Characterization of Mouse and Human Embryonic Stem Cell-Derived Neural Progenitors

by

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Abstract

Embryonic stem cells (ESCs) are an exciting candidate for treating many degenerative diseases, because they retain the ability to become any desired cell type. We do not know enough to safely use embryonic stem cell therapies in human neurodegenerative diseases such as epilepsy, however. In our research, we transplant mouse or human embryonic stem cell-derived neural progenitors (mESNPs or hESNPs) into the hippocampus of control mice and those subjected to prior seizure experience. By characterizing these transplants, we can better understand the interaction between the ESNPs and the host brain. We are currently using immunohistochemistry to characterize the differentiation of transplanted m and hESNPs. Our results show that after 28 days, transplanted mESNPs predominantly become the neural cell types found in the upper blade of the dentate gyrus of the hippocampus. We also show that interneuron progenitors can be generated from hESNPs and transplanted into the mouse hippocampus where they become mature interneuron subtypes after 3 weeks. Since interneurons are responsible for inhibiting over-excitation and are susceptible to cell death in temporal lobe epilepsy (TLE), this finding is particularly significant for developing cell replacement therapies for seizure reduction. Results from both projects will help us better understand the interactions between transplanted ESNPs and the host brain and bring us closer to being able to use ESCs in neurodegenerative disease therapies.
Acknowledgements

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Chapter One: Introduction

ESC derivatives are an exciting option for cell-based therapies. Many diseases, disorders, and injuries involving the central nervous system (CNS) ultimately result in cell death or the loss of a certain cell type. We now know that adult neurogenesis exists in discrete areas of the brain, but it is not sufficient in repairing large-scale damage or cell death (Fuentealba et al., 2012). While damage or dysfunction in the CNS can take many forms, ESCs not only have the potential to become any cell type in the body, but also reliably differentiate into neuronal and glial derivatives in vitro. Using one of multiple in vitro cell culture systems, we can induce the ESCs to differentiate along a particular lineage to become neural progenitors and even mature neurons. This process of neural differentiation of ESCs, in many key ways, closely parallels the development of the CNS in vivo. One important application of this system is our ability to document, manipulate, and test treatments for human developmental disorders in vitro. A second application of this system gives us the ability to produce a desired neuronal cell type for transplantation into a patient suffering from a neurodegenerative disease or CNS injury.

Culture of Embryonic Stem Cells

There are multiple in vitro systems for both maintaining and differentiating ESCs. In order to derive neuronal subtypes, the ESCs are first differentiated into neural stem cells (NSCs). Figure 1 outlines various protocols used to differentiate ESCs into NSCs. It is important to understand the differences between these protocols
in order to accurately interpret the results of others as well as choose the most appropriate differentiation protocol when designing an experiment.

Figure 1. Protocols for the Maintenance and Neuronal Differentiation of ESCs
The figure shows schematic outlines of various protocols used for maintenance of ESCs and to direct the differentiation of ESCs into NSCs. Abbreviations: embryoid body-retinoic acid (EB-RA), medium conditioned by the human hepatocellular carcinoma cell line HepG2 (MEDII CM), embryoid body (EB), embryonic stem cells (ESCs), neural stem cells (NSCs). (Cai et al., 2007)

Protocols 1-5 show day 0 of neuronal induction beginning when the ESCs are removed from their feeder layer culture and maintenance medium. The three embryoid body (EB) protocols are distinguished by the presence of the EB intermediate. An EB is an aggregate of differentiating ESCs in suspension culture that produces derivatives of all three primary germ layers (Itskovitz-Eldor et al., 2000). Each of the three EB protocols uses different media to induce differentiation. The first
protocol treats with retinoic acid (RA) at day 4 of differentiation, the second uses the human hepatocellular carcinoma cell line HepG2 conditioned medium (MEDII CM), and the third uses an EB-defined medium followed by serum-free selection for NSCs. Though all three protocols produce NSCs, the second protocol is distinct in that it does not support the differentiation of visceral endoderm. Of the EB protocols, this would be advantageous when the experiment hopes to derive a pure population of NSCs. The first two protocols are also a little over a week shorter. One advantage to the third, longer protocol is that there are no primitive NSC aggregates by the end of the protocol. The fourth and fifth protocols described in figure 1 also follow removal of the ESCs from their feeder layer, but do not include the formation of EBs and take a little less time to develop NSCs. Protocol 4 co-cultures the ESCs with bone marrow stromal cells, which have been shown to induce neural differentiation. In this protocol, no aggregates are formed and NSCs are present by day 5, but, by the nature of a co-culture, the NSCs would have to be isolated from the stromal cells in order to obtain a pure population (i.e. for transplantation). Protocol 5 takes advantage of the default pathway for ESCs to differentiate along the neural lineage. When plated at very low densities, the ESCs form primitive NSC aggregates in the presence of the leukemia inhibitory factor (LIF). Though in aggregates, this protocol does produce a pure population of NSCs. The final procedure outlined in figure 1 shows a protocol in which the ESCs are first cultured in monolayer with no feeder layer and then simply transitioned into a serum free differentiation medium. This protocol eliminates the need for a feeder layer and relatively quickly produces a fairly pure population of NSCs. When using a fluorescent reporter line driven by an early NSC marker, this
final population can be sorted using fluorescence activated cell sorting (FACS) to select a highly pure population of NSCs. (Cai et al., 2007)

