EX VIVO GENE THERAPY FOR LYSOSOMAL STORAGE DISEASE USING IPSC-DERIVED NEURAL STEM CELLS

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ABSTRACT

EX VIVO GENE THERAPY FOR LYSOSOMAL STORAGE DISEASE USING IPSC-DERIVED NEURAL STEM CELLS

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Diseases affecting the central nervous system (CNS) pose a formidable obstacle to the delivery of effective therapeutics. A tight-knit collection of cells and macromolecules known as the blood-brain-barrier (BBB) prevents most substances from entering the brain. One intriguing approach to overcoming this obstacle involves transplanting neural stem cells (NSCs), the precursor cells to neurons and glia in the brain, as vehicles for the delivery of therapeutic proteins in their native environment. Notably, this strategy has already been successfully applied to several lysosomal storage diseases caused by genetic deficiencies in one of the many lysosomal hydrolases expressed throughout the body. A major drawback to this approach is that foreign NSCs, e.g. immortalized cell lines and primary fetal NSCs can be tumorigenic and immunogenic. Recently developed induced pluripotent stem cell (iPSC) technologies, combined with pluripotent stem cell differentiation techniques, have the potential to overcome these obstacles. This approach was evaluated using a comprehensive strategy targeting a prototypical lysosomal storage disease, Sly disease (MPS VII). MPS VII patient fibroblasts were transduced with retroviral vectors expressing the transcription factors Oct4, Sox2, Klf4, and c-Myc. Patient fibroblasts were reprogrammed into embryonic stem cell-like iPSCs that demonstrated hallmarks of pluripotency. Patient iPSCs, alongside iPSCs derived from an unaffected individual, were subjected to a stepwise differentiation protocol, yielding a relatively homogenous population of NSCs. Following *in vitro* characterization, patient iPSCs were genetically corrected using a DNA transposon-based vector. Transplantation of NSCs into neonatal MPS VII mice revealed that these cells could migrate long distances and survive for several months. However, corrected grafts expressing physiological

levels of the missing enzyme, β-glucuronidase, were too sparse to significantly ameliorate pathology. In contrast, the same cells transplanted into the post-symptomatic adult MPS VII striatum were restricted to the injection site. Corrected, but not uncorrected patient iPSC-NSCs, were able to restore pathologically activated microglia to a normal quiescent state in a zone surrounding the graft. Together, these results provide evidence that *ex vivo* NSC gene therapy may be a viable option for many lysosomal storage diseases using easily attainable, non-neural patient tissue.

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General Introduction

I. Lysosomal Storage Disease and Mucopolysaccharidosis

Optimal cellular health requires a balance between the metabolism and catabolism of macromolecules within the cell. In concert with the ubiquitin-proteasome system, the lysosome is responsible for the breakdown and recycling of macromolecules directly from the cytoplasm and via fusion with vesicles such as phagosomes, autophagosomes, and endosomes. The discovery of the lysosome was a seminal event in the history of cell biology, winning Christian de Duve the Nobel Prize in Medicine or Physiology in 1974. It is now increasingly recognized that the autophagosome-lysosome system acts as a central point of convergence for many cellular processes, reacting dynamically to the environment and contributing to overall cellular homeostasis (Behrends et al. 2010). The lysosome consists of many soluble acid hydrolases (~50) along with dozens of membrane bound proteins (~25 discovered so far) responsible for trafficking, nutrient sensing, membrane fusion, cytoplasmic import/export, and generation of an immense pH gradient (Saftig et al. 2009).

Loss-of-function mutations in lysosomal genes are responsible for many diseases related to defective catabolism within the cell, the lysosomal storage diseases (LSDs). Individually, these diseases are rare, but as a group they represent one of the most common causes of monogenetic disease affecting humans today (~1:7500 live births) (Meikle et al. 2004). The majority of LSDs are the result of genetic mutations that negatively impact the enzymatic function of a soluble lysosomal hydrolase. Depending on which hydrolase is affected, the consequent accumulation of the primary substrate/s involved can result in myriad indirect pathologies which are incompletely understood (Walkley et al. 1998, Settembre et al. 2008, Ohmi et al. 2009).

While many of the pathological cascades accompanying lysosomal dysfunction remain a mystery, there remains tremendous hope for treating soluble hydrolase deficiencies based on some of their unique physiological properties. Lysosomal hydrolases are translated in the endoplasmic reticulum and processed in the golgi to include a terminal mannose-6-phosphate (M6P) moiety on specific asparagine residues (Brown et al. 1984). M6P receptors concentrated in the cis-golgi direct bound enzymes through pre-lysosomal compartments towards the lysosome proper (Griffiths et al. 1988). A minor fraction of M6P-tagged enzymes do not traffic to the

lysosome but rather escape the cell via endosomal fusion with the cell membrane. Now in the extracellular space, soluble enzymes can bind M6P receptors on the surface of neighboring cells, triggering endocytosis and trafficking towards the lysosome (Coutinho et al. 2012). The sensitive pH dependence of lysosomal hydrolases (~4.5) prevents premature activation prior to reaching the lysosome. (Roederer et al. 1987). Importantly, this pathway can be exploited to treat the majority of lysosomal storage diseases.

Definitive evidence for the inter-cellular trafficking of lysosomal hydrolases came from a series of landmark studies, led by Elizabeth Neufeld's lab, using mixed fibroblast cultures derived from two patients with different lysosomal sorage diseases. The cells from both patients contained pathologically high levels of mucopolysaccharides (known today as glycosaminoglycans, or GAGs) (Fratantoni et al. 1968). One patient had Hurler's syndrome (MPS I), while the other had Hunter's syndrome (MPS II). The two diseases are clinically related and both phenotypes exhibit elevated levels of the GAGs heparan sulfate and dermatan sulfate throughout the body (Wraith 1995). Remarkably, co-culturing MPS I and MPS II fibroblasts reduced the GAG burden in cells from both patients, a process now referred to as cross-correction. Neufeld's group went on to demonstrate that the corrective factors were functional soluble hydrolases (α-L-iduronidase and iduronidate-2-sulfatase, respectively) absent in diseased cells (Fratantoni et al. 1968, Fratantoni et al. 1969).

The mucopolysaccharidoses (MPSs) are an important group of LSDs caused by mutations affecting one of 11 enzymes responsible for the step-wise degradation of GAGs (Platt et al. 2004). All but one of these diseases are autosomal recessive (MPS II is X-linked) (Berg et al. 1968). GAGs are a heterogenous group of linear polysaccharides broadly categorized into groups defined by one of the following component disaccharide units: heparin/heparan sulfate, chondroitin/dermatan sulfate, keratan sulfate, or hyaluronan. Depending on which enzyme is defective, patients can accumulate one or more classes of GAG (Esko et al. 2009).

Since the early days of Neufeld's pioneering studies, the MPSs have been studied extensively, yielding diverse insights into lysosome biogenesis, autophagy, intracellular trafficking, neuron dendritogenesis, and gene expression networks (Goodman et al. 1996,

McGlynn et al. 2004, Settembre et al. 2008, Cox et al. 2012). MPS diseases are utilized not only for their contributions to basic cell biology, but also to evaluate novel treatment strategies, in part because of a well-understood therapeutic mechanism (the M6P pathway) and numerous animal models that recapitulate human LSDs.

Enzyme replacement therapy (ERT) was first developed for Type I Gaucher disease, the most common LSD (Barton et al. 1990). Unique among enzyme therapeutics, currently approved formulations of β-glucocerebrosidase for Gaucher disease are modified such that enzyme glycans terminate in mannose residues. The rationale for this approach was to increase uptake in macrophages, preferentialy affected in Type I Gaucher disease, via the mannose receptor rather than the M6P receptor (Brady 2006). Although the benefit of this particular approach is unproven, ERT is nevertheless effective in treating nonneuronopathic forms of Gaucher disease. ERT is now available for several LSDs including MPS I, MPSII, and MPS IVA. However, there remains a fundamental obstacle to treating LSD pathology in the CNS: the blood-brain-barrier. Gaucher disease patients with the acute neuronopathic form of the disease (L444P) showed no improvement when treated with recombinant enzyme even very early in life (Prows et al. 1997). Most LSDs, including the MPSs, have some level of CNS involvement (Platt et al. 2004).

While it has been reported that extremely high levels of IV recombinant enzyme (20/mg/kg/week) can have modest success in clearing storage lesions in the brains of MPS VII mice, it is clear that additional strategies are needed (Vogler et al. 2005). Some groups have reported enzyme activity in the brain following bone marrow transplantation, presumably mediated by bone marrow derived cells entering the brain (Zheng et al. 2003, Zheng et al. 2004). However, success requires lethal irradiation or intense chemotherapy, creating a niche into which bone marrow derived monocytes can enter the brain to replace resident microglia (Morganti et al. 2014). It has since been demonstrated that the yolk-sac, and not the bone marrow, is the normal developmental origin of microglia and other tissue resident macrophages (Perdiguero et al. 2014). Microglia are normally self-renewing, they are replaced by bone marrow derived precursors at a very low rate under normal circumstances and when the brain is shielded during

whole-body irradiation (Derecki et al. 2012). These studies demonstrate an unmet need for effective strategies targeting LSD pathogy in the CNS.

II. Neuropathology of Mucopolysaccharidosis

In order to develop effective CNS therapies, well characterized animal models are crucial. Naturally occurring and genetically modified animals with MPS have served as some of the best models for developing therapies targeting the CNS. True homologues of human disease, MPS animal models have been extensively characterized (Levy et al. 1996, Haskins et al. 2002). Large animal models of MPS are particularly important for evaluating CNS-targeted therapies. When compared to mice, animals such as cats and dogs have very large brains, more accurately representing the challenge of achieving widespread enzyme delivery in humans (Wolfe et al. 2000). Local treatments that work well in mice, such as the injection of viral vectors encoding lysosomal enzymes, are generally ineffective when it comes to treating global CNS pathology in large animals and LSD patients (Heuer et al. 2002, Cearley et al. 2007, Ponder et al. 2007, Worgall et al. 2008).

In order to effectively evaluate corrective therapies, it is important to understand the complicated pathological sequelae of primary substrate accumulation. As long lived non-dividing cells, neurons are particularly susceptible to the buildup of GAGs. Consequently, MPS diseases often involve neurological impairment. Patterns of neuropathology, common to a wide range of LSDs, provide insights into disease mechanisms and may help guide treatment strategies.

While the primary feature of all MPSs is the intracellular accumulation of undegraded GAGs, there is a secondary accumulation of products for which GAG degrading enzymes are not required (Walkley 2004). Primary GAG storage affects the expression and distribution of many lysosomal enzymes in MPS patients (Kint et al. 1973, Hollak et al. 1994). The GAGs heparan sulfate and chondroitin sulfate can bind many lysosomal enzymes, decreasing their activity in vitro (Avila et al. 1975). Interestingly, secondary storage products often do not co-localize with GAGs, implicating broader defects in endosomal sorting pathways (McGlynn et al. 2004).

The exact composition of secondary storage products depends on the particular disease. The brains of mice with MPS I, IIIA, IIIB, and VII were all found to accumulate the gangliosides GM2 and GM3 in varying amounts. Cholesterol storage was shown in MPS I, IIIA, IIIB, but not in MPS VII (McGlynn et al. 2004). Specific morphological alterations can be observed in cells accumulating certain primary or secondary storage products. Alterations include the enlargement of axon hillocks (meganeurites) and the formation of ectopic dendrites, observed in cortical pyramidal neurons subsequent to GM2 accumulation (Walkley 2004). Meganeurites are found in many LSDs including Tay-Sachs, α-mannosidosis, fucosidosis, Batten disease, Niemann-Pick type C, MPS I, and MPS VI.

GM2 ganglioside is thought to be particularly important in the neuropathology of many LSDs. Ectopic dendrites, often protruding from the meganeurite, are found exclusively in neurons accumulating GM2 ganglioside (Goodman et al. 1991). High levels of GM2 ganglioside are not commonly seen in healthy mature neurons, but they are present in developing neurons undergoing normal dendritogenesis. One theory is that GM2 may be inappropriately activating dendritogenesis in susceptible neuronal subtypes, thus contributing to neurological dysfunction (Goodman et al. 1996).

Another common feature of LSD neurons is the presence of axonal spheroids. These large swellings, distal to the cell body, are common to a wide range of neurodegenerative diseases. They contain a dense collection of autophagosomal-like and multivesicular-type bodies (Platt et al. 2004). Axonal spheroids are most common in GABAergic neurons, especially purkinje cells in the cerebellum, which are particularly vulnerable to cell death in several LSDs (March et al. 1997). A characteristic of MPS neurons are “zebra bodies”, so-called for their multilamellar striped appearance (Bhaumik et al. 1999).

Inflammation is a significant component of many LSDs, including MPS diseases. Gene expression profiling of the MPS VII mouse brain revealed that many inflammatory-related transcripts are significantly altered relative to unaffected littermate controls (Parente et al. 2012). There is evidence that GAGs may directly contribute to this pro-inflammatory state. Heparan sulfate oligosaccharides can activate microglia *in vitro* via TLR4, and the extent of microglial

activation correlates with disease progression in MPS mice (Ohmi et al. 2003, Ausseil et al. 2008). However, deletion of TLR4 or the adapter protein MyD88 did not decrease other markers of disease progression in MPS IIIB mice, indicating that microglial activation is not a major determinant of neurodegeneration in this disease (Ausseil et al. 2008). Recently, an MPS VII/TLR4/complement component 3 (C3) (MPS VII$^{-/-}$/TLR4$^{-/-}$/C3$^{-/-}$) mouse was reported, but the CNS phenotype was not described (Xing et al. 2015). Neurological function and lifespan in mice with the LSD Sandhoff disease (GM2 gangliosidosis) are improved when crossed to mice lacking Macrophage-inflammatory protein 1 alpha (MIP-1α) (Wu et al. 2004). GAGs can bind and enhance the acitivity of several pro-inflammatory cytokines, including MIP-1α (Ali et al. 2000). MPS IIIB mice have high levels of perforin and granzyme B transcripts in the brain, suggesting inappropriate NK or CD8 T cell activity (Villani et al. 2009).

Autophagy is a process involving the selective and non-selective degradation of cytosolic proteins and entire organelles. Autophagy becomes grossly dysregulated in many LSDs because lysosomal hydrolases are ultimately responsible for much of the actual degradation following autophagosome-lysosome fusion (Settembre et al. 2008). Mutant mice lacking key autophagic proteins gradually accumulate undegraded proteins within large poly-ubiquitinated inclusions, leading to cell death and severe neurodegeneration (Komatsu et al. 2006). Interestingly, poly-ubiquitinated inclusions and other hallmarks of defective autophagy are common to many neurodegenerative diseases including dementia, Parkinson's, Huntington's, and Alzheimer's (Damme et al. 2014). All the evidence is consistent with a central role for autophagy in the neurodegenerative process, suggesting that insights gleaned from genetically tractable LSDs may also apply to more common, idiopathic forms of neurodegenerative disease.

Autophagy and lysosomal biogenesis are tightly regulated at the transcriptional level by the transcription factor TFEB (Sardiello et al. 2009). mTORC1-dependent phosphorylation of TFEB results in cytoplasmic sequestration, whereas dephosphorylated TFEB translocates to the nucleus, greatly enhancing lysosome biogenesis (Roczniak-Ferguson et al. 2012, Settembre et al. 2012). The coordinated action of TFEB, a master regulator of lysosomal gene expression,

may explain why some pathological features (e.g., secondary enzyme elevation) are common to many or all LSDs.

Much of the data regarding neuropathology in the MPS VII mouse brain comes from electron microscopy and immunohistochemical studies (Levy et al. 1996, Heuer et al. 2002). Electron micrographs show that lysosomal storage is established by three weeks of age and gradually increases thereafter. Storage is distributed uniformly in most non-neuronal cells, and varies among neurons depending on subtype and location. Neurons in the CA_2, CA_3, and CA_4 regions of the hippocampus are heavily distended by cytoplasmic vesicles whereas neurons in CA_1 appear relatively unaffected. Brain regions with a high storage burden correspond to regions of weak β-glucuronidase activity in normal mice (Levy et al. 1996). Immunohistochemical staining showed a significant difference in the number of ubiquitin-positive inclusions in MPS VII and normal mice at 3 months of age (Heuer et al. 2002). By 5 months, additional markers of neuropathology are present including neurofilament inclusions, Fluoro-Jade positive cells, and astrogliosis. Following administration of an adeno-associated viral (AAV) vector expressing β-glucuronidase, markers of neuropathology were restored to normal levels in the transduced region (Heuer et al. 2002). Currently, viral vectors are limited in their ability to transduce cells throughout the brain. This limitation might be overcome, perhaps in the near future, through a strategic choice of vector serotypes and optimized routes of administration (Cearley et al. 2008, Hinderer et al. 2014).

Another promising approach to achieving widespread enzyme delivery throughout the brain is neural stem cell (NSC) transplantation. Over 20 years ago, a mouse NSC line was shown to migrate widely and secrete therapeutic levels of β-glucuronidase in MPS VII mice (Snyder et al. 1995). Since that time, key discoveries have made it possible to derive NSCs from easily accessible patient tissue, but *ex vivo* gene therapy using this approach has yet to be demonstrated.

III. Induced Pluripotent Stem Cells

Experiments by Briggs and King in the 1950s demonstrated that nuclei from frog blastocysts could be transferred to enucleated oocytes, giving rise to a healthy adult frogs (Briggs et al. 1952). John Gurdon later showed that nuclei from fully differentiated skin cells could accomplish the same feat (Gurdon et al. 1975). The clear implication of these studies was that nuclei removed from fully differentiated cells retained the epigenetic plasticity necessary to derive the entire embryo. Dolly the sheep famously demonstrated that this phenomenon was not just a quirk of frog biology (Wilmut 2003). Other milestones of reprogramming include the development of mouse and human embryonic stem cells (ESCs), derived from the inner cell mass of mice (Evans et al. 1981, Martin 1981) and men (Thomson et al. 1998).

Less than a decade ago, Shinya Yamanaka demonstrated that retroviral expression of 4 genes (the transcription factors Oct4, Sox2, Klf4, and c-Myc) was sufficient to reprogram somatic cells to a pluripotent state (Takahashi et al. 2006). When these induced pluripotent stem cells (iPSCs) were transplanted into a mouse blastocyst they were capable of forming chimeras and contributing to every cell type in an adult mouse, including germ cells (Wernig et al. 2007). Shortly thereafter, this same strategy was successfully applied to human cells using the same set of factors (Takahashi et al. 2007) or a different set (Oct4, Sox2, Nanog, and Lin28) (Yu et al. 2007). With these developments, the field of transcription factor-based nuclear reprogramming was born.

iPSCs can be generated using relatively simple procedures, leading to the rapid adoption of this technology as a means to study embryogenesis, create *in vitro* models of disease, and develop novel stem cell therapeutics. For extensive reviews on the rapid advances following Yamanaka's original discovery, see (Robinton et al. 2012) and (Stadtfeld et al. 2010). There has been debate over exactly how faithful these iPSCs are to blastocyst-derived ESCs, with some groups showing that the cell-of-origin biases iPSC gene expression, leaving behind an "epigenetic memory" (Mikkelsen et al. 2008, Kim et al. 2010, Sullivan et al. 2010). However, most of these studies used early passage iPSCs, and a preponderance of evidence has shown that iPSCs gradually lose this epigenetic memory over time. With sufficient passaging under the right

conditions and cultured in the same lab, iPSCs fall well within the normal variation of ESC lines in terms of DNA methylation status, histone modifications, and differentiation capacity (Bammler et al. 2005, Guenther et al. 2010), While some debate remains (Chin et al. 2010), iPSCs and ESCs can, for most practical purposes, be considered epigenetically and functionally equivalent (Smith et al. 2009, Zhao et al. 2009, Guenther et al. 2010).

