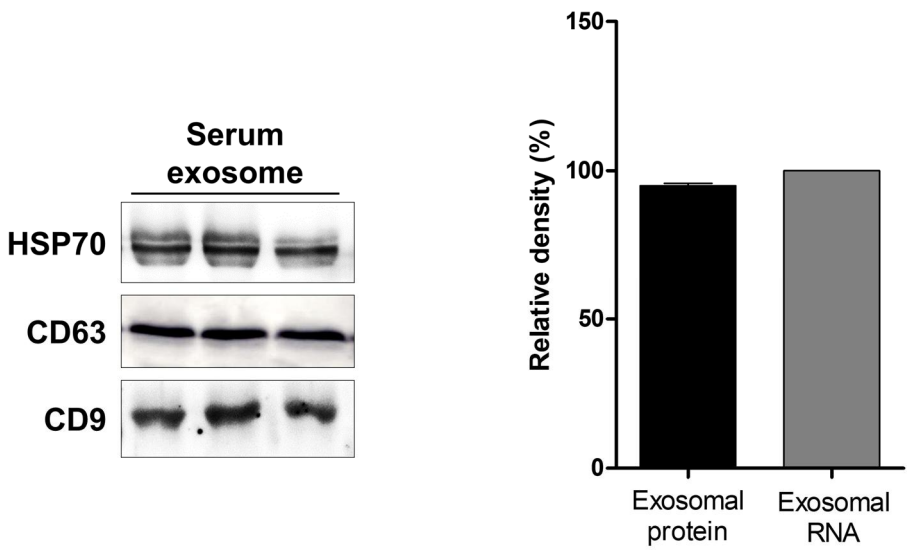


Figure 8.18 Isolation of blood serum derived exosomes. Exosomes from blood serum were isolated using Exo-Quick and ultracentrifuge method. Extracted exosomal proteins were immunoblotted with antibodies for representative

exosome markers, HSP70, CD63 and CD9. We thus confirmed the presence of exosomes.

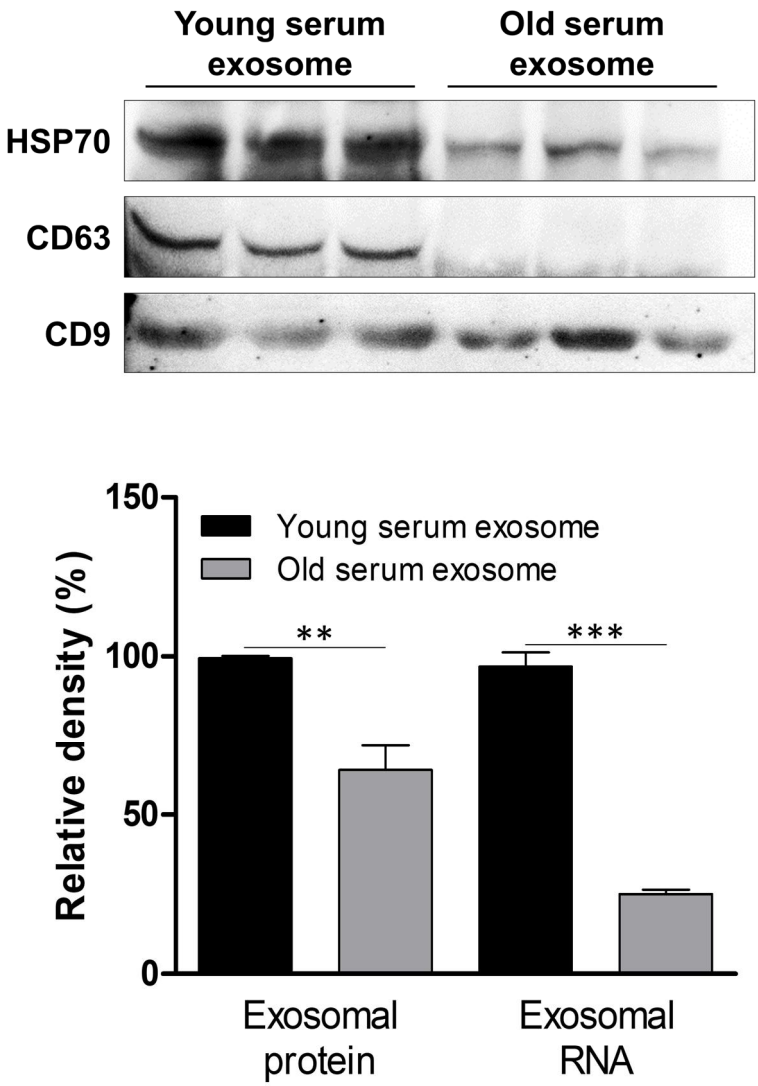


Figure 8.19 Identification of markers confirm exosomes isolation from serum and High concentraction in Exosomal fractions isolated from serum of young

blood versus old blood. To investigate exosomal protein and RNA concentration in young blood and old blood, proteins and total RNA were extracted by protein extraction buffer and total RNA isolation kit respectively after isolating exosomes from young blood and old blood. Exosomes from blood serum were isolated using Exo-Quick method. Extracted exosomal proteins were immunoblotted with antibodies for representative exosome markers, HSP70, CD63 and CD9. Interestingly, somewhat higher exosomal protein and RNA revels were observed in young blood. Two representative methods for exosome isolation are serial ultracentrifugation and Exo-Quick reagent. We identified increased yield of exosomal protein by young blood using ultracentrifugation method. These results suggest that exosomal protein and RNA from serum was significantly greater in young blood than old blood. (n=6)

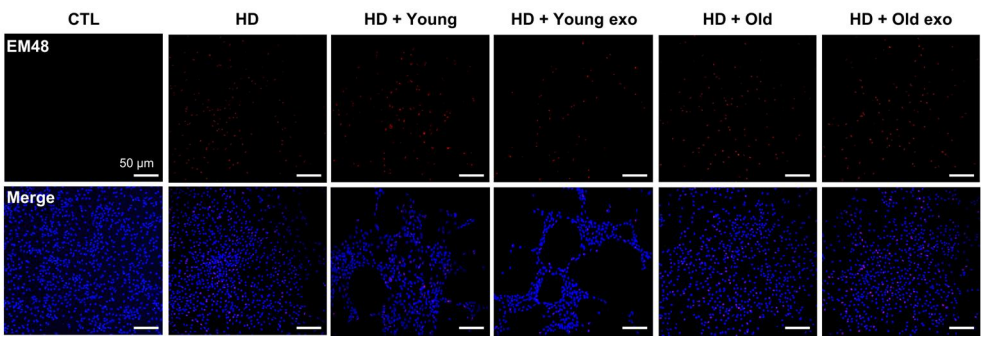


Figure 8.20 mHtt aggregates were analyzed by immunocytochemistry and western blotting. For immunocytochemistry, cells were stained with Em48 (red) and DAPI (blue) and the ratios of Em48 (+) to DAPI (+) cells were calculated.

