Induced neural stem cell transplantation reduced behavioral deficits and ameliorated neuropathological changes in YAC128 Mouse Model of Huntington's Disease

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Abstract

Huntington's disease (HD) is a genetic neurodegenerative disorder characterized by neuronal loss and motor dysfunction. Although there is no effective treatment, stem cell therapy provides one of the most promising therapeutic strategies, but the safety and efficacy of this approach needs to be optimized. The purpose of this study was to test the potential of intra-striatal transplantation of induced neural stem cells (iNSCs), which were derived from induced pluripotent stem cells (iPSCs), for treating HD. For this purpose, we developed mouse adenovirus-generated iPSCs, differentiated them into neural stem cells *in vitro*, labeled them with Hoechst, and transplanted them bilaterally into striata of 10-month old wild type (WT) and HD YAC128 mice. We assessed the efficiency of these transplanted iNSCs to reduce motor deficits in YAC128 mice by testing them on an accelerating rotarod task at one day prior to transplantation, and then weekly for 10 weeks. Our results showed an amelioration of locomotor deficits for YAC128 mice that received iNSC transplantation. Following testing, the mice were sacrificed, and their brains were analyzed using immunohistochemistry and Western blot (WB). The results from our histological examinations revealed no signs of tumors and evidence that many iNSCs survived and differentiated into region-specific neurons (medium spiny neurons) in both WT and HD mice, as confirmed by co-labelling of Hoechst-labelled transplanted cells with NeuN and DARPP-32. Also, counts of Hoechst-labeled cells revealed that a higher proportion were co-labelled with DARPP-32 and NeuN in HD-, compared to WT- mice, suggesting a dissimilar differentiation pattern in HD mice. Whereas significant decreases were found in counts of NeuN- and DARPP-32-labeled cells, and for neuronal density measures in striata of HD controls, such decrements were not

observed in the iNSCs-transplanted-HD mice. WB analysis showed significant increases of BDNF and TrkB levels in striata of transplanted HD mice. Collectively, our data suggest that iNSCs may provide a safe and effective option for neuronal replacement therapy in HD.

Introduction

Huntington's disease (HD) is a progressive neurodegenerative genetic disorder characterized by choreic movements, behavioral and cognitive disturbances and dementia (Craufurd et al., 2001). The disease is caused by an autosomal dominant mutation in *huntingtin* gene (*HTT*) and the mode of inheritance is dominant with almost full penetration. The genetic basis of HD was discovered in 1993 (MacDonald et al, 1993), and it was found to be caused by an elongated Cystosine-Adenine-Guanine (CAG) repeats on the short arm of chromosome 4p16.3 in the *HTT* gene. Although symptoms of the disease are variable among patients at the beginning, the course of the disease becomes predictable, and all patients will eventually develop similar pathology (Walker, 2007). The life expectancy for HD patients ranges between 15-20 years after the appearance of symptoms (Foroud et al., 1999; Landles & Bates, 2004). The most apparent and earliest damage is seen in the neostriatum of the basal ganglia, which is composed of the caudate nucleus and putamen (Walker, 2007). Medium spiny neurons (MSNs) in the striatum appear to be the most vulnerable neurons to the damage in HD (Albin et al., 1990).

Different animal models, including those which are either chemically or genetically induced, have been developed to study various aspects of HD. One of these models is the YAC128 HD mouse, which contains the full-length human mutant *HTT* (m*HTT*) inserted into its genome, resulting in the expression of m*HTT* with 128 CAG repeats (Slow et al., 2003). YAC128 mice show selective, age-dependent, striatal and cortical atrophy and neurodegeneration, and develop progressive deterioration of motor and cognitive functions (Van Raamsdonk et al., 2005; Gray et al., 2008; Ehrnhoefer et al., 2009). In addition, a progressive deficit on the rotarod is observed, which correlates with neuronal loss in the striatum (Slow et al., 2003).

Different approaches to treat HD are being explored by several laboratories worldwide. The goal of most of those studies is to decrease the level of the mutant HTT protein and to improve the survivability of neurons, or to replace the affected neurons. Stem cell transplantation offers a promising approach for treating neurodegenerative diseases, including HD (Cundiff & Anderson, 2011). Many studies have been conducted on HD animal models, as well as human clinical trials, to test the efficacy of stem cell transplants (Clelland et al., 2008). Although some clinical studies using fetal tissue for transplantation have shown promising results (Bachoud-Levi et al., 2000, 2006; Reuter et al., 2008; Gallina et al., 2010), others have revealed serious complications (Hauser et al., 2002), including signs of degeneration of the graft (with evidence that HTT aggregates can invade transplanted cells), brain hemorrhage complications (Cicchetti et al., 2014), and multiple solid and cystic lesions in brain (Keene et al., 2009). Similarly, transplants of embryonic stem cells (ESCs) in mice and rat HD models have shown variable results with signs of immune response in the brain tissue (Bernreuther et al., 2006), and over-proliferation of tumors after the transplantation (Aubry et al., 2008).