Most labs currently working with iPSCs have been interested in one of three primary areas of research: basic mechanisms of reprogramming and the epigenetic basis of cell fate, disease modeling using patient-derived iPSCs, and cell therapy using patient-derived iPSCs (Kanawaty et al. 2009, Okano et al. 2014, Theunissen et al. 2014). The first labs to isolate iPSCs were focused on the mechanisms of how the pluripotent state arises and is maintained. iPSCs are valuable tools for addressing these questions and much has been learned regarding the molecular mechanisms of reprogramming. How exactly can so few TFs induce a genome-wide pluripotent state? Can small molecules speed up the process or make it more efficient? How closely related are pluripotent cells *in vitro* to their inner cell mass counterparts? Can any differentiated cell type be converted directly into any other through the proper combination of TF overexpression? Some of these questions have been answered using iPSC technology, and many questions remain (Hanna et al. 2010).

Application of high-throughput sequencing techniques yields insights related to global transcriptional and epigenetic networks and how these change during reprogramming (Kim et al. 2008, Ang et al. 2011). Studies comparing ESCs and blastocysts demonstrated that transcription factors involved in the reprogramming process, such as Oct4 and Nanog, are the same factors responsible for maintaining pluripotency in the early blastocyst (Marson et al. 2008). Global reorganization of gene expression, histone status, and DNA methylation has been found to be a highly coordinated process. Many factors are involved including polycomb repressor complexes (Boyer et al. 2006), DNA methyltransferases (Li et al. 2007, Deng et al. 2009), histone modification complexes (Lessard et al. 2010), microRNAs (Wang et al. 2013) and gene regulatory networks (Muraro et al. 2013). Using techniques such as ChIPseq, high-throughput DNA and RNA sequencing, and high-throughput analysis of global DNA methylation and histone status in

ESCs and iPSCs, we understand much more about the pluripotent state and cell differentiation than we did only a decade ago (Robinton et al. 2012).

Since the first derivation of human ESCs (Thomson et al. 1998), many labs have focused on developing protocols to generate specific cell types from pluripotent cells (Odorico et al. 2001). The advent of iPSC technology allows for the derivation of patient-specific cell types, useful for disease modeling and cell therapy, which would have been difficult or impossible to obtain otherwise.

IV. Generating and Utilizing Neural Lineages from Pluripotent Stem Cells

By the early 1990's, methods were established for the identification of NSCs, as well as the culture conditions and growth factors required to isolate and maintain primary fetal and adult NSCs *in vitro* (Cattaneo et al. 1990, Reynolds et al. 1992, Kilpatrick et al. 1993, Davis et al. 1994, Palmer et al. 1995). Shortly thereafter, the first therapeutic studies were carried out, using the MPS VII mouse as a disease model (Snyder et al. 1995). NSCs isolated from the cerebellum of neonatal mice, immortalized with the oncogene v-Myc (C17.2 cell line) (Ryder et al. 1990, Snyder et al. 1992), were successfully transplanted into the ventricles of neonatal MPS VII mice (Snyder et al. 1995). Not only did the cells survive but they migrated widely, delivering sufficient levels of the missing enzyme, β-glucuronidase (GUSB), to prevent storage lesions throughout the brain (Snyder et al. 1995). Unfortunately, the use of immortalized cell lines for transplantation has clear disadvantages, namely the strong likelihood of tumorigenesis or immune rejection in the host. The MPS VII mouse has served as a useful model for developing new, more clinically relevant gene and cell therapies in the brain thanks to its well characterized pathology, sensitive and quantitative enzyme assays, and a viable mechanism for correcting every cell in the brain (cross-correction) (Wolfe et al. 1990).

Tissue isolated from fetal brains has been proposed as an alternate source of NSCs and several small trials have evaluated this strategy, with mostly disappointing results (Bjorklund et al. 2000, Aboody et al. 2011, Thomsen et al. 2014). A rare example of success was recently published following up 2 patients with Parkinson's disease that had been injected with fetal

ventral mesencephalic tissue over 15 years ago. Both patients showed moderate motor improvement over time and were able to discontinue dopaminergic therapy (Kefalopoulou et al. 2014). Many similar trials involving fetal grafts for Parkinson's disease over the past 30 years have ended in failure, either because the grafted cells quickly took on the Parkinsonian phenotype of the surrounding cells (Kordower et al. 2008, Li et al. 2008) or because the grafted cells actually induced additional dyskinesias (Aboody et al. 2011, Lindvall 2013).

Clearly there is not enough basic knowledge of NSCs, regarding their diversity and their interactions with diseased environments, to proceed with large-scale clinical trials. More animal studies are needed to determine the optimal source of transplantable NSCs. If the fetal brain is not an optimal source, than what is? Pluripotent stem cells have the ability to differentiate into any cell type in the body, and can now be derived from a patient's own cells, making them particularly attractive candidates for NSC therapy.

Many protocols have been developed for the differentiation of pluripotent stem cells (primarily ESCs) into multipotent neural progenitors as well as mature neurons and glia. While specific protocols come with advantages and disadvantages, they all rely on similar mechanisms to recapitulate early neural development (Lanza et al. 2004, Abranches et al. 2009). Nearly every protocol that successfully induces neural lineage differentiation relies on a stepwise series of culture conditions, utilizing combinations of signaling molecules at specific dosages and time points to mimic *in utero* development. Confirming their functionally similar nature, ESCs and iPSCs respond the same way to various neuralization protocols (Hu et al. 2010).

Neuralization procedures first recapitulate early neurogenic signaling events involving molecules such as FGFs, Wnts, and BMPs (Wilson et al. 2001). The first example of directed neural differentiation from ES cells comes from (Bain et al. 1995). ESCs were detached from their mouse embryonic fibroblast (MEF) feeder layer to generate spherical aggregates, or "embryoid bodies" (EBs), which were then exposed to retinoic acid. Many cells within the EBs differentiated into neuron-like cells characterized by the firing of action potentials and expression of tetrodotoxin (TTX)-sensitive sodium channels, voltage-gated potassium channels, and calcium channels (Bain et al. 1995). Exposure to conditioned media from the HepG2 cell line also

increases the efficiency of EB conversion towards neural lineages (Rathjen et al. 2003). EBs can be dissociated and grown as a monolayer of neural progenitors, which led to the co-culture of ESCs with various cells lines in an attempt to convert ESCs even more efficiently. It was found that certain mouse stromal lines, e.g. PA6 (Kawasaki et al. 2000) or MS5 (Barberi et al. 2003), support neural differentiation from ESCs quite efficiently.

A crucial refinement to the basic EB protocol by (Okabe et al. 1996) involved plating 4 day-old EBs in fetal calf serum to promote attachment, followed by growth in a minimal neural induction media. Further passaging of these cells in an optimized NSC media, plus the addition of cytosine arabinose to inhibit astrogliogenesis, yields relatively pure populations of transplantable NSCs (Okabe et al. 1996).

One of the easiest and most efficient neural differentiation protocols is known as “dual SMAD inhibition” (Chambers et al. 2009), which involves the application of only two crucial morphogens (Noggin, an inhibitor of TGF-β proteins including BMP-4, and the small molecule SB431542, an inhibitor of several activin receptor-like kinases [AKTs]) (Lamb et al. 1993, Laping et al. 2002). Following the application of these 2 morphogens, over 80% of ESC or iPSCs become $PAX6^+$ early neural progenitor cells that can be further differentiated towards various mature neuronal subtypes (Chambers et al. 2009).

Another important development in the generation of transplantable NSCs does not involve pluripotent cells at all. Combinations of transcription factors were shown by several groups to directly generate neural progenitors, thus bypassing the pluripotent stage (Han et al. 2012, Lujan et al. 2012, Sheng et al. 2012). This method has several advantages, specifically a lower risk of tumorigenesis following transplantation, as well as the speed at which these cells can be generated and characterized. It remains to be seen how faithful these cells are to endogenous and pluripotent cell-derived NSCs, but so far the data seem promising (Lujan et al. 2012).

One particularly interesting feature of neural differentiation is that it seems to be the default pathway for differentiating pluripotent cells (Munoz-Sanjuan et al. 2002). This indirectly led to the discovery that BMP-4 is the primary inhibitor of neural induction (Wilson et al. 1995). If

human ESC cultures, in the absence of exogenous factors, remain in the same culture dish for several weeks, neural cells begin to emerge (Reubinoff et al. 2001). The addition of FGF and EGF enhance this induction, allowing NSCs to be cultured as a relatively homogenous population expressing the neural markers nestin, vimentin, and PAX6 (Reubinoff et al. 2001).

While there are differences in neurodevelopmental signaling pathways between mice and humans, they appear to be more alike than different (Moon et al. 2006). Similar protocols yield remarkably similar results and human pluripotent stem cell-derived neural progenitors can functionally integrate into cortical circuits in mice, although this process can take several months (Espuny-Camacho et al. 2013). One significant example of a species-specific difference is that Sox1 is the first neural-associated gene to be expressed in mice while Pax6 (expressed before Sox1) is both necessary and sufficient for neurectodermal formation in humans (Zhang et al. 2010).

There are several different protocols that give rise to early neural progenitors, but every method has drawbacks, depending on the application in question. Most protocols generate neural lineages by recapitulating early neural development. In doing so, they suffer the same fate as endogenous neural progenitors, demonstrating a shift from a neuronogenic to a gliogenic bias after many cell divisions, mimicking the situation seen in normal brain development (Abranches et al. 2009, Edri et al. 2015). Several groups have tried to overcome this bias by selectively culturing cells at an early “rosette” stage of neural development, reminiscent of the early neural tube. It has been shown that such cells can retain a broad and consistent differentiation potential over many passages (Elkabetz et al. 2008, Koch et al. 2009).

Large scale clinical application of ESC/iPSC-derived NSCs will require a level of consistency that is difficult to obtain using cells that change significantly with passage number. It would require redifferentiating each batch of cells and extensively testing them for batch-to-batch variability. So far, a protocol developed by Koch et al. is unique in that it produces cells that maintain a consistent gene expression profile and differentiation capacity over 100 passages at an NSC stage (Koch et al. 2009). This method was developed using ESC lines but also works with iPSCs (Falk et al. 2012). A protocol with similar properties may be ideal for transplantation

studies or future clinical use. These “long-term self-renewing NSCs” do show a bias towards generating GABAergic neurons of the hindbrain upon passive withdrawal of growth factors, but the cells remained responsive to regionalization cues across time, generating both dopaminergic and motor neurons in appropriate culture conditions (Koch et al. 2009).

V. Rationale

We have devised and evaluated a comprehensive strategy for the treatment of lysosomal enzyme deficiencies in the brain involving the reprogramming, differentiation, and genetic correction of diseased somatic cells. We chose MPS VII as a model system because it has been well characterized and for its long history as a platform for the development of novel therapeutics, particularly in the brain (Snyder et al. 1995). We used recently developed techniques to reprogram somatic tissue into pluripotent cells with the rationale that patients would be less likely to mount a counter-productive immune response against autologous cells. We decided that NSCs would be most appropriate for cell-based therapy in the brain based on previous studies demonstrating that NSC cell lines and primary NSCs can respond appropriately to developmental cues and migrate widely (Flax et al. 1998, Gage et al. 2013). Importantly, immortalized NSCs have already proven to be effective following transplantation into neonatal and adult MPS VII mice (Snyder et al. 1995, Buchet et al. 2002).

We hypothesized that a comprehensive strategy, beginning with diseased MPS VII patient fibroblasts and ending with corrected patient NSCs, would effectively treat an immunodeficient mouse model of MPS VII. To test this, we derived patient iPSCs from frozen fibroblasts using a modification of the Park et al. protocol (Park et al. 2008), omitting 2 of the 6 reprogramming factors (hTERT and the SV40 large T antigen) to reduce the risk of tumogenesis. We then used a protocol previously shown to convert ESC/iPSCs into a stable long-term self-renewing population of NSCs (Koch et al. 2009). We reasoned that such a population of patient-derived iPSC-NSCs would be ideal for expansion, genetic correction, and transplantation. This combination of cellular reprogramming and genetic engineering techniques is a logical step towards developing personalized cell-based therapies in the brain.

CHAPTER 2

Genetically Corrected Neural Stem Cells

Generated from MPS VII Patient Fibroblasts

This chapter is adapted from Griffin TA, Anderson HC, and Wolfe JH. Ex Vivo Gene Therapy Using Patient iPSC-Derived NSCs Reverses Pathology in the Brain of a Homologous Mouse Model, Stem Cell Reports (Griffin et al. 2015).

I. Introduction

Induced pluripotent stem cells are promising candidates for treating many diseases in the brain. Unfortunately, disease-related mutations can impede this process. It may not be possible to generate iPSCs from all patients, and differentiation towards therapeutic cell types can be impeded as well (Hamasaki et al. 2012, Ogawa et al. 2013). It has been reported that iPSCs from mouse models of some LSDs (Sandhoff and MPS VII) are defective in their ability to differentiate towards neural lineages, which may severely hamper efforts to generate patient specific iPSC-derived NSCs suitable for transplantation (Meng et al. 2010, Ogawa et al. 2013). We hypothesized that β-glucuronidase (GUSB) deficiency should not be an obstacle to the reprogramming of MPS VII patient cells because of the cross-correction process. Exogenous GUSB derived from the mouse embryonic feeder (MEF) layer upon which iPSCs are generated should serve as an adequate source of enzyme for deficient cells (Fratantoni et al. 1968). However, differentiation requires that iPSCs be removed from the MEF feeder layer, and it was unclear whether differentiation towards neural lineages would be impaired relative to control iPSCs.

The current study demonstrates that fibroblasts from a female patient with MPS VII, frozen for ~30 years, can be reprogrammed through retroviral overexpression of Oct4, Sox2, Klf4, and c-Myc. The GUSB mutations in these fibroblasts and consequent MPS VII phenotype has been extensively described elsewhere (Wu et al. 1994). We show that these MPS VII iPSCs display stringent correlates of pluripotency including teratoma formation. MPS VII iPSCs were successfully differentiated alongside control iPSCs into long-term self-renewing NSCs using a protocol developed for ESCs described previously (Koch et al. 2009). Finally, a functional version of the GUSB gene was introduced into MPS VII iPSC-derived NSCs using a DNA transposon-based vector (PiggyBac), demonstrating a comprehensive strategy that may be useful for generating clinically relevant transplantable NSCs in the future.

II. Results

Generation and characterization of MPS VII iPSCs

Frozen dermal fibroblasts from a patient with MPS VII (GM02784, Coriell Institute) were thawed, expanded, and transduced with VSV-G pseudotyped retroviral vectors expressing OCT4, SOX2, KLF-4, and c-MYC to initiate reprogramming. This patient was previously reported to have 1.4% of normal β-glucuronidase activity in cultured fibroblasts (Wu et al. 1994). Despite the fact that these fibroblasts had been frozen for ~30 years, colonies of ESC-like cells emerged alongside aggregates of partially reprogrammed cells, consistent with previous reports (Chan et al. 2009). One line was selected for further characterization.

To confirm the pluripotency of MPS VII iPSCs, standard *in vitro* and *in vivo* assays were performed. The putative MPS VII iPSC line displayed typical ESC/iPSC-like colony morphology **(Fig. 2.1a)** and expressed multiple markers of pluripotency including SSEA4, Tra-1-60, and Tra-1-81 **(Fig. 2.1b-d)**. Cells injected into immunodeficient mice formed teratomas containing the three primary germ layers. Hematoxylin and eosin stained teratoma sections revealed structures typical of endoderm, ectoderm, and mesoderm **(Fig. 1e-h),** and immunostained teratoma sections were positive for markers of each lineage **(Fig. 2.1i-k)**. iPSCs were passaged >40 times with no change in morphology or pluripotency marker expression.

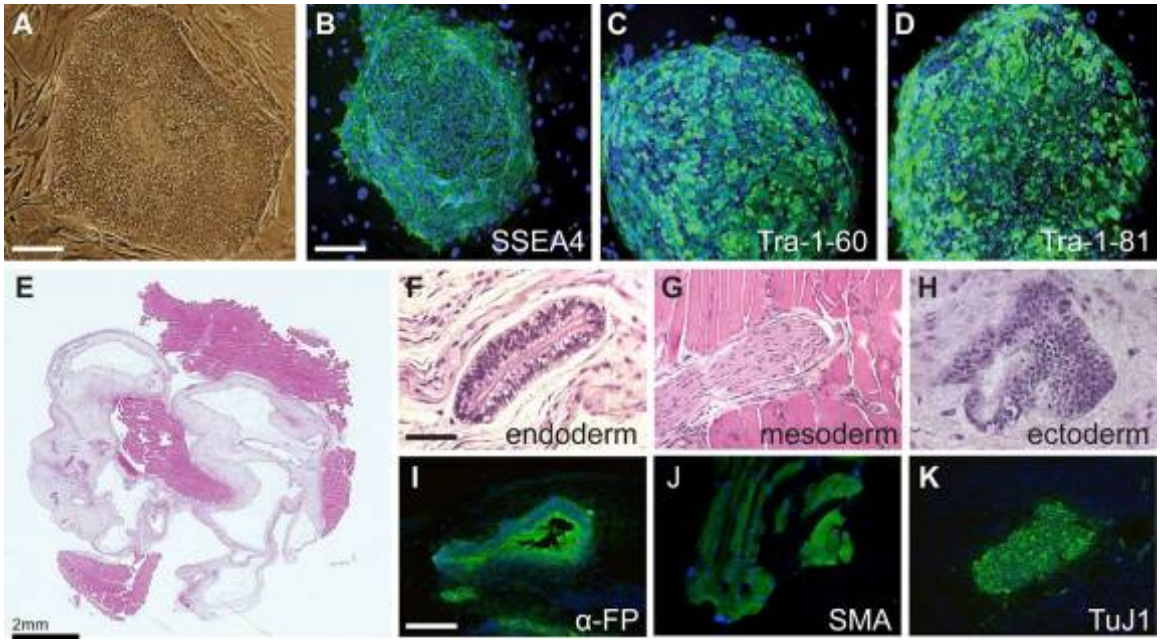

Fig. 2.1 Generation of iPSCs from an MPS VII patient. Fibroblasts from a female patient with MPS VII (GM02784) were transduced with retroviral vectors expressing the reprogramming factors Oct4, Sox2, Klf4, and c-Myc. A putative iPSC line was isolated and expanded under serum-free conditions on a feeder layer of mouse embryonic fibroblasts. MPS VII iPSC colonies, seen in the phase contrast image **(a)**, expressed markers of pluripotency **(b-d)**. After injection into immunodeficient mice, MPS VII iPSCs formed teratomas **(e)** that differentiated into the three primary germ layers as shown by H&E stained sections **(e-h)** and germ layer specific marker expression **(i-k)**. α-FP = α-fetoprotein, SMA = smooth muscle actin, Tuj1 = βIII-tubulin, scale bars = 200µm (except in **e**).

