Gene Therapy Combined with IPC Cell Induction to Treat Huntington's Disease

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Summary

Huntington's disease (HD) results from expansion of CAG repeats in the huntingtin gene in chromosome 4, resulting in misfolded and aggregated huntingtin protein inside medium spiny neurons. In HD, these neurons ultimately die, resulting in the classical symptoms of the disease. Current treatments for HD are ineffective, as none attempt to treat the source of the disease. A new method that attempts to correct the expanded huntingtin gene is needed for effective, long term treatment of the disease. Recent advances in the field of induced pluripotent stem cells and gene therapy have shown that induction of pluripotency combined with homologous recombination can correct genetic disease in mice. We propose the hypothesis that fibroblasts from HD model mice and human HD patients can be transformed into IPS cells. After IPS cell formation, the huntingtin gene will be repaired using homologous recombination, and the cells will be directly differentiated into medium spiny neurons. Transplantation of the neurons into HD mice models representing healthy, mild, and severe forms of the disease will follow, and improvements in HD related symptoms will be evaluated. Perfection of our methods is vital to future use of IPS cells and gene therapy to treat disease.

Background

Neurodegenerative disease is a broad term used to describe a state of selective neuronal death. The death of neurons results in the symptoms of the disease, and these symptoms are usually characteristic of the specific disease the patient is suffering from (1). For example, in Parkinson's disease (PD), dopaminergic neurons of the substantia nigra are lost, resulting in movement based symptoms (2). In Alzheimer's disease (AD), the memory deficits seen in patients are the consequence of neuronal death in the hippocampus (3). The reason for the neuronal degeneration, however, is complex and varied. While many of the currently known neurodegenerative diseases are of sporadic origin, a number of these neurodegenerative diseases can be traced to genetic mutations, such as the familial mutations known to cause PD (4). Current treatments for these diseases are insufficient, as they do not fix the source of the disease and only seek to ease symptoms. Since there is no way to slow or stop disease progression, treatment typically consists of physical rehabilitation to improve movement symptoms and drug use to treat emotional symptoms (5).

One such neurodegenerative disease of specific interest is Huntington's disease (HD). HD is a late onset autosomal-dominant neurodegenerative disorder characterized by death of neurons in the caudate nucleus (5, 6). Symptoms include psychiatric disturbances, motor impairment, and cognitive decline and typically present themselves in midlife. The disease is a trinucleotide repeat disease caused by a CAG repeat expansion in the *IT15* gene on the short arm of chromosome 4, resulting in a long stretch of polyglutamine in the encoded huntingtin (htt) protein (7). Adults with between six and thirty-five CAG repeats in the *IT15* gene have been found to be asymptomatic. When more than thirty-six CAG repeats are present, HD will most likely develop. The length of the CAG expansion is inversely correlated with age of disease onset, and juvenile cases of HD are characterized by expansions of more than sixty repeats (5). The polyglutamine expansions in HD lead to characteristic patterns of neurodegeneration, including the death of γ-aminobutyric (GABA) acid-releasing spiny-projection neurons of the striatum as well as loss of neurons in the hippocampus and cortex (6). Protein aggregation has also been shown to play a role in HD as in some other neurodegenerative diseases, such as PD and AD. There is characteristic formation of intranuclear inclusions (NIIs) of the huntingtin protein in the striatum which stain positively for ubiquitin, indicating that protein degradation occurs. The timing of appearance of NIIs parallels the cognitive and motor decline in mice. This indicates that protein aggregation plays an important role in the progression of Huntington's disease. However, exact mechanisms by which progression takes place remain to be identified (8).

No effective treatment currently exists to prevent or slow the loss of these neurons, although previous research on tissue transplantation from unaffected patients into HD patients has shown promise (9). Additionally, the use of histone deacetylase inhibitors is being investigated as a potential treatment method (4). Other small molecules that prevent aggregation of huntingtin protein are currently being studied as potential HD treatments as well (4). However, most of these treatments are still being conducted in model organisms, and the move to clinical trials is time consuming and expensive. Other alternatives should be pursued.