Mesenchymal stem cells (MSCs) have also been used in transplantation studies. Many of these studies showed that MSC transplants improve cognitive, motor and psychiatric deficits (Edalatmanesh et al., 2010; Rossignol et al., 2011, 2014, 2015; Jiang et al., 2011; Hosseini et al., 2015), as well as reducing neuronal loss and enhanced neurogenesis (Jiang et al., 2011; Fink et al., 2013a; Rossignol et al., 2015). However, MSCs do not readily differentiate into neurons (Przyborski et al., 2008). It is suggested that the therapeutic effect of MSCs is mediated by anti-inflammatory cytokines and/or neurotrophic factors such as brain derived neurotrophic factor (BDNF), which are released from these MSCs, and can protect neurons and exert favorable immunomodulatory effects in the region of transplant (Rossignol et al, 2009, 2011; Lin et al., 2011; Sadan et al., 2012; Sánchez et al., 2014; Dey et al., 2010).

Neural stem cells (NSCs), which can be obtained from embryonic or adult sources, provide another option for stem cell therapy for HD. Neural stem cells were shown to improve motor functions and

increase life span in R6/2 mice (Yang & Yu, 2009) and to differentiate into neurons in the quinolinic acid (QA) rat model (Vazey et al., 2006). In addition, transplants of NSCs have been shown to decrease striatal loss in the striatum of HD model (Yang & Yu, 2009). In contrast, Johann and colleagues (2007) found that intrastriatal transplants of embryonic NSCs into QA mouse model were short-lived and showed signs of rejections at 14 days post-transplant. More recently, it was found that adult NSCs derived from rats which were transplanted into the 51 CAG transgenic model of HD, induced some immune response after transplantation (Rossignol et al., 2014).

Unfortunately, lack of availability, presence of immune response, the potential of tumor formation, in addition to the ethical concerns of using embryos has limited the clinical utility of ESCs and NSCs. However, a new source of stem cells emerged when Takahashi and Yamanaka (2006) generated pluripotent stem cells by reprogramming somatic cells through inserting 4 genes (*OCT4, SOX2, Klf4* and *c-Myc*) into skin cells. These cells defined as induced pluripotent stem cells (iPSCs) were able to differentiate into almost any cell type in the body (Takahashi & Yamanaka, 2006). iPSCs and ESCs appear to be functionally equivalent, but there are genetic and epigenetic differences between them (Robinton & Dalley, 2012). Interestingly, studies have indicated that induced pluripotent cells could offer a viable alternative to ESCs and circumvent the issues of the availability and ethical concerns of using embryos (Verma & Verma, 2011). Work in our laboratory indicated that transplantation of rat-derived iPSCs into the striata of rats given 3- nitropropionic acid to model HD, revealed improvements in the motor function, and differentiation of transplanted cells into region-specific neurons in the striatum (Fink et al., 2014). Subsequently, improved motor functions observed in a study using NSCs, that were derived from iPSCs (iNSCs) generated from a patient with juvenile onset HD, and transplanted into the striata of YAC128 mice, which carry the full-length human *HTT* gene (Jeon et al., 2014).

Based on these findings, we hypothesize that iNSCs could provide a useful alternative for HD treatment because these cells can be readily obtained, can provide a more personalized treatment (via reduced rejection when transplant recipient donates the cells be used), and integrate to the host tissue. In addition, as these iNSCs are restricted to neuronal lineages, we believe that they confer less risk of over-proliferation and tumor formation. As such, the purpose of the this study was to test the efficacy of transplantation of iNSCs as a therapy for HD after intrastriatal transplantation in YAC128 HD mice by (1) assessing their effects on motor function and (2) the survivability and differentiation capabilities after 10 weeks.