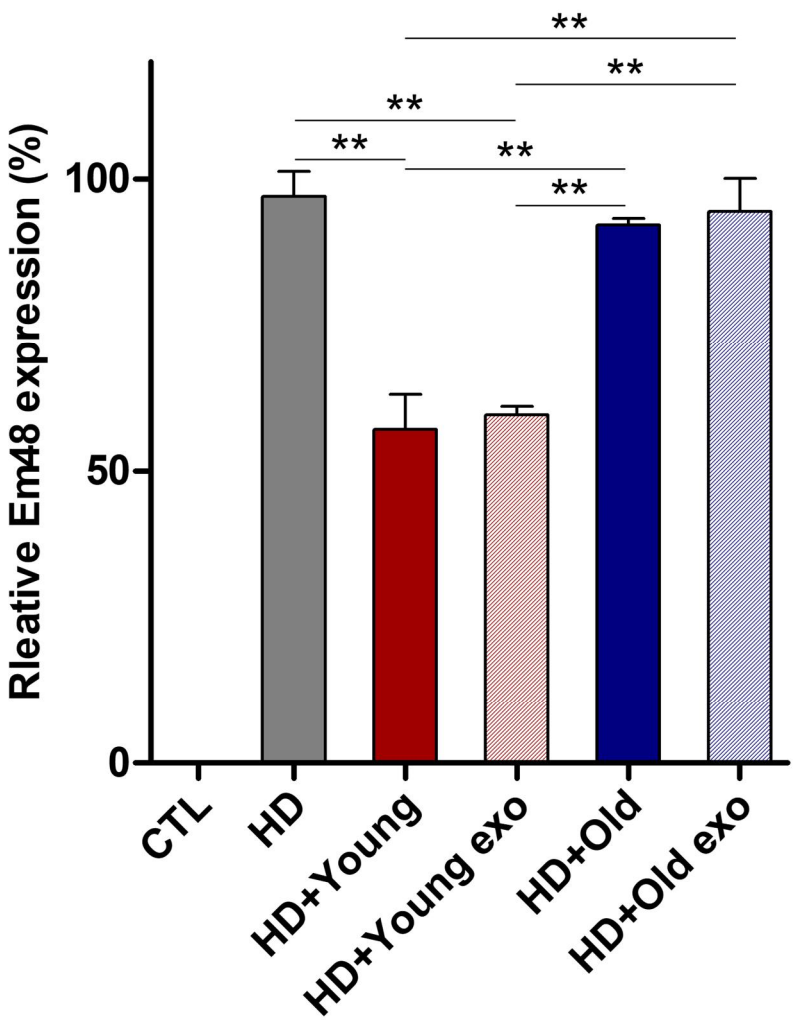


Figure 8.21 The young blood serum and serum exosome HD group showed less mHtt aggregates than the HD group. Scale bar = 50 um. *P < 0.05. (n=3)

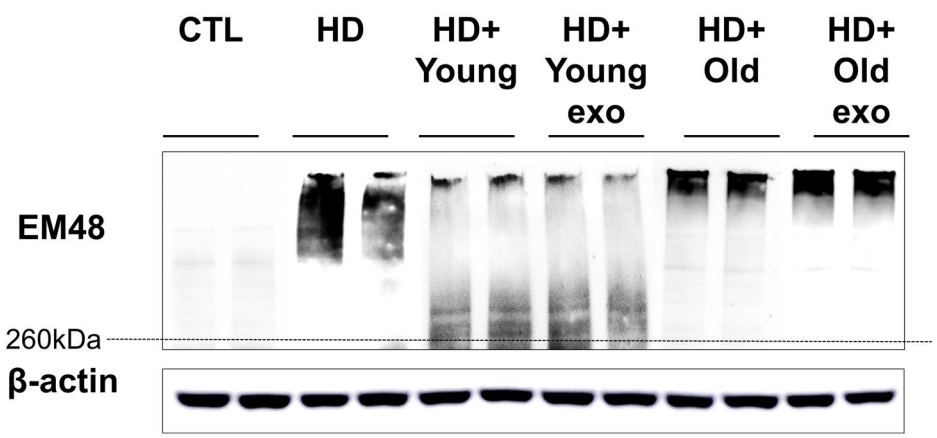


Figure 8.22 Western blotting confirmed that treatment with young blood serum and serum exosome HD decreased the accumulation of mHtt aggregates.