One potential treatment involves the use of stem cells to replace dying neurons. Embryonic stem cells (ES) are a type of pluripotent cells located in the inner mass of a developing blastocyst. These cells are capable of dividing repeatedly while still retaining the ability to differentiate into tissue from any of the three germ layers (10). This capacity for self renewal and tissue specific differentiation makes ES cells promising tools in regenerative medicine. ES cells derived from developing mice embryos successfully integrated into the striatum and reduced symptoms in a drug induced PD mice model (11). Application of ES cell therapy to treat human disease is limited by rejection of donor cells and the current techniques used to harvest the cells. Modern methods require the destruction of a developing embryo to obtain human ES cells, raising a moral debate in the political arena (12). Consequently, the pressure to develop a new approach to obtain pluripotent cells led to the discovery of induced pluripotent stem cells (IPS).

IPS cells are derived from somatic cells that have been reprogrammed to an ES cell-like state using a cocktail of four transcription factors delivered by retroviruses. The transcription factors c-Myc, Oct3/4, Sox2, and Klf4 are sufficient to reprogram a human fibroblast into an IPS cell that has the ability to differentiate into all three germ layers (13). The source of these pluripotent cells bypasses any ethical opposition, and thus is a promising technique to use in disease treatment. The patient-specific origin of these

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cells also circumvents immune rejection. IPS cells have been used successfully to treat rats in a PD model, and IPS cells from human patients with a number of genetic diseases have also been generated. More specifically, fibroblasts cells from two Amyotrophic Lateral Sclerosis (ALS) patients were transformed into IPS cells and directly differentiated into the motor neurons affected by the disease (10, 14). The genetic mutation that causes ALS was present in the genome of these disease specific IPS cells (dsIPS). The differentiation techniques used to generate the motor neurons involved manipulation of the sonic hedgehog (SHH) pathway combined with retinoic acid treatment (14). It has been suggested that the timing and concentration of retinoic acid and SHH protein addition influences the type of resulting neuronal differentiation (15, 16).

In addition to IPS cells, gene therapy has emerged as a powerful tool to correct genetic mutations for use in regenerative medicine. Previously, genetic defects in mice ES cells have been successfully treated using homologous recombination gene therapy (17). Recently, generation of IPS cells from a rat sickle cell anemia model and subsequent homologous recombination gene therapy to correct the sickle cell mutation improved symptoms in the rats (17, 18, 19). This proof of principle experiment furthers the possibility of using IPS cell induction combined with gene therapy to treat the disease. However, generation of the IPS cells using potentially tumorigenic oncogenes delivered by retrovirus is undesirable (20). Recent advances have utilized a plasmid based delivery system that circumvents the integration of genes into the host genome (21). The ability to combine this plasmid based delivery with gene therapy to correct disease genetics and how plasmid delivery differs from retroviral delivery, however, is currently not known. Additionally, the feasibility of differentiating these corrected IPS cells into the desired tissue and using them for disease specific treatment has not been assessed. Therefore, we hypothesize that HD fibroblasts can be genetically repaired and differentiated into neurons for treatment in HD. Thus, we propose to induce the formation of IPS cells using fibroblasts from a mouse HD model and human HD patients followed by successive homologous recombination gene therapy to correct the HD gene in the newly formed IPS cell. Directed differentiation of the IPS cells into GABAergic releasing spiny projection neurons and insertion of corrected mice and human neurons into the mouse HD model will follow.

Relevance

The expansion of biological knowledge in the past century has been immense. Discoveries such as penicillin and vaccines have lead to a dramatic reduction in deaths caused by infectious disease. However, the genetic basis for inherited diseases and our inability to treat them effectively reflects a serious deficit in our treatment arsenal. Perfecting the methods we intend to utilize in our studies and successfully treating HD symptoms using IPS cells opens the door to application in a plethora of other genetic diseases, and in the process, benefit all of mankind. Development of an effective HD treatment could potentially save the lives of the 30,000 people currently suffering from the disease in the United States alone (4).

