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Examination of mesenchymal stem cell-mediated RNAi transfer to Huntington's disease affected neuronal cells for reduction of huntingtin

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Abstract

Huntington's disease (HD) is a fatal, autosomal dominant neurodegenerative disorder caused by an expanded trinucleotide (CAG) repeat in exon 1 of the huntingtin gene (Htt). This expansion creates a toxic polyglutamine tract in the huntingtin protein (HTT). Currently, there is no treatment for either the progression or prevention of the disease. RNA interference (RNAi) technology has shown promise in transgenic mouse models of HD by reducing expression of mutant HTT and slowing disease progression. The advancement of RNAi therapies to human clinical trials is hampered by problems delivering RNAi to affected neurons in a robust and sustainable manner. Mesenchymal stem cells (MSC) have demonstrated a strong safety profile in both completed and numerous ongoing clinical trials. MSC exhibit a number of innate therapeutic effects, such as immune system modulation, homing to injury, and cytokine release into damaged microenvironments. The ability of MSC to transfer larger molecules and even organelles suggested their potential usefulness as delivery vehicles for therapeutic RNA inhibition. In a series of model systems we have found evidence that MSC can transfer RNAi targeting both reporter genes and mutant huntingtin in neural cell lines. MSC expressing shRNA antisense to GFP were found to decrease expression of GFP in SH-SY5Y cells after co-culture when assayed by flow cytometry. Additionally MSC expressing shRNA antisense to HTT were able to decrease levels of

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mutant HTT expressed in both U87 and SH-SY5Y target cells when assayed by Western blot and densitometry. These results are encouraging for expanding the therapeutic abilities of both RNAi and MSC for future treatments of Huntington's disease.

Keywords

Mesenchymal stem cell; Huntington's disease; RNAi; Cellular therapy

Introduction

Huntington's disease (HD) is a dominant neurodegenerative disease caused by polyglutamine repeat expansions in exon 1 of the huntingtin protein (HTT), which leads to a toxic gain of function (Huntington's Disease Collaborative Research Group, 1993). HD characteristics include neuronal inclusions, striatal and cortical neurodegeneration, chorea, and cognitive and behavioral changes (Ross and Tabrizi, 2011; Shannon, 2011). The expansion occurs as a mutation of a naturally occurring trinucleotide (CAG) repeat in exon 1 of the huntingtin gene, also known as IT15, normally encoding a 350-kDa protein (Huntington's Disease Collaborative Research Group, 1993). Htt alleles containing more than 35 CAG repeats generally cause HD. The disease usually develops in midlife, but juvenile-onset cases can occur with CAG repeat length over 60 (Langbehn et al., 2010). Current treatments for HD are limited to managing behavior and chorea. There are no existing therapies that prevent the death of striatal neurons or improve the long-term clinical outcome of affected patients.

RNA interference (RNAi) technology employs the use of short pieces of RNA that are complementary anti-sense to specific regions of mRNA. The RNAi, when introduced intracellularly, complex with the RNA Induced Silencing Complex (RISC) and subsequently bind to the targeted mRNA. The double stranded RNA is then destroyed, effectively silencing the expression of the gene. RNAi targeting mutant HTT has proven effective in transgenic mouse models of HD, where the RNAi expression can easily be turned on in selected tissues (Boudreau et al., 2009; DiFiglia et al., 2007; Harper et al., 2005; Maxwell, 2009). Viral vector delivery of short hairpin RNAs (shRNA) has also been performed in mouse models, and has shown reduced neuropathy and motor deficits (DiFiglia et al., 2007). However, RNAi therapy is difficult to translate into human clinical trials due to poor uptake and transient effects of RNAi when delivered systemically (Dykxhoorn et al., 2006). The use of RNAi presents a difficulty in that extracellular RNA has a very short half-life and is rapidly cleared and degraded in the body. Additionally, as a charged molecule it has difficulty crossing both the blood brain barrier and cell membranes (Boudreau et al., 2011; Davidson and McCray, 2011).

Previously only described in plants, (Kehr and Buhtz, 2008; Waigmann and Zambryski, 1994) recent studies have found that nucleic acid structures and RNA species can be passed from a mammalian donor cells to target cells through gap junctions, exosomes, virtosomes, or tunneling nanotubules (Gahan and Stroun, 2010; Gerdes and Carvalho, 2008; Simons and Raposo, 2009). Feeder cells of mesodermal origin engineered to express an siRNA against GFP were found to affect GFP expression in the ES cells with which they were co-cultured (Wolvetang et al., 2007). Additionally, RNAi molecules were found to be secreted in microvesicles, which later fused with other cells (Bruno et al., 2009; Quesenberry and Aliotta, 2008; Skinner et al., 2009; Taylor and Gercel-Taylor, 2011). Ratajczak et al. described horizontal transfer of mRNA from cell to cell through embryonic stem cell-derived microvesicles (Ratajczak et al., 2006). Virtosomes were recently described to be complexes of RNA-lipoprotein which can readily enter other cells where they can modify

the biology of the recipient cells (Gahan and Stroun, 2010). Tunneling nanotubes have also been described for transferring RNA species and organelles between cells (Gerdes and Carvalho, 2008). Therefore, we chose to explore a cell-based platform for producing and delivering RNA interference moieties targeted to the Huntingtin mRNA as a potential therapeutic avenue for treating HD.