Induction of Neural Differentiation

The process of generating neural progenitors from ESCs in vitro utilizes many of the same pathways seen in the endogenous process of neurogenesis. We use both mESCs and hESCs to derive the progenitor populations for transplantation. The research in the second chapter outlines the procedures used to generate and characterize the neural progenitors from two different mESC lines: the YC5 GFP line and the Sox1-GFP/Ubi-RFP line. The YC5 GFP line was induced to differentiate along the neural lineage using an EB protocol, similar to the third protocol in figure 1, in which the EBs were first formed in suspension culture and then in a serum free adherent culture. These ESNPs were expanded using a medium containing fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF) (Hartman, 2010). In order to induce the differentiation of the mESCs into mESNPs, the Sox1-GFP/Ubi-RFP mESCs were cultured in a monolayer system with a serum free medium. This mESC line has a green fluorescent protein (GFP) reporter at the Sox1 gene locus (Ying, 2003). Essentially, this means that whenever the Sox1 gene is turned on, the cell also expresses GFP. Sox1 is expressed in early NSCs and can be localized at the nucleus using immunofluorescence (Pevny, 1998). During differentiation, GFP can be localized throughout the live cells, which indicates whether or not they are becoming neural progenitors. The Sox1-positive progenitors are still immature. A benefit of transplanting immature mESNPs is that they generally survive better when transplanted and retain the ability to become multiple neuronal subtypes depending
on the molecular signaling they receive, as shown in figure 2. Once transplanted, the signaling in the area where they have been delivered supports further differentiation.

The research in the third chapter focuses on hESNPs both \textit{in vitro} and \textit{in vivo}. The Hes3 Nkx2.1-GFP/Ubi-RFP hESCs were cultured on a feeder layer of mouse embryonic fibroblasts (MEFs) and mechanically passaged. To induce neural differentiation, hESCs were cultured in an N2B27 differentiation medium with noggin, a bone morphogenetic protein (BMP) signaling antagonist. Treatment with noggin promotes the maintenance of the ESC population rather than differentiation (Chaturvedi et al., 2009). To ventralize the progenitor population, the differentiation cultures also received a treatment of recombinant human sonic hedgehog (SHH). (Germain et al., 2013)

![Diagram](image.png)

**Figure 2. Directed Differentiation of Embryonic Stem Cells into Neural Derivatives**

(Left) The schematic shows generic mESCs directed to differentiate into neural progenitors using a serum free differentiation medium and growth factors. Box indicates the stage at which we transplant the mESNPs. (Right) The schematic shows hESCs directed to differentiate into first generic neural progenitors and then ultimately, with SHH treatment, into ventral forebrain progenitors (i.e. interneuron...
progenitors). Box indicates the stage at which we transplant the hESNPs. Sonic hedgehog (SHH), bone morphogenic protein (BMP), fibroblast growth factor (FGF), retinoic acid (RA). (Modified from Petros et al., 2011)

**Transplanting Embryonic Stem Cell Derivatives**

Once we have generated the m and hESNPs in vitro, they are transplanted into the mouse hippocampus. The hippocampus is a brain structure in the medial temporal lobe. It is part of the limbic system and is involved in learning and memory processes. Our previous research has looked at multiple aspects of characterizing transplanted ESNPs, including region specific differentiation, migration, and teratocarcinoma formation. Carpentino, et al. characterized transplanted mESNPs and showed that the fate of the transplant was dependent on where it was in the brain. Specifically, when mESNPs were transplanted into the fimbria, an area medial to the hippocampus and dense with axon tracts, they co-localized with markers for oligodendrocytes, a glial subtype (Carpentino et al., 2007). A subsequent project looked at the migration of transplanted mESNPs in the upper blade of the dentate gyrus. This research demonstrated that the chemokine CXCL12 plays a role in the migration of transplanted mESNPs and that blocking the interaction between CXCL12 and its receptor CXCR4 with AMD3100 truncates the migration of the transplanted cells. Understanding the mechanisms by which transplanted mESNPs migrate is important as we move toward designing cell replacement therapies. Some disease models will require focal administration of the transplant (i.e. inhibiting migration) and in other models it would be useful to be able to direct the migration of transplanted ESNPs to a certain area of interest (Hartman et al., 2010). One concern in using cell based therapies for treating neurodegenerative diseases is the possibility of teratocarcinoma
formation. Tumor formation can inhibit the therapeutic benefits of the transplant by producing unwanted cell types and/or growing to a lethal size. Germain, et al. set out to look at reducing the incidence of teratocarcinoma formation by increasing the purity of the transplanted population. They found that FACS isolated mESNP populations did indeed form fewer teratocarcinomas when transplanted. Though there is a lot of variation between ESNP batches, we can continue to transplant the purest populations possible in order to reduce tumor formation (Germain et al., 2012).