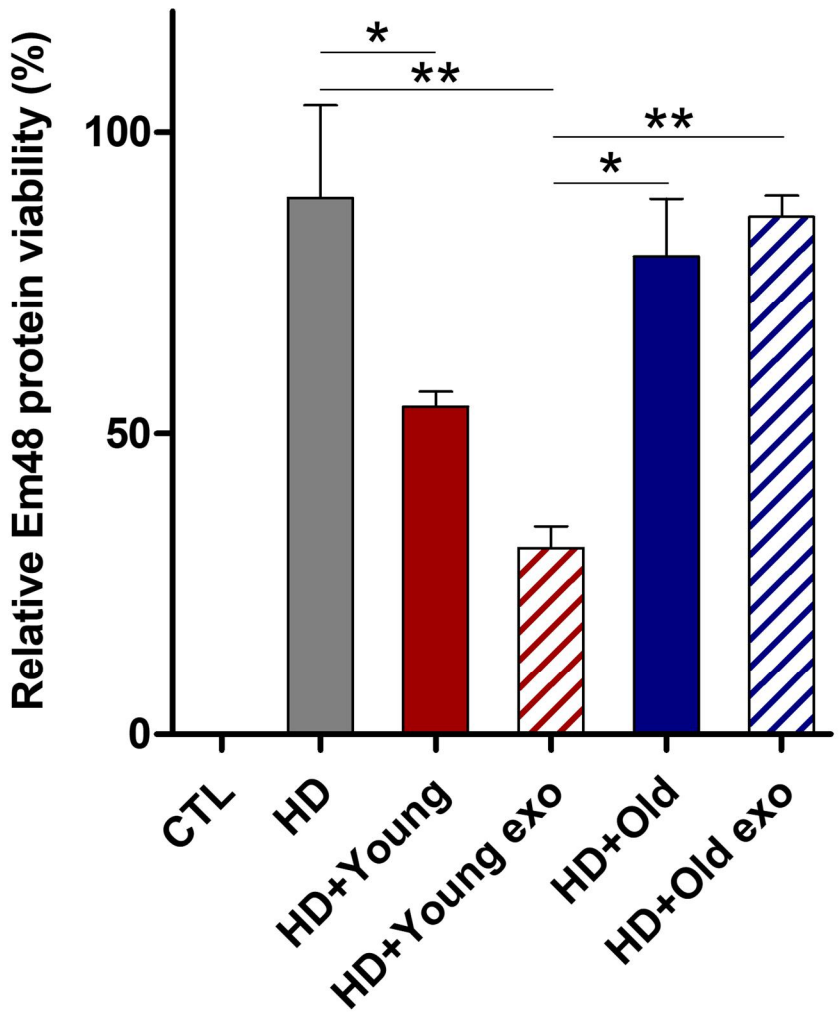


Figure 8.23 Normalized mutant huntingtin level was represented as bar graph. *P < 0.05, **P < 0.01 (n = 3).

8.6. Increase of cell survival by serum exosomes

To test whether young serum and serum-exo has cell survival roles, we investigated the WST-1. The result showed that treatment of young serum and serum-exo significantly increases cell survival (Figure 8.24). Cell survival was investigated using the WST-1. Old blood serum and serum exosome no significantly cell survival (Figure 8.25). To examine the effects of young serum and serum-exo on the PGC1a-p-CREB pathway, cells were treated with control medium or young serum and serum-exo for 3 days after day 2 of differentiation. Treatment with young serum and serum-exo promoted expression of p-CREB (**$p<0.01$, ***$p<0.001$ vs. no treatment HD cells) and PGC1α showed no significant difference compared to no treatment HD cells (Figure 8.26). As shown by western blotting, treatment with Old blood serum and serum exosome no increased the levels of PGC1α and p-CREB (Figure 8.27). To investigate whether young serum and serum-exo protect against apoptosis in vitro HD model, we determined the levels of apoptosis-related proteins by western blotting. We found that p53, Bax, and cleaved caspase-3 levels were lower in the HD young serum and serum-exo groups than in the HD control groups. Bcl-2 levels were upper in the HD young serum and serum-exo groups than in the HD control groups (Figure 8.28). Also, to confirm the anti-apoptotic effect of young serum and serum-exo on the in vitro HD model, NSCs derived from the HD mice model were differentiated and treated the young serum and serum-exo for 3days, and flow cytometry analysis was performed using Annexin-V and propidium iodide. Cell populations were analyzed as viable/early apoptotic/late apoptotic/necrosis cells, and this calculation was conducted using the Annexin-V and propidium iodide positive cell count. HD neuronal cells were found to have a more apoptotic population a less viable population. However,

Young serum and serum-exo treated HD cells showed significant reduction of the apoptotic cell population and an increase in the viable cell population (Figure 8.29). As shown by western blotting, Treatment with Old blood serum and serum exosome no decreased the levels of p53, Bax, and cleaved caspase-3 compared with HD cells and no increased the levels of Bcl-2 (Figure 8.30). Taken together, young serum and serum-exo treatment results in more cell survival and less cell death, accompanied by a reduction of mHtt aggregation protein and apoptotic signaling. As the results, young serum and serum-exo improves mitochondrial activation and cell survival in vitro HD model.

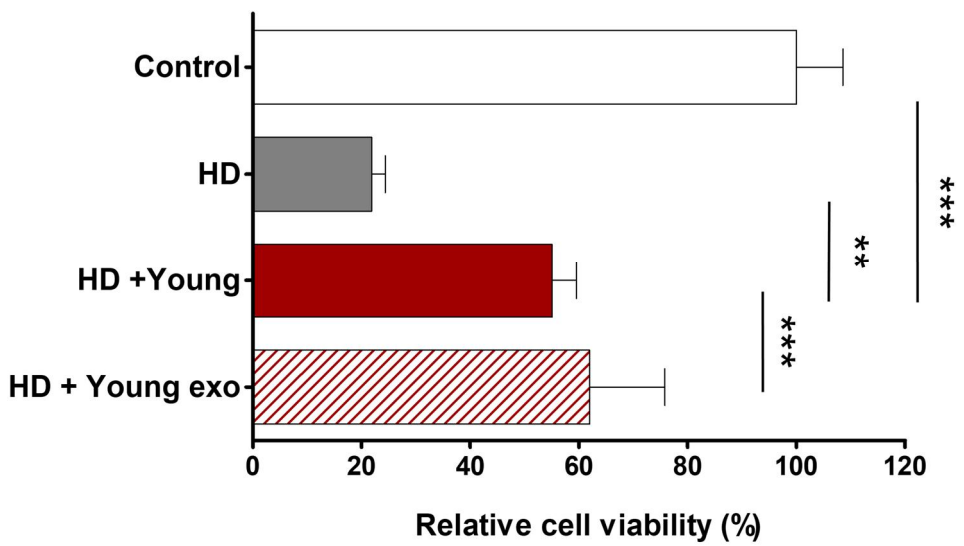


Figure 8.24 Cell survival was investigated using the WST-1. Young blood serum and serum exosome significantly increased cell survival. **P < 0.01, ***P < 0.001 (n = 5).

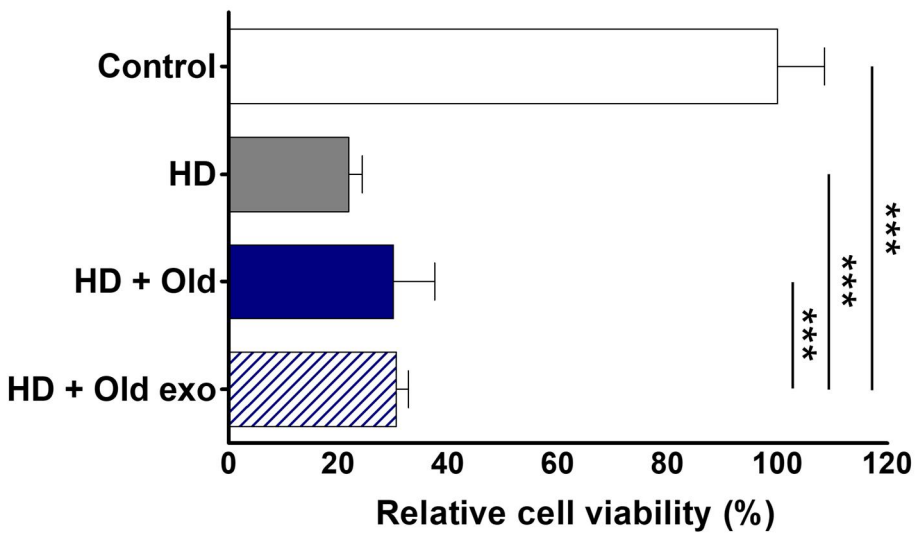


Figure 8.25 Cell survival was investigated using the WST-1. Old blood serum and serum exosome no significantly cell survival. (n = 5).