1 Materials and Methods

1.1 Cell generation and culture

1.1.1 iPSC culture

iPSCs used in this study, were generated and characterized as described previously (Fink et al., 2013b). In brief, iPSCs were generated from fibroblasts that were isolated from tails of adult wild type mice. These fibroblasts were reprogrammed into iPSCs by using two adenoviruses: one contains *Oct4, Sox2, and Klf4* and another contains *c-Myc* which are all considered pluripotent factors. The generated iPSCs were confirmed to express pluripotency by using immunocytochemistry (ICC) and flow cytometry. The cells were then cryopreserved in 10% dimethyl sulfoxide. The generated iPSCs were thawed and plated on 0.1% gelatin coat, and cultured in iPSC media [Dulbecco's Modified Eagles Media (DMEM; Life Technologies, Carlsbad, CA) supplemented with 10% knock-out serum, β-mercaptoethanol (Life Technologies, Carlsbad , CA), 1% 1X non-essential amino acids (NEAA; Life Technologies, Carlsbad, CA), 20 ng/mL basic fibroblast growth factor (bFGF; Life Technologies, Carlsbad, CA), 2 μM L-glutamine (Sigma, St. Louis, MO), 5 mg/mL streptomycin and 5 UI/mL penicillin, and 10 ng/mL leukemia inhibitory factor (LIF; Life Technologies, Carlsbad, CA)]. Cells were passaged by

dissociating them in Accutase (Sigma, St. Louis, MO), centrifuging at 250 g for 5 minutes at 4°C, and plating them on 0.1 % gelatin coat.

1.1.2 iNSCs Generation

The iNSCs were generated by differentiation of iPSCs following the first stage in a published protocol with some modifications (Niclis et al., 2013). Briefly, iPSCs were expanded to 80% confluency, and then, the iPSC media were replaced by neuronal induction media [Neurobasal-A (Life Technologies, Carlsbad, CA) supplemented with 1X B27-A (Life Technologies, Carlsbad, CA), 1X N2 (Life Technologies, Carlsbad, CA), 1X NEAA (Life Technologies, Carlsbad, CA), 1X Glutamax (Life Technologies, Carlsbad, CA), and 5 mg/mL streptomycin and 5 UI/mL penicillin]. Half of the media was changed every three days, and the cells were kept in culture until they detached and formed neurospheres. The media containing detached cells were centrifuged at 100 g for 5 minutes at 4°C, and the pellet was dissociated in 1 mL Accutase (Sigma, St.louis, Mo) for 5 minutes at 37°C, then suspended in 5 mL phosphate buffer saline (PBS) and centrifuged another time. Cells were then re-plated in neural stem cell media [Neurobasal-A supplemented with 1X B27-A, 1X N2 (Life technologies, Carlsbad, CA), 1X NEAA, 1X Glutamax (Life Technologies, Carlsbad, CA), 20 ng/mL epidermal growth factor (EGF; Life Technologies, Carlsbad, CA) and 10 ng/mL bFGF (Life Technologies, Carlsbad, CA), and 5 mg/mL streptomycin and 5 UI/mL penicillin] (Fig 1).

1.1.3 Characterization of iNSCs

The iPSC-derived neurospheres were passaged every week for three weeks and then characterized through ICC for neural lineage specific antibodies (Nestin, Sox2, β-tubulin-III and NeuN). Briefly, the cells were grown on poly-L-lysine-coated, 20 mm glass coverslips for 2 days, after which cells were washed with PBS (0.01M at pH 7.4) three times and fixed using 4% paraformaldehyde for 10 minutes at 4°C. Then, a blocking solution (10% normal goat serum in PBS) was added to the coverslips and

incubated for one hour at room temperature. After that, the primary antibodies (Nestin, Sox2 and NeuN; 1:500; Abcam, Cambridge, U.K, β-Tubulin III; 1:300; Aves Labs Inc., Tigard, OR) diluted in PBS containing 0.1% Triton X-100 were added to the assigned wells and incubated at 4°C overnight. The primary antibodies were then aspirated and the coverslips were rinsed 3 times in PBS. Secondary antibodies with either AlexaFluor488 or AlexaFluor594, (1:500, Invitrogen, Carlsbad, CA) were then added and incubated at room temperature for 1 hour. After that, the coverslips were rinsed 3 times in PBS. Hoechst-33342 (1:1000; Sigma, St. Louis, MO) was added to each coverslip for 5 minutes at room temperature, and then, the coverslips were rinsed 3 times and mounted onto glass slides using Fluoromount reagent (Sigma, St louis, MO). Slides were visualized using a fluorescence microscope (Leica, Germany).

1.2 Animals

All procedures involving animals that were used in this study are approved by the Central Michigan University Institutional Animal Care and Use Committee. Twenty eight, 10-month-old male and female wild type and YAC 128 mice were randomly assigned to groups and housed in cages on a continuous 12-hour day/night cycle (from 1100 to 2300 h). Mice had access to water and food, *ad libitum*, and they were kept at the same conditions of temperature and humidity.