Many others have investigated the use of fetal, embryonic, adult, induced pluripotent, and mesenchymal stem cell derivatives for disease therapies. The neurodegenerative diseases which could potentially benefit from these cell based therapies include spinal cord injury, amyotrophic lateral sclerosis, Parkinson’s disease, Huntington’s disease, multiple sclerosis, stroke, traumatic brain injury, and epilepsy, to name a few. In some cases, therapies involve deriving a specific cell type or progenitor in vitro in order to replace the disease/injury-related cell loss. Others have found therapeutic benefits from transplanting ESNPs as a result of their general anti-inflammatory and immunosuppressive effects. (Naegele et al., 2010)

One example of using hESC derivatives to treat a degenerative disease in the CNS is the work from Aharonowiz, et al. published in 2008. It is often difficult or impossible to manipulate human diseases in vivo. Thus, we rely on animal models for testing new therapies. In this case, they used the mouse model for multiple sclerosis (MS). Even though we often work in animal models, we can move our research closer to clinically applicable therapies by transplanting hESC derivatives into disease models. Through these experiments, we can characterize hESC behavior in vivo, hone
techniques, and design protocols for treating these diseases in humans. MS in mice and humans is characterized by demyelination in the CNS. They generated hESNPs \textit{in vitro} and transplanted them into the ventricles of mice with MS. The transplanted hESNPs migrated through the white matter of the cortex, reduced inflammation, and lessened axonal damage. Not only did the transplanted hESNPs reduce demyelination, but they also saw amelioration of clinical signs of MS in animals that received the transplants. (Aharonowiz et al., 2008)

In another example, hESCs were differentiated into a more mature neuronal subtype. Patients who suffer from Alzheimer’s Disease show a significant loss in a specific neuronal subtype: basal forebrain cholinergic neurons (BFCNs). Okabe, et al. generated BFCNs from hESCs with the goal of treating an animal model of Alzheimer’s Disease. They developed an \textit{ex vivo} set up in which they showed that the hESC-derived BFCNs make functional synapses with endogenous neurons. (Okabe, 1996) Neurodegenerative diseases, such as Alzheimer’s Disease, affect a large portion of the population. Further research used an mESC derivative to promote the phagocytosis of and reduce aggregation of the peptide amyloid-β (Aβ) in a mouse model of AD. Once Napoli et al. differentiated the mESCs into glial precursors (ESCdM) \textit{in vitro}, they were transplanted into a mouse model of AD. Mice who received transplanted ESCdMs showed fewer Aβ aggregates. (Napoli et al., 2008)

\textit{Disease Models: Temporal Lobe Epilepsy}

TLE is a particularly appropriate neurodegenerative disease for cell-based therapy research for a number of reasons (Figure 3). The disease is characterized by recurring seizures and cell death, particularly of the inhibitory interneurons (Figure
4). A seizure event is the result of excessive excitatory activity in the neural circuitry due in part to the loss of the inhibitory interneurons (Stief et al., 2007). Seizures can disrupt daily activities and be debilitating (Shackleton et al., 2003). Currently, the seizure phenotype is treated using pharmacology. Unfortunately, a third of patients do not respond to the anticonvulsants prescribed for patients who suffer from recurrent seizures. The ability to model the phenotypes of TLE in the mouse is an important step towards addressing the need for alternative therapies.

Figure 3. Characteristics of Temporal Lobe Epilepsy
At the top left, the schematic shows the human brain. The temporal lobe is highlighted in green. The black circle represents the focus of a seizure. Arrows indicate the direction in which the over excitation event propagates through the circuitry. The flow chart describes the stages and symptoms of TLE. (Naegele et al., 2010)

Figure 4. Neurodegeneration in the Hippocampus of Human Patients with TLE
All three images show NeuN labeling in the granule cell layer (G) of the dentate gyrus and the polymorph (PM) region, just dorsal to the hilus. A shows NeuN in a control brain. B and C show NeuN in patients who suffered from TLE. Scalebar = 100um. (Swartz et al., 2006)

One model for neurodegeneration in the temporal lobe is the upper blade replacement model. An injection, of either cells or fluid, targeted to the upper blade of the dentate gyrus will follow a path of least resistance and disperse along the ventral surface of the upper blade (Figure 5). On the day of the injection, 0 Day, the dense population of
endogenous neurons in the upper blade is intact (Figure 5.A). After 3 and 7 days in vivo, these transplanted GFP mESNPs begin to populate the area of the upper blade and there is progressive disorganization of the endogenous neurons of the upper blade (Figure 5.C-F). By day 7, some of the transplanted mESNPs express the neuronal marker NeuN (Figure 5.F inset). After 28 days, there is extensive degeneration of the endogenous neurons of the upper blade and repopulation of that area by the transplanted mESNPs that have continued to mature into NeuN positive neurons (Figure 5.G-H). This model gives us a way to reliably transplant cells in a somewhat focal location, directly adjacent to the neurogenic niche of the SGZ, where we can characterize their differentiation over time and follow their repopulation of the neuronal loss due to the injection. (Hartman et al., 2010)