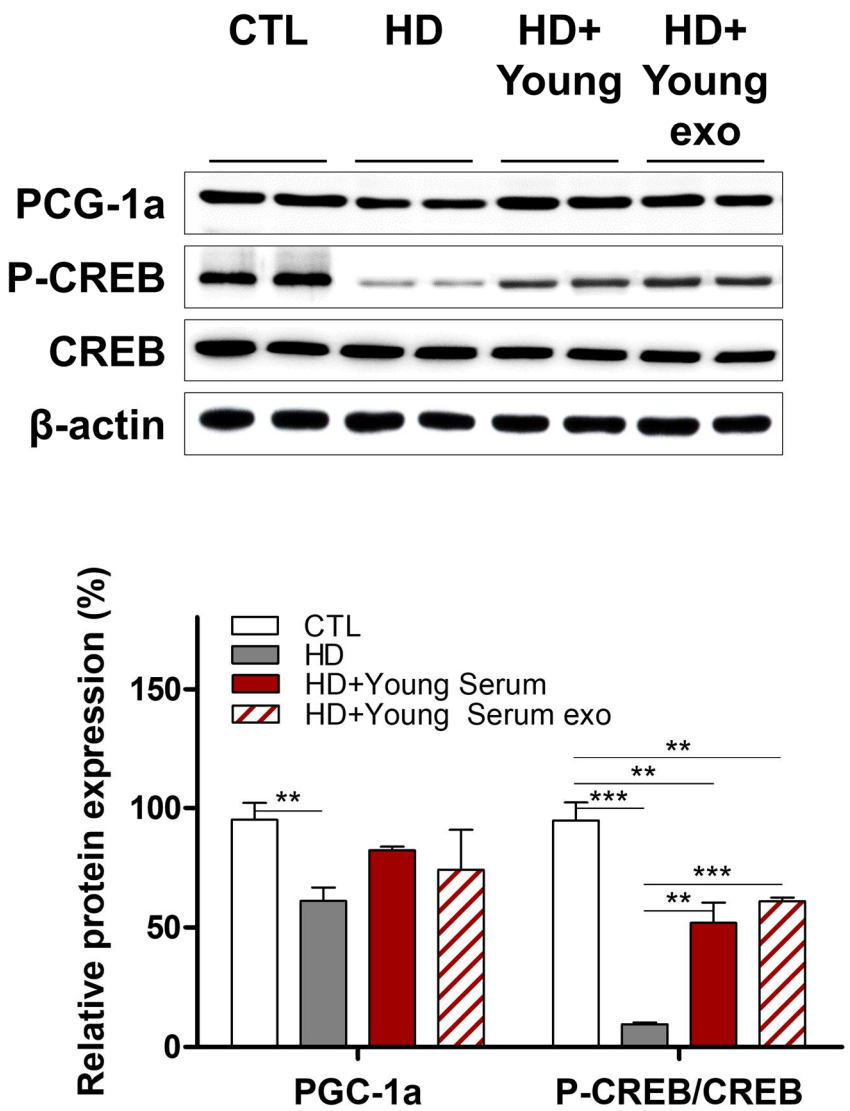


Figure 8.26 As shown by western blotting, treatment with young blood serum and serum exosome increased the levels of PGC1a and p-CREB. **P < 0.01, ***P < 0.001 (n = 3).

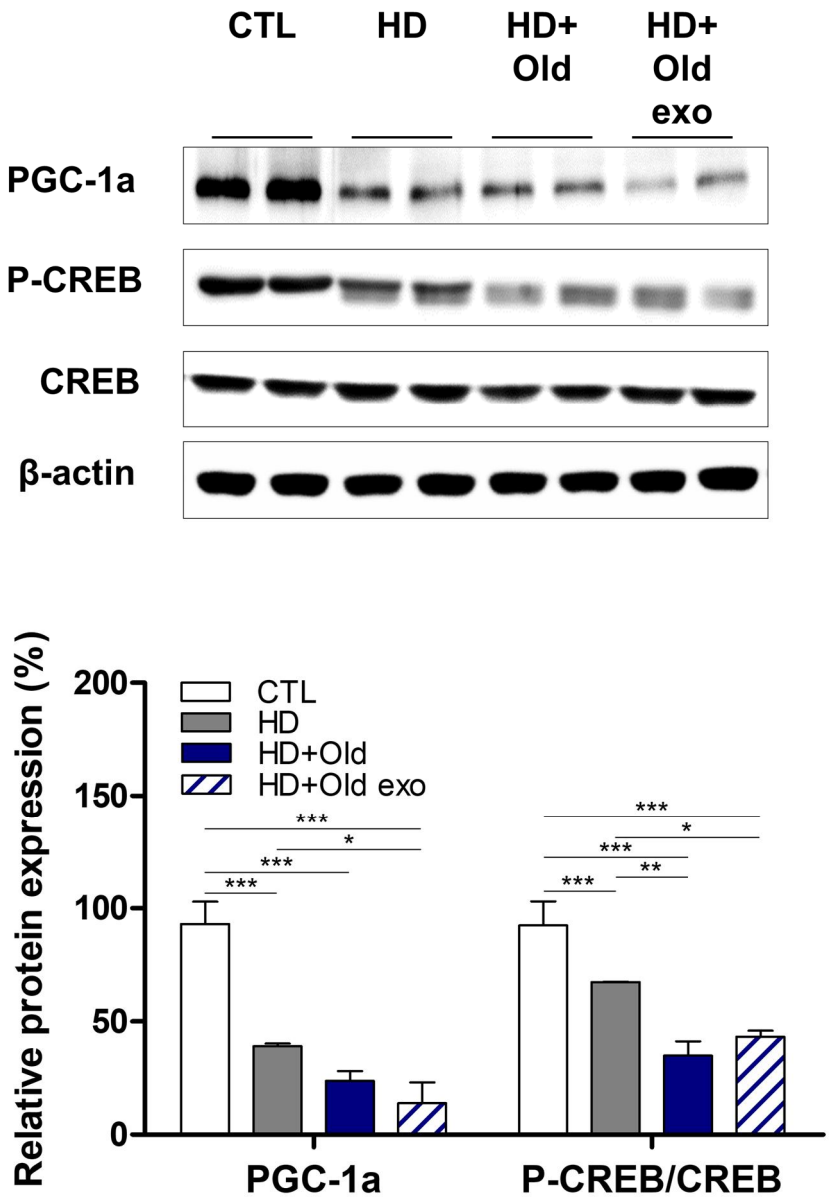
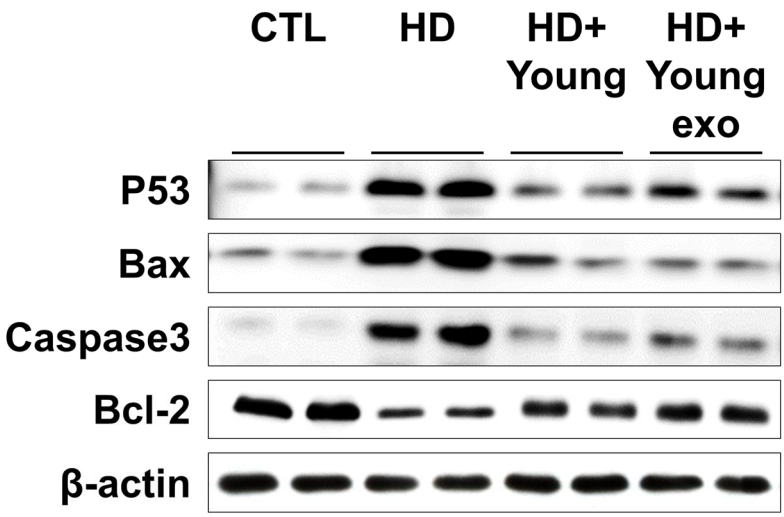