1.3 Transplantation

1.3.1 Preparation of iNSC for transplantation

On the day of surgery, iNSCs were pre-labeled with 5 µg/mL of Hoechst 33342 (Sigma, St Louis, MO), and re-suspended at a density of 200,000 cells/µL in Hanks' Balanced Salt Solution (HBSS).

1.3.2 Surgeries

Surgeries were performed on all mice in the study at 10 months of age. Mice were randomly assigned into one of the following groups (n=7): HD+HBSS, HD+iNSCs, WT+HBSS, and WT+iNSCs. The surgery was conducted under aseptic conditions. Mice were anesthetized using 2.0% isoflurane with 0.8 L/min oxygen maintenance throughout the procedure. The mice were continuously monitored throughout surgery, and adjustments of isoflurane and oxygen supply were made as needed. The back of the head of each mouse was shaved from the line between ears to the frontal part. After that, each anesthetized mouse was placed into the stereotaxic device (Kopf Instruments, Tujunga, CA), and the surgical site of the head was cleaned with chlorhexidine (Molnycke Healthcare, Norcross, GA). Then, a midline incision was made on the scalp, and skin was retracted. Two burr holes were made over the neostriatum (coordinates relative to bregma: anterior +0.5 mm; lateral ± 1.75 mm; with the tooth bar set at -3.3 mm). The iNSCs or HBSS were loaded into a 10 µL Hamilton micro-syringes and every mouse received bilateral injections of cells and/or vehicle at a constant rate of 0.33 µL/minute. Each hemisphere was injected with 200,000 cells at 2.5 mm ventral to the dura. After a 3-minute rest period, the micro-syringe was moved 0.1 mm dorsally and another 200,000 cells were injected, followed by another 3-minute rest period. The syringe was withdrawn slowly and re-positioned over the contralateral hemisphere and the procedure was repeated. Each hemisphere received total of 400,000 cells, while the vehicle control group received 2 µL HBSS. Incisions were closed by using 7-mm sterile wound clips, and analgesic ointment was applied to the incision site. Following surgeries, mice were monitored in recovery cages and transferred to their home-cages when they were fully recovered.

Postoperative care over a 5-day period included monitoring of vital signs, weight, movement, amount of food and, water ingested as well as the status of the tissue at the incision site. Intra-peritoneal injections of physiological saline were given for mice showing signs of dehydration during the second post-surgical day. Clips were removed 10 days following the surgery.

1.4 Accelerating rotarod testing

The motor activity of the mice was assessed using the accelerating rotarod (San Diego Instruments; San Diego, CA). Mice were first trained at increasing speed starting from 0 rpm up to 40 rpm on five consecutive trials for five days before receiving the treatment. For testing, the mice were placed on the rod, which started rotating at 0 rpm and accelerated at 0.5 rpm/second until they fell. Mice were given 5 trials with a 45-second inter-trial interval. Baseline measurements were performed one day before the transplantation, and then, testing was done once each week, for 10 weeks after surgery. Motor function was measured by latency to fall (sec) from the accelerating rotarod.

1.5 Histology

1.5.1 Immunohistochemistry

Four mice from each group were anesthetized with sodium pentobarbital by intraperitoneal injection, and then, were transcardially perfused first with 0.01 M cold PBS (pH 7.4), followed by 4% paraformaldehyde for fixation of the brains and their brains were extracted and kept in 4% paraformaldehyde for 24 hours at 4°C. The brains were then transferred to 30% sucrose in PBS for 48 hours at 4°C and then flash-frozen in 2-methylbutane (Sigma, St. Louis, MO) on dry ice for 3 minutes and stored at -80°C until processing. The brains were sectioned coronally on a cryostat at 40 µm thickness and placed in 6 serial wells.

For immuno-histochemical (IHC) analysis, primary antibodies were used for double labeling of (1) mature neurons (mouse NeuN, 1:500; Millipore, Billerica, MA) and (2) medium spiny neurons (rabbit DARPP32, 1:500; Abcam, Cambridge, U.K). Equally spaced sections from every brain was used for labelling of astrocyte response (GFAP, 1:500; Abcam, Cambridge, U.K). Tissue sections were blocked using 10% normal goat serum in PBS for one hour at room temperature, and then transferred to wells containing the primary antibodies in PBS with 0.1% Triton X-100, and incubated at 4°C overnight with

continuous agitation. On the following day, the brain sections were rinsed three times in Tris-buffer saline with 0.1% Tween-20 (TBST) and transferred to wells containing the appropriately conjugated secondary antibodies [Alexa Fluor 488 (1:1000), and AlexaFluor594 (1:1000); Invitrogen] for 1 hour at room temperature. Finally, the sections were rinsed in TBST and mounted onto positively charged glass slides, using Fluoromount media (Sigma, St. Louis, MO).