We chose human MSC to produce siRNA, as we and others have previously shown them to be excellent *in vivo* delivery vehicles for enzymes and proteins (Meyerrose et al., 2008, 2010). MSC can be easily isolated from the bone marrow or adipose tissue and subsequently expanded in culture (Meyerrose et al., 2006, 2010). MSC are immunoprivileged in that they normally elicit no immune response when used allogeneically. They are anti-inflammatory and secrete a broad range of trophic factors, including BDNF, NGF, IGF-1, and others (reviewed in Joyce et al., 2010). For these reasons, MSC show therapeutic potential for the treatment of many neurodegenerative diseases (Joyce et al., 2010). MSC have been shown to be capable of transferring mitochondria to damaged cells. Spees and Olson et al demonstrated that the active transfer of mitochondria from MSCs could rescue aerobic respiration in mammalian cells with nonfunctional mitochondria (Spees et al., 2006). The ability to easily and safely genetically engineer adult MSC (Bauer et al., 2008; Joyce et al., 2010; Meyerrose et al., 2010), as well as the ability of these cells to directly transfer structures as large as mitochondria to target cells (Spees et al., 2006), to release microvesicles (Bruno et al., 2009; Simons and Raposo, 2009) and virtosomes (Gahan and Stroun, 2010), and to form gap junctions with target cells *in vitro* and *in vivo* (Matuskova et al., 2010), made MSC an ideal cellular delivery system for further examination.

The traditional gene therapy approach, where the neurons would be directly infected *in vivo* by live lentiviral or AAV vectors carrying the shRNA gene of interest suffer from a number of safety concerns. Integrating virus can pool at the injection site, superinfecting neighboring cells and limiting distribution beyond a small area. Viral integrations can be controlled *ex vivo* with MSC, and verified by LAM PCR that there is an average of one to two viral integrants per MSC genome, as suggested by the FDA for stem cell gene therapy trials. Using MSCs as the delivery vehicle, a “suicide gene” such as thymidine kinase can be used to eliminate a graft if anything went wrong. This would not be possible with vector-mediated delivery since it would destroy the neuron into which the gene had integrated. We can also use the natural reparative characteristics of MSCs synergistically, and could potentially use their capacity to migrate to injured cells to potentially better deliver siRNA.

In this study, we utilized viral vectors to create a model system to detect the transfer of RNAi molecules between MSC and a targeted cell (Fig. 1). MSC were transduced to express shRNA antisense to either GFP or HTT mRNA and a scrambled shHTT shRNA (shSCRAM), as well as a red fluorescent transduction reporter. The MSC were then co-cultured with neural target cells expressing GFP or mutant HTT with a GFP transduction reporter. Measurable decreases in GFP expression, measured by flow cytometry, and HTT, measured by densitometry were detected in some of the co-cultures assayed. These findings support the future possibility of MSC-mediated delivery of therapeutic RNAi to treat HD.

Results

Characterization of MSC engineered to express shRNA

MSC appeared to be unburdened by either the lentiviral transduction or the expression of any of the shRNAs. As a precaution, we conducted a series of assays to ensure that transduction had not fundamentally altered the cells by comparing shRNA-transduced MSC from different donors to wild type MSC by growth, capacity to differentiate, and by karyotype analysis.

MSC from donor 2628 were transduced with shHTT and shSCRAM vectors at an MOI of 80 at efficiencies of 33.09% and 27.32%, relatively, as measured by dsRed expression, and plated at initial densities of 1000 cells/cm² for growth analysis. Viable cells were then counted using an MTT assay on sample cultures over time (Fig. 2A). All cultures demonstrated logarithmic growth without any significant differences between shRNA expressing cells and their wild type counterparts.

The capability of transduced MSC to undergo osteogenic and adipogenic differentiation was also confirmed using MSC from donor 2627 transduced with shHTT, shGFP, and shSCRAM at an MOI of 100. Differentiation was performed for a total of 17 days. Osteogenic medium induced the formation of calcified extracellular matrix, which stains by Alizarin red, indicating successful bone deposition (Fig. 2C, bottom center). Adipogenic medium induced the accumulation of large lipid droplets in cells that retained Oil Red O dye, a hallmark of adipocytes. Lentiviral integration and prolonged time in culture could potentially introduce the possibility of genomic instability. Therefore, karyotypic analysis was performed on MSC after transduction with shHTT at an MOI of 80 with three different donors (Fig. 2B) and results were compared to both non-transduced MSC controls and normal karyotypes. All karyotypes were analyzed by a cytogeneticist and no abnormalities were detected on any of the slides.

These results indicate that lentiviral-transduced and culture-expanded MSC engineered to express shHTT retained the hallmarks of mesenchymal stem cells, being capable of extended self-renewal and differentiation into multiple cell lineages. Additionally, the genetic manipulations and expansion did not cause any changes in growth rate, chromosomal instabilities or anomalies.

Target protein expression decreases following direct transduction with shRNA

The effect of the shRNA lentiviral vectors on the expression of HTT and 4dGFP was analyzed using flow cytometry and densitometry. U87 cells were transduced with a lentiviral vector constitutively expressing mutant HTT and GFP (U87HTT142gfp). Cultures were then transduced with shGFP or shHTT and protein expression was monitored over time.