Differentiation of MPS VII iPSC-NSCs

MPS VII and control iPSCs were passaged at least 20 times before being subjected to an adapted NSC differentiation protocol (Koch et al. 2009) **(Fig. 2)**. After iPSCs were removed from the MEF feeder layer and grown in suspension culture containing FBS, they formed large spherical aggregates. These "embryoid bodies" were plated and grown in a minimal neural induction medium. A variety of cell types grew outward from the plated aggregates, including many with neurite-like extensions. After approximately two weeks, neural tube-like structures began to form on some cell aggregates, consisting of a raised ring surrounding a central lumen (Elkabetz et al. 2008). These rosette structures were isolated and grown as neurospheres for two days, after which they were dissociated and plated, yielding an adherent monolayer. No obvious differences were observed between normal and MPS VII iPSCs during the differentiation procedure at any point and both yielded a relatively homogenous population of putative neural stem cells **(Fig. 2.2)**.

Characterization of MPS VII iPSC-NSCs

The majority of the iPSC-NSCs (89.9 ± 2.9%) retained expression of the neural stem cell marker nestin **(Fig. 3a)**. The rate of spontaneous differentiation into MAP2-positive neurons (9.5 ± 0.8%) and GFAP-positive astrocytes (0.4 ± 0.6%) was low **(Fig. 2.3a,b).** Importantly, no cells expressed the pluripotency marker Tra-1-60 or the reprogramming factor Oct4 **(Fig. 2.3a,b)**. The generation and culture of iPSCs from frozen MPS VII fibroblasts and the subsequent differentiation and propagation of iPSC-NSCs did not introduce any gross chromosomal abnormalities, as shown by a normal 46,XX karyotype **(Fig. 2.3c).** To test the differentiation capacity of these cells, they were grown in terminal differentiation medium without growth factors for one month. Differentiating conditions yielded neurons and astrocytes **(Fig. 2.3d)**, as measured by MAP2 (86.4 ± 1.6%) and GFAP (13.9 ± 8.2%) expression, respectively **(Fig. 2.3e)**. The majority of cells (74.8 ± 5.2%) were positive for the inhibitory neurotransmitter GABA, while there was no evidence of tyrosine hydroxylase-positive dopaminergic neurons **(Fig. 2.3d,e)**.

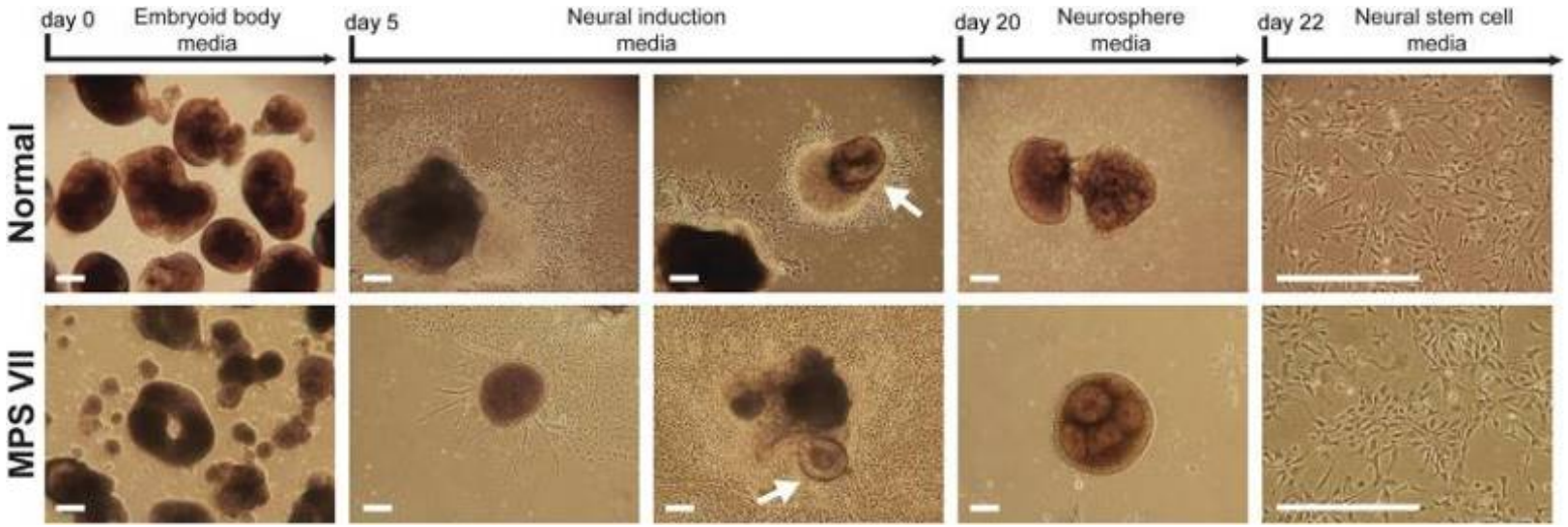

Figure 2.2 Differentiation of NSCs from MPS VII and normal iPSCs. Control and MPS VII iPSCs were removed from their MEF feeder layer and grown in suspension culture with FBS for five days to induce embryoid body formation. The cell clusters were plated onto poly-ornithine coated dished and grown in neural induction media. Neural tube-like structures (white arrows) began to appear and were manually isolated at day 20 and cultured in suspension in neurosphere media for two days. Neurospheres were trypsinized and plated onto poly-ornithine/laminin coated dishes. Trypsinized cells were passaged as nestin-positive NSCs indefinitely.

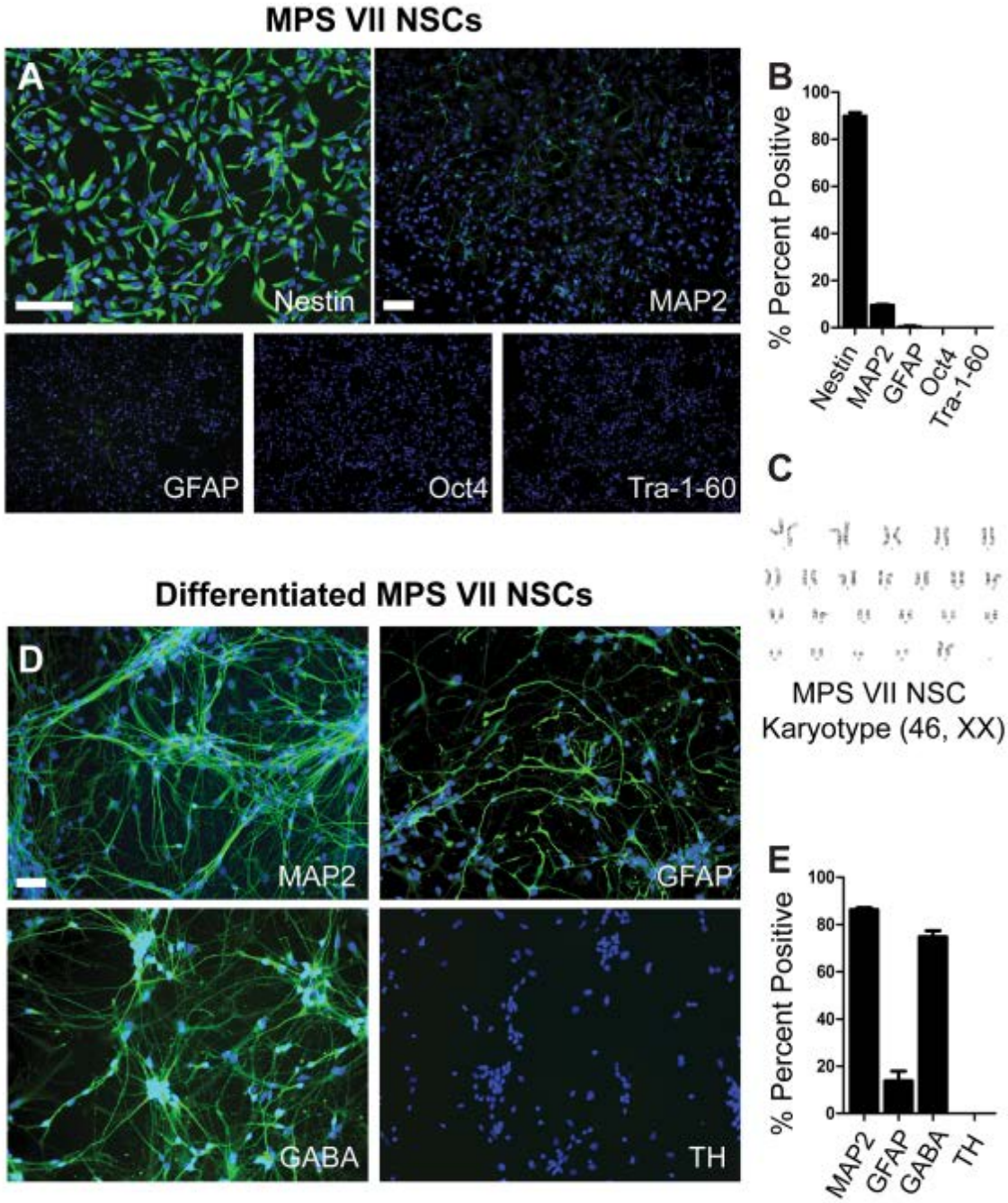

Figure 2.3 Characterization and terminal differentiation of MPS VII iPSC-NSCs

Figure 2.3 Characterization and terminal differentiation of MPS VII iPSC-NSCs.

(a,b) MPS VII NSC cultures expressed nestin (89.9 ± 1.5%) and showed low levels of spontaneous differentiation towards neurons (MAP2, 9.5 ± 0.4%) and astrocytes (GFAP, 0.4% ± 0.3%). No cells expressed the reprogramming factor Oct4 or the pluripotency marker Tra-1-60. **(c)** MPS VII iPSC-NSCs had a normal 46,XX karyotype. **(d,e)** NSCs were subjected to terminal differentiation by the removal of growth factors and the addition of cAMP for one month. MAP2-positive neurons comprised 86.4% ± 0.8% of all differentiated cells. The majority of those cells (74.8% ± 2.6%) were GABA-positive and none of the cells stained positive for the dopaminergic marker tyrosine hydroxylase (TH). GFAP-positive astrocytes comprised 13.9% ± 4.1% of all differentiated cells. Data are represented as the mean ± SEM, n=4 independent cultures. Scale bars = 200µm

Generation of GFP labeled and GUSB expressing MPS VII iPSCs

To introduce GFP into NSCs for the purpose of tracking their engraftment following transplantation, we first evaluated previously optimized transduction conditions using VSV-G pseudotyped lentiviral vector preps that had been extensively validated both *in vitro* and *in vivo*. Surprising, iPSC-derived NSCs proved exquisitely sensitive to lentiviral vectors. Multiple attempts with different pseudotypes consistently resulted in cell death at MOIs >10 and were ineffective at transducing cells when the MOI < 10. We therefore decided to switch to a PiggyBac transposon-based system consisting of 2 plasmids. The first plasmid contains terminal repeats flanking two promoter units in tandem. The 5' promoter (CMV) drives expression of GUSB, followed by the EF1α promoter, which drives expression of both GFP and a puromycin resistance gene via a 2A polypeptide linker. The second plasmid transiently expresses a transposase capable of integrating plasmid sequence bounded by terminal repeats into the genome at TTAA sites. The use of a Lonza nucleofector and proprietary transfection reagents was very efficient at introducing vectors into iPSC-derived NSCs **(fig..2.4a)**. However, when we placed a gene downstream of the CMV promoter (or CAG, switched to avoid promoter shutdown in the brain (Gray et al. 2011)), we observed very high levels of the transgene driven by the upstream

promoter, but expression of GFP and the puro resistance gene driven by EF1α was barely detectable.

The iPSC-NSCs were electroporated with a PiggyBac vector containing *GUSB* cDNA driven by the CAG promoter. A separate culture of the same passage of NSCs was prepared as a negative control by electroporating them with a mock-correction vector containing *GUSB* cDNA in the reverse orientation (revGUSB). Following puromycin selection, mock-corrected MPS VII iPSC-NSCs had negligible GUSB activity (1.3 ± 1.3 nmol 4-MU/µg protein/ hr) while the corrected cells showed strong GUSB activity (116.8 ± 2.7 nmol 4-MU/µg protein/hr, $p<0.01$), comparable to an iPSC-NSC line derived from a healthy control (112.3 ± 3.1 nmol 4-MU/µg protein/hr, $p >0.05$) **(Fig. 2d)**.

To evaluate the issue of low expression from the downstream promoter, several transgenes or non-coding sequences were placed under the control of the upstream CAG promoter (GUSB, revGUSB, sialidase, EGFRVII, reprog), all of which had the same effect on GFP expression. Expression of the puromycin resistance gene was good enough to select for transduced cells **(fig. 2.4d)** thanks in part to the sensitivity of iPSC-NSCs to puromycin, but no GFP could be visualized, even after immunohistochemistry for GFP protein. We therefore used three populations of cells for future transplantation experiments: uncorrected/GFP-positive NSCs, corrected/GFP-negative NSCs, and mock-corrected/GFP-negative cells. Further experiments using 293t cells demonstrated that replacing CAG with the minimal GUSB promoter results in much higher levels of GUSB as well as restoring strong expression of GFP and puromycin resistance from the EF1α promoter **(fig. 2.5)**.

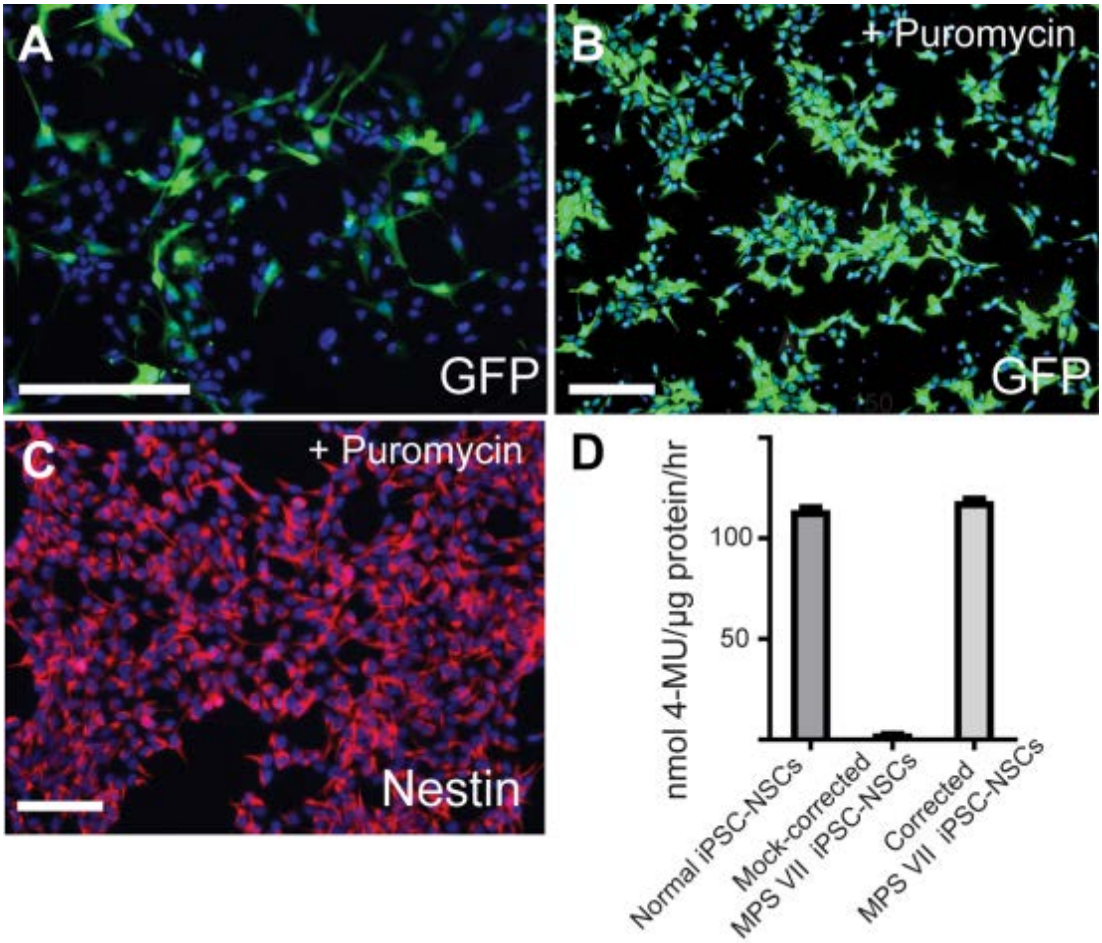

Figure 2.4 A PigyBac transposon vector efficiently introduces GFP or GUSB into MPS VII iPSC-derived NSCs. **(a)** A PiggyBac transposon-based plasmid encoding GFP and a puromycin resistance gene was electroporated into MPS VII iPSC-NSCs. **(b)** Puromycin was applied for 7 days in order to select for NSCs that had been stably transduced. **(c)** MPS VII iPSC-NSCs retained their nestin expression and growth capacity after electroporation and puromycin selection. **(d)** Levels of β-glucuronidase expression in iPSC-derived NSCs from a normal control compared to MPS VII iPSC-derived NSCs receiving PiggyBac vector containing GUSB cDNA in the reverse orientation (mock-corrected) or GUSB in the forward orientation (corrected)**.**