1.5.2 Imaging and analysis

For NeuN and DARPP-32 slides from each mouse, both striatum from 4 randomly selected tissue sections were imaged under fluorescent microscopy, while maintaining a consistent exposure time and fluorescent intensity for each slide. Exposure settings were maintained at 360-, 460-, and 380-millisecond, respectively, for images obtained from Hoechst, Alexafluor-488, and Alexafluor-594 labeled sections. A 20-µm Z-stack of images was collected from seven individual depths, spaced 3 µm apart. Each striatum was imaged in its entirety by tiling each individual region under a 20-x objective. Following image acquisition, the complete Z-stack from each image was processed using ZEN 2.3 (Carl Zeiss AG; Oberkochen, Germany) through the extended depth of focus to flatten the acquired images, and the tiled image was stitched. Each color channel was exported as individual TIFF-files and subsequently analyzed, and estimation of total neuronal profiles and area were calculated using stereological procedures with the MBF Stereo Investigator software (MBF Bioscience; Williston, VT). Counts of DARPP-32 and/or NeuN labelled cells was done within 200 X 200 counting frames spaced evenly throughout the striatum (grid size was 1000 X 1000 mm). For GFAP, transplants site from each mouse was imaged using a fluorescence microscope (Leica, Germany).

1.6 Western Blot

Three brains from each group were used for Western blot (WB) analysis at 54-weeks of age. Mice were sacrificed by cervical dislocation and their brains were extracted and dissected. For every brain, striata

were isolated, and lysed in cold radio-immuno-precipitation assay (RIPA) buffer [10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 0.1% SDS, 140 mM NaCl, 0.1% sodium deoxycholate, 1% Triton X-100, with protease inhibitors (Sigma, St. Louis, MO)]. The homogenate was centrifuged at 20 g at 4°C for 30 min. The supernatant was taken and aliquoted in PCR tubes and stored at -80°C until use. Protein concentrations for each sample were determined using the Pierce BCA protein assay (Thermo Scientific, Rockford, IL). Samples were mixed with equal amount of 2X SDS-sample buffer (125 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol, and 10% 2-mercaptoethanol) and boiled for 2 min. For assessment, equal amount of protein of each sample was loaded and separated on gradient gel (4-20 % SDS-PAGE). The SDS-PAGE was run at 100 V with running buffer (25 mM Tris-Base, 192 mM glycine, 0.1% SDS, and 1 mM EDTA). The proteins from gel were transferred overnight to the PVDF membrane (Millipore, Billerica, MA) in an ice cold buffer containing 25 mM Tris– Base, 192 mM glycine and 10 % methanol. Following transfer, the blots were rinsed three times in TBST, and the membranes were blocked with 5% fat-free milk in TBST for one hour. Then, the blots were incubated with primary antibody BDNF (1:1000; Sigma, St. Louis, MO) or TrkB (1:1000; Cell Signaling Technology, Danvers, MA), and β-tubulin, (1:1000; Abcam) in 5% fat-free milk powder dissolved in TBST for overnight at 4°C. Membranes were then rinsed three times with TBST, and incubated with the respective horse radish peroxidase (HRP) conjugated secondary antibodies (diluted 1:10,000) in 1.5 % fat-free milk powder in TBST for 1 hour. The membranes were then washed three times with TBST. The blots were then developed with Immobilon^{TM} Western Chemiluminescent HRP-substrate (Millipore, Billerica, MA), and scanned. The optical density of each lane of the blot was measured using ImageJ software (NIH, Bethesda, MD).

1.7 Statistics

All statistical analyses were performed using SPSS v24. Accelerating rotarod data was analyzed using repeated measures analysis of variance (ANOVA). Fall latency, including the baseline and ten weeks

following transplantation, was used for statistical comparisons between wild type and YAC128 mice treated with iNSCs, or received HBSS. One way ANOVA was performed to analyze differences of weekly fall latency amongst all groups. Histological and WB data were analyzed using one way ANOVA. Tukey's Honest Significant Difference (HSD) *posthoc* test, was performed when the omnibus F-values were significant. The alpha level is set at $p \leq 0.05$ for all analyses.