Our proposal has significant relevance to the scientific field as it will give insight into the genetic mechanisms of HD. Although the disease is well studied, the actual cause of the CAG repeats is not well understood. While investigating methods to correct the HD gene, discoveries that further our knowledge of the disease will inevitably be made. Our study will also contribute heavily to the molecular knowledge of IPS cell therapy and differentiation techniques. In addition, successful use of gene therapy will help the technique become more widely utilized in regenerative medicine.

Specific Aims

Our ultimate goal is to develop an effective treatment for Huntington's disease. Mouse fibroblasts from an HD mouse model, as well as human fibroblasts will be used to create IPS cells. Homologous recombinant gene therapy will be applied to correct the HD CAG repeats in the IPS cell genome. Based on previously established methods, the corrected IPS cells will be differentiated into medium spiny neurons and then implanted into the brains of HD mice. We hypothesize that implanting these neurons into the diseased mice will alleviate their symptoms.

1. Treating diseased Huntington mice models with corrected IPS cells from mice

Fibroblasts from adult HD mouse models will be reprogrammed into IPS cells using a retrovirus or a plasmid carrier. Homologous recombination will replace the diseased huntingtin gene in IPS cells with a healthy gene. Furthermore, we will directly differentiate recombinant IPS cells into the specific neurons affected in HD. These neurons will then be injected into the striatum of mouse brains, the location of neurodegeneration in HD. Finally, we will use immunostaining to assess pluripotency, PCR to evaluate successful gene replacement after homologous recombination, and GFP expression to confirm successful differentiation and implantation of neurons.

2. Treating diseased Huntington mice models with corrected IPS cells from humans

Fibroblasts from adult HD humans will be reprogrammed into IPS cells by use of a retrovirus or a plasmid carrier. Homologous recombination will replace the diseased huntingtin gene in IPS cells with a healthy gene. Furthermore, we will directly differentiate recombinant IPS cells into the specific neurons affected in HD. In order to avoid rejection, neurons will be injected into the striatum of immunosuppressant mouse brains. Finally, we will use immunostaining to assess pluripotency, PCR to evaluate successful gene replacement after homologous recombination, and GFP expression to confirm successful differentiation and implantation of neurons.

3. Treating diseased Huntington mice models with healthy IPS cells from humans

Healthy fibroblasts from adult humans will be reprogrammed into IPS cells by use of a retrovirus or a plasmid carrier. We will then directly differentiate IPS cells into the specific neurons affected in HD. In order to avoid rejection, neurons will be injected into the striatum of immunosuppressant mouse brains. Finally, we will use immunostaining to assess pluripotency and GFP expression to confirm successful differentiation and implantation of neurons.

Research Method and Design

1. Treating diseased Huntington mice models with corrected IPS cells from mice

1. Rationale: To evaluate the therapeutic potential of the proposed method, Huntington mice models will be treated with their own neurons containing a huntingtin gene repaired using homologous recombination. Retroviral reprogramming of fibroblasts is a proven method for reestablishing pluripotency in adult mice fibroblast cells (13). Due to possible tumorigenesis from retroviral

vector integration, insertion of the reprogramming factors will also be done using a plasmid (21). Homologous recombination can correct gene mutations in mice. Thus, we will use this method to replace the HD gene with a normal, low repeat gene.