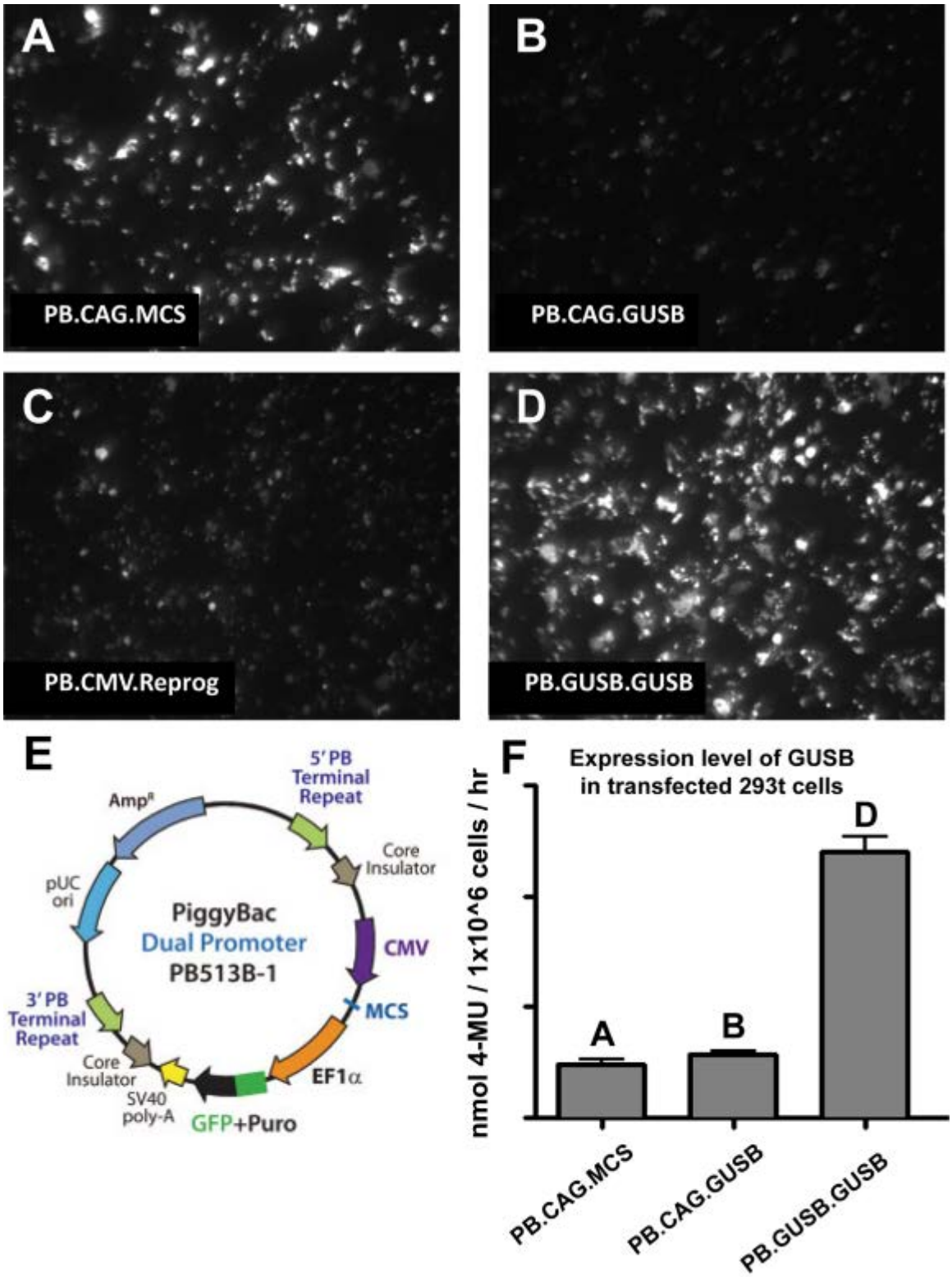

Figure 2.5 Effect of Promoter Choice on GUSB and GFP levels

Figure 2.5 The Effect of Promoter Choice on GUSB and GFP levels. The dual promoter PiggyBac system (Systems Biosciences) vector suffers from a major flaw. When a coding sequence (or non-coding sequence [revGUSB]) **(b,c)** is inserted into the multiple cloning site downstream of the CMV or CAG promoter, GFP expression from the EF1α promoter is severely reduced. This is consistent with either promoter-shutdown or frank toxicity, both of which have been described previously (van den Pol et al. 1998, Papadakis et al. 2004). **(e)** Map of the dual expression plasmid vector. **(f)** Transient transfection of 293t cells with PB.GUSB.GUSB shows improved GFP expression from the second promoter and much higher levels of GUSB expression. PB.CAG.MCS = PiggyBac driven by the CAG promoter with nothing inserted into the multiple cloning site. PB.CAG.GUSB = PiggyBac driven by the CAG promoter with GUSB cDNA inserted into the multiple cloning site. PB.CMV.Reprog = PiggyBac driven by the CMV promoter with a quadrivalent set of reprogramming cDNAs inserted into the multiple cloning site. PB.GUSB.GUSB = PiggyBac driven by the minimal GUSB promoter (~500 bp upstream of the start site) with GUSB cDNA inserted into the multiple cloning site.

III. Discussion

First and foremost, these results demonstrate that a deficiency in GUSB does not preclude the generation of iPSCs or neural stem cells. Given that deficient fibroblasts have access to exogenous enzyme from the MEF feeder layer during the reprogramming process, we predicted that GUSB deficiency would not be an obstacle to reprogramming. It would have been preferable to use fresh patient cells, but the scarcity of MPS VII patient samples precluded this possibility. Because our patient sample had been frozen so long ago, and given how extensively it has been utilized, it's unclear how many divisions these cells have gone through. Both of these factors (long-term freeze and high passage number) are known to reduce the efficiency of reprogramming. As somatic cells divide, several factors decrease the efficiency of reprogramming, notably telomerase length (Huang et al. 2011). We modified the iPSC generation protocol developed by Park et al. through the omission of the reprogramming factors telomerase (hTERT) and the SV40 large T antigen (Park et al. 2008). Potential consequences of including these factors include tumorigenesis and chromosomal instability (Ouellette et al. 2000, Ali et al. 2001). Many groups have derived iPSCs without these factors, and we decided to omit them at the price of reprogramming efficiency.

Subsequent to the generation of our MPS VII iPSCs, an article was published reporting the generation of iPSCs from three mouse models of LSD, including MPS VII (Meng et al. 2010). Of the LSDs examined, MPS VII iPSCs displayed defects in their differentiation capacity as measured by a significant decrease in the number and size of embryoid bodies generated when iPSCs were removed from their MEF feeder layer. Exogenous application of recombinant β-glucuronidase partially corrected this defect (Meng et al. 2010). Another paper using a mouse model of the LSD Sandhoff disease showed specific defects in neural differentiation, raising further doubts about the ability to generate neural lineages from our human MPS VII iPSCs (Ogawa et al. 2013).

In seeming contradiction to the Meng et al. study, when our MPS VII iPSCs were removed from the MEFs alongside control iPSCs, we saw no obvious differences in the ability to

generate neural lineages or long-term self-renewing NSCs. Many factors could explain this discrepancy, including subtle differences in differentiation protocols, species-specific differences, or low levels of GUSB activity in the human iPSCs. While undetectable using fluorometric methods in our lab, the patient fibroblasts were previously reported to express 1.4% normal levels of β-glucuronidase (Wu et al. 1994). Human GUSB mutations resulting in the complete absence of β-glucuronidase activity usually result in pre-term or neonatal mortality (Vervoort et al. 1997, Vervoort et al. 1997, Tomatsu et al. 2009). In contrast, MPS VII mice can be viable in the complete absence of detectable β-glucuronidase activity, indicating the possibility of species-specific differences (Sands et al. 1993). Whatever the reason, our results clearly show that MPS VII patient fibroblasts can be reprogrammed. Furthermore, they can be removed from all potential sources of exogenous enzyme, and differentiated into long-term self-renewing NSCs capable of generating mature neurons and glia.

CHAPTER 3

MPS VII iPSC-Derived Neural Stem Cells Engraft Widely in Neonates

and Correct Microglial Pathology in Adult MPS VII Mice

This chapter is adapted from Griffin TA, Anderson HC, and Wolfe JH. Ex Vivo Gene Therapy Using Patient iPSC-Derived NSCs Reverses Pathology in the Brain of a Homologous Mouse Model, Stem Cell Reports (Griffin et al. 2015).

I. Introduction

A combination of iPSC generation from easily accessible patient tissue, NSC differentiation protocols, *ex vivo* gene therapy, and transplantation directly back into the patient, may be an effective strategy for treating many LSDs. In this chapter we transplant MPS VII iPSC-derived NSCs into the brains of neonatal and adult mice with MPS VII. To prevent immune rejection of human cells we used the immunodeficient NOD/SCID/MPS VII model previously described (Hofling et al. 2003). GFP-labeled MPS VII iPSC-derived NSCs were transplanted in the ventricles of neonatal NOD/SCID and NOD/SCID/MPS VII littermates to evaluate the patterns and persistence of engraftment over the course of 4 months. Corrected or mock-corrected MPS VII NSCs were also injected into the post-symptomatic striatum of 2 month old mice to evaluate the extent of disease correction. We identified CD68-positive, activated microglia as an easily quantifiable biomarker of CNS pathology in these animals. This strategy permitted us to visualize engrafted cells and neuropathology in the same samples. We further validated this method using an adeno-associated viral vector expressing β-glucuronidase in MPS VII mice.

Following neonatal intraventricular transplantation, iPSC-NSCs engraft throughout the rostrocaudal axis of the CNS, primarily within white matter tracts, and survive for at least four months with little evidence of differentiation. Genetically corrected and mock-corrected cells, while dispersed widely, were unable to correct microglial pathology. Transplanted cells exited the cell-cycle rapidly and no differences were found between MPS VII and normal iPSC-NSCs engrafted in normal and MPS VII mice. Genetically corrected MPS VII iPSC-NSCs transplanted post-symptomatically into the striatum of adult NOD/SCID/MPS VII mice reversed neuropathology in a zone surrounding the grafts, while control mock-corrected grafts did not. MPS VII and control iPSC-NSCS did not migrate from the injection site, more consistent with data from primary NSCs than immortalized NSC lines. The results suggest that improvements are needed to increase survival, enzyme expression levels, and migration, especially in adult animals. However, the dramatic amelioration of microglial pathology surrounding the corrected grafts provides a

compelling argument for *ex vivo* gene therapy in the brain using human NSCs from autologous, non-neural tissues.

II. Results

MPS VII patient iPSC-derived NSCs engraft widely in the neonatal brain

To assess the engraftment potential of MPS VII iPSC-NSCs, GFP-labeled cells were injected intraventricularly into neonatal mice, which provide a more hospitable environment for engraftment relative to the adult brain (Snyder et al. 1995). Over 100 NOD-SCID neonates were injected with iPSC-NSCs between passages 15-25 with no evidence of deleterious effects in behavior or gross brain structure. By 1 month post-transplant, cells had engrafted along the rostrocaudal axis of the brain and were primarily found in periventricular regions and white matter tracts **(Fig. 3.1)**. Regardless of location, the engrafted cells expressed nestin and had an immature morphology **(Fig. 3.1, lower panels)**. GFP-labeled MPS VII iPSC-NSCs were also transplanted into NOD/SCID/MPS VII neonates. At 4 weeks post-transplant the distribution of normal and MPS VII iPSC-NSCs engrafted cells was similar to the distribution in non-MPS VII NOD/SCID littermates, with cells found predominantly in the ventricles surrounding the hippocampi **(Fig. 3.2)**. Thus the disease state of the mice did not alter the engraftment properties of donor cells. The iPSC-NSCs survived for at least four months **(Fig. 3.3)** with the donor cells predominantly located in the white matter at that time point. Engrafted iPSC-NSCs remained in an immature stage even after four months *in vivo*, as indicated by human-specific nestin immunostaining **(Fig. 3.3)**. Interestingly, the same cells injected into the striatum of neonatal mice did reveal some evidence of differentiation to DCX-positive and β-III tubulin-positive neuronal cells **(Fig. 3.4)**. Despite their immature phenotype, transplanted iPSC-NSCs quickly exited the cell cycle. At 1 week post-transplant, only a few cells expressed the cell proliferation marker Ki67 and no Ki67-positive cells were seen at 4 or 16 weeks post-transplant **(Fig. 3.5)**.

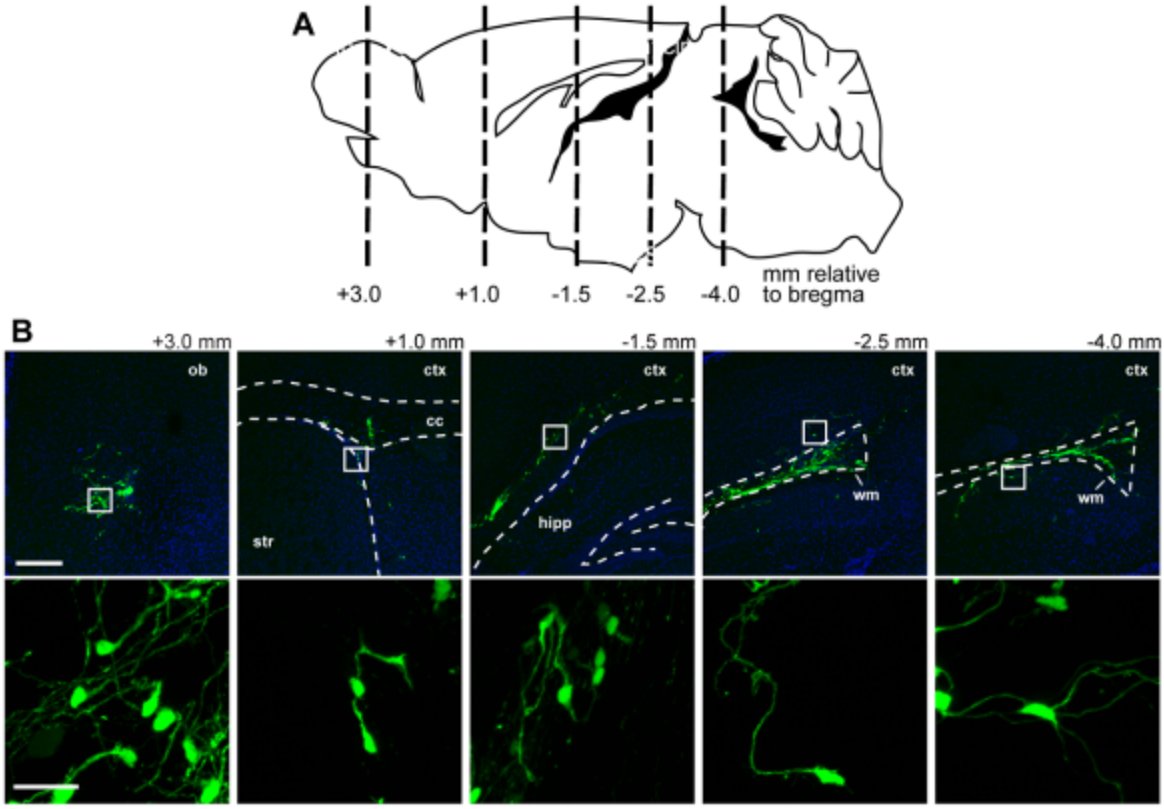

Fig. 3.1 Distribution of Engrafted MPS VII iPSC-NSCs. 100,000 GFP-labeled iPSC-NSCs were injected into the lateral ventricles of neonatal NOD/SCID mice. **(a)** Diagram of the brain shows the relative rostral-caudal positions of the images below. **(b)** One month post-transplant, cells were found along the rostral-caudal axis of the brain, often adjacent to ventricles and within white matter tracts. The areas within the white boxes are enlarged in the bottom panels, showing the morphology of engrafted iPSC-NSCs. Scale bars = 500µm in upper panels of **(b)** and scale bar = 200µm in lower panels of **(b)**.

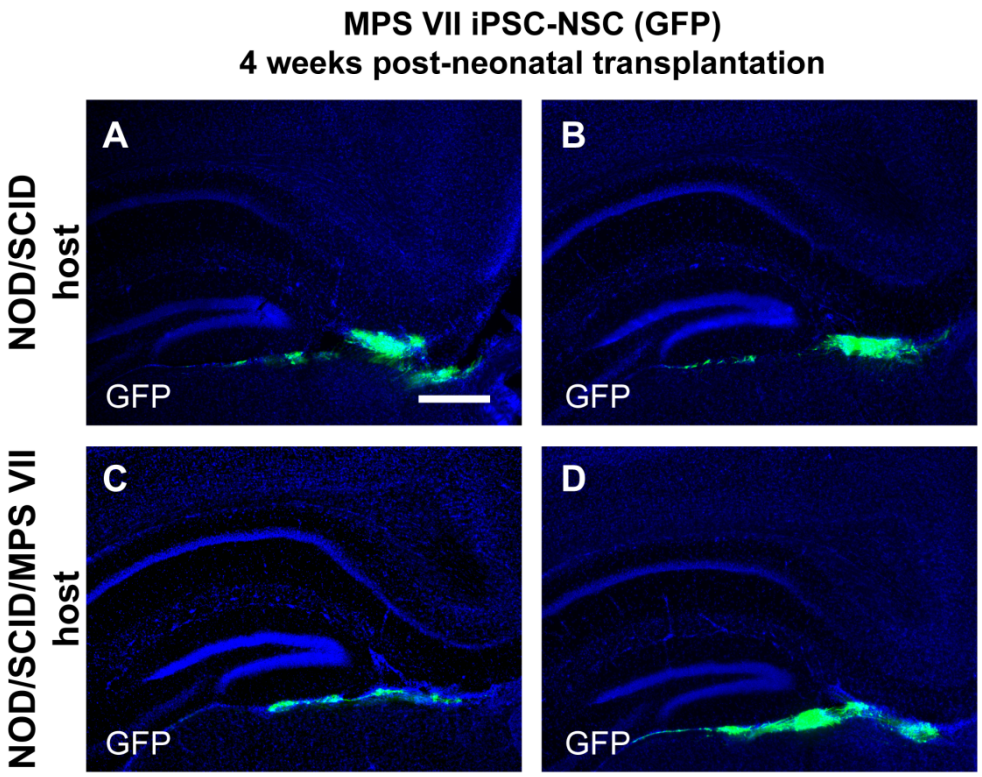

Figure 3.2 Host Genotype does not Affect Neonatal Engraftment.

(a,b) Representative sections from two NOD/SCID animals transplanted with GFP labelled MPS VII iPSC-NSCs 4 weeks following neonatal transplantation. **(c,d)** Similar sections from 2 NOD/SCID/MPS VII animals. Regardless of host genotype, transplanted cells are predominantly found in near ventricles, often surrounding the hippocampus. Scale bar = 200µm.

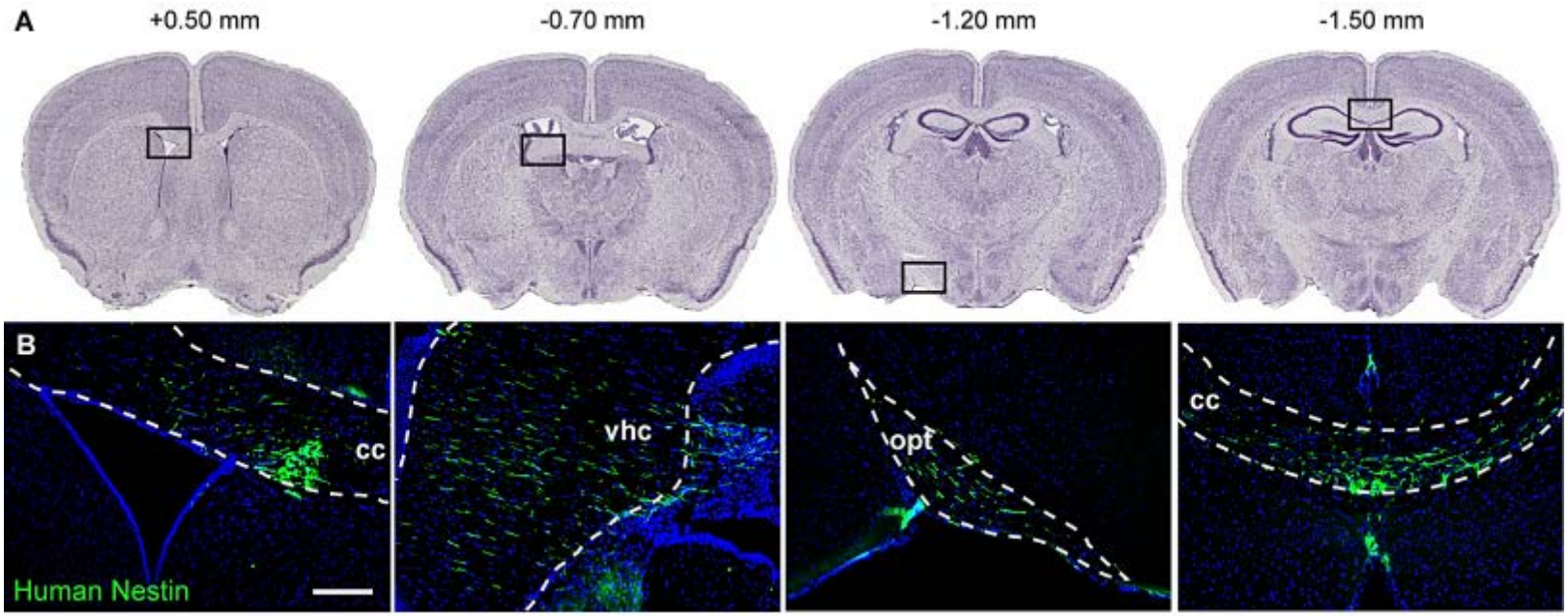

Figure 3.3 MPS VII iPSC-NSCs survive and retain nestin expression four months following intraventricular injection. The presence of engrafted iPSC-NSCs was revealed by immunostaining four months after neonatal transplantation. The highest concentrations were found in and along white matter tracts. **(a)** Black boxes over Nissl stained coronal sections show the locations of engrafted MPS VII iPSC-NSCs seen below. The location relative to bregma (in mm) is shown above each section. **(b)** Engrafted MPS VII iPSC-NSCs remained nestin-positive even after four months *in* vivo. Scale bar = 200µm, cc = corpus callosum, vhc = ventral hippocampal commissure, opt = optic chiasm. Nissl stained sections from the Allen Mouse Brain Atlas (Allen Institute for Brain Science).