2. Results

2.1 iNSC Characterization

ICC results confirmed that neurospheres showed positive expression of neural stem cells markers (SOX2 and Nestin; Fig 2A). Single cells from the neurospheres showed positive expression of immature neuronal marker (β-Tubulin-III; Fig 2B), confirming the status of immature neuron cells of the iNSCs.

2.2. Accelerating Rotarod

Repeated measures ANOVA of accelerating rotarod (accelerod) data demonstrated that there was an overall significant group effect in the performance of the mice [F (3, 24) =8.461, p=0.001]. Tukey Post HSD test revealed a significant between-group difference in performance on the accelerod, with WT groups demonstrates longer latencies than mice in the HD vehicle control group (p<0.05).No significant differences were found between WT groups and iNSCs-treated HD mice (p>0.05), nor between HD vehicle-control and the iNSCs-treated HD mice group (p>0.05) (Fig 3).

Although the one way ANOVA of weekly accelerod data showed that there was a significant difference at baseline between WT and HD groups (p<0.05), by week 4 after transplantation, iNSCs-treated HD mice were not significantly different from WT groups (p>0.05).

2.3. Histological results

2.3.1. Survivability and Differentiation

Hoechst-labelled cells were found in both WT and HD brains 10 weeks post-transplantation. However, less transplanted cells (Hoechst-labelled cells) in iNSCs-treated WT mice were observed in the striatum in comparison to iNSCs-treated HD mice (Fig 4 A).

We observed that Hoechst-labeled cells co-localized with NeuN and DARPP-32 in both WT and HD mice (Fig 5). However, a higher proportion of Hoechst-labeled cells were co-labelled with DARPP-32 and/or NeuN in HD- (m=46.3%, SD=3.56) compared to WT- mice (m=27.88%, SD= 13.39).

Analysis of the stereologically acquired cell count -data revealed significant between-group differences in DARPP-32 and NeuN stained cells counts in striatum [$F(3, 12) = 9.512$, p=0.002, $F(3, 12) = 8.573$, p=0.003, respectively]. Specifically, *posthoc* analysis showed that there was a significant difference between WT groups and HD vehicle control in DARPP-32 and NeuN stained cells ($p<0.05$). However, there was no significant difference between WT and the iNSCs- treated HD groups ($p>0.05$). Furthermore, there was a significant difference between HD vehicle control and iNSCs-treated HD mice. ($p<0.05$; Fig 4 A, B &D).

In addition, results of stereologically acquired optical density measures revealed significant between-group differences in the density of NeuN and DARPP-32 in striata ($F(3, 12) = 5.031$, p=0.017, $F(3, 12) = 7.392$, p=0.001). *Posthoc* analysis showed a significant reduction in NeuN- and DARPP-32 labelled cell densities in HD vehicle-control mice in comparison to the WT mice ($p<0.05$). However, these significant differences were not seen between iNSCs-treated HD and WT control groups ($p>0.05$). Moreover, NeuN- and DARPP-32 labelled cells densities were significantly increased in iNSCs-HD treated mice, in comparison to HD vehicle controls ($p<0.05$) Fig (4 A, C & E)

2.3.2. Astrocyte Response

Reactive astrocytes were observed at the transplantation site. Higher astrocytic (GFAP; red) responses were observed in iNSCs (Hoechst labelled cells; blue) transplanted brains, in comparison to vehicle controls. Also, higher astrocytes response was observed in iNSCs-transplanted WT mice in comparison to iNSCs-transplanted HD mice (Fig 6).

2.4. Western Blotting

Analysis of WB data showed that there was a significant between-group differences in BDNF and TrkB levels in striata [F (3, 8) = 4.250, $p = 0.045$ & F (3, 8) = 4.739, $p = 0.035$, respectively]. *Post hoc* analysis showed that there is a significant reduction in BDNF and TrkB in HD vehicle-control mice ($p<0.05$). However, there was no significant difference between iNSCs-treated HD mice and WT groups ($p>0.05$) (Fig 7).

3. Discussion

The primary findings of the present study were that iNSCs survived for at least 10 weeks after transplantation in both WT and HD mice brains. We also found that iNSCs differentiated into mature neurons and region-specific neurons (medium spiny neurons), which was confirmed by co-localizing of Hoechst-labelled cells (transplanted iNSCs) with NeuN and DARPP-32, respectively. Interestingly, we noticed a dissimilar differentiation pattern in HD and WT mice, in which a higher percentage of Hoechst-labelled cells were co-localized with NeuN and DARRP-32 in the HD brains.