**NeuN**

<table>
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<tr>
<th>0 Days</th>
<th>3 Days</th>
<th>7 Days</th>
<th>28 Days</th>
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Figure 5. Upper Blade Replacement Model
The figure shows a time course of transplanted mESNPs in the upper blade of the mouse dentate gyrus. The timeline is measured in days post transplantation. A, C, E, and G show immunohistochemistry images for localization of the antibody Neuronal nuclei (NeuN). B, D, F, and H show the same NeuN localization merged with GFP+ transplanted mESNPs and Hoechst which labels all nuclei. Arrows indicate cells which are co-labeled with NeuN and GFP. (Hartman et al., 2010)

We are currently working toward using the pilocarpine model of TLE, characterized by both the recurring seizures and interneuron cell death seen in human patients with TLE. The initial insult of the administration of pilocarpine is followed by a latent period, the development of chronic seizures, neural degeneration, mossy fiber sprouting, and reorganization of the neural circuitry (Mello et al., 2005). Pilocarpine primarily affects the muscarinic receptors (M1 and M2), subsequently increases neuronal activity via N-methyl D-aspartate (NMDA) receptors and ultimately leads to neurodegeneration (Curia et al., 2008). Other research has shown that transplanting interneuron progenitors derived from the fetal mouse brain, specifically the medial ganglionic eminence (MGE), cells engineered to express certain anticonvulsant neurotransmitters, hESCs, or mESNPs can repair some of the characteristic loss of inhibition in the endogenous circuitry (Maisano et al., 2012), (Chu et al., 2004), (Boison et al., 2009), (Raedt et al., 2007), and (Calcagnotto et al., 2010). Reproducing this effect using hESC derivatives is an important step in the process of going from bench to bedside, because it shows the viability of the actual cells that can be used to treat human patients.
Chapter Two: Characterization of Mouse Embryonic Stem Cell-Derived Neural Progenitor Transplants

*Background*

While investigating cell-based treatments for neurodegenerative diseases, it is important to understand the interactions between the transplanted cells and their environment once transplanted into the brain. Characterizing these interactions helps us understand how to get the transplant to best integrate and survive with the endogenous cells and the mechanism by which the transplant may or may not elicit a response. Typically, this research is first performed in a model system. The ideal model system closely resembles the disease phenotype and mechanisms seen in the organism of interest, which can then be reproduced in a lower order species. For the purposes of studying neurodegenerative diseases, the mouse acts as a good model system, because it is a small mammal with a short gestation period and fairly similar nervous system to humans. Even before we induce the neurodegenerative disease in the mouse, we are able to transplant the ESNPs and study the interactions between the transplanted ESNPs and the endogenous brain. Since we transplant neural progenitors, characterizing their differentiation once *in vivo* gives us key insights into the signaling environment in the area receiving the transplant. We need to gather data on the reaction of the host brain to the transplant and the differentiation of the transplant over time in order to begin experimenting with treating a disease model.
**Adult Neurogenesis**

Since in our studies the ESNPs are transplanted into one of the sites of adult neurogenesis, it is particularly useful to survey the characteristics of this endogenous process. In the adult brain, there are two sites where new neurons are born: the subventricular zone (SVZ) and the subgranular zone (SGZ) (Doetsch, 2003). Figure 6 outlines the process of maturation as the progeny of an astrocyte like neural stem cell differentiates into a mature granule cell in the SGZ. Directly adjacent to the hilus, the neural stem cell divides asymmetrically. This asymmetric division results in one intermediate progenitor daughter cell and one astrocyte like neural stem cell. The intermediate progenitor cells begin to adopt markers of immature neurons, migrate radially, and eventually integrate into the circuitry of the upper blade of the dentate gyrus as mature granule cells (Fuentealba et al., 2012). After transplanting mESNPs into this region, we investigated whether they adopt a fate similar to or different from the endogenous neurons.
Figure 6. The Cell Stages of Neurogenesis in the Dentate Gyrus
The top left image shows a coronal section of the mouse brain. The inset on the top right is a magnification of the dentate gyrus from the coronal section. Darker lines represent the densely populated blades of the dentate gyrus. The lower image is a magnification of a small section of the upper blade of the dentate gyrus, boxed in inset. The schematic shows radial astrocytes (RA) in blue with a primary cilia and process extending into the granule cell layer (GCL). RA divide asymmetrically to produce one RA daughter and one intermediate progenitor cell (IPC) daughter in green. As the IPC matures, it migrates radially along the RA and become immature granule cells (IGC) in red. Ultimately, these cells mature into granule cells (GC) in
brown. The RA are in close contact with blood vessels (BV). RA domains I, II, and III indicate distinct reference areas of the RA population. Domain I is where the cilium projects into the hilus and RA are in close contact with BV. Domain II includes the cell body and process extending into the GCL. Domain III is where the processes of the RA come in contact with other cell types and glia. (Fuentealba et al., 2012)