Following transduction of U87HTT142gfp cells with shGFP, expression of GFP was demonstrated to decrease over 6 days when measured by flow cytometry (Fig. 3A). As GFP expression decreases in the FL1 channel, dsRed expression increases in the FL2 channel (Fig. 3B). The shGFP vector effectively silenced GFP expression in 78.5% of the population, from 91.4% GFP-positive to 12.5% GFP-positive cells on day 6 of the culture (Fig. 3C) measured by gating on GFP-negative populations. In contrast, transduction of U87HTT142gfp had a negligible effect on the number of GFP-positive cells.

To ensure expression of HTT142, cell lysates from U87 and U87HTT142gfp were collected and subsequently analyzed by Western blot. Fig. 4A shows a 92 kDa protein band recognized by an anti-HTT antibody present in the U87HTT142gfp cells and not in control U87 cells. The change in this 92 kDa band was then measured using densitometry. After transduction with shHTT, mutant HTT expression quickly decreased, as evidenced by the decreasing intensity of the 92 kDa HTT142 fragment. Levels of HTT142 fell to 80% of its starting level by day 9, normalized to actin (Fig. 4B). In contrast, the control shGFP vector had no effect on HTT142 throughout the time course.

Target protein expression is reduced following co-culture with MSC engineered to produce RNAi

In order to detect the transfer of RNAi between MSC and recipient cells, a series of co-cultures were performed. Initial experiments focused on reducing the expression of EGFP as

a reporter (not shown). We hypothesized that the long half-life of EGFP (Indraccolo et al., 2002) was preventing detection of any reduction in fluorescence over the time-course of the co-cultures. We began using a destabilized GFP variant with a half-life of only 4 h (4dGFP) to ensure that any reduction in mRNA would become rapidly apparent as a reduction in fluorescence. MSC expressing shRNAs, targeted to 4dGFP and HTT as a control, and a red fluorescent protein reporter (dsRed-mito) were co-cultured with SH-SY5Y expressing 4dGFP and fluorescence was assayed by flow cytometry after 4 days and 10 days in co-culture (Fig. 5). There are 4 different cell populations easily discernable by different fluorescence profiles in the dot plots (Supplementary Fig. S1): non-transduced MSCs and SH-SY5Ys (bottom left), dsRed-positive GFP-negative shRNA expressing MSC (top left), dsRed-negative SH-SY5Y expressing moderate levels of 4dGFP (bottom middle), and dsRed-negative SH-SY5Y expressing high levels of 4dGFP (bottom right). After 4 days, the two co-cultures were indistinguishable by fluorescence, with 4dGFP expression in the SH-SY5Ys being almost identical when the MSCs were gated out regardless of whether the MSC expressed an shRNA targeting 4dGFP or HTT (Fig. 5B left histogram). In contrast, when the co-cultures were analyzed after 10 days, the green fluorescence profile of the SH-SY5Ys had decreased significantly in the shGFP MSC co-culture as compared to shHTT MSC (Fig. 5B right histogram). We next expanded our system and altered techniques to better optimize the co-culture system.

The fluorescence based reporter systems had flaws that were demanding increasingly complicated systems to overcome. In order to focus our efforts on HD, we instead measured shRNA-mediated decreases of lentivirally overexpressed mutant HTT levels directly by Western blot and densitometry. U87HTT142gfp were co-cultured six times with MSC expressing either shGFP (as a control) or shHTT. We found mutant HTT142 expression decreased through day 9 in the target cells (Fig. 6). The U87HTT142gfp cells were pretreated with mitomycin C to prevent excessive proliferation. This decrease was present to some extent in both shHTT MSC and shGFP MSC co-cultures, however, the target protein HTT142 decreased more when co-cultured with the shHTT –expressing MSC.

Next, SH-SY5YHTT142gfp cells were co-cultured a total of five times with MSC expressing either shHTT or shSCRAM as a control in a variety of culture conditions. Two of five co-cultures showed that target protein expression decreased at day 5 after co-culture with MSC expressing shHTT, quantified by densitometric analysis of Western blots (Fig. 7). These co-cultures were performed in reduced serum (5%) and serum free conditions to better limit MSC and SH-SY5Y proliferation. Mutant HTT142 fragment levels were normalized to GFP expression in order to remove any protein contribution by MSC and to control for any variance in integrated vector expression due to silencing. The decrease in target protein at day 5 did not occur in co-cultures with MSC expressing shSCRAM. Mutant HTT142 levels began to recover by day 7. This late culture recovery could be due to culture conditions deteriorating late in the time-course, or could be due to an initial transfer of RNAi that was not sustained in the co-culture as cells were depleted of nutrients or became contact inhibited. A better understanding of the molecular mechanisms allowing this transfer will be needed to make the system more highly reproducible. Future studies will examine the efficacy of the RNAi transfer by MSCs in the rodent brain rather than in the confines of cell culture. Our data demonstrate that specific protein reduction in a targeted cell population can be achieved through intercellular transfer of RNAi from MSC to neuronal cell.