2. Design and Method: Two sets of three groups of HD model mice will be used. The three groups will consist of mice with a healthy/control number of CAG repeats, a low number of CAG repeats (40-50, moderate disease), and a high number of CAG repeats (70, severe disease). One set of mice will be treated with their own genetically *corrected* and differentiated GABAergic neurons and the other will be treated with *uncorrected* and differentiated neurons. Furthermore, each group will be divided into two subcategories for a total of six mouse groups per set. The purpose of the division is to compare reprogramming technologies; one subcategory will be transduced with the retrovirus and the other with the plasmid carrier. The healthy mouse groups will serve as a control for the moderately diseased, low repeat models and the severely symptomatic, high repeat models.

a) Development of transgenic mice*:* Mice will be generated that are transgenic for the 5' end of the human HD gene carrying less than 40, 40 to 50, and more than 70 CAG repeat expansions as previously described (7). In this experiment it was shown that mice with a particular amount of CAG repeats will acquire an HD phenotype and show late onset neurodegeneration of the dorsal striatum.

b) Creating IPS cells: Fibroblasts will be extracted from control, high, and low repeat mice and disease genotype will be verified using DNA sequencing. Transgenes encoding *KLF4*, *SOX2*, *OCT4*, and *c-Myc* will be introduced into mice fibroblasts from all three groups by means of vesicular stomatitis glycoprotein (VSVg) pseudotyped Maloney retrovirus (9, 15). At this point, fibroblast will be tagged with green fluorescent protein (GFP) in order to provide a clear outline of cells once they have been implanted into a mouse model. This will allow us to determine their location and shape. Fibroblasts will be transduced twice over 72 hours, cultured for 4 days in standard fibroblast medium, and then passaged onto a feeder layer of mouse embryonic fibroblasts in an ES cell supportive medium. After two weeks, we expect to see a small number of colonies with morphology similar to ES cells. Several lines will be selected and clonally expanded. Mice fibroblasts from each group will also be reprogrammed by means of a plasmid-based delivery system as previously described (20). DNA fingerprinting analysis will be performed to verify that each of the IPS cell lines created matches that of the mouse from which they were derived, has the mutation in the huntingtin protein, and the correct number of CAG repeats (15). Furthermore, polymerase chain reaction (PCR) analysis of genomic DNA will be performed in order to verify that they all carry integrated copies of the four retroviral transgenes for which they were transduced (15, 22). Also, to verify that the reprogramming of the fibroblasts is successful and that the putative IPS cells are pluripotent, their similarity to embryonic stem cells will be determined. To accomplish this, the expression of several ES cell associated antigens (SSEA-3, SSEA-4, TRAI-60, TRAI-81, and NANOG) will be verified (15). We will also confirm that the IPS lines created are not immunoreactive for the fibroblast associated antigen TE-7. Quantitative reverse transcription PCR will be used to measure transcript levels of several genes expressed in pluripotent cells. We will look at the levels of *REX1/ZFP42, FOXD3, TERT, NANOG,* and *CRIPTO/TDGF1* to confirm that these are transcribed at levels comparable to ES cells in each of our created lines (22). Pluripotent cells are, by definition, able to readily form embryoid bodies composed of cells representative of the three embryonic germ layers. Immunostaining will be performed to verify this. Staining for desmin, α-fetoprotein, and β-tubulin IIIb will be performed to assess pluripotent ability (15, 22).

c) Gene correction: Custom plasmids with a healthy IT-15 gene encoding the huntingtin protein will be inserted into one set of diseased IPS cells by electroporation; the second set will be left untouched. Diseased genes in the presence of the plasmid will be corrected with the healthy gene via homologous recombination. Corrected cells will be transferred to hygomycin and gancyclovir containing media for 2 weeks. Custom primers and PCR will be used to identify homologous recombinants in IPS cells derived from diseased mice (9).

d) Motor Neuron differentiation: The hallmark of HD is depletion of GABA releasing spiny projection neurons in the striatum. Differentiation of such neurons relies on the sonic hedgehog (SHH) pathway and retinoic acid treatment. Corrected and uncorrected IPS cells will be differentiated into GABAergic neurons by manipulation of the SHH pathway and exposure to retinoic acid as previously described (15, 23). 100,000 to 300,000 GABAergic spiny-projection neurons from the IPS lines expressing GFP will be transplanted into the lateral brain ventricles of mice (9).