In order to distinguish these stages of differentiation, from the progenitor to the mature neuron, there needs to be a way to characterize the intermediate cell types. Throughout the process of differentiation, there are changes in gene expression. Figure 7 shows that certain genes are turned on and off during the differentiation process; these genes serve as markers for specific stages. Through various techniques, these markers can be used to determine what intermediate subtypes the ESNPs have become. After the ESNPs have been transplanted, immunohistochemistry is used on frozen brain sections to track the differentiation of the transplanted ESNPs in vivo. Characterizing the ESNP transplants at various time points allows us to determine their rate of maturation and eventually what subtypes of neurons they become. The ESNPs are characterized using immunocytochemistry and reverse transcriptase polymerase chain reaction (RT-PCR) before transplantation and with immunohistochemistry after transplantation. Before transplantation, the cells are still proliferative and capable of differentiating along multiple neural lineages. The goal is for the ESNPs to mature once they are transplanted and become mature neurons, which integrate into the endogenous circuitry.
One of the advantages to transplanting the mESNPs into the upper blade of the dentate gyrus in the hippocampus is that it is directly adjacent to the SGZ, which already supports the well documented process of differentiating the endogenous neural progenitors, as seen in figure 6 (Fuentealba et al., 2012). Figure 7 outlines a more general progression of neural differentiation. The type-1 cells are analogous to the radial astrocytes in figure 6. Similarly, the type-2 and type-3 are analogous to the IPCs, the immature neuron to the immature granule cell, and the mature neuron to the mature granule cell (Kim et al., 2011). In characterizing the marker expression of the mESNPs in the upper blade, we can track their differentiation. In following this maturation process of the transplanted mESNPs, we can better understand the pathways for neuronal differentiation supported by the SGZ and how the transplanted cells respond to the environment of the upper blade.
Methods

Culture of Sox-1-GFP/Ubi-RFP 46C mESCs in vitro and induction of neuronal differentiation

Sox-1-GFP/Ubi-RFP mESCs were cultured using a monolayer protocol for maintenance of the mESCs and differentiation into mESNPs. To induce differentiation along the neural lineage, the mESCs were transferred onto laminin-coated cell culture dishes in N2B27 differentiation medium. (Ying et al., 2003; Germain et al., 2012)

Culture of YC5 GFP mESCs in vitro and induction of neuronal differentiation

YC5 GFP mESNPs were cultured using an EB protocol in which the mESCs were transferred into suspension with fetal bovine serum and in the absence of leukemia inhibitory factor (LIF). Finally, mESNPs were expanded in adherent culture with a serum free medium. (Hartman et al., 2010)
**Transplantation**

All experiments involving live animals were carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals and the National Institutes of Health. mESNPs were transplanted into the dentate gyrus using stereotaxic surgery. The surgery was performed as previously described (Hartman et al., 2010). Using a stereotaxic frame fitted with a rodent isoflurane gas mask, coordinates targeted the dentate gyrus in the hippocampus, and approximately 50,000 cells were deposited in one microliter of expansion medium. After 3, 7, or 28 days, the animal was perfused and the brain frozen in tissue freezing medium. Later, a cryostat was used to make 25 micrometer sections, which were then stored at -80°C.

Another source of variation in these experiments comes from the fact that not all of the animals experienced the same conditions pre transplantation. Some of the brains which received the GFP mESNP transplants had been previously treated with kainic acid for a different experimental protocol. Since there are documented neurochemical and histopathological changes in the brain as the result of the administration of kainic acid, we cannot say that the mESNP transplants were put into identical host environments (Sperk et al., 1983).

<table>
<thead>
<tr>
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<th>YC5 GFP</th>
<th>Sox1-GFP/Ubi-RFP</th>
</tr>
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<td><strong>28 days</strong></td>
<td>020409-03 020409-06 020409-09</td>
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Table 1. Case Numbers for mESNP Transplants Analyzed
Case numbers are named for the day on which the animal received the transplant (month, day, year-number on that day). Only transplanted mESNPs in the upper blade were analyzed. The following case numbers received kainic acid treatment: 020409 and 082809.