Figure 8.27 As shown by western blotting, treatment with Old blood serum and serum exosome no increased the levels of PGC1a and p-CREB. (n = 3).



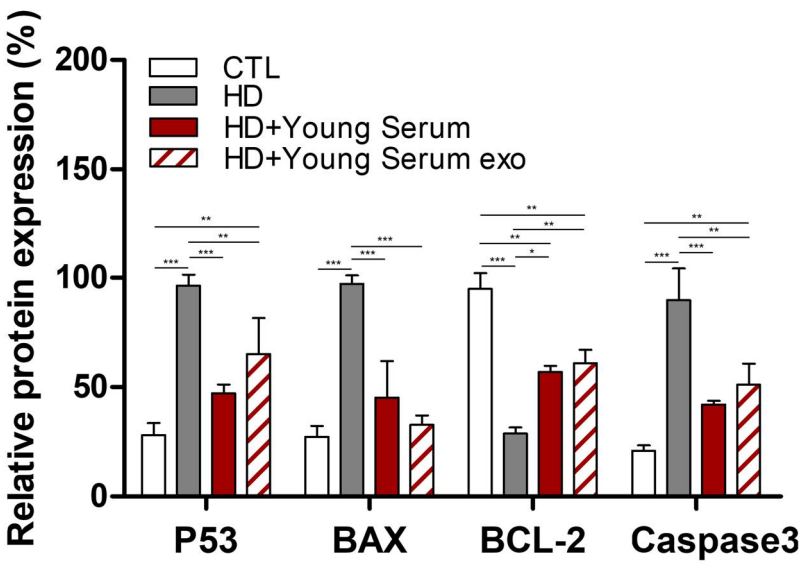


Figure 8.28 As shown by western blotting, Treatment with young blood serum and serum exosome decreased the levels of p53, Bax, and cleaved caspase-3 compared with HD cells and increased the levels of Bcl-2. *P < 0.05, **P < 0.01, ***P < 0.001 (n = 3).

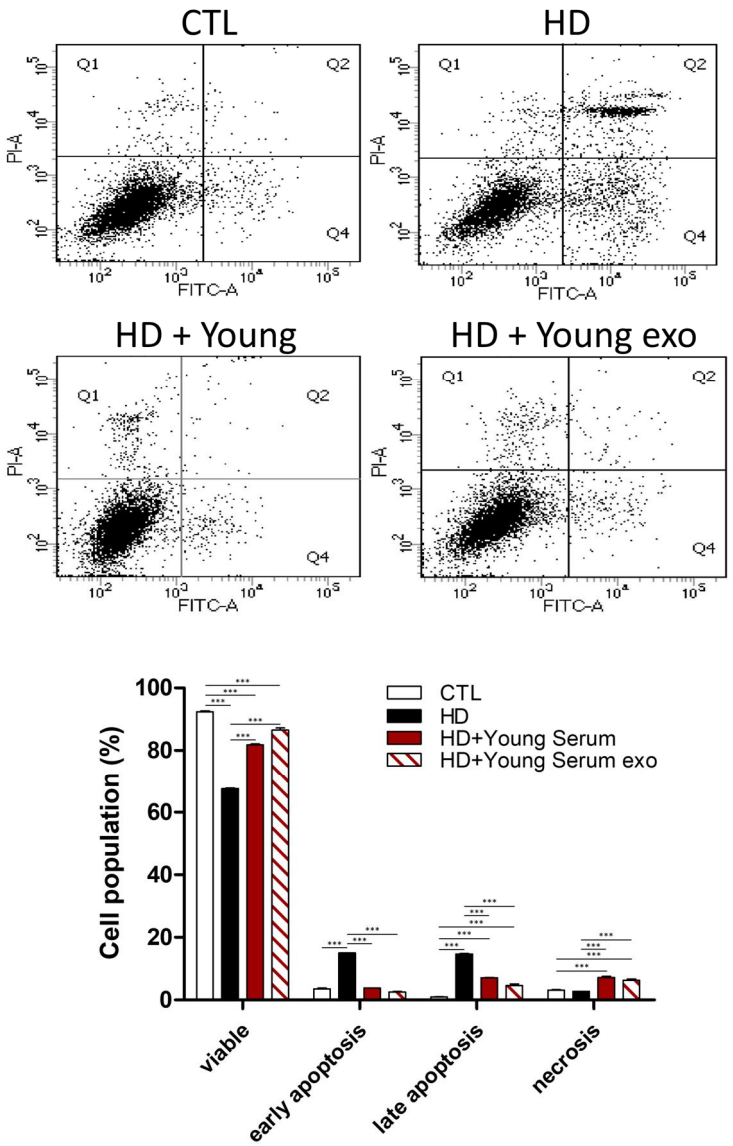


Figure 8.29 Young blood serum and serum exosome ameliorates the apoptosis of HD cells. NSCs from the subventricular zone of WT and HD transgenic mice were cultured and differentiated using a differentiation medium. NSCs from HD mice were cultured in the presence of young blood serum and serum exosome

(200 lg/mL) on day 2 after differentiation. young blood serum and serum exosome treatment was conducted for 3 days and cell were then harvested and incubated with annexin V-FITC for 30 min at room temperature. Propidium iodide was added to all samples just before the analysis. The viable (lower-left), early apoptotic (lower-right), late apoptotic (upper-right), necrotic (upper-left) cell populations were analyzed using flowcytometry. The cell percentage was analyzed according to annexin V and propidium iodide staining and presented as a bar graph. This result used young serum and serum exosome from three different volunteers and similar result was obtained. *** $p < 0.001$.