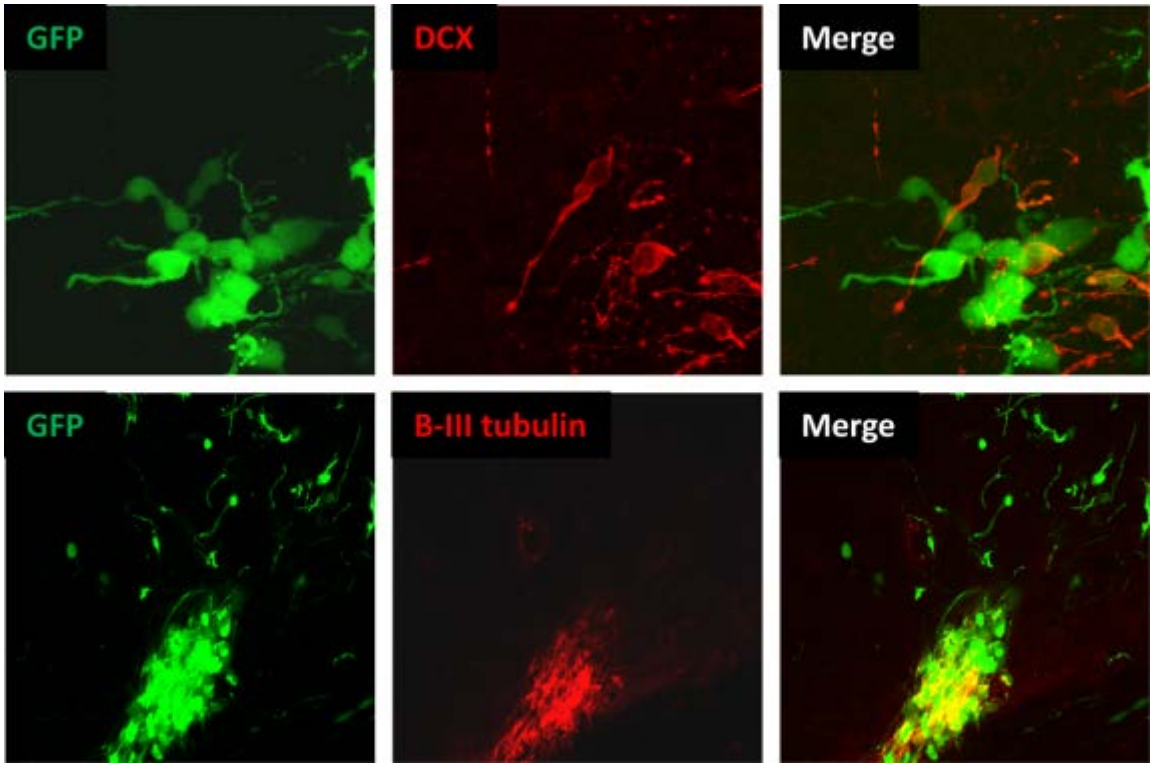

Figure 3.4 Differentiation of MPS VII iPSC-derived NSCs in neonatal striatum.

When uncorrected GFP-labeled cells were injected into the stratum of NOD/SCID mice, we saw very little migration after one month. However, there was some evidence of differentiation. DCX-positive neuronal precursors with leading processes could be seen migrating away from the injection site **(top row)**, while β-III tubulin-positive cells remained at the injection site **(bottom row)**. Mouse neurons are not seen in the background because the antibody binds much more strongly to human β-III tubulin. This result shows that environmental signals play a role in determining the differentiation status of engrafted cells within the neonatal mouse brain.

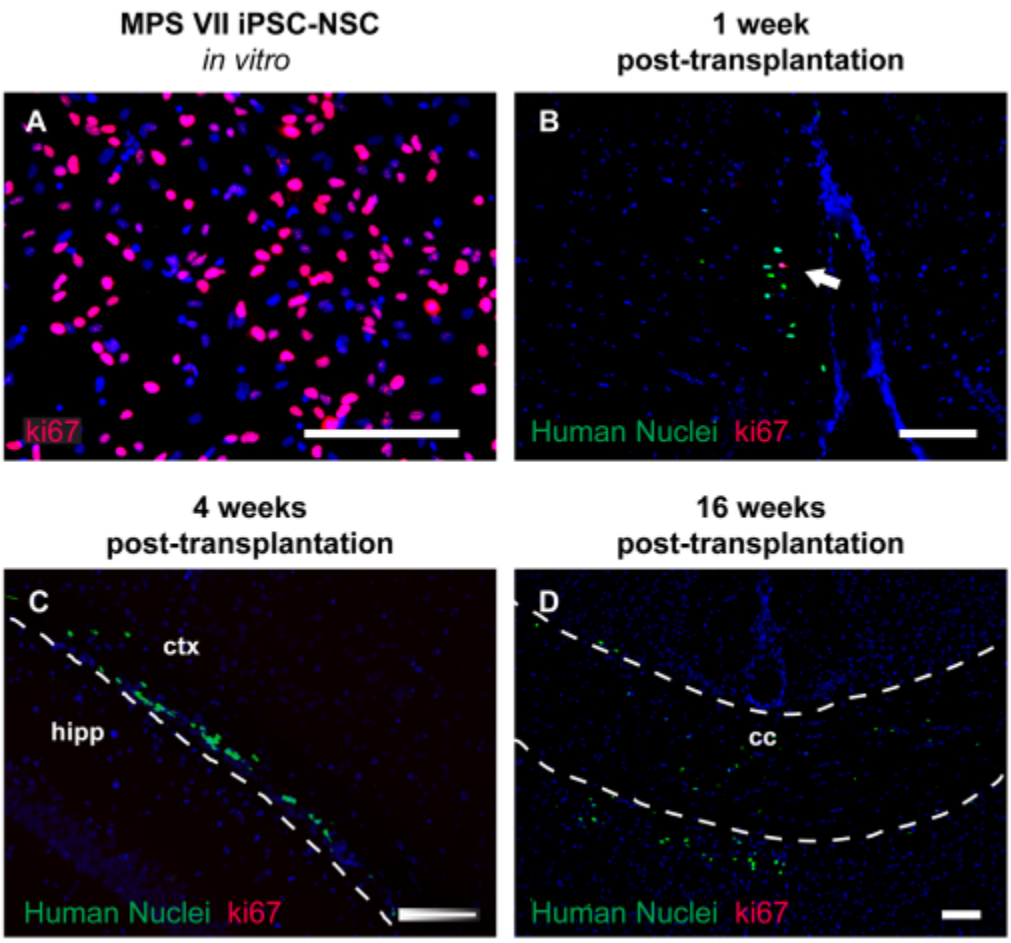

Figure 3.5 MPS VII iPSC-NSCs Quickly Exit the Cell Cycle Following Neonatal Transplantation. **(a)** MPS VII iPSC-NSCs *in vitro* prior to transplantation. The majority of cells stain positive for Ki67, a marker of cell division. **(b)** Vibratome section of NOD/SCID brain, one week following neonatal transplantation. Very few transplanted cells were co-labeled with both human specific nuclei and Ki67. **(c,d)** No cells were found co-labeled with both human specific nuclei and Ki67 4 weeks or 16 weeks post-transplantation, indicating that transplanted cells are no longer dividing at these time points. Scale bars = 200µm.

Therapeutic potential of corrected MPS VII iPSC-NSCs

MPS VII is a progressive disease with extensive pathology present by two months of age (Snyder et al. 1995, Levy et al. 1996). Therefore, two-month-old NOD/SCID/MPS VII mice were injected bilaterally into the striatum with 50,000 corrected MPS VII iPSC-NSCs in one hemisphere and 50,000 mock-corrected cells in the contralateral hemisphere. Animals were sacrificed one month later. Engrafted cells survived, remained nestin-positive, and did not migrate away from the injection site. GUSB enzymatic activity, detected by a histochemical reaction (Snyder et al. 1995) was limited to the injection site of the hemisphere receiving corrected cells **(Fig. 3.6)**.

To test whether the lack of differentiation and migration was unique to our MPS VII line or to the diseased environment, we transplanted iPSC-NSCs derived from a healthy human control, as well as MPS VII iPSC-NSCs, into NOD/SCID (non-MPS VII) adult mice. At 1 month post-transplant, engrafted cells from both lines remained at the injection site and stained positive for human-specific nestin **(Fig. 3.7)**. iPSC-NSCs from a normal and diseased source had the same properties when transplanted into adult brain parenchyma, demonstrating that the MPS VII iPSC-NSC line used in our studies was not unique in this respect.

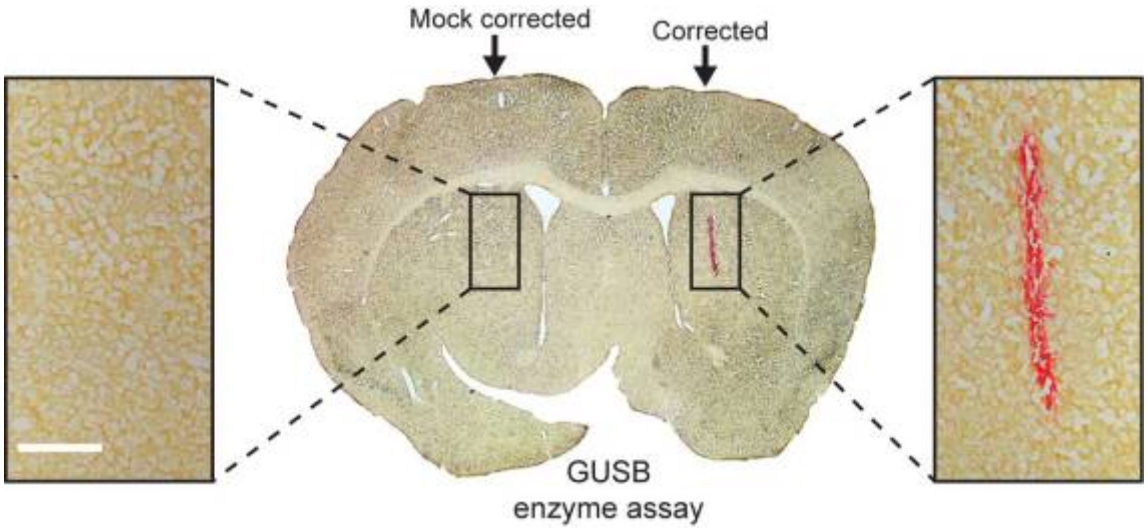

Figure 3.6 Corrected and Uncorrected MPS VII iPSC-NSCs Transplanted into Adult MPS VII Mouse Brain. 50,000 cells were injected bilaterally into the striatum of two month old NOD/SCID/MPS VII mice. One hemisphere received corrected MPS VII iPSC-NSCs and the other hemisphere received mock-corrected MPS VII iPSC-NSCs. One month following transplantation, enzyme activity was present at the injection tract in the hemisphere receiving corrected cells, but not in the hemisphere receiving mock-corrected cells. n=4 mice, scale bar = 0.25mm.

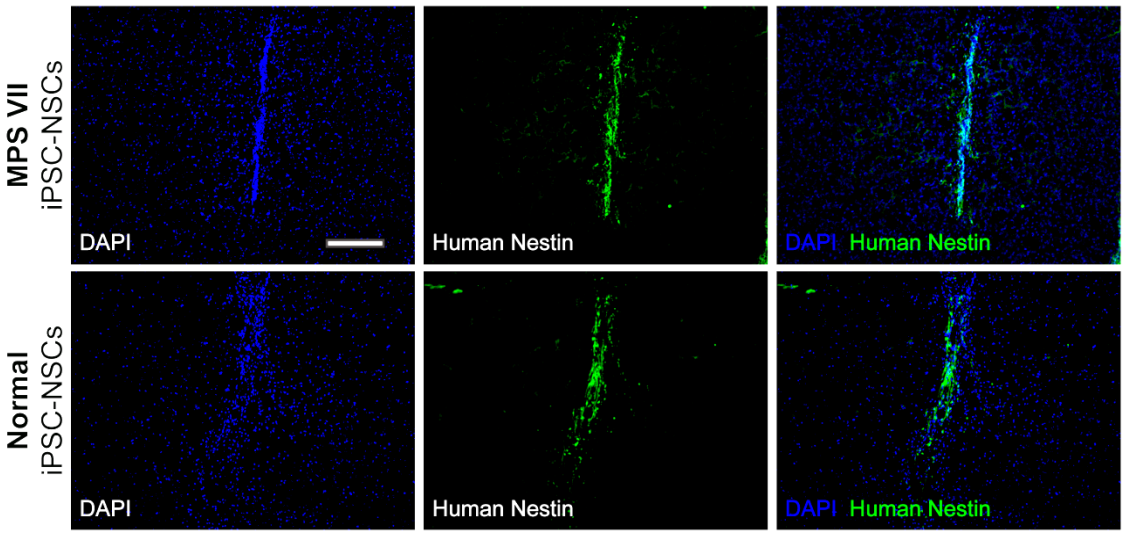

Figure 3.7 MPS VII and Control iPSC-NSCs Transplanted into Adult Mouse Striatum. **(top row)** Human specific nestin staining of sections from adult NOD/SCID mice one month post-transplantation with MPS VII iPSC-NSCs into adult striatum at two months of age (n = 7 mice). **(bottom row)** Human specific nestin staining of sections from adult NOD/SCID mice one month post-transplantation with iPSC-NSCs derived from a normal control (n = 5 mice). MPS VII and normal iPSC-NSCs remain at the injection site and stain positive for the NSC marker nestin. Scale bar = 200µm.

Immunofluorescent Analysis of MPS VII animals at 3 Months of Age

Given that MPS VII is a gradually progressive neurodegenerative disease, we evaluated a number of antibodies in frozen 20µm thick NOD/SCID/MPS VII brain sections at 3 and 8 months of age to determine the best markers of pathology at 3 months, the age at which our experimental animals were sacrificed **(Fig. 3.8)**. We evaluated a number of markers known to be pathologically upregulated late in the disease process, but it was unknown to what extent these markers would be useful in 3 month old animals. We examined lysosomal distention (LAMP2), astrogliosis (GFAP), secondary substrate accumulation (gangliosides GM_2 and GM_3), and a non-specific fluorescent stain commonly used to mark degenerating neurons (Fluoro-Jade C) (Schmued et al. 2000). We also evaluated the extent of microglial activation (via CD68 immunostaining) as extensive microglial pathology has been reported in some, but not all LSDs (e.g. MPS IIIB, less so in MPS I) (Wada et al. 2000, Ohmi et al. 2003). Neuro-inflammation is a common finding in MPS diseases (Archer et al. 2014), and microarray analysis of MPS VII brains from our lab showed that CD68, a major marker of microglial activation (Kreutzberg 1996), is transcribed at very high levels in the brain relative to normal mice (~10-fold higher across all brain regions) (Parente et al. 2012). In fact, 25 of the top 36 genes upregulated relative to normal brains were associated with the manually curated “immune/inflammation” theme using DAVID functional annotation analysis (Huang da et al. 2009, Parente et al. 2012). We confirmed via immunostaining that CD68 was highly upregulated in NOD/SCID/MPS VII animals early in the disease process, preceding the appearance of other commonly used markers of neuropathology. CD68-positive microglia were uniformly distributed throughout the MPS VII brain by three months of age whereas CD68 was limited to small intracellular punctae within microglia of normal mice **(Fig. 3.8 and 3.11d)**.

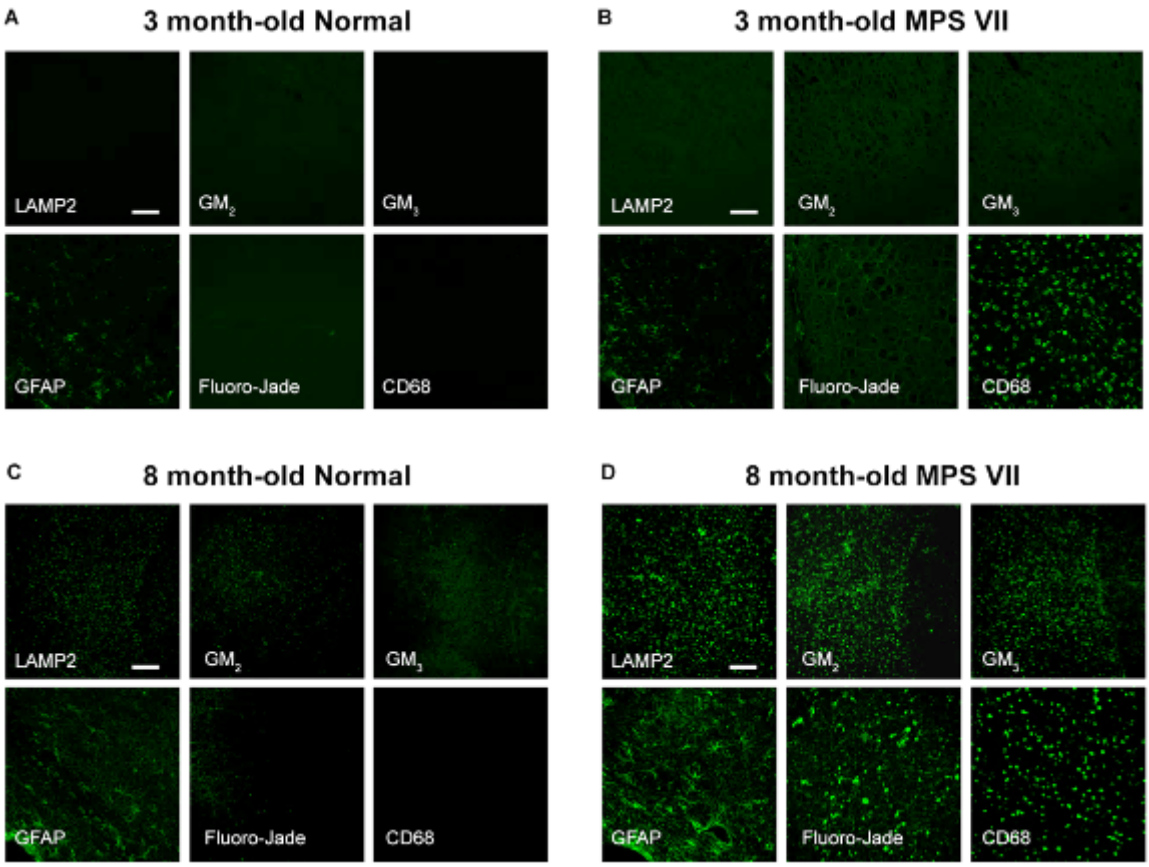

Figure 3.8 CD68-Positive Activated Microglia Precedes the Appearance of Other Markers of Neurodegeneration. NOD/SCID and NOD/SCID/MPS VII mice brain sections stained at three and eight months of age. **(a,b)** Markers of neuropathology in three month-old mice and **(c,d)** eight month-old mice. Lysosomal distension (LAMP2), secondary substrate accumulation (gangliosides GM_2 and GM_3), astrogliosis (GFAP), and neurodegeneration (Fluoro-Jade C) occur after the appearance of $CD68^+$ microglia. LAMP2 = Lysosome-Associated Membrane Glycoprotein 2, GFAP = Glial Fibrillary Acidic Protein GM_2 = GM_2 ganglioside, GM_3 = GM_3 ganglioside. Scale bar = 200µm.