Immunohistochemistry

All immunohistochemical analysis was performed using the following protocol for frozen tissue sections as described in Hartman, et al., 2010. Slides with tissue sections were removed from the -80°C freezer, warmed on a slide heater, and briefly rehydrated with phosphate-buffered saline (PBS). For the antibodies requiring an antigen retrieval step for optimal staining, slices were incubated in an EDTA solution for 8-13 minutes at 65°C before undergoing the following steps. After blocking for one hour at room temperature in a humidified chamber with 5% natural goat serum (NGS) in .1% triton in PBS (PbTr) blocking buffer. Slices were then incubated in blocking buffer and primary antibodies overnight at 4°C in a humidified chamber. After rinsing and washing with PbTr, goat secondary antibodies were added to blocking buffer at a concentration of 1:1,000 and incubated on the slices for 90 minutes at room temperature in a humidified chamber. Slices were then again rinsed and washed with PbTr. Finally, they were incubated in Hoechst 33342 (Molecular Probes, Eugene, OR), diluted to 1:10,000. After another rinse and wash in PbTr, slides were mounted in gelvitol and left to dry overnight. Slides were imaged using a Nikon fluorescent microscope. NIS Elements imaging and cell counting software were used to analyze the images. Images were counted for the number of transplanted cells and then the number of cells also labeled for one of the cell specific markers.
Table 2. Primary Antibodies Used to Characterize mESNP Transplants

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<th>Antibody Type</th>
<th>Company</th>
<th>Dilution</th>
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<tbody>
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<td>ms anti-Neun</td>
<td>Chemicon</td>
<td>1:400</td>
</tr>
<tr>
<td>ms anti-Prox1</td>
<td>Chemicon</td>
<td>1:100</td>
</tr>
<tr>
<td>rb anti-S100B</td>
<td>Abcam</td>
<td>1:1000</td>
</tr>
<tr>
<td>ms anti-CNPase</td>
<td>Chemicon</td>
<td>1:750</td>
</tr>
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<td>rt anti-SST</td>
<td>Millipore</td>
<td>1:100 (Antigen Retrieval)</td>
</tr>
<tr>
<td>rb anti-PV</td>
<td>Sigma</td>
<td>1:1000</td>
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Note: The abbreviations ms, rt, rb, and gp denote the species in which the antibody was made: mouse (ms), rat (rt), rabbit (rb), and guinea pig (gp).

Results

Characterization of YC5 GFP mESNP 7 and 28 Days Post Transplantation

We observe from previous research that transplanted mESNPs respond to endogenous signaling, because they exhibit region specific differentiation (Carpentino et al., 2007). We also see that YC5 GFP mESNPs mature over time, when transplanted into the upper blade of the dentate gyrus (Hartman et al., 2010). Hartman et al. were also investigating the migration of transplanted mESNPs in the kainite mouse model of TLE. Thus, it is important to remember that the GFP mESNP transplants were made into animals that had received an injection of kainic acid and...
had experienced a seizure event. To characterize the YC5 GFP mESNP transplants, Hartman et al. used immunohistochemistry to identify what percent of the transplanted cells co-localized with markers for neural stem cells (nestin), immature neural progenitors (DCX), mature neurons (NeuN), and mature granule neurons (Prox1). Over time, a smaller percent of the transplanted cells expressed immature neuronal markers and more expressed mature markers. They reported that over 90% of the transplanted mESNPs were nestin positive on day 0, whereas less than 10% were nestin positive by day 28. Concurrently, 0% were NeuN positive on day 0 and almost 30% were NeuN positive by day 28 (Hartman et al., 2010).

In this study, we extend the immunohistochemistry analysis to further characterize these YC5 GFP mESNP transplants. The goal of this analysis was to determine which neuronal subtypes the transplanted mESNPs had become in vivo and to see how the subtype composition of the transplants differed over time between the 7 and 28 day time points. Various neural and glial markers were chosen to obtain as comprehensive a characterization profile as possible. The markers used to analyze the 7 day transplants include NeuN, which labels the neuron specific hexaribonucleotide binding protein-3, which is present in the nucleus at various later stages of maturity. β-III-tubulin (Tuj1), which recognizes a neuron-specific tubulin, is one of the earliest markers indicating committed neuronal specification of neurepithelial cells. The third marker used on the 7 day transplants, PSA-NCAM, also localizes immature neurons and is expressed in migrating neuroblasts. After 7 days in vivo, the transplanted mESNPs are approximately 60% Tuj1+, 40% PSA-NCAM+, and 20% NeuN+. These data suggest that at 7 days the mESNPs are primarily immature neurons (Figure 9 top
right graph). These results do not provide an in-depth analysis, however. Using previously collected data limited the number of transplants and range of time points available for analysis.