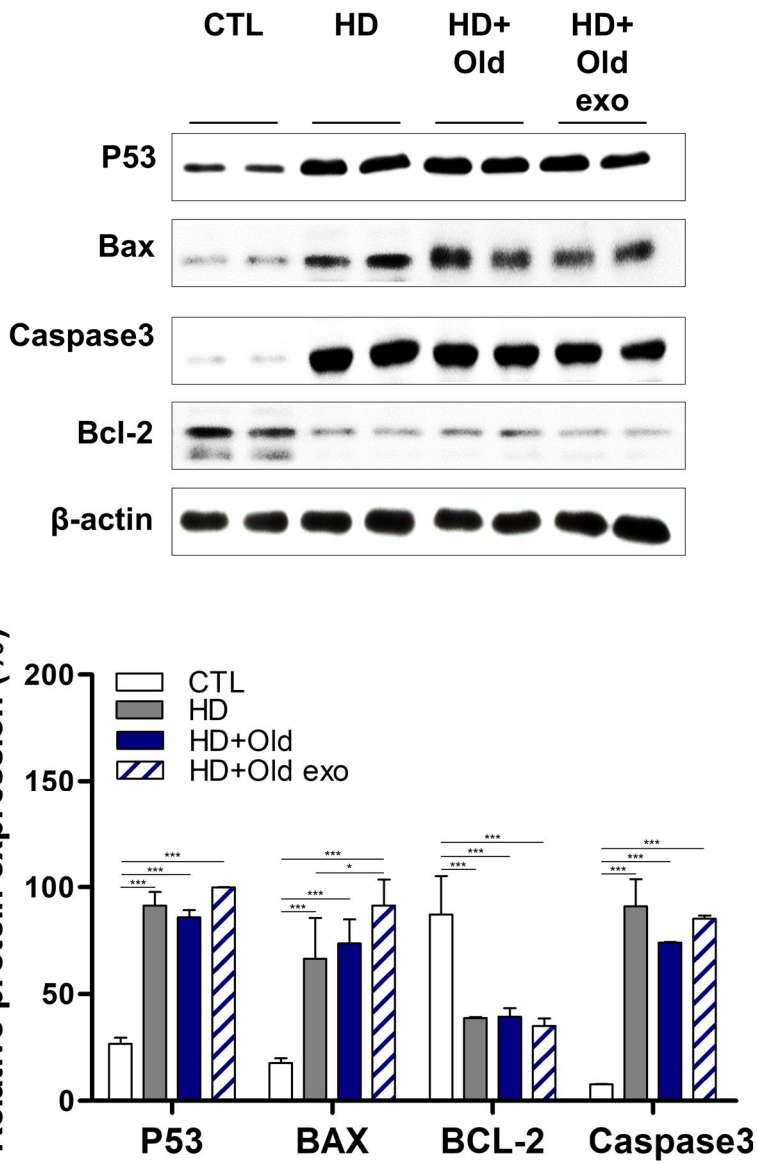


Figure 8.30 As shown by western blotting, Treatment with Old blood serum and serum exosome no decreased the levels of p53, Bax, and cleaved caspase-3 compared with HD cells and no increased the levels of Bcl-2. (n = 3).

8.7. Summary

In this study, our finding demonstrated that blood from young mice contains soluble factors that sufficient to ameliorate depletion of neuronal proteins that have served as sensitive markers for neurodegeneration in HD and R6/2 transgenic mouse models and restore mitochondria dysfunction and cell death observed in R6/2 mice. Although our mice model misses aspects of the disease, we demonstrated therapeutic properties of young serum by ameliorating aspects of the disease that are present in patients. Many clinical trials with drug targeting mHtt aggregation proteins have failed to slow the progress of HD. Also, we investigated the preventive role of young serum-Exo in the HD related phenotypes within the in vitro HD model. NSCs were isolated from R6/2 mice and differentiated into neuronal cells. Differentiated HD neuronal cells showed increased mHtt aggregation protein and mitochondria dysfunction. The cells also showed an increase of apoptotic molecules and decrease of anti-apoptotic protein accompanied with cellular apoptosis, which are the major phenotypes of HD in vivo. Our results showed that increased mHtt aggregation protein of HD cells were decreased by young serum-Exo treatment and restored mitochondria dysfunction. The increase of apoptotic signaling and the apoptotic/necrotic cell population of HD cells were normalized by young serum-Exo treatment. Our results suggest that young serum□exo decreases accumulation of mHtt aggregates, activates the p□CREB□ PGC1α pathway, and modulates the expression of apoptotic proteins. As a results, young serum exosome was able to exert beneficial effects in HD.

Heterochronic pabiosis is a surgical union of 2 organisms of different age that results in a shared blood circulation between, eg, 2 mice. The model thus allows us to study whether circulating factors from the young mice can alter tissue function of the

disease parabiont or vice versa. Tissues from heterochronic pairs are compared with those of isochronic pairs, 2 organisms of the same disease sharing a blood circulation, as a control. our results demonstrate that heterochronic parabiosis is effective in modulating HD through the reduction of cellular apoptosis and the HD pathology. mHtt aggregation protein accumulation and mitochondrial dysfunction are considered as a major biomarker and key step of HD. [15-19] In addition, reducing mHtt aggregation protein accumulation and mitochondrial oxidative stress have been studied to date as a beneficial strategy for HD. [20-22] Our R6/2 mice exhibit increased level of mHtt aggregation protein and mitochondrial superoxide as representative phenotypes with a typical mHtt aggregation protein. Our study demonstrated that these phenotypes were attenuated by heterochronic parabiosis, therefore, therapeutic potential of heterochronic parabiosis on HD phenotypes was confirmed by in vivo. Parabiosis has led to remarkable biological and medical discoveries over the last decades and over the past few years in particular. Given its success, it is surprising that this model is not used more extensively. Considering the remarkable rejuvenating impact young mice have on neurodegenerative disease in heterochronic pairings, we will hopefully accelerate our progress towards curing the most devastating diseases of our time.