Confirmation of CD68-Positive Microglia as a Marker of MPS VII Pathology

To confirm that CD68 staining is a reliable marker for MPS VII neuropathology in 3 month-old animals, we injected 2 month-old BL6/MPS VII mice bilaterally with an adeno-associated viral vector encoding GFP (AAV1-GFP) in one hemisphere and a 1:1 mixture of AAV1-GFP and AAV1-GUSB in the other hemisphere **(Fig. 3.9)**. The total dose of vector was held constant at $1x10^{11}$. We confirmed via immunostaining that CD68-positive microglia were already widespread by 2 months of age (data not shown). The mice were sacrificed at three months and frozen sections were stained for enzyme activity and CD68. The spherical nature of CD68-positive microglia combined with their relatively uniform distribution allowed us to develop a straightforward method of schematizing CD68 in whole brain sections.

Using Photoshop to merge individual immunofluorescent images, combined with ImagePro software to identify and dilate all objects with an area greater than 20 pixels, we created schematics of CD68 that were easily amenable to quantification. Adjacent sections were stained for enzyme activity using a sensitive colorimetric enzyme assay for the localization of β-glucuronidase activity based on the cleavage of Naphthol AS-BI β-D-glucuronide and its subsequent reaction with a pararosaniline dye to create an insoluble product that is easily visualized (the HPR assay) (Wolfe et al. 1990, Gallagher 1992) **(Fig. 3.9a)**. Co-immunostaining of GFP and CD68 clearly shows a reduction in the number of CD68-positive cells surrounding the region that received the mixture of AAV1-GFP and AAV1-GUSB but not in the region receiving AAV1-GFP alone **(Fig. 3.9b)**.

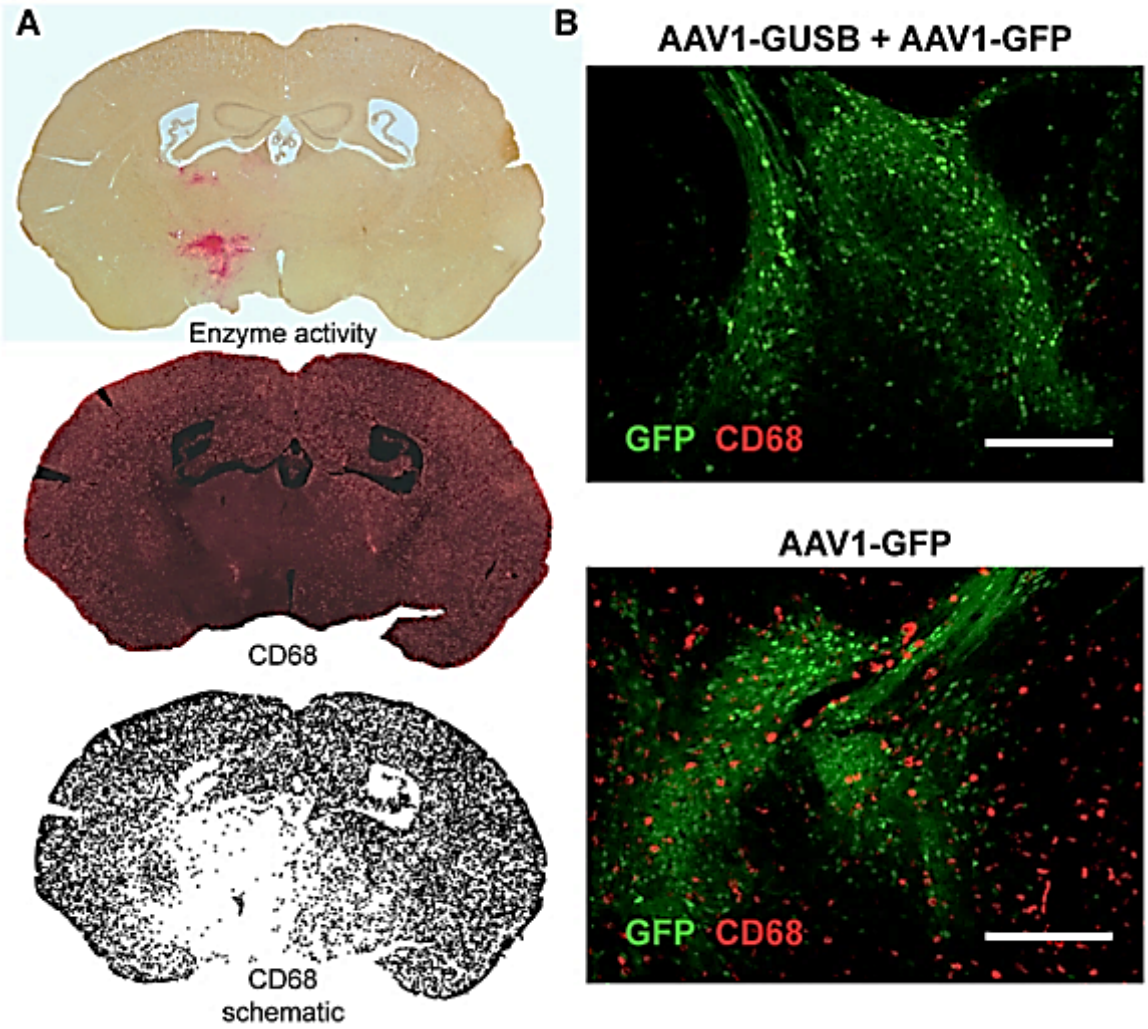

Figure 3.9 Absence of CD68-Positive Microglia Correlates with β-glucuronidase Activity in MPS VII animals. MPS VII mice were injected with a mixture of AAV1-GFP and AAV1-GUSB in one hemisphere, while the contralateral hemisphere was injected with AAV2/1-GFP alone (total of $1x10^{11}$ vector genomes, n=4 mice). **(A)** A schematic of a CD68 immunostained section shows a lack of CD68-positive microglia in the area surrounding the site injected with AAV1-GUSB one month post-injection. **(B)** GFP and CD68 immunostaining shows a reduction in activated microglia surrounding the injection site of AAV1-GUSB + AAV1-GFP but not around the injection site of AAV1-GFP alone. Scale bar = 200µm.

Evaluation of Pathology Following Neonatal Intraventricular Transplantation

To determine if corrected or mock-corrected MPS VII iPSC-NSCs affected neuropathology in MPS VII mice, changes in activated microglia were used as a marker of neuropathology. Three months following transplantation, engrafted cells could be found across the brain, but at low densities, similar to those seen following primary NSC grafts **(Fig. 3.1 and 3.3)**. The HPR assay reveled cells clustered primarily in periventricular regions between the hippocampus and corpus callosum. CD68-positive microglia were not reduced even in the regions directly adjacent to transplanted cells **(Fig. 3.10)**.

Evaluation of Pathology Following Adult Striatal Transplantation

One month following adult transplantation, there was a striking reduction in CD68 immunoreactivity surrounding the corrected MPS VII iPSC-NSC grafts, but not the mock-corrected grafts **(Fig. 3.11).** The density of CD68-positive cells was quantified in a 0.5 mm^2 region of interest (ROI) surrounding each injection tract **(Fig. 11c)**. There was a significant difference between the region surrounding corrected grafts (49.2 ± 9.9 cells/mm^2) and the region surrounding mock-corrected grafts (145.2 ± 13.2 cells/mm^2, $p<0.0001$) **(Fig. 3.11c)**. There was not a significant difference between the region surrounding mock-corrected grafts and a comparable region of untreated MPS VII striatum (166.1 ± 13.4 cells/mm^2, $p>0.05$). Immunostaining for CD68 and the pan-microglial marker Iba1 showed that the microglia in untreated MPS VII mice as well as the microglia surrounding mock-corrected iPSC-NSCs had a distended, amoeboid-type morphology **(Fig. 3.11d)**. The region surrounding corrected iPSC-NSCs contained smaller, ramified-type microglia, which closely resembled the microglia in normal control brains **(Fig. 3.11d)**.

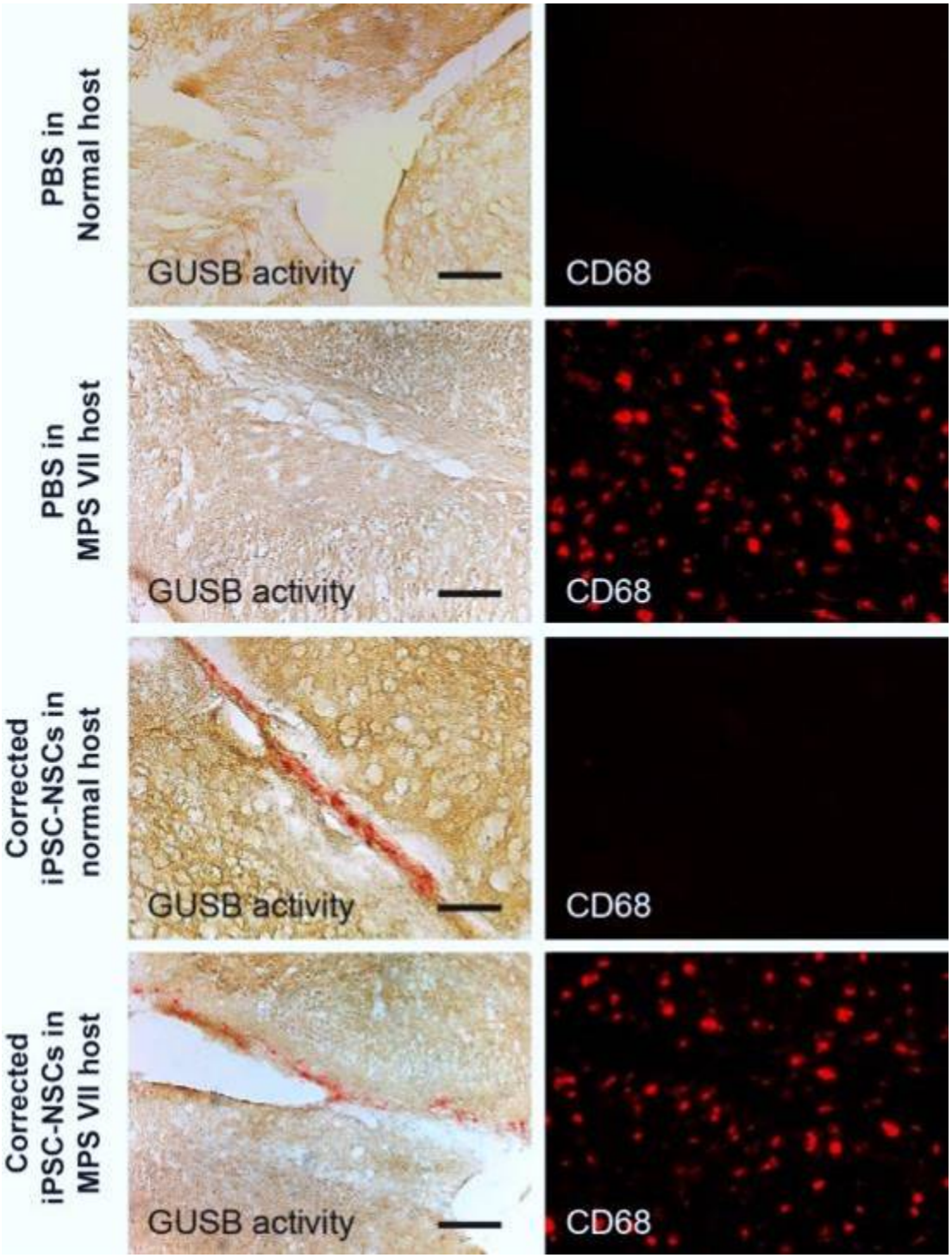

Figure 3.10 Low Levels of Engraftment Following Neonatal Transplantation do not Reduce CD68-positive Microglia

Figure 3.10 Low Levels of Engraftment Following Neonatal Transplantation do not Reduce CD68-positive Microglia. NOD/SCID/MPS VII neonates and their non-MPS VII littermates were injected intraventricularly with 50,000 corrected MPS VII patient iPSC-derived NSCs or PBS. The sensitivity of the HPR assay **(left column)** allowed for the detection of sub-therapeutic levels of levels of β-glucuronidase activity in engrafted cells (red stained areas). CD68-positive cells were widespread in mice receiving PBS or corrected iPSC-derived NSCs, even in areas directly adjacent to corrected cells **(right column)**. Normal littermates showed no signs of CD68-positive microglia at this suddestin, Anlaysins of.

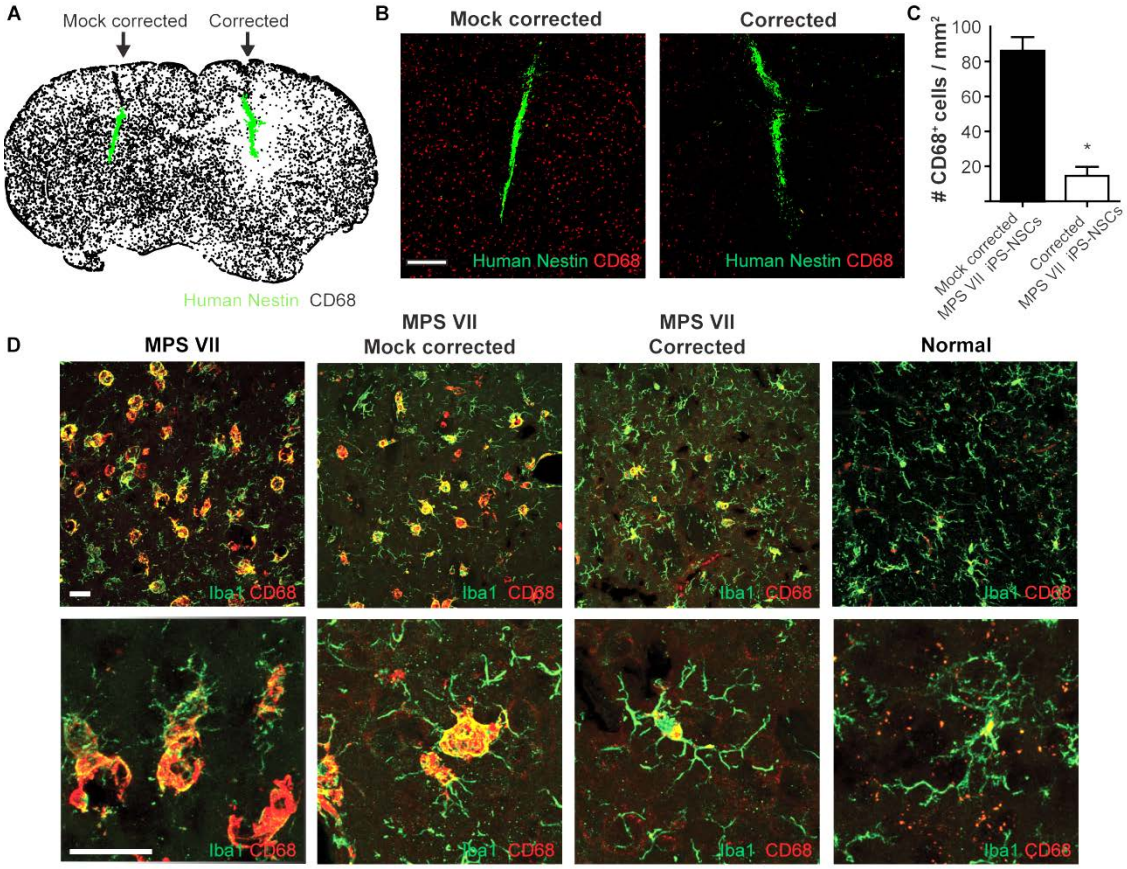

Figure 3.11 Correction of Microglial Pathology Following Adult iPSC-derived NSC Transplantation.

Figure 3.11 Correction of Microglial Pathology Following Adult iPSC-derived NSC Transplantation. Corrected and mock-corrected iPSC-NSCs (50,000 cells/condition) were injected intrastriatally into the right and left hemisphere respectively, of adult NOD/SCID/MPS VII mice. **(a)** Schematic of a representative coronal section showing engrafted MPS VII iPSC-NSCs and CD68-positive microglia one month post-transplantation. **(b)** Immunofluorescent images of nestin-positive iPSC-NSCs at the site of injection. The region surrounding the corrected MPS VII iPSC-NSCs contained significantly fewer activated microglia than in the region surrounding mock-corrected MPS VII iPSC-NSCs or in a comparable region of untreated MPS VII striatum. CD68-positive microglia with an area greater than 20 pixels was quantified **(c).** **(d)** Confocal images of Iba1 and CD68 immunoreactivity in resident microglia. The brains of MPS VII mice showed marked microglial activation, as evident by upregulated CD68 expression and amoeboid morphology. Microglia adjacent to corrected, but not mock-corrected, MPS VII iPSC-NSCs exhibited a ramified morphology and low CD68 expression typical of the quiescent microglia found in normal mice. Mean ± SEM, n=3 sections/mouse, n=4 mice, scale bar = 200µm in **(b)** and 30µm in **(d).**

III. Discussion

In Chapter 2 we showed that MPS VII patient-derived iPSCs can be differentiated into long-term self-renewing NSCs, similar to iPSCs from an unaffected control. In order to evaluate the engraftment potential of these cells we transplanted them within the ventricles and striatum of neonatal NOD/SCID mice. We then tested the therapeutic potential of these cells by quantifying microglial pathology surrounding corrected and mock-corrected grafts in MPS VII mice.

Cells transplanted intraventricularly into neonatal mice survived and engrafted throughout white matter tracts in the brain. However, they remained nestin-positive even 4 months post-transplantation. In contrast, Koch et al. found mature engrafted neurons at the same time point following neonatal telencephalic injections using similar ESC-derived NSCs (Koch et al. 2009). We ruled out the possibility that reactivation of the retroviral transgenes was responsible for the lack of differentiation by co-immunostaining using antibodies against human nuclei and Oct4. Our experiments primarily utilized intraventricular injections to maximize the spread of engraftment, and we hypothesized that the site of injection might explain the lack of differentiation. We found that injecting cells into the striatum of neonatal mice resulted in some neuronal differentiation one month post-transplantation. In contrast to the cells found in the white matter after intraventricular injection, we found DCX-positive neuronal precursors and β-III tubulin-positive neurons with an immature morphology near the injection site. The engrafted cells failed to migrate far from the striatal injection site. This is consistent with previous studies showing that the location of engraftment makes a difference in the differentiation and migration potential of transplanted NSCs (Watson et al. 2006).