e) Behavioral tests: In order to assess recovery of Huntington's disease, two behavioral models will be used to compare mice treated with the corrected and uncorrected cells. The first test, the RotaRod test involves the placement of the mouse on a spinning rod which moves at a speed slightly faster than walking speed (24, 25). Performance will be scored by the amount of time the mouse is able to stay on the moving rod. This test is used in order to evaluate an animal's sense of balance and coordination, two abilities that are affected in Huntington's disease. The second test used will be the footprint test. HD patients frequently experience abnormalities in gait and impairments in sensorimotor gaiting. This test is frequently used to determine the extent of these impairments in a mouse model of HD. We will perform the footprint test in order to compare the gait of HD mice with non HD mice. The front and hind limbs of animals will be painted two different colors. Mice will walk down a 50 cm long and 10 cm wide runway. Stride length, hind-base width, front-base width, and front footprint/hind footprint overlap. (26). Diseased mice are expected to spend less time on the RotaRod and are expected to show more irregularities in gait.

f) Neuropathology of disease: After performance in the behavioral tests, all mice will be sacrificed.

Brains will be removed and cut into coronal sections. First, we will seek evidence of integration of transplanted IPS cells. Before the development of IPS cell colonies, reprogrammed fibroblasts will have been tagged with GFP. Immunofluorescent staining will allow us to verify that grafted cells moved to the striatum, and will provide a clear outline of incorporated cells, with definition of their shapes and neuronal processes (27). Electrophysiological recordings will also be performed to examine the functionality of grafted cells. Synaptic inputs and outputs will be determined for several cells from each animal and compared to normal ranges (27).

To determine the recovery of HD mice, several neuropathological stains will be performed on the tissue. Density of GABAergic neurons in the striatum and DARPP-32 expressing striatal projection neurons will be determined. We will also assess cell death in the striatum and perform a TUNEL stain, a marker for apoptotic cells, to determine levels of cell death (28). NIIs are shown to be present in the striatum of transgenic mice. Ab 1 is an antiserum against huntingtin and produces intense labeling for huntingtin localized to neuronal intranuclear inclusions. Therefore, it can be used to indentify NIIs (29). Levels of NIIs will be compared in mice from different groups. Size of the striatum as well as other central nervous system structures has previously been shown to be smaller in transgenic animals than in wild-type mice (7). We will compare the size of coronal sections of the normal, low, and high CAG repeat mice to one another to confirm these changes in size. Ultimately, performance of the behavior test will be correlated with data from neuropathology in order to determine the effects of IPS cell implantation. We expect to see an increase in GABAergic spiny-projection neurons in the striatum as well as other regions, possibly correlating with a decrease in symptoms (30, 31).

Prediction: We predict that the mildly diseased mice treated with fixed neurons will show the biggest improvement in symptoms. Severely diseased mice will also show improvements, albeit not to such a large extent as the mildly diseased. The mice models implanted with unfixed neurons will not show any improvement.