Exosomes are small extracellular vesicle and ideal carriers for delivery of therapeutic agents to tissues that are difficult to access, such as those in the brain. Methods of isolating exosomes from blood, urine, or saliva in vivo and conditioned medium in vitro have been developed. Exosomes contain various molecular components of its cell of origin, such as proteins, mRNAs, and microRNAs to maintain or change environmental conditions. [23] Many studies have reported the biological and medical significance of exosomes. Cancer cell exosomes mediate

cancer progression by inducing a cancer favorable microenvironment. [24-25] Ab protein seems to be associated with the exosomes of AD neurons, resulting in the propagation of the amyloid beta protein. [26] Furthermore, prion protein of exosomes in Creutzfeldt-Jakob disease contributes to the progression of the disease. [27-28] In addition to its pathological roles, exosomes have been regarded as a biomarker of various diseases. [29-32] Several reports have suggested that young blood, including young serum exosome, secrete various cytokines and vesicles, which can modulate a hostile pathological environment. Our results prove that the secretory vesicles contain some of the beneficial components of young serum exosome. An exosome is an extracellular vesicle released from most blood types. Young serum has beneficial effects on Cognitive improvement, ischemic injury, vascular function, and tissue regeneration. Recent evidence shows that young serum results in improving cognitive function and neuropathology through different underlying mechanisms. [33-34] Furthermore, young serum secretory factors also ameliorate AD phenotypes by Ab degradation [35] and a Cognitive improvement. [36] In addition to these previous reports of the therapeutic potential of young serum on AD, our results further demonstrate that young serum-Exo is effective in modulating HD through the reduction of cellular apoptosis and the HD pathology. Although our results show that young serum-Exo improves the in vitro HD phenotype, young serum exosome therapy may also vary in efficacy by cell donor. Typically, the age or gender of the donor may influence the efficiency, characteristics, and differentiation potential of young serum. For future clinical application, thorough investigation of young serum-Exo according to the donor characteristics will be required. We confirmed mitochondria dysfunction, anti-apoptotic effect and HD pathology through young serum exosome obtained from three different mices, and same results were obtained

in three independent experiments. Thus, although there may be slight difference in efficiency, it has been confirmed that young serum-Exo has anti-apoptotic and neurogenesis effect in common. MHtt aggregation protein accumulation and mitochondrial dysfunction are considered as a major biomarker and key step of HD. In addition, reducing mHtt aggregation protein accumulation and mitochondrial oxidative stress have been studied to date as a beneficial strategy for HD. Our primary neuronal stem cells from R6/2 mice exhibit increased level of mHtt aggregation protein and mitochondrial superoxide as representative phenotypes with a typical mHtt aggregation protein. Our study demonstrated that these in vitro phenotypes were attenuated by young serum-Exo treatment, therefore, therapeutic potential of youn serum-Exo on HD phenotypes was confirmed by in vitro. Further studies will have to determine the importance of the identify key factors responsible for these effects and elucidate their mechanisms of action. This could lead to development of potential small molecule in serum exosome interventions. However, a group of soluble factors in young serum exosome targeting several pathways may be necessary for therapeutic benefits. Therefore, intravenous administration of serum exosome in humans, a low risk procedure that is already offered as a therapy with limited complications, is feasible to test the efficacy of young serum exosome in patients with HD and possibly other forms of mitochondria dysfunction and neurodegeneration. This is a preclinical study done in an HD mice model, which might have limited translation to the human disease. Young mice serum exosome had beneficial effects on a number of disease parameters in this model, but it did not reverse all aspects of the mice disease. For example, we did not carry out pharmacological studies optimizing the frequency or duration of serum administration or volume given per injection. Last, it is possible that continuous administration of young serum in an old recipient might have detrimental

effects, although we have not observed any at this point. This preclinical study shows encouraging beneficial effects of young serum exosome administration on molecular and functional outcomes in a mice model of HD. serum did reduce mHtt aggregation proteins and appears to target molecular pathways involved in mitochondria, cell death. Young serum exosome administration is quite safe in humans and it would be possible to translate these findings to patients relatively quickly, pharmacological parameters and clinical end points will have to be established and safety in order patient needs to be demonstrated.

In summary, our results show that young serum-Exo have modulating effects on the in vitro model of HD. Neuronal cells from HD transgenic mice show an accumulation mHtt aggregation protein, mitochondria dysfunction, apoptotic molecules, and cellular apoptosis. young serum-Exo reduces these pathological phenotypes of the HD in vitro model. 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.

CHAPTER 9

Conclusion

9.1. Summary of Results

The aim of this study is to develop a methodology that can obtain the high-quality exosomes and adopt exoeomes to the various neurodegenerative diseases (HD, AD, and ALS). To obtain high-quality exosomes, the effect of pH on the yield of exosome isolation and the temperature condition for the storage is studied. The concentrations of exosomal protein and nucleic acid were increased after incubation in the acidic medium, and the termperature below -70 ^{o}C was favorable condition for preservation of exosomes at fresh condition while incubation at 4 ^{o}C and RT resulted in major loss of CD63.

By treating ASC-exo to the HD in vitro model, the phenotype of HD was ameliorated. ASC-exo decreased mHtt aggregates in R6/2 mice-derived neuronal cells, and it up-regulates PGC-1, phospho-CREB, and abnormal apoptotic protein level. Next, ASC-exo was treated to the neuronal stem cells isolated from G93A ALS mice model. ASC-exo alleviated aggregation of SOD1 and decreased the phospho-CREB and PGC-1α. Also, The β-amyloid pathology and apoptosis of neuronal cells derived from the transgenic mouse model of AD was reduced by ASC-exo. ASC-exo was treated to the neuronal stem

cells from the brains of TG2576 AD mice. ASC-exo treatment resulted in reduced Aβ42 levels, Aβ40 levels, and the Aβ42/40 ratio of AD cells. Also, apoptotic molecules, p53, Bax, pro-caspase-3 and cleaved-caspase-3, and Bcl-2 protein level were normalized by ASC-exo treatment. Furthermore, by developing animal models for the heterochronic parabiosis (HP) of Huntington's disease, the alleviation of HD pathology was observed after the parabiosis. The exosomes extracted from the young wild type mouse showed the alleviation of HD pathology in vitro model, indicating that the exosome is a messenger unit for the positive factors during the heterochronic parabiosis.