Discussion

Here we present a series of experiments designed to detect transfer of RNAi molecules from MSC to neighboring cells of neuronal or glial lineage. RNAi therapies are difficult to

translate, mainly due to the difficulties in delivering RNAi molecules to the affected cells, and their transient effects. MSC are currently being considered for the treatment of neurodegenerative diseases due to their innate homing abilities, established safety profile, anti-inflammatory effects, and the broad array of cytokines that they secrete into the microenvironment (Joyce et al., 2010). For these reasons, we believe that MSC could make an excellent delivery vehicle for RNAi molecules to treat disorders such as Huntington's disease, combining their innate reparative abilities with the disease silencing power of targeted siRNA.

Several groups have reported the use of RNAi targeted to single nucleotide polymorphisms (SNPs) to specifically silence a single HTT allele, used to knockdown HD HTT and spare normal HTT genes (Boudreau et al., 2009; Davidson and Paulson, 2004; Lombardi et al., 2009; Pfister et al., 2009; Rodriguez-Lebron et al., 2009; van Bilsen et al., 2008; Zhang et al., 2009). Additionally, other researchers have shown that while HTT knockouts are embryonic lethal in mice, conditional knockouts in adult mice have little effect (Boudreau et al., 2009; DiFiglia et al., 2007; Drouet et al., 2009; Harper et al., 2005). For this report, we chose to use a shRNA sequence that would knock down all HTT as an initial proof of concept study. The shRNA sequence could be easily switched to a sequence specific to a common familial HTT allele.

Lentiviral vectors were constructed to create both shRNA donor and target cells in a co-culture system. shRNA vector expression did not result in any significant effect on MSC viability, growth or differentiation potential, as assayed by growth curve analysis and differentiation into adipogenic and osteogenic lineages. Genetically transduced and expanded MSC retained a normal karyotype without any detected chromosomal aberrations. As a potential human therapy it was imperative for the shRNA to be well tolerated by MSC, and we demonstrated that the MSC were essentially unaffected by the RNAi production.

The direct activity of the shRNA vectors was established by transducing U87 cells previously transduced with a vector that carried both the mutant HTT fragment and EGFP (U87HTTgfp). shGFP significantly reduced the expression of GFP as measured by flow cytometry. Likewise, shHTT was effective in reducing mutant HTT levels as assayed using densitometry after gel electrophoresis and Western blot. No changes in protein levels were detected with the scrambled control (shSCRAM), demonstrating that the reduction in protein was due to specific shRNA activity and not a byproduct of either lentiviral integration or shRNA expression.

Specific protein reduction in a targeted cell population was detected in co-culture systems. MSC expressing either a shRNA targeting GFP or HTT as a control were co-cultured with SH-SY5Y cells expressing a short-lived GFP variant and GFP expression per cell was quantified using flow cytometry on day 4 and day 10. The shGFP and shHTT cultures went from indiscernible at day 4 to very different on day 10, with GFP expression sharply reduced in the shGFP culture. A separate co-culture featured MSC expressing either shGFP as a control or shHTT mixed with U87 cells expressing a mutant HTT fragment and an eGFP marker that had been pretreated with mitomycin C to decrease proliferation. HTT levels were assayed over time by Western blots quantified using densitometry, revealing a decrease in HTT in both cultures when normalized to actin, but a larger decrease from the shHTT containing co-culture. We found that normalizing to actin, a common control protein, is insufficient for our conditions due to the presence of otherwise undetected MSC in the culture. To address this, we constructed new co-cultures using shSCRAM as a control instead of shGFP in order to normalize HTT expression to the GFP used as a transduction marker and co-expressed with HTT. In co-cultures conducted simultaneously in either

reduced serum or serum-free conditions, we found a reduction of mutant HTT through day 5 of cultures, as compared to GFP expression.

The sporadic successes of our co-cultures are due, in part to the technical challenges of keeping two very different cell populations alive and healthy for prolonged periods of time while maintaining a cell density high enough to encourage substantial exchange of RNAi by either secreted microvesicles or direct cell to cell transfer. Approximately 5 days after being seeded at the moderate density of 1000 cells/cm² most cultures were completely confluent. As the cultures overgrew, the medium would become toxic to one or both cell populations. The more tolerant cell population would then dominate the culture and cause spurious measurements late in the time-courses. In order to address these obstacles, we examined a number of culture conditions and treatments; changing cell types, assays, sera concentration, and using both mitomycin C and gamma-irradiation to induce growth arrest. While U87 and MSC co-cultures exhausted themselves quickly, MSC and SH-SY5Y often persisted a few additional days before becoming errant. Mitomycin C treatment of MSC was largely ineffective at preventing overgrowth, but did manage to reduce U87 growth in the cultures. We conducted kill-curves to optimize gamma radiation doses for the individual cell types to cause growth arrest without apoptosis/necrosis, but found that irradiation did not produce reliable cultures or positive outcomes. These data might indicate that the mechanisms that MSC can use to transfer macromolecules to neighboring cells are adversely affected by radiation.