After nearly a month *in vivo*, immunohistochemistry and antibody markers were again used to characterize the differentiation of the transplanted mESNPs. Again, I looked at the localization of NeuN, Tuj1, and PSA-NCAM in order to compare the maturity of the transplant’s cell subtypes over time. I also looked at other markers for mature cell subtypes. S100Beta is a glia-specific protein, which primarily localizes to astrocytes (Abdel-Rahman et al., 2004). 2’,3’-Cyclic-nucleotide 3’-phosphodiesterase (CNPase) is an enzyme related to myelin and is exclusively found in the glial subtype, oligodendrocytes. I also looked at two mature interneuron subtype markers, parvalbumin (PV) and somatostatin (SST). Interneurons are the primary inhibitory, gamma-aminobutyric acid (GABA)-ergic neurons of the CNS. Finally, I investigated the mature granule cell marker prospero homeobox protein 1 (Prox1). Prox1 is localized to the mature neuronal subtype, granule cells. Granule cells populate the dense blades of the dentate gyrus, including surrounding the SGZ where the mESNPs are transplanted. After counting the number of GFP+, transplanted mESNPs from three different brains, I counted the number of transplanted cells that were positive for each marker (Figure 9). The data showed that, for the glial subtypes, the transplants were .08% CNPase+ and 5% S100Beta+. The interneuron markers, PV and SST, were expressed in 2% of the transplanted cells (Figure 9 bottom right graph). The immature neuronal markers, Tuj1, NeuN, and PSA-NCAM, comprised 18%, 22.3% and 24%, respectively, of the transplanted
population. 12.63% of the transplants were Prox1+. These data suggest that at 28 days \textit{in vivo}, the transplanted mESNPs have differentiated primarily into immature and mature neuronal subtypes with very few interneurons or glial subtypes present. Since some of these markers are expressed during multiple stages of differentiation and there can be a lot of variation from transplant to transplant, it is difficult to determine what percent of the transplanted population has been accounted for. I estimate that over 50% of the transplanted population has been accounted for in this analysis.

Figure 9. Characterization and Quantification of GFP mESNP Transplants (upper right graph) The graph shows the quantification of neural markers using immunohistochemical analysis on 7 and 28 day YC5 GFP mESNP transplants. The marker localization is shown as a percentage of positive cells per the number of transplanted cells (GFP+) in the upper blade. N=2 for 7 day transplants and N=3 for 28 day transplants. (lower right graph) The graph shows quantification of immunohistochemical data from 28 day YC5 GFP mESNP transplants. Glial and
Characterization of RFP mESNPs 3 and 7 Days Post Transplantation

Since the RFP mESNPs are generated from a different mESC line than the YC5 GFP mESNPs, it is important to confirm that they also follow a similar differentiation process when transplanted into the same environment. In preliminary experiments addressing this question of whether or not the cells are maturing along the neural lineage while in vivo, the localization of the marker polysialyted-neural cell adhesion molecule (PSA-NCAM) was quantified. PSA-NCAM marks immature and migrating neurons (Quartu et al., 2008). The percentage of PSA-NCAM/RFP+ transplanted cells in the UDG were counted. Figure 10 shows this quantification data as a percent of the transplanted population, which is PSA-NCAM+. 38% of the 3 day RFP transplants were PSA-NCAM+, whereas 7% of the 7 day RFP transplants were PSA-NCAM+ (Figure 10). Prior to transplantation, the majority of the cells were primitive neural progenitors, as indicated by being Sox1-GFP positive (Becker, unpublished). The presence of immature neural markers and the lower percentage of immature neurons over time suggest that the mESNPs are maturing in vivo. When comparing the GFP and RFP mESNP transplants, we see that at day 7 30% of the GFP transplants are PSA-NCAM positive, whereas 7% of the RFP transplants are PSA-NCAM (Figure 9 and 10). This variation can likely be attributed to differences
in cell lines, the different stages at which the mESNPs were transplanted, and the variation in the differentiation protocols used *in vitro*. Future research will need to also confirm the presence of more mature neural markers over time.
Marker Expression of RFP mESNP Transplants: PSA-NCAM

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</tr>
<tr>
<td>PSA-NCAM/RFP+</td>
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</tr>
</tbody>
</table>

**DAY 3**

A. **Hoechst/RFP**

B. **Hoechst/RFP**

**DAY 7**

C. **Hoechst/PSA**

D. **Hoechst/PSA**

E. **RFP/PSA**

F. **RFP/PSA**
Figure 10. Characterization and Quantification of RFP mESNP Transplants

(Graph) The graph shows quantification of RFP mESNP transplants in the upper blade of the dentate gyrus after 3 and 7 days in vivo. 38% of the RFP+ cells in the 3 day transplants were also PSA-NCAM+. Error bar shows standard error of 13 (n=3). 7 percent of the RFP+ cells in the 7 day transplants were also PSA-NCAM+. Error bar shows standard error of 3 (n=2). A and B) show RFP+ transplanted mESNPs in 3 and 7 day transplants, respectively, with the nuclear stain, hoechst. C and D) show the same fields corresponding to A and B, respectively. Hoechst is again in blue and PSA-NCAM is shown in green. E and F) are merges of the RFP+ in red and PSA+ in green cells from A/C and B/D, respectively. Yellow cells are co-labeled for both PSA and RFP. Scalebars are 25 um.

Discussion

From the immunohistochemical analysis, we show that both the mESNPs generated from the Sox1-GFP/Ubi-RFP and YC5 GFP ESC lines in vitro continue to mature and primarily differentiate along the neuronal lineage after transplantation in vivo. The small percentages of interneuron and glial subtypes found in these transplants can be attributed to the fact that, when transplanted, the ESNPs are still general neural progenitors. Since the mESNPs were transplanted into the UDG, directly adjacent to the neurogenic niche of the SGZ, the results speak to the ability of this region to support primarily neuronal maturation and differentiation into the neuronal subtypes of this endogenous environment of the SGZ.