2. Treating diseased Huntington mice models with corrected IPS cells from <u>humans</u>

1) Rationale: Huntington mice models will be treated with human HD neurons containing a huntingtin gene repaired using homologous recombination. To avoid rejection, immunosuppressant mice will be used. Retroviral reprogramming is a proven method for establishing pluripotent IPS cells from adult human fibroblast cells (13, 20, 21). Due to possible tumorigenesis from retroviral vector reactivation, the factors will also be inserted using a plasmid carrier (21).
2) Design and Method: Two sets of three groups of immunosuppressant mice will be used to model a non-diseased HD patient, a low CAG repeat patient and a high CAG repeat patient. One set of mice will be treated with genetically *corrected* and differentiated human GABAergic neurons and the other will be treated with *uncorrected* and differentiated neurons. The remainder of the design will correspond to that of aim 1.
   a) Development of transgenic mice: Mice will be developed that are transgenic for the 5' end of the human HD gene with less than 40, 40-50, or more than 70 CAG repeats as described in aim 1.
   b) Creating IPS cells: Activation of c-*Myc*, KLF4, OCT-4 and Sox2 transcription factors in adult human fibroblasts with retrovirus or plasmid carriers will follow the protocol previously described (15, 20). IPS cell pluripotency will be verified as stated in aim 1.
   c) Gene correction: Control, low and high repeat HD genes in human IPS cells will be replaced with healthy HD genes. Plasmids containing the healthy IT-15 gene will be inserted into one set of diseased IPS cells for correction and the second set will be left uncorrected. Custom primers and PCR will be used to identify homologous recombinants in IPS cells derived from diseased humans (9).
   d) Motor Neuron differentiation: Corrected and uncorrected IPS will be differentiated into GABAergic neurons and inserted into the mouse brains as explained in aim 1.
   e) Behavioral tests: Two behavioral tests called the RotaRod and the footprint test will be used to assess progression of Huntington's disease in the mice. The tests will be performed as explained in aim 1.
   f) Neuropathology of disease: After performance of the behavioral tests, mice will be sacrificed. Brains will be removed and cut into coronal sections. Immunofluorescent labeling for GFP and electrophysiological recordings will be performed as explained in aim 1 to verify successful integration and function of engrafted cells. To assess recovery from disease, density of GABAergic and DARPP-32-ir neurons will be quantified. Size of striatum and levels of NIIs and cell death will be determined as explained in aim 1.

Prediction: We predict that the mildly diseased mice treated with fixed human neurons will show the biggest improvement in symptoms. Severely diseased mice will also show improvements, albeit not to such a large extent as the mildly diseased. The mice models implanted with unfixed human neurons will not show any improvement.

3. Treating diseased Huntington mice models with <u>healthy</u> IPS cells from humans

1) Rationale: Aims one and two rely on homologous recombination and directed differentiation to create neurons capable of treating HD mouse models. To evaluate whether our method works with non-diseased cells, we will inject an HD mouse model with neurons transformed from humans *without* HD. Comparing both procedures will give an indication of how reliable homologous recombination is for correcting IPS cells and treating HD.
2) Design and Method*:* Two sets of three groups of immunosuppressant mice will be used to model a non-diseased HD patient, a low CAG repeat patient and a high CAG repeat patient. Mice will be treated with differentiated human GABAergic neurons derived from *healthy human* fibroblasts. The remainder of the design will correspond to that of aim 1.
   a) Creating IPS cells: Activation of c-*Myc*, KLF4, OCT-4 and Sox2 transcription factors in adult human fibroblasts with retrovirus or plasmid

carriers will follow the protocol previously described (15, 20). IPS cell pluripotency will be verified as stated in aim 1.

b) Motor Neuron differentiation: Corrected and uncorrected IPS will be differentiated into GABAergic neurons and inserted into the mouse brains as explained in aim 1.

c) Behavioral tests: Two behavioral tests called the RotaRod and the footprint test will be used to assess progression of Huntington's disease in the mice. The tests will be performed as explained in aim 1.

d) Neuropathology of disease: After performance in the behavioral tests, mice will be sacrificed. Brains will be removed and cut into coronal sections. Immunofluorescent labeling for GFP and electrophysiological recordings will be performed as explained in aim 1 to verify successful integration and function of engrafted cells. To assess recovery from disease density of GABAergic and DARPP-32-ir neurons will be quantified. Size of striatum and levels of NIIs and cell death will be determined as explained in aim 1.

Prediction: The mildly diseased mice models will show the largest improvement in HD related symptoms upon transplantation of differentiated GABAergic neurons. The severely diseased HD models will also show improvement but to a lesser extent.

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