A number of different basal media (both catering to MSC and U87/ SH-SY5Y) were used with varying amounts of serum to abrogate overgrowth of the cultures. We found that as little as 1% FBS induced sufficient growth to overwhelm the culture, probably due to the large number of autocrine and paracrine growth factors produced by MSC. The varied conditions with which we experienced successful outcomes speak to the transient and delicate nature of the transaction. A large number of culture variables were created in an effort to control cell growth with irradiation and chemical treatments. These treatments could have inhibited RNAi transfer either directly, through their effect on the cells or, indirectly through something as small as pH changes in the medium. A better understanding of the nature of intercellular RNAi transfer from MSC to target cell at the molecular level will allow our group and others to design better platforms for RNAi delivery in the future. Knowledge of the molecular processes involved in the RNAi transfer, whether by gap junction, nanotubes, exosomes, virtosomes, or some other mechanism, could allow screening of MSC batches to find those most likely to be robust RNAi donors. Additionally, we are currently optimizing mouse models in which we can assay RNAi transfer from human MSC *in vivo*. In an animal model there is no need for extensive cell manipulations to arrest cell overgrowth, and there is greater cell-cell contact as MSC are surrounded by tissue, and the physiological conditions may encourage more native interactions.

We believe that the data presented here is compelling evidence that RNAi molecules can be transferred from one cell population to another. The results presented in this report demonstrate that specific protein reduction in a recipient cell population can be achieved through intercellular transfer of RNAi from MSC to target cell. These findings are novel, are encouraging and warrant further study to describe and optimize mechanisms of RNAi exchange. The ability to use MSC as a vector for intercellular delivery of RNAi molecules holds promise for a wide variety of cellular therapies. A number of genetic diseases that are currently incurable and often untreatable could benefit greatly should MSC, or another cell type, be able to deliver efficacious amounts of RNAi directly to affected cells over a prolonged period of time. The potential benefits of cellular therapies and RNAi therapies combined may be sufficient to delay or even halt disease progression, which could

dramatically improve quality of life for patients suffering from degenerative diseases like HD.

Experimental methods

Lentiviral vector design and production

Several viral vectors were designed for these experiments (Fig. 1B). For the preparation of the shRNA containing vectors, a pSuper (Oligoengine Cat #VEC-pBS-0002) was used as a template, which contained the H1 promoter. The following primers were designed to PCR the H1 driven shHTT, shGFP and shSCRAM: H1p forward primer 5' CTGCAGGAATTCGAACGCTGACGT 3', shHTT reverse primer 5' AAAAAACCTGGAAAAG CTGATGAATCTCTTGAATTCATCAGCTTTTC-CAGGGGGGGATCTGTGGTCTCATAACAG 3', shSCRAM reverse primer 5' AAAAAACCGTGGACAAGCTAAGGAATCTCTTGAATTCCTTAGCTTGTCCACG GGGGGGATCTGT GGTCTCATAACAG 3'. The PCR products were then cloned into pCR2.1-TOPO (Invitrogen Cat #K4500). H1p-shHTT was excised with BamH1-Xho-1 and cloned into the BamH1-Xho1 sites of pCCLc-X. H1p-shSCRAM was excised with EcoRV-BamH1 and cloned into the EcoRV-BamH1 sites of pCCLc-X. A Cla1 blunted WPRE fragment was subsequently cloned into the blunted Kpn1 site of these vectors. These vectors were later excised with Xho-1 and an Xho-1 dsRed-mito fragment (Clontech) which encodes a fusion of a red fluorescent protein extracted from *Discosoma* sp. coral with a mitochondrial localization signal from human cytochrome c oxidase, was inserted.

The HD HTT142 fragment used was a 1.9 kb Sac1-EcoR1 fragment, containing approximately 1 kb of 5' UTR sequences, exon 1 with approximately 100 CAG repeats and the first 262 bp of intron 1. It was excised from pSKE4.0 (kindly provided by Gillian Bates, UMDS, UK) blunted and cloned into Sma1 site of the pCCLc-MNDU3-X-WPRE lentiviral vector. A Sma1-Nru1 PGK-EGFP cassette was then cloned into a blunted Sal1 site downstream of the HD fragment for constitutive GFP expression.

A destabilized EGFP vector (4dGFP) was constructed by inserting an EcoR1/blunted Not-1 d4EGFP fragment excised from pd4EGFP-N1 (Clontech Cat #6072-1) into the EcoR1/Sma-1 sites of pCCLc-MNDU3-X-WPRE lentiviral vector to provide a rapid response reporter for RNAi-mediated silencing.

To produce live, replication-incompetent lentivirus, Lenti-X 293 T cells (Clontech) were transfected with 25 µg of the lentiviral transfer vectors described above, 25 µg of pCMV-8.9 (packaging plasmid expressing gag and pol) and 5 µg of pMDG-VSVG (vesicular stomatitis virus glycoprotein-pseudotyping envelope) using TransIT Reagent (Mirus), following manufacturer's instructions. Two days post-transfection vector supernatants were collected and concentrated either by ultracentrifugation (22,000 rpm for 1.5 h) for the dsRedMito containing vectors or by ultrafiltration using Centricon Plus 70 filter units (Millipore). The vectors were aliquoted and stored at -80 °C. The titer was calculated by performing serial dilutions of virus on U87 cells and assaying for fluorescent reporter expression after 3 days.