We confirmed that YAC128 mice have significant motor deficits as manifested by a decrease in the latency of fall on the accelerating rotarod, a result which is consistent with previous studies (Slow et. al., 2003) and represents a dysfunction which is usually preceded by striatal neuronal loss (Van Raamsdonk et al., 2005). The HD mice which received iNSCs had a significant increase in the total striatal neuronal counts and in the number of medium spiny neuron in comparison to the HD vehicle-

control mice. In addition, the iNSC-transplanted HD mice showed behavioral sparing, as evidenced by their performance on the accelerating rotarod.

We also confirmed that total and medium spiny- neuronal densities in the striatum decreased in HD vehicle-controls at 54 weeks of age in comparison to WT mice, a finding which is in line with the trends observed by Slow and colleagues in one-year-old YAC mice (2003). In our study we found that YAC128 HD controls showed a significant decrease in the neuronal density at 54 weeks of age, but iNSCs transplantation into the striata seems to ameliorate this neuronal loss.

For the location of the transplanted cells, iNSCs were mainly found in the striata in HD mice, while in WT mice, we observed fewer of the iNSCs in striata, and these were mainly near the injection site. We found some cells distributed near the corpus callosum, and some in the cortex suggesting some migration of the transplanted cells in the brains. We observed a more reactive astrocytic response in transplanted brains in comparison to vehicle controls. We also observed more reactive astrocytes in the brains of iNSC-transplanted-WT mice in comparison to iNSC-transplanted-HD mice. These observations could be explained by the immune response having a stronger effect on cells transplanted into the brains of WT mice, in which the cells were not needed and which the immune system may have been less compromised. This also accounts for the fewer number of transplanted iNSCs observed in WT mice. Future studies will focus on the immune responses at different time points following transplantation of iNSCS using a wider range of immunological markers and cells.

The importance of the BDNF for MSNs, the neurons most vulnerable for degeneration in HD, is well documented (Strand et al., 2007; Zuccato & Cattaneo 2007). A decrease in the BDNF levels, and in the number and activity of TrkB receptors have been confirmed in the striatum of mouse models of HD, as well as in HD patients (Gines et al., 2006; Zuccato at al., 2008). We also showed that BDNF and TrkB decreased significantly in the brains of HD mice, however, levels of BDNF and TrkB were

increased/preserved in iNSC transplanted brains. Although we have not tested whether or not these iNSCs-derived neurons are fully functional, the increase in BDNF and TrkB that are accompanied by amelioration of motor deficit suggest that those neurons are functioning. However, focusing on the connections of these cells and electrophysiological analysis in a long term survivability experiments is a goal for future experiments. Another important finding in our study was the lack of tumors, as this is major concern in stem cell transplantation. Since the iNSCs we used are more restricted in their neuronal lineages than most embryonic cells and iPSCs, we think that there is less risk of tumor formation in the protocol used in this study.

Collectively, our findings support the hypothesis that iNSCs may prove to be a viable cell-replacement strategy with a high potential for therapeutic benefit. As one of the first studies to describe functional and neuropathological improvements after transplantation of neural stem cells that are derived from iPSCs in HD transgenic mice models, our results suggest that future research along these lines may provide an effective treatment for this devastating disease.

Abbreviations

ANOVA- analysis of variance, BCA- bicinchoninic acid assay, BDNF- brain derived neurotrophic factor, bFGF- basic fibroblast growth factor, DARPP32- Dopamine- and cAMP-regulated phosphoprotein of 32 kDa, DMEM- Dulbecco's Modified Eagles medium, EDTA- Ethylene-di-amino-tetra-acetic-acid, EGF- epidermal growth factor, GFAP- glial fibrillary acidic protein, HBSS- Hank's balanced salt solution, HD- Huntington's disease, HRP- Horseradish peroxidase, iNSC-, induced neural stem cells, iPSC- Induced pluripotent stem cells, Klf4-Kruppel-like factor 4, MSN- Medium spiny neuron, NEAA, non-essential amino acids, NeuN- Neuronal nuclei, NSC- Neural stem cell, OCT4- Octamer-binding transcription factor 4), OD- Optical density, PBS- Phosphate buffer saline, PFA- Paraformaldehyde, PVDF- Polyvinylidene fluoride, QA- Quinolinic acid, RIPA- Radio immunoprecipitation assay, SDS- Sodium dodecyl sulfate, SDS-PAGE- sodium dodecyl sulfate polyacrylamide gel electrophoresis, SOX2- SRY (sex determining region Y)-box 2, TBS- Tris buffer saline, TrkB- Tropomyosin-related kinase B, YAC- Yeast artificial chromosome