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요약(국문초록)

엑소좀은 직경이 30-100 nm 인 작은 세포 외 소포이며 많은 종류의 세포에서 유래한다. 엑소좀은 endosomal membrane 으로부터의 소포의 내부 발아 및 절단에 의해 세포 간 전달 단위의 역할을 하며, 세포외 배출 시 MVB 루멘에서 세포 외 환경으로 방출된다. 분비된 엑소좀은 체외에서 혈액, 소변, 생체 내 타액 및 조정 배지에서 분리되고 분석 될 수 있다. 여기에는 단백질, mRNA 및

마이크로 RNA 를 비롯한 세포 기원의 다양한 분자 구성 요소가 포함된다. 따라서, 엑소좀은 치료제, 바이오 마커 및 약물 전달체로서 광범위하게 연구되어왔다.

정상 생리 및 질병의 진행에서의 중요성에도 불구하고, 일반적으로 exosome 의 수득 및 저장 조건의 표준 기준은 불명확하고 논쟁의 여지가 있어 왔다. 따라서 본 연구에서는 다양한 pH 조건에서 엑소좀을 배양 한 후 분리하고 회수율을 분석하였으며, 다양한 저장 온도에서 엑소좀의 안정성을 조사하였다. 본 연구에서는 산성 조건이 엑소좀 분리에 대하여 높은 수율을 얻는 데에 유리하다는 것을 발견했으며, 임상 및 기본 분야 응용을 위하여 엑소좀을 신선하게 보존하기 위해서는 −20 ℃ 이하의 온도가 중요하다는 것을 발견하였다.

엑소좀에 대한 분리 및 저장 조건을 최적화 한 후 우리는 인간지방줄기세포에서 유래한 엑소좀이 (ASC−exo) 알츠하이머 병 (AD), 헌팅톤 병 (HD), 근 위축성 측삭 경화증 (ALS) 등의 질병의 대표적인 세포 표현형을 조절하는 것을 확인하였다. 지방 줄기 세포 (ASCs)는 재생 의학의 잠재적 원천으로 여겨지고 있는데, 지방 줄기 세포는 파라크린 효과에 대해 여러 인자를 내포하고 있으며, 다른 조직 유래 줄기 세포보다 낮은 노화로 높은 증식률을 나타낸다고 알려져 있다. 비록 줄기 세포에서 유래된 엑소좀은 많은 질병에서 치료적 잠재력을 가지고 있지만, ASC−exo 가 HD, AD 및 ALS 의 진행에 미치는 정확한 기전은 연구되지 않았다.

이에 본 연구에서는 R6/2 유래 신경 세포의 세포 사멸 및 미토콘드리아 기능 장애 및 mHtt 응집체 수준을 비롯한 주요 HD 표현형에 ASC−exo 가 미치는 영향을 조사하였다. 또한, 본 연구는 G93A 신경 세포에서 SOD1 집합체, 증가된 SOD1 수준 및 비정상적인 미토콘드리아 단백질 수준을 포함하는 ALS 의 주요 특징에 대해 ASC−exo 의 효과를 조사 하였다. 또한, AD 질병 모델에 대표적으로 알려진 트랜스 제닉 생쥐 라인인 TG2576 에서 유래한 줄기 세포 (NSCs)를 사용하여 시험관내 AD 모델을 개발하였다. Aβ 병리 및 증가된 세포

사멸을 포함하는 AD 의 주요 표현형을 나타내는 AD 마우스에서 분화된 NSC 는 ASC-exo 에 의해 질병이 개선됨을 확인하였다. 또한, 신경 돌기가 중추 신경계의 시냅스 형성에 관여하기 때문에, 신경 돌기 성장의 변화를 조사하였다. 이를 통해 ASC-exo 가 AD 에서 Aβ 유도성 신경 세포의 진행을 완화시키고 ASC-exo 가 SOD-1 응집과 미토콘드리아 기능 장애를 조절하여 AD 의 치료적 후보 물질이 될 수 있다는 것을 증명하였다. HD 에서는 ASC-exo 가 PGC1, phospho-CREB 를 상향 조절하고 비정상적인 세포 사멸 단백질 수준을 개선하고 mHtt 응집체 수준을 감소 시킨다는 것을 확인하였다. 또한, MitoSOX Red, JC-1 및 세포 생존능 분석 결과, ASC-exo 는 미토콘드리아 기능 장애 및 세포 사멸을 감소시키는 것을 확인하였다. 이러한 결과는 ASC-exo 가 대표적인 세포 표현형을 조절함으로써 신경 퇴행성 질환을 치료할 수 있는 치료적 잠재력을 가지고 있음을 시사한다.

다음 주제는 Heterochronic parabiosis (HP)를 통한 젊은 혈액 순환을 통한 노출은 정상적인 노화로 관찰되는 인지 장애를 개선시킴을 확인한 결과입니다. R6 / 2 마우스의 HD 유사 질환에 대한 젊은 순환기 요인의 영향을 알아보기 위해 본 연구에서는 R6 / 2 마우스와 함께 젊은 야생형 동물의 순환계에 HP 방법을 통하여 합류시켰다. Heterochronic parabiosis 후 질병이 확립된 R6 / 2 마우스의 뇌에서 단백질 발현의 변화를 분석하였고, p-CREB-PGC-1a 경로, 세포 사멸 단백질 및 세포 사멸을 포함한 HD 의 주요 표현형에 젊은 혈장 exosome 의 영향을 조사하였다. 그 결과, 헌팅톤 병의 대표적인 돌연변이형 헌팅틴 응집 단백질의 병리 수준이 회복됨을 확인하였고, 미토콘드리아 기능 장애가 개선되었으며, 인지 부분이 개선 됨을 확인할 수 있었다. 또한, 젊은 혈액에 노출된 후에 세포 사멸 감소를 확인하였다. 마지막으로 젊은 혈청 및 혈청 엑소좀으로 처리한 시험관 내 HD 모델 세포는 mHtt 응집, 미토콘드리아 기능 장애 회복 및 세포 증식을 확인하였으며, 세포 사멸을 감소 시킴을 확인하였다.

이를 통해 헌팅톤 병 개선을 시키는 물질이 젊은 혈장 내 exosome 내 물질일 가능성을 제시하였다.

이 연구는 엑소좀의 분리 및 저장을 위한 최적 조건을 이해하고 엑소좀을 질병 개선에 긍정적인 요소를 전달할 수 있는 메신저 단위로 확인함으로써 유용한 정보를 제공하고, 다양한 신경 퇴행성 질환에 대한 엑소좀을 치료제로 채택 할 수 있는 가능성을 열어준다.

표제어: 퇴행성 신경질환, 인간 지방 줄기 세포, 엑소좀, 저장 조건, 온도, 산성 미세 환경, 병체결합

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PUBLICATIONS

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