Lentiviral transductions

For each cell type used, transduction efficiency as a function of MOI was measured using a GFP expressing vector in order to adjust for differences in susceptibility to lentivirus between the cell types. Transductions were performed by plating a known number of cells at 5000 cells/cm². The following day, titrated lentivirus was added to culture medium to create the desired MOI in the presence of 10 µg/ml protamine sulfate (APP Pharmaceuticals), used to increase non-specific viral uptake by cells (Cornetta and Anderson, 1989). The next day

the medium was replaced and cells were subsequently subcultured as described below or directly used for assay. Transduction efficiency was determined by flow cytometry for vectors containing fluorescent markers during subculture.

Culture of U87 and SH-SY5Y cell lines

Target neural cell lines, U87, a human derived glioblastoma cell line (Ponten and Macintyre, 1968) and SH-SY5Y, a human derived neuroblastoma cell line (Biedler et al., 1978), were purchased commercially (ATCC) and cultured according to ATCC's guidelines. In brief, cells were cultured in DME/F12 1:1 (HyClone) supplemented with 10% Fetal Bovine Serum (FBS) (Hyclone, Atlanta Biologicals), 1% penicillin/streptomycin (HyClone), and 1% L-glutamine (HyClone). All cells were grown at 37 °C and 5% CO₂. Cultures were lifted by trypsinization and subcultured when they reached approximately 80% confluence.

Isolation and culture of bone marrow mesenchymal stem cells (MSC)

Whole bone marrow was purchased commercially (All Cells, Emeryville, CA) from four different donors. To isolate human MSCs, nucleated cells were recovered from the 10 ml bone marrow aspirates with a density gradient by Ficoll-Paque (GE Healthcare) and resuspended in complete culture medium (CCM). CCM is composed of MEM modification media supplemented with 20% Premium Select Fetal Bovine Serum (Atlanta Biologicals) which was lot selected to achieve maximum growth, with additives of 1% Penicillin/ streptomycin, and 1% L-Glutamine. After 24 h, non-adherent cells were discarded, and adherent cells were washed twice with PBS, then incubated in fresh CCM (Meyerrose et al., 2006; Sekiya et al., 2002). Cells were grown in 37 °C and 5% CO₂. When MSC reached approximately 60% confluence, cultures were lifted with trypsinization and reseeded at 1000 cells/cm². Medium was changed every third day.

Growth analysis by MTT assay

MSC were plated at 1000 cells/cm² in 24-well plates and cultured normally. After 1, 3, 5, and 7 days in culture, MTT dye (Promega) was added directly to culture medium. The plate was returned to the incubator for 2 h before halting the reaction with stop solution. Samples were stored at 4 °C until completion of the time course, at which time the plates were returned to 37 °C for 2 h until the dye crystals had fully dissolved. Then, 100 µl from each well was loaded into a 96-well plate, which was then analyzed for absorbance at 570 nm with a reference at 650 nm on a microplate spectrometer (Emax, Molecular Devices). The number of cells was calculated from a standard curve generated from known numbers of cells treated identically to the above using a linear regression.

Osteogenic and adipogenic differentiation

MSC were differentiated into an osteogenic lineage by culture for 17 days at 70–80% confluence in MEM containing 10% FBS, 1× L-glutamine, 0.2 mM ascorbic acid, 0.1 µM dexamethasone, and 10 mM β-glycerophosphate with medium changes every 3 days. For adipogenic differentiation, MSC were cultured at 70%–80% confluence for 17 days in MEM containing 10% FBS, 1× L-glutamine, 0.5 mM isobutylmethoxyxanthine, 50 µM indomethacin, and 0.5 µM dexamethasone with medium changes every 3 days (Ogawa et al., 2004).

Karyotypic analysis of cultured MSC

To determine the chromosomal and genetic stability of the isolated BM-MSC, karyotyping was performed. The MSC were treated with ethidium bromide (BioRad) for 30 min at 37 °C, then colcemid (Gibco) for 30 min at 37 °C to arrest the cells in metaphase followed by treatment with 0.25% Trypsin (Cellgro) for 10 min at 37 °C. The cell suspension was then

treated with potassium chloride hypotonic solution and subsequent 3:1 methanol:acetic acid fixative solutions. Karyotyping slides were made and Geimsa banded. Karyotyping was performed on an Olympus CX41 microscope (Olympus) equipped with a 100× 1.4NA UPlanSApo objective (Olympus) and images were captured with a DP25 5MP camera (Olympus). Analysis was performed by a cytogeneticist.

Co-cultures

Where specified, U87 when cultured to 80% confluence were treated with 10 µg/mL Mitomycin C (Cat #475820, Calbiochem) for 3 h at 37 °C in 5% CO₂. Cells were then washed with PBS and subsequently trypsinized in preparation for co-culture with MSC. MSC and U87 or SH-SY5Y cells were mixed and seeded in culture flasks in equal numbers unless otherwise specified. Cultures were maintained in MEM supplemented with 0% or 5% FBS with 1% penicillin/streptomycin, and 1% L-glutamine or CCM. All cells were grown at 37 °C in 5% CO₂. Co-cultures were collected at days 0, 3, 5, and 7 for Western blot analysis by trypsinization. Cell cultures were collected at days 4 and 10 for assay by flow cytometry. Co-cultures were imaged using an inverted fluorescence equipped microscope (Eclipse Ti-U, Nikon) with 4× and 10× objectives in the phase, FITC and TRITC channels.