The mouse sub-ventricular zone (SVZ) provides a niche for stem cells in the mouse brain, and this may serve to inhibit NSC differentiation (Ghashghaei et al. 2007). Autoattraction of transplanted cells likely plays a role in the lack of migration. The same group that first demonstrated the ESC to NSC protocol used in the Koch et al. study later published a report demonstrating that NSCs transplanted onto live brain slices failed to migrate (Ladewig et al. 2014). They used cells expressing GFP under the control of the DCX promoter to distinguish

between NSCs and neural progenitor cells (NPCs). After sorting DCX-positive cells they transplanted a pure population of NPCs and found that they migrated very well. They demonstrated that blockade of VEGF and FGF2 receptors on the surface of neural progenitor cells (NPCs) allowed for greater migration in the presence of NSCs. This was accomplished using antibodies against VEGF and FGF2 receptors or a tyrosine kinase inhibitor, nintedanib (Ladewig et al. 2014). This is consistent with our results and the results of other studies showing that chemoattractant signaling plays a large role in NSC migration. NSCs are known to migrate towards areas of injury, such as tumors or even the needle track created during the surgical procedure (Aboody et al. 2000, Boockvar et al. 2005). Chemokines such as PDGF and stromal cell-derived factor 1α (SDF-1α, or CXCL12) are likely to play a role in attracting NSCs expressing CXC chemokine receptor 4 (CXCR4) (Imitola et al. 2004, Ladewig et al. 2014).

One week following neonatal transplantation, cells were found primarily within and adjacent to ventricles. After one month, cells were widely distributed throughout white matter tracts. This is consistent with many studies showing that NSCs from various sources display a tropism for white matter in both normal and pathological contexts (Tabar et al. 2005, Maciaczyk et al. 2009, Carney et al. 2011, Gupta et al. 2012). This affinity for white-matter tracts may be useful for treating leukodystrophies or as a pathway for NSC dissemination. The limited migration of transplanted NSCs within grey matter may be a barrier to widespread delivery for some diseases with global CNS pathology; however, axonal transport can facilitate wider distribution of lysosomal proteins within this group of diseases (Passini et al. 2002).

Another factor limiting engraftment is donor cells exiting the cell-cycle. While iPSC-NSCs grew prolifically in culture, they stopped dividing shortly after transplantation. Very few Ki67-positive cells were found one week post-transplantation and no Ki67-positive cells were found at one or four moths post-transplantation. Ideally, a population capable of limited or controlled cell division will strike the delicate balance between total engraftment levels and the risk of tumorigenesis.

Due to the intrinsic variation between ESC and iPSC lines, we were concerned that the results we found following transplantation were specific to our MPS VII iPSC line. As a control,

we transplanted an iPSC line derived by an independent source (the CHOP iPSC core) using a different reprogramming method (Cre-excisable constitutive polycistronic lentivirus) (Sommer et al. 2010). Based on the similar behavior of the lines, we concluded that the results were not an artifact of our particular line.

A major hurdle we overcame was the evaluation of pathology in treated brains at 3 months-of-age. Neuropathology of MPS VII is often evaluated in very thin sections (0.5-5µm), necessitating that brains be embedded in plastic (e.g. JB4), epoxy (e.g. Epon), or paraffin (Heuer et al. 2002, Liu et al. 2005, Mikula et al. 2012). We opted to use thicker (~20µm) frozen sections to evaluate pathology for several reasons: 1) The antibodies used in these studies were validated and optimized using frozen sections 2) paraffin, plastic, or epoxy sectioning of so many brains (>100) was impractical, and 3) The use of plastic or epoxy sections generally precludes IHC, reducing our ability to co-localize engrafted cells and pathology (Shi et al. 1991, McCluggage et al. 1995, Cai et al. 2005).

We systematically evaluated antibodies validated in older animals in addition to antibodies against candidate proteins identified by microarray. We found that only CD68 was highly upregulated in MPS VII animals at 3 months of age, and therefore decided to use this as our primary readout of pathology.

Neonatally engrafted cells migrated widely, and could be visualized immunofluorescently using antibodies against GFP, human nuclei, and human-specific nestin. They could also be visualized using the highly sensitive HPR assay. Unfortunately, there was no difference in CD68-positive microglia, even directly surrounding the transplanted cells. In contrast, our adult transplantation experiments unambiguously show that a high local concentration of corrected MPS VII iPSC-NSCs cleared CD68-positive microglia in an area surrounding the graft. Ramified microglia in the corrected region had small soma and extended processes, consistent with a quiescent microglial state (Kreutzberg 1996).

CHAPTER 4

Conclusions and Future Directions

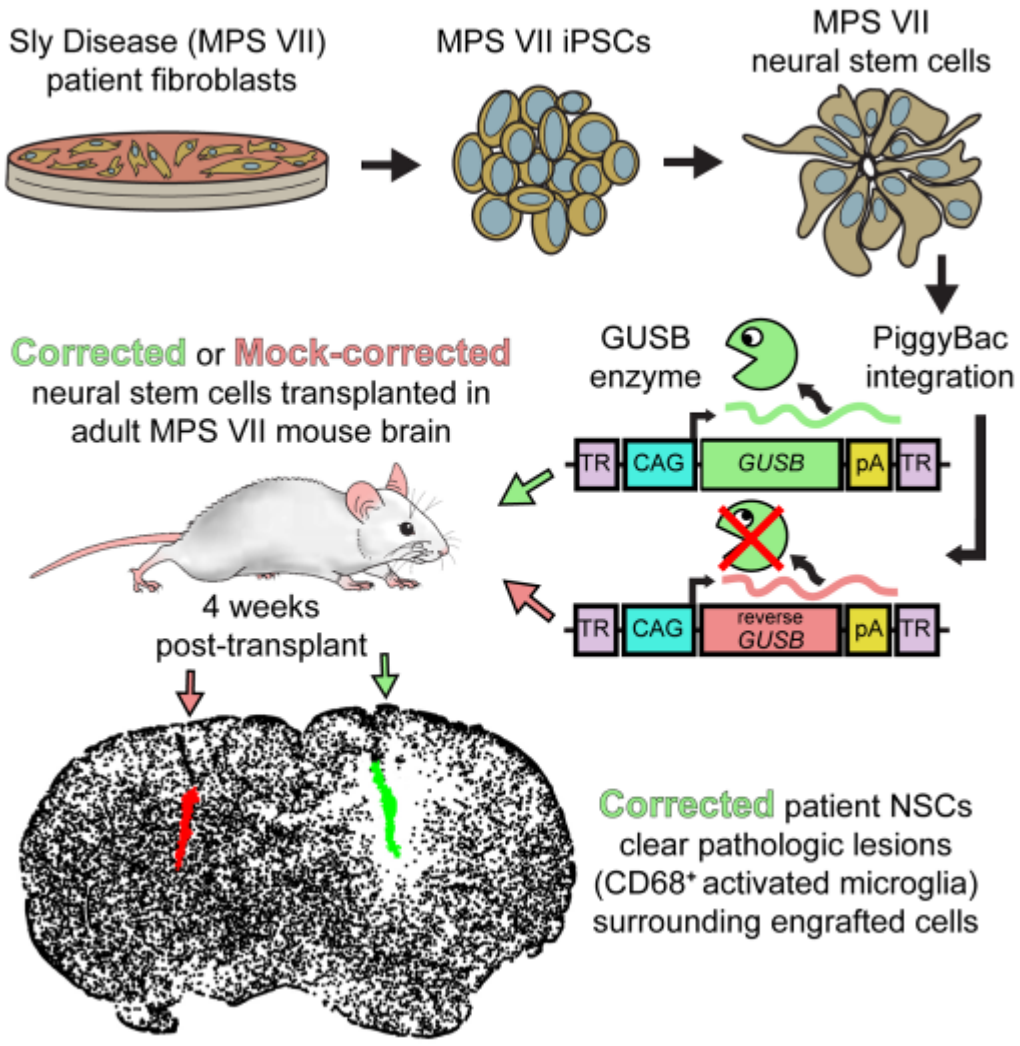

Figure 4.1 Graphical Outline of an iPSC-derived NSC-based Strategy for β-glucuronidase Replacement in the Brain using a Xenograft Model of MPS VII.

The general use of this strategy involves removing somatic cells from the patient, reprogramming them via introduction of the transcription factors Oct4, Sox2, Kl4, and c-MYC. Using differentiation protocols developed for ESCs/iPSCs, pluripotent cells can be converted to long-term self-renewing neural stem cells. Introduction of the GUSB gene via DNA transposon-mediated (PiggyBac) gene delivery rescues the mutant phenotype in cells. Following

transplantation in the brain, β-glucuronidase is secreted by corrected cells and taken up by nearby affected cells, thereby reducing pathology *in vivo.*

I. Conclusions

Here we demonstrate the feasibility of *ex vivo* gene therapy for the treatment of neuropathology accompanying metabolic disease using patient-derived somatic cells from a readily accessible source (e.g. frozen skin biopsy). By reprogramming patient fibroblasts into pluripotent stem cells and subsequently generating genetically corrected tissue-specific stem cells, we evaluated a therapeutic strategy that can be applied to many genetic diseases affecting the brain. We show, via xenotransplantation into a mouse homologue of the human disease, that such a strategy can reverse pathologic lesions surrounding engrafted cells.

The success of NSC-based therapy will depend on protocols that yield well-characterized and expandable lines suitable for transplantation. We chose an NSC differentiation protocol for its ability to generate a self-renewing population of relatively homogenous NSCs from ESC and iPSC lines (Koch et al. 2009, Falk et al. 2012). The *in vitro* characteristics of MPS VII iPSC-NSCs generated here were consistent with reports utilizing similar ESC-based protocols in regards to the immunophenotype and ability to generate predominately GABAergic neurons upon withdrawal of growth factors (Koch et al. 2009).

We found that GUSB deficiency did not compromise the ability of human MPS VII iPSCs to generate embryoid bodies or differentiate towards neural lineages, in contrast to a previous report on mouse MPS VII iPSC lines (Meng et al. 2010). Disease-related phenotypes have been reported, *in vitro*, in iPSCs derived from patients with other LSDs such as Niemann-Pick type C or MPS IIIB (Lemonnier et al. 2011, Bergamin et al. 2013), and in primary canine MPS VII NSCs (Walton et al. 2007). However, there was no evidence in our study that the MPS VII iPSC-NSCs or their progeny had a disease-related phenotypic difference *in vitro.* We directly compared corrected and mock corrected NSCs by immunofluorescent analyses and found no significant differences. We also co-cultured corrected or mock-corrected iPSC-NSCs with primary cortical cells derived from MPS VII or normal littermate controls (data not shown). After loading the co-

cultures with a calcium dependent fluorescent dye, we were able to visualize several network properties in real time (Patel et al. 2015). While these experiments were preliminary, we found no differences in network firing properties between corrected iPSC-NSC/MPS VII cortical co-cultures and mock-corrected iPSC-NSC/control cortical co-cultures. Furthermore, the GUSB deficiency did not impair engraftment as we observed no apparent differences in the number or distribution of genetically corrected and mock-corrected MPS VII iPSC-NSCs following transplantation into MPS VII mice. More GUSB-negative cell lines are needed to determine if this phenomenon is specific to our MPS VII line or merely reflects the subtlety of the disease.

Transplantation of iPSC-NSCs within the neonatal brain yielded stable engraftment across the neuroaxis for at least four months, but only within and adjacent to white matter tracts. Although the precise mechanisms are unclear, NSCs from various sources display a tropism for white matter in both normal and pathological contexts (Tabar et al. 2005, Maciaczyk et al. 2009, Carney et al. 2011, Gupta et al. 2012). The affinity for white-matter tracts may be useful for treating leukodystrophies and as a pathway for NSC dissemination. The limited migration of transplanted NSCs within grey matter precludes widespread delivery for some diseases with global CNS pathology, however axonal transport can facilitate wider distribution of lysosomal proteins within this group of diseases (Passini et al. 2002).

We transplanted iPSC-NSCs into neonatal mice in order to evaluate their behavior in a more appropriate developmental context. Many of the cues required for survival and migration of NSCs are present in neonates but not in healthy adults (Guzman et al. 2007). We found that iPSC-NSCs engrafted widely but sparsely, which is consistent with neonatal transplants of primary mouse NSCs (Chaubey et al. 2013). Following transplantation directly into grey matter (adult striatum), the iPSC-NSCs remained localized to the injection tract. As a consequence, microglial pathology was corrected in a zone surrounding the graft, corresponding to the distribution of enzyme in three dimensions in the brain parenchyma (Taylor et al. 1997). In contrast, neonatal transplants engrafted throughout the white matter but were too sparse to significantly reduce the number of CD68-positive cells, even in regions directly adjacent to corrected cells. Thus, substantial improvements to increase both the density and distribution of

donor cells within the brain will be needed to deliver the therapeutic enzyme to more areas of the brain to advance clinically relevant NSC therapy further for LSDs.

The engrafted iPSC-NSCs showed very little evidence of differentiation, even four months post-transplant. The absence of mature neurons and glia following neonatal and adult transplantation stands in contrast to the efficient differentiation of iPSC-NSCs *in vitro*. This result underscores the need for a better understanding of the environmental signals governing NSC differentiation. However, for use in correcting most LSDs the differentiation status of engrafted cells is not critical, provided that they do not cause deleterious effects in the brain. Undifferentiated cells may in fact be advantageous, as inappropriate neurotransmitter release from mature engrafted neurons can be harmful in some settings. One example of this is the graft-induced dyskinesias resulting from cell replacement therapy in Parkinson's disease patients (Lane et al. 2010).

Gene and cell based therapies that successfully deliver lysosomal enzymes in the brain reduce pathology in many animal models of LSD (Simonato et al. 2013). To test whether genetically corrected patient iPSC-NSCs could deliver corrective levels of GUSB, we evaluated pathology in the adult striatum surrounding the graft. We assayed disease-associated neuroinflammation as a biomarker, using CD68-positive activated microglia. Evidence for a microglial contribution to MPS VII pathology was previously shown via microarray analysis of normal and diseased brains (Parente et al. 2012). Microglial involvement has been well documented in some, but not all, storage diseases including MPS IIIB and Sandhoff disease (Wada et al. 2000, Ohmi et al. 2003), and activated CD68-positive microglia have been used as a biomarker for correction of pathology (Lee et al. 2007). We show here that microglial activation is a significant and early component of MPS VII neuropathology. CD68-positive microglia are particularly useful as a quantifiable biomarker of MPS VII neuropathology because of their distended size, spherical morphology, and uniform distribution.

We observed no differences in the engraftment capacity of corrected and mock-corrected MPS VII iPSC-NSCs. Activated microglia adjacent to mock-corrected grafts appeared distended and amoeboid, indistinguishable from microglia in comparable regions of the untreated MPS VII

brain. In contrast, the area surrounding corrected grafts contained significantly fewer CD68-positive cells. The microglia were reduced in size and adopted a more ramified-type morphology consistent with the phenotype of resting microglia seen in normal animals.

Mock-corrected grafts did not provide any therapeutic benefit, suggesting that GUSB alone was responsible for correcting neuropathology, rather than anti-inflammatory or other factors expressed by the iPSC-NSCs themselves. The observation that GUSB over-expression from an AAV vector similarly reduces CD68-positive microglia corroborates the conclusion that GUSB activity is solely responsible for the correction of neuropathology.

The spatially restricted nature of correction following cell transplantation indicates the need for new differentiation protocols or other strategies capable of increasing NSC migration within the brain parenchyma. Overall, our results suggest that a comprehensive strategy involving somatic reprogramming and gene therapy could benefit patients with neuropathology from storage diseases affecting the brain.

II. Issues Related to Measuring Neuropathology in MPS VII mice.

The amount of storage present in 3 month-old MPS VII animals proved insufficient to reliably distinguish from controls. The presence of storage lesions at this time point has been established via electron microscopy (Levy et al. 1996) and immunostaining of poly-ubiquitin inclusions in paraffin sections (Heuer et al. 2002). However, the limited distribution of our grafts combined with the small percentage of NOD/SCID/MPS VII offspring from heterozygous breeding pairs made these strategies impractical on a scale needed to adequately power our studies. Our original plan was to stain frozen cryosections with antibodies that had been validated on 8 month-old animals. However, there was very little difference between 3 month-old normal and mutant mice using these antibodies, including the same poly-ubiquitin antibody used previously on paraffin embedded sections of 3 month-old mice. Standard immunoflourescent detection of pathology becomes problematic as autofluorescent material accumulates over time in mutant animals. By 8 months-of age there is extensive autofluorescent material accumulated throughout

the brain, preventing all but the best antibodies from discriminating between real signal and autofluorescence. Of all the antibodies we tested using cryosections of 3 month-old NOD/SCID/MPS VII brains, α-CD68 clearly stood out. The pan-microglial markers Iba1 and CD11b confirmed that microglial activation precedes other hallmarks of pathology. The spherical, uniformly distributed nature of activated microglia in the MPS VII brain made it possible to develop an automated method of identifying and quantifying these cells in the areas surrounding the iPSC-NSC grafts. Given that activated microglia are known to be present at an early age in many lysosomal storage diseases, we hope that this simple method of software-based quantitative fluorescent image analysis may prove useful for the field. Importantly, this activation is not strain-specific, and is readily detectible in both NOD/SCID/MPSVII and BL6/MPS VII by 2 months of age, the earliest time point examined.

III. Future Aims

By genetically correcting MPS VII iPSCs at the NSC stage we limited ourselves to using only these cells for all transplantation experiments. Genetically manipulating cells at the iPSC stage would allow us to test multiple differentiation protocols. To this end, we modified the iPSC culture conditions allowing us to transfect cells and select stably transfected iPSCs with improved versions of our PiggyBac vectors that contain the GUSB promoter **(Fig. 4.2)**. Another concern is line-to-line variability among individual ESC and iPSC lines. Given that there is always some level of variability in terms of gene expression and differentiation potential, it is desirable to generate more MPS VII lines. Given the rarity of the disease, and the fact that we had to derive our MPS VII iPSC line from frozen fibroblast samples (a very inefficient process), it may not be worth the time and effort to derive more lines directly from patient samples.

The use of recently developed gene editing technologies (e.g. TALEN and CRISPR) may be a more attractive option for generating GUSB-deficient pluripotent cell lines (Christian et al. 2010, Ran et al. 2013). Knocking out GUSB in well-established ESC lines such as H9 (Amit et al. 2000) or iPSC lines provides several advantages. Most MPS VII patients have some small but

residual expression of β-glucuronidase, and eliminating all enzymatic activity may yield additional insights into the function of the enzyme and the pathophysiology of disease. Another advantage to using well established lines is that we can mitigate the effect of line-to-line variability inherent to pluripotent stem cell lines. Our current plan is to knock out GUSB in the normal iPSC line used in these studies as well as in the well-studied H9 ESC line. CRISPR guide RNAs have been created and transfected into a normal iPSC line, and clones are currently undergoing genotyping and enzymatic analysis to confirm that no β-glucuronidase is present. Multiple $GUSB^{-/-}$ ESC and iPSCs lines compared to their parental $GUSB^{+/+}$ lines would provide further evidence that patient-specific iPSC-NSCs (or other progenitor cell types) can be a safe and effective therapy for LSDs in the brain.