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Abeer Al-Gharaibeh: conception and design, collection and/ or assembly of data, data analysis and interpretation, manuscript writing. Rebecca Culver: participated in the design of the study, collection and/or assembly of data. Andrew Stewart, Bhairavi Srinageshwar, Kristin Spelde, Laura Frollo, Nivya Kolli, Darren Story, Leela Paladugu, and Sarah Anwar : collection and/ or assembly of data. Andrew Crane and Robert Wyse: generation of iPSCs. Panchanan Maiti: design, administrative support, data analysis and interpretation, manuscript writing. Gary L. Dunbar and Julien Rossignol: conception and design, financial support, administrative support, data analysis and interpretation, manuscript writing, final approval of manuscript.

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Figure legends

Figure 1: Differentiation of iPSCs into iNSCs. iPSCs were expanded to 80% confluency, and then, the iPSC media were replaced by neuronal induction media [Neurobasal-A, 1X B27-A, 1X N2, 1X NEAA, 1X Glutamax, and 5 mg/mL streptomycin and 5 UI/mL penicillin]. The cells were kept in culture until they detached and formed neurospheres. The media containing detached cells were centrifuged, and the pellet was dissociated in Accutase. Cells were then re-plated in neural stem cell media [Neurobasal-A, 1X B27-A, 1X N2, 1X NEAA, 1X Glutamax, 20 ng/mL EGF, 10 ng/mL bFGF, and 5 mg/mL streptomycin and 5 UI/mL penicillin]. Cells were passaged three times and then characterized using ICC.

Figure 2: Characterization of iNSC through ICC. (A): Hoechst-labeled iNSCs (blue) in neural stem cell media, showed positive expression of neural stem cells markers; SOX2 (green) and Nestin (red). Upper row shows a neurosphere (Scale bar is 100µm) and lower row shows individual cells (scale bar is 100µm). (B) Hoechst-labeled iNSCs (blue) showed positive expression of immature neuronal marker, β-tubulin-III (green).

Figure 3. Accelerating rotarod testing. Accelerod testing showed a significant decrease of the fall latency in HD vehicle control mice but no significant decrease in the iNSCs treated HD mice. Weekly testing on the accelerod revealed a significant difference between HD mice and WT mice at baseline. iNSCs treated HD mice showed no significant difference from WT mice starting from Week 4 after transplantation. * indicates significant different from WT+HBSS, $p<0.05$

Figure 4: Striatal neuronal and medium spiny neurons count and density. (A) Stitched images for striatum in each group showing markers of iNSCs (Hoechst labelled cells; blue), mature neurons (NeuN; green) and medium spiny neurons (DARPP-32, red). (B&D) Analysis of NeuN and DARPP-32 labelled cells (mature neurons and medium spiny neurons respectively) in striata showed that HD+HBSS mice are significantly different from WT mice ($p<0.05$), but HD+iNSCs are not significantly different from WT mice ($p>0.05$). (C&E) Analysis of NeuN and DARPP-32 labelled cells density (mature neurons and medium spiny neurons, respectively) in striata showed that

565 HD+HBSS mice are significantly different from WT mice (p<0.05), but HD+iNSCs are not
566 significantly different from WT mice (p>0.05). * indicates significant different from WT+HBSS,
567 p<0.05

568 Figure 5: Transplanted iNSCs survived and differentiated into mature neurons and medium spiny.
569 Hoechst labelled iNSCs (blue) were found in striata 10 weeks post transplantation. Also, Hoechst
570 labelled iNSCs (blue) show co-expression of mature neurons marker (NeuN; green) and region
571 specific neurons (DARPP-32; red).

572 Figure 6: Astrocyte response at the injection site following transplantation. Higher astrocytes
573 (GFAP; red) response was observed in iNSCs (Hoechst labelled cells; blue) transplanted brains in
574 comparison to vehicle controls. Also, higher astrocytes response was observed in iNSCs transplanted
575 WT mice in comparison to iNSCs transplanted HD mice.

576 Figure 7: Western blot analysis of BDNF and TrkB. (A&B) Western blot analysis showed a
577 significant decrease of BDNF in striata of HD+HBSS mice (p<0.05), but HD+iNSCs are not
578 significantly different from WT mice (p>0.05). (A&C) Western blot analysis showed a significant
579 decrease of TrkB in striata of HD+HBSS mice (p<0.05), but HD+iNSCs are not significantly
580 different from WT mice (p>0.05). * indicates significant different from WT+HBSS, p<0.05.