Western blot analysis

Total protein was isolated using RIPA buffer (Thermo Scientific) and quantified by Coomassie Plus Protein Assay Reagent (Pierce) from cultures containing at least 500,000 cells. To detect the fragment of mutant HTT protein, 40 µg total protein was run on 4%–20% pre-cast gradient gel (BioRad) at 80 V for 20 min and then 150 V for 40 min and transferred to a PVDF membrane for 2 h at 100 V. Membranes were immunostained with primary anti-poly-glutamine expansion disease marker antibody (1:500; clone 5TF1-1C2, Millipore), primary anti-human α -actin (1:5000; Clone AC-15, Sigma), or anti-human huntingtin antibody (1:1000; MAB5492, Millipore) followed by secondary goat anti-mouse IgG-HRP (1:1000; Cat #sc-2005, Santa Cruz Biotech). Immunostaining was also performed using primary anti-human EGFP (1:2500; Clone ab290, Abcam) followed by secondary goat anti-rabbit IgG-HRP (1:1000; Cat #sc-2004, Santa Cruz Biotech). Blots were developed by using Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific) and imaged using a ChemiDoc XRS+ and ImageLab software (BioRad). Additional densitometry was performed using ImageJ analysis software.

Flow cytometry

Cultures were lifted by trypsinization, pelleted by centrifugation, and resuspended in PBS at concentrations from 1 to 5×10⁵ cells/ml and analyzed on a FC500 (Beckman Coulter). GFP was measured on the FL1 photomultiplier tube between 515 and 535 nm. DsRed fluorescence was detected using FL2 between 567.5 and 582.5 nm. At least 25,000 events were recorded for each sample. Data were acquired and analyzed using Cytomics RXP (Beckman Coulter) at time of acquisition and further analyzed using the FlowJo software package (TreeStar Inc, Ashland OR).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MSC	Mesenchymal stem cells
HD	Huntington's disease
HTT	huntingtin protein
HTT142	huntingtin fragment containing exon 1 and containing approximately 100 CAG repeats
U87	glioblastoma cell line
SH-SY5Y	neuroblastoma cell line
GFP	enhanced green fluorescence protein
HTT142gfp	lentiviral vector containing both HTT142 and a GFP reporter
4dGFP	destabilized EGFP variant with 4 h half-life
shRNA	short hairpin RNA
shHTT	shRNA antisense to HTT exon 1
shGFP	shRNA antisense to GFP
shSCRAM	shRNA of same composition of shHTT with several minor alterations
dsRed-mito	red fluorescence protein reporter with a mitochondrial localization sequence
ES	embryonic stem cells

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Fig. 1. Overview of co-culture system and vectors. **A.** MSC and U87 or SH-SY5Y cells were transduced to express shRNAs with a dsRed reporter or HTT142 with a GFP reporter respectively. The cell populations were then co-cultured, allowing the RNAi to transfer from the MSC to the other cell by either direct cell to cell contact or indirect contact, where the shRNA subsequently reduces protein expression. **B.** The shRNA expression and target vectors are shown here with viral elements colored dark gray, shRNA expression elements light blue, constitutive promoters dark blue, fluorescent markers green and red, HTT142 yellow, and WPRE light gray.

Fig. 2.

MSC characterization. **A.** MSC from donor 2628 were transduced with shSCRAM or shHTT at an MOI of 80 and expanded. Representative transduced and wild type cultures at passage 4 were then analyzed for proliferation using an MTT assay and cell counts were calculated using a standard curve. The results demonstrate logarithmic growth of all the cultures, with no single culture exhibiting any significant difference compared to any other.

B. Karyotypes were generated from all MSC donors used here both before and after lentiviral transduction. Shown here is a representative karyotype from MSC donor 2627 transduced with shHTT at an MOI of 80, demonstrating lack of any chromosomal changes.

C. MSC from donor 2627 were transduced with shGFP, shHTT, and shSCRAM at an MOI of 100 and were then differentiated, along with wild type MSC, using osteogenic and adipogenic inducing media for 17 days. All cultures demonstrated robust differentiation into adipocytes and osteoblasts. Shown are representative images from MSCshHTT stained with either Oil Red O (top row) at 200 $\times$ magnification or Alizarin Red (bottom row). Adipogenic

differentiation is indicated by the presence of red lipid-containing vesicles in the cell (top row, right), while osteogenic differentiation is confirmed by the presence of calcified extracellular matrix stained red by Alizarin Red dye (bottom row, middle). There was no appreciable accumulation of lipid or calcified matrix in the non-induced wells (left).

Fig. 3.

Reduction in GFP from shGFP as measured by flow cytometry. To test the activity of GFP targeted shRNA, U87HTT142gfp cells were transduced with shGFPdsRed-mito. Cell cultures were collected at day 0, 2, 4 and 6 and were assayed by flow cytometry. **A.** The green fluorescence of each culture is shown in this histogram overlay. Reduced GFP expression is evident after only two days (yellow) compared to starting levels at day 0 (green). GFP expression continues to decrease at day 4 (orange) after which only a slight decrease of 4dEGFP expression is seen on day 6 (red). **B.** Overlaid dot plots of the four time points reveals the progressive decrease in green fluorescence (x-axis) while expression of dsRed increases (y-axis) causing the populations to shift from bottom right to upper left of the plot. **C.** When compared to no shRNA (green triangle), and shHTT (red square), shGFP (blue diamond) shows a progressive and dramatic protein reduction. Populations were gated for GFP expression (shown in **A**) on day 0 and percent GFP-positive was subsequently measured.