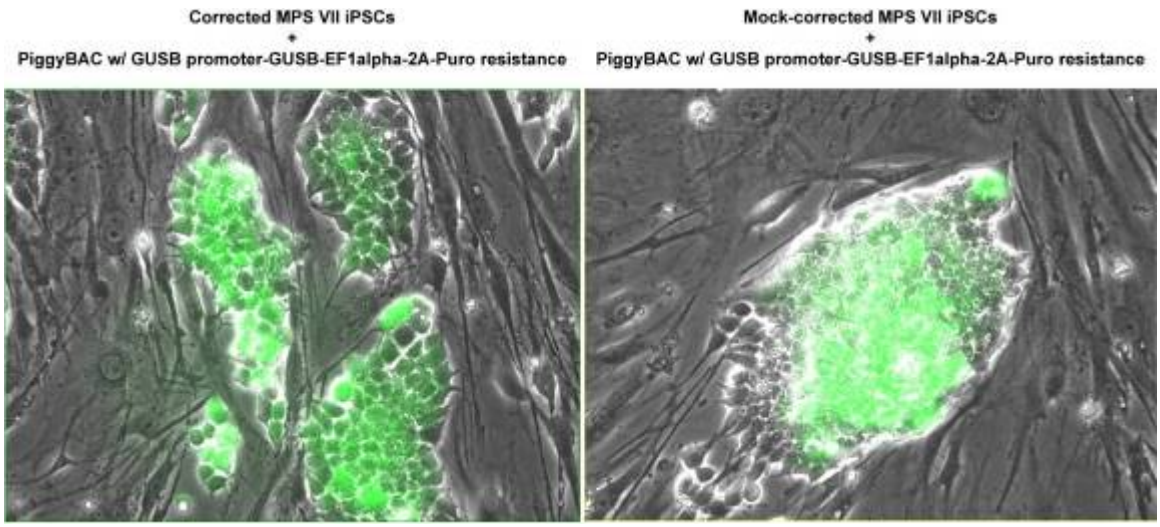

Figure 4.2 Genetically Corrected and Mock-Corrected MPS VII Patient iPSC labeled with GFP. MPS VII iPSCs were dissociated into single cells and transfected with one of two optimized PiggyBac transposon vectors described previously **(Fig. 2.5)**. The use of the GUSB promoter (rather than CMV or CAG) resulted in much higher β-glucuronidase levels in 293t cells while maintaining GFP expression from the downstream EF1α promoter. Transfecting and plating iPSCs on a high concentration of Matrigel in the presence of the ROCK inhibitor Y-27632 resulted in many colonies surviving, a small percentage of which were GFP positive. Several clones from each condition were isolated and expanded. Correction of patient iPSCs allows for the evaluation of multiple differentiation methods to increase survival and migration following transplantation.

IV. Future CNS Cell Therapy

The studies undertaken here, combined with results from many other groups, demonstrate that the exact method of deriving NSCs, the source, the age of transplantation, the transplantation site, and the pathological state of the host brain can dramatically alter the fate of transplanted NSCs (Snyder et al. 1992, Snyder et al. 1995, Taylor et al. 1997, Boockvar et al. 2005, Tabar et al. 2005, Corti et al. 2008, Koch et al. 2009, Maciaczyk et al. 2009, Chaubey et al. 2013, Jensen et al. 2013, Mazzini et al. 2015). Our data suggest that early-stage NSCs may not be ideal for widespread cell therapy in the brain. Several promising pluripotent stem cell-derived alternatives have been recently developed that deserve to be more thoroughly evaluated in a therapeutic context.

One approach involves the migratory progeny of long-term self-renewing NSCs similar to the iPSC-NSCs used in our experiments. After initiating the differentiation process, Laedewing et al. showed that DCX-positive neuroblasts, derived from non-migratory NSCs, migrated extensively in slice culture (Ladewig et al. 2014). Another ESC-derived cell type shown to migrate widely in the brain has been generated by mimicking the developmental cues encountered by cells in the medial ganglionic eminence (MGE). The MGE is a subcortical structure adjacent to the embryonic lateral ventricles. It is the birthplace of most cortical interneurons, which must migrate long distances before functionally integrating into cortical circuits (Wonders et al. 2006). Highly migratory MGE-like cells derived from human ESC/iPSCs can be selected based on their expression of the transcription factor NKX2.1 (Maroof et al. 2013). A drawback of these approaches is the requirement of fluorescent reporters to purify the cell-type of interest from a heterogeneous population. Refinements to differentiation protocols or the identification of cell surface markers will be required before these cell types can be used clinically.

Currently, one of the most promising approaches to achieving widespread CNS engraftment involves glial progenitor cells (GPCs) rather than NSCs. GPCs, also known as oligodendrocyte precursor cells or $NG2^+$ glia, comprise the majority of mitotic cells in the adult

human brain and as much as 5-10% of total cells, depending on the region (Dimou et al. 2015). In adults, GPCs are primarily responsible for generating oligodendrocytes for the purpose of mylenating axons, but are capable of differentiating into astrocytes and even neurons (Rivers et al. 2008, Sim et al. 2009). GPCs can be purified from primary human tissue using surface markers including NG2, A2B5, and PDGFRA (Goldman et al. 2012). They can also be generated *in vitro* from ESC/iPSCs or via direct reprogramming (Wang et al. 2013, Yang et al. 2013).

Human GPCs, whether derived from primary tissue or pluripotent cells, have the rather amazing quality of actively displacing murine glia following adult transplantation (Windrem et al. 2014). Over the course of a year, transplanted human GPCs migrated throughout the white matter and gradually advanced into the cortex, eliminating endogenous glia and supplanting their function. Furthermore, engrafted mice demonstrated increased synaptic plasticity and performed better on several cognitive tasks. Relative to mice engrafted with murine GPCs and unengrafted mice, animals engrafted with human cells showed enhanced long-term potentiation (LTP), maze performance, fear conditioning, and object-location memory (Han et al. 2013). Engrafted glia were morphologically and functionally human, and unknown species-specific factors conferred a clear competitive advantage. When the mechanism/s responsible are elucidated, it may be possible to alter GPCs *in vitro*, giving the cells a similar advantage in an autologous host.

Microglia are major effectors of catabolism in the brain, and their correction or replacement could restore a critical scavenger function in storage disease patients. With the hope that bone marrow-derived stem cells would replace microglia and other macrophages while providing a plentiful source of enzyme for the body, bone marrow transplantation (BMT) has been performed on ~1000 LSD patients with generally disappointing results (Rovelli 2008). With the exception of very young pre-symptomatic patients, BMT is usually not worth the significant risks. Relative to other LSDs, BMT has been most successful in treating MPS I, and trials have shown modest results for treating Krabbe disease and metachromatic leukodystrophy (Escolar et al. 2005, Biffi et al. 2013, Aldenhoven et al. 2015). A more thorough understanding of the factors governing success and failure will facilitate the development of more effective cell therapies targeting the brain.

One important variable is the enzyme's effective therapeutic dose. In the case of MPS I, just 0.4% of normal catalytic activity can be enough to confer a relatively mild phenotype, partially explaining the efficacy of BMT for this disease (Bunge et al. 1998). It's important to maximize the amount of enzyme produced by each engrafted cell. Take for example the recent *ex vivo* gene therapy trial for metachromatic leukodystrophy (MLD) (Biffi et al. 2013). Lentiviral overexpression of arylsulfatase A in conjunction with autologous BMT was very effective in preventing disease, at least in a small group of pre-symptomatic patients.

Microglia and other tissue resident macrophages can self-renew, and they are not normally replaced by cells from the bone marrow. In order to get significant numbers of bone marrow-derived cells into the brain a niche must first be created. After killing resident myeloid precursors in the brain with radiation or chemotherapy, bone marrow-derived cells entered the brain and differentiated into microglia (Capotondo et al. 2012). This effect was seen with busulfan, which crosses the blood-brain-barrier, but not with theosulfan, which does not (Capotondo et al. 2012). Clinical trials that reported enzyme activity in the brain almost all used busulfan as the primary chemotherapeutic (Rovelli 2008). PLX3397 is an inhibitor of the CSF-1 receptor (CSF1R) and it has the remarkable ability to kill all resident microglia over the course of a few weeks. This compound was recently used to show that long-term microglial depletion in adults has very little effect on behavior and that the microglial repopulating cells are likely perivascular cells that transiently express nestin (Elmore et al. 2014). PLX3397 or similar drugs may be important for creating a niche in which blood-borne donor cells have a real competitive advantage in the brain.

The exact source of cells greatly influences the outcome as well. Umbilical cord stem cells actually engrafted better than peripheral blood stem cells and bone marrow-derived stem cells for MPS I (Boelens et al. 2007). iPSC-derived microglial precursors may be an ideal source of enzyme in the brain capable of being delivered IV. Under the right conditions, it's plausible that myeloid progenitors could even replace resident macrophages throughout the body. A microglial differentiation protocol has already been established for murine pluripotent stem cells, and should be relatively straightforward to adapt it for human cells (Beutner et al. 2010). The success of cell

transplantation for LSDs will depend on advances in differentiation techniques and transplantation biology, which in turn rely on a broad understanding of the origins and behavior of cells in both normal and pathological circumstances.

Materials and Methods

Generation of MPS VII iPSCs

Frozen fibroblasts were obtained from a 3 month old female patient (GM02784, Coriell Institute for Medical Research) with no detectable β-glucuronidase activity. Fibroblasts were maintained in DMEM containing 15% FBS. Reprogramming was performed as previously described with some modifications (Park et al. 2008). Retroviral vectors expressing Oct4, Sox2, Klf4, and c-Myc were produced in 293t cells using the pMXs backbone (courtesy of Dr. Shinya Yamanaka, Addgene plasmids 13366, 13367, 13375, and 17219) and pseudotyped with VSV-G (Takahashi et al. 2006). MPS VII fibroblasts were transduced with the reprogramming vectors and after five days were split onto mitomycin-C inactivated mouse embryonic fibroblasts (MEFs). The cells were maintained in iPSC media containing ROCK inhibitor Y-27632 (10µM, Sigma) until colonies were ready to be picked. Putative iPSC colonies were manually picked and transferred to fresh MEFs. Subsequently, iPSC cultures were passaged either manually or enzymatically with dispase (Gibco). Control iPSCs were obtained from the ESC/iPSC core of the Children's Hospital of Philadelphia, and generated using the Cre-excisable STEMCAA lentiviral vector (Sommer et al. 2010).

Teratoma formation

MPS VII iPSCs ($1x10^6$) were resuspended in 140µL DMEM/F12 and mixed with 60µL Matrigel (BD biosciences). The cell suspension was injected subcutaneously in NOD/SCID mice (Jackson Labs). Teratomas were removed 6 weeks later and either frozen for immunostaining or embedded in paraffin for H&E staining.

Differentiation and culture of NSCs

MPS VII and control iPSCs were differentiated based on a protocol for generating long-term self-renewing NSCs from ESCs (Koch et al. 2009). Briefly, iPSC colonies were removed from the

MEF feeder layer and 20% FBS was added in order to generate embryoid bodies. At day five the embryoid bodies were attached to poly-ornithine (Sigma) coated dishes and switched to a minimal neural induction media containing insulin, transferrin, selenium, and fibronectin (Sigma) until neural tube-like structures appear. At day 20 these tube-like structures were manually removed from the surrounding cell mass using a dissecting microscope and transferred to a suspension culture containing N-2 supplement (1:100, Gibco), bFGF (10 ng/mL, Gibco), and EGF (10 ng/mL, Sigma). Cells were trypsinized at day 23 and plated on poly-ornithine/laminin (BD biosciences) coated plates in NSC media containing bFGF (10ng\mL), EGF (10ng\mL), insulin (20µg/mL, Gibco), N-2 (1:100), and B-27 (1:1000, Gibco). iPSC-NSCs were passaged 1:2 every three to four days by trypsinization.

Characterization of iPSC-NSCs

G-banded karyotyping of iPSC-NSCs (p9) was performed by Cell Line Genetics. Terminal *in vitro* differentiation was carried out in Neurobasal:DMEM/F12 (Gibco) containing N-2 (1:100), B-27 (1:100), and cyclic AMP (300ng/mL, Sigma) for one month. Quantification of the percentage of undifferentiated and terminally differentiated iPSC-NSCs expressing cell-type specific markers was performed by manual counting of immunofluorescently labeled slides. Percentages are represented as means ± S.E.M. (n=4).

Gene transfer in MPS VII iPSC-NSCs

GFP labeling of iPSC-NSCs was achieved using a PiggyBac expression vector containing a puromycin resistance gene (PB-513B-1, Systems Biosciences). The CMV promoter sequence was replaced with the CAG promoter sequence using *SpeI* and *EcoRI*. *GUSB* was cloned into the PiggyBac plasmid using *SmaI* sites flanking the *GUSB* cDNA and a *SwaI* site within the multiple cloning site of the PiggyBac plasmid. Blunt-end ligation produced a correction vector as well a mock-correction vector containing *GUSB* in the reverse orientation. For transfection of iPSC-NSCs, we used the Lonza Kit for Mouse Neural Stem Cells according to the manufacturer instructions. $4x10^6$ cells were electroporated with 3µg of the GUSB expression vector or GFP

control + 1µg of the transposase expression plasmid (PB200PA-1, Systems Biosciences) using the Lonza Nucleofector set to program A-33. After allowing the cells a few days to recover, transfected cells were selected with 0.5µg/mL puromycin for one week.

Neonatal NSC Transplantation

Neonatal NOD/SCID mice were cryoanesthetized prior to transplantation. iPSC-NSCs were trypsinized and resuspended in PBS at 50,000 cells/µL. 2µL of the cell suspension was injected into each lateral ventricle using a pulled glass micropipette. All procedures were approved by the Institutional Care and Use Committee at the Children's Hospital of Philadelphia.

Adult NSC Transplantation

Prior to injection, 8 week old NOD/SCID or NOD/SCID/MPS VII mice (breeding stock was a kind gift of Drs. M. Sands and J. Nolta) were anesthetized with isofluorane and secured in a sterotaxic frame (Kopf). Burr holes were drilled into the skull and 50,000 corrected or mock-corrected cells in 1µL PBS were infused at a rate of 0.5µl/minute. The striatal coordinates were: 0.50mm caudal to bregma, 1.5mm left or right of midline, and 3.0mm ventral to the dural surface.

Adult AAV Injection

Prior to injection, 8 week old C57BL/6/MPS VII mice (n=4) were anesthetized with isofluorane and secured in a sterotaxic frame (Kopf). Burr holes were drilled into the skull and AAV2/1 vector in 1µL of PBS was infused at a rate of 0.5µl/minute. One hemisphere was injected with AAV2/1-GFP, and the contralateral hemisphere was injected with a 1:1 mixture of AAV2/1-GFP and AAV2/1-GUSB (total of $1X10^{11}$ vector genomes). Packaging, purification, and tittering were performed by The University of Pennsylvania Vector Core as previously described (Passini et al. 2003) The coordinates used for injection were: 0.50mm rostral to bregma, 1.5mm left or right of midline, and 3.0mm ventral to the dural surface.

Immunostaining

Brains were embedded in 2% agarose prior to vibratome sectioning and immunostaining. 60µm coronal sections were stored in PBS + 0.1% Na azide at 4º until ready for use. Brains were cryoprotected with 30% sucrose prior to embedding in OCT (Tissue-Tek). 20µm-thick frozen sections were cut using a cryostat (Leica). Vibratome and frozen sections were post-fixed in 4% paraformaldehyde and permeabilized in 0.3% Triton X-100. After blocking in 4% goat serum, the sections were incubated in primary antibody containing 1% serum for 1 hour at RT. Antibodies used and their concentrations are listed in Supplemental Table 1. Images were acquired using an epifluorescence microscope (DM6000 B, Leica) or a confocal-scanning laser microscope (FluoView1000, Olympus).

Image analysis and statistics

To visualize the distribution of CD68-positive microglia in MPS VII mice following adult iPSC-NSC engraftment, a montage of immunofluorescent images was combined using the photomerge tool in Photoshop CS6 (Adobe). ImagePro Plus 3.0 (Media Cybernetics) was used to identify and dilate all objects with an area greater than 20 pixels. CD68-positive microglia were quantified within 0.25mm of engrafted iPSC-NSCs. The density of microglia adjacent to corrected and mock-corrected cells was compared by two-tailed t-test using Prism 5.0 (Graphpad). Microglia with a CD68-positive area greater than 20 pixels were counted. Frozen sections (20µm) were quantitated for each condition, and data are represented as means ± S.E.M. (n=7).

β-glucuronidase assays

Napthol-AS-BI-β-D-glucuronide *was used to determine the presence of* GUSB activity in 20µm thick frozen sections as previously described (Snyder et al. 1995). For quantitation of β-glucuronidase activity in MPS VII and control NSCs, the fluorescence of hydrolyzed 4-methylumbelliferyl-β-D-glucuronide was measured as previously described (Cervera 2005). Three independent cultures were quantified, and data are represented as means ± S.E.M.

Gene transfer in iPSCs

MPS VII and normal iPSCs, normally passaged using dispase, were dissociated into single cells using TrpLE in the presence of 10µm Y-27632. The cells were plated on mitomycin-C inactivated MEFs which were in turn plated on a 1:3 dilution of Matrigel. X-tremeGENE 9 (Roche) was used to introduce PiggyBac (GUSB or revGUSB) and transposase plasmids 24 hr after iPSCs had been plated. After one week, green colonies were picked and expanded.

Figure 5.1 List of Antibodies used the Study

Antibody	Vendor	Catalog number	Concentration
mouse anti-SSEA4	Millipore	MAB4304	1:100
mouse anti-Tra-1-60	Millipore	MAB4360	1:100
mouse anti-Tra-1-81	Millipore	MAB4381	1:100
mouse anti-human specific nestin	Millipore	MAB5326	1:250
mouse anti-human specific nuclei	Millipore	MAB1281	1:200
goat anti-DCX	Santa Cruz	sc8066	1:100
mouse anti-β-III tubulin	Neuromics	MO15013	1:1000
mouse anti-MAP2	Sigma	M4403	1:500
rabbit anti-GABA	Sigma	A2052	1:1000
rabbit anti-GFAP	Millipore	AB5804	1:1000
mouse anti-Tyrosine hydroxylase	Sigma	T1299	1:1000
rabbit anti-KI67	Leica	NCL-ki67p	1:200
rabbit anti-S100β	Immunostar	22520	no dilution
mouse anti-Oct4	Abcam	Ab18976	1:100
mouse anti-c-Myc	Sigma	C3956	1:500
mouse anti-O4	Millipore	MAB345	1:100
rabbit anti-Iba1	Wako	019-19741	1:1000
rat anti-CD68	Abd Serotech	MCA1957	1:200
rabbit anti-α-Fetoprotein	Abcam	ab9372	1:100
mouse anti-Smooth muscle actin	Santa Cruz	sc-53142	1:200
goat anti-mouse Alexafluor 488 or 594	Life Technologies	A-11001/A11005	1:250
goat anti-rat Alexafluor 594	Life Technologies	A-11007	1:250
donkey anti-goat Alexafluor 594	Life Technologies	A-11058	1:250
goat anti-rabbit Alexafluor 488 or 594	Life Technologies	A-11008/A-11012	1:250

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