Fig. 4.

Detection and silencing of HTT142 by shHTT. U87 cells were transduced to express HTT142 and GFP as a transduction reporter. **A.** Cell lysates from both wild type U87 cells and U87HTT142gfp cells were collected and proteins were analyzed by Western blot. Anti-HTT antibody revealed the expression of HTT142 as an approximately 92 kDa band present only in U87HTT142gfp cells. **B.** U87HTT142gfp cells were then transduced with either shHTT or shGFP lentivirus, and culture lysates were collected on day 2, 3, 5, and 9 and compared by Western blot. **C.** Protein levels were quantified using densitometry and HTT142 expression was normalized to actin. shHTT transduction progressively silenced HTT142 expression through day 9 (gray diamond), while the control shGFP transduction had no appreciable effect (black square).

Fig. 5.

RNAi transfer in co-culture reduces 4dGFP reporter expression. SH-SY5Y cells expressing 4dGFP as an RNAi activity reporter were co-cultured with either MSC expressing shGFPdsRed or shHTTdsRed as a control (see Supplemental Fig. 1). **A.** Dot plots of day 4 cultures (left) contain a distinct dsRed-positive 4dGFP-negative shRNA expressing MSC (parallel to the y-axis) and dsRed-negative 4dGFP-positive SH-SY5Ys in both moderate and high subpopulations (parallel to the x-axis). The MSCshHTT (red) and MSCshGFP (blue) cultures are indistinguishable at this point. However, on day 10 (right), the culture containing MSCshGFP had a marked decrease in green fluorescence, evident by the complete loss of the 4dGFP-high and a shift left in the more moderately expressing SH-SY5Ys as well. **B.** The co-cultures were then gated to analyze only dsRed-negative GFP-positive SH-SY5Ys and GFP expression was plotted on overlaid histograms (gates shown on the respective dot plots in **A**). At day 4 (left) the overlaid histograms show identical

peaks. At day 10, however, the cultures had become significantly different, as the culture containing MSCshGFP (blue) had a remarkable reduction in GFP expression when compared to MSCshHTT (red). RNAi transfer in co-culture reduces 4dGFP reporter expression. SH-SY5Y cells expressing 4dGFP as an RNAi activity reporter were co-cultured with either MSC expressing shGFPdsRed or shHTTdsRed as a control (see Supplemental Fig. 1). **A.** Dot plots of day 4 cultures (left) contain a distinct dsRed-positive 4dGFP-negative shRNA expressing MSC (parallel to the y-axis) and dsRed-negative 4dGFP-positive SH-SY5Ys in both moderate and high subpopulations (parallel to the x-axis). The MSCshHTT (red) and MSCshGFP (blue) cultures are indistinguishable at this point. However, on day 10 (right), the culture containing MSCshGFP had a marked decrease in green fluorescence, evident by the complete loss of the 4dGFP-high and a shift left in the more moderately expressing SH-SY5Ys as well. **B.** The co-cultures were then gated to analyze only dsRed-negative GFP-positive SH-SY5Ys and GFP expression was plotted on overlaid histograms (gates shown on the respective dot plots in **A**). At day 4 (left) the overlaid histograms show identical peaks. At day 10, however, the cultures had become significantly different, as the culture containing MSCshGFP (blue) had a remarkable reduction in GFP expression when compared to MSCshHTT (red).

Fig. 6.

Reduction in HTT142 after co-culture with shHTT-expressing MSC. U87HTT142gfp cells were treated with mitomycin C to induce growth arrest prior to being seeded with either MSC expressing shHTT or shGFP as a control. Cell lysate was then harvested from co-cultures on day 0, 2, 3, 5, and 9. **A.** Lysates were analyzed for expression of HTT142, GFP, and actin using Western blot. A progressive reduction of HTT142 is visible over time. **B.** Densitometry was used to quantify HTT142 normalized to actin. Cultures containing MSCshHTT (black diamond) demonstrated a progressive and consistent decrease in HTT142 over the time course. In contrast, cultures containing MSCshGFP only exhibited a slight decrease in HTT142.

Fig. 7.

Transient decrease in HTT142 after culture with shHTT-expressing MSC. SH-SY5YHTT142gfp cells were co-cultured with MSCshHTT or MSCshSCRAM in either serum free medium or medium containing only 5% FBS in order to slow cell proliferation. Cultures were then harvested for protein on days 0, 3, 5, and 7 and assayed using Western blot for HTT142, GFP, and actin (not shown) as a loading control for both 0% and 5% FBS cultures (A and B respectively). C. Protein expression for the two sets of co-cultures was then analyzed using densitometry. Both sets of co-cultures show a dramatic dip in HTT142 expression on day 5, followed by a recovery to near start levels in the cultures containing MSCshHTT (black diamond) when normalized to the co-expressed GFP. However, the control cultures containing MSCshSCRAM (gray square) showed no such pattern, with HTT142 levels either slightly increasing or decreasing.