It is important to recognize that the transplanted populations of mESNPs were somewhat heterogeneous in that not all progenitors were at the same stage when transplanted, derived using the same protocol, or put into the same host environment. Since prior to transplantation mESNPs were not sorted, we are likely transplanting a population that is not comprised of only neural progenitors. Cells in culture do not all differentiate synchronously. Starting with a heterogeneous population nearly ensures heterogeneity as the transplants mature in vivo. We can also not make a direct
comparison between the YC5 GFP transplants and the Sox1-GFP/Ubi-RFP transplants, since they were derived using different protocols. Both differentiation protocols use serum free medium to enhance the population for neural progenitors, but the EB protocol used to differentiate the GFP mESCs takes longer, uses different signaling, and produces more intermediates than the monolayer protocol used for the RFP mESCs. Prior to transplantation, over 90% of the GFP mESNPs were nestin positive and less than 10% were Tuj1 positive (Hartman et al., 2010). The RFP mESNPs were transplanted once a high percentage of the population was Sox1-GFP positive (Becker and Lassiter, Unpublished). As previously mentioned, the endogenous environments into which the mESNPs were transplanted varied. Due to the data available, the animals that received the GFP mESNP transplants had been subjected to kainic acid treatment, whereas the RFP mESNP transplants were performed on naïve animals. In future experiments, we hope to better control for and track progenitor transplants from the same run of differentiation, and we will continue to characterize the transplanted mESNPs in naïve animals and eventually work toward transplanting into a disease model in the hopes of attenuating certain symptoms of neurodegeneration.

Note that this characterization data has been restricted to transplanted mESNPs in the UDG. Neurodegenerative diseases can affect all different regions of the CNS, which are each characterized by different signaling environments (Okabe et al., 1996) and (Aharonowiz et al., 2008). In order to better understand how transplanted mESNPs will behave in these different environments, future studies will need to continue characterizing transplants in diverse regions. For example, we know
from previous research that transplanted mESNPs in the fimbria tend to differentiate into oligodendrocytes rather than the populations of granule neurons we see in the UDG (Carpentino et al., 2007). Furthermore, others have shown that mESNPs transplanted into a mouse model of Parkinson’s disease can differentiate into dopaminergic neurons and ameliorate disease symptoms (Bjorklund et al., 2001). We also know some of the molecules that play a role in the migration of transplanted mESNPs (Hartman et al., 2010). Combining a better understanding of region specific differentiation and molecular migratory cues could prove to be a powerful tool for directing the differentiation and localization of transplanted mESNPs for cell based therapies. Currently, the characterization results showing neuronal maturation in UDG mESNP transplants gives us a better understanding of the interactions between the endogenous environment and the transplanted cells. Ultimately, this brings us closer to being able to use ESC-derivatives as cell based therapies for neurodegenerative diseases.
Chapter Three: Characterization of Human Embryonic Stem Cell-Derived Interneuron Progenitors

Background

The larger goal of the experiments in this project is to continue to produce and characterize the hESC-derived interneuron progenitors in vitro and transplant them into the hippocampus where they differentiate into mature interneurons and integrate into the endogenous neural circuitry. Specifically, we investigated the use of hESC-derived interneuron progenitors as a cell based therapy for temporal lobe epilepsy (TLE).

In order to derive these interneuron progenitors from hESCs, we look to the endogenous process by which interneurons are generated. During development, the interneurons are born in ventral structures and migrate tangentially to populate the cortex and the hippocampus. Labeled in figure 11, these structures are called the lateral, caudal, and medial ganglionic eminences (LGE, CGE, and MGE) (Wichterle et al., 1999). Though the vast majority of interneurons use the neurotransmitter gamma-aminobutyric acid (GABA) and provide inhibitory input to the neural circuitry, there are many distinct subtypes of interneurons (Xu et al., 2004; Tricoire et al., 2011). In TLE, the somatostatin (SST)-positive interneurons and other MGE derived interneuron subtypes are more susceptible to cell death (Choi et al., 2007). Though the cause for increased vulnerability in these interneurons of the hilus is unknown, Choi, et al. demonstrated that levels of striatal-enriched protein tyrosine phosphatase (STEP) and the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway are implicated. They found that the presence of
STEP inhibits the neuroprotective MAPK pathway, leaving the interneurons more vulnerable to status epilepticus (SE)-induced excitotoxicity (Choi et al., 2007).

Figure 11. In Vivo Origin of Hippocampal Interneurons
This schematic illustrates the transient embryonic structures of the ganglionic eminences and the paths taken by the migrating interneurons to the cortex. The blue region is the medial ganglionic eminence (MGE). Blue arrows show migration pathways of somatostatin (SST) and parvalbumin (PV) expression interneurons, derived from this region. The lateral ganglionic eminence (LGE) is labeled in green and red. The dorsal and ventral caudal ganglionic eminence (d and vCGE) span the posterior portion of these regions. (Wonders et al., 2006)
Figure 12. Fate of MGE versus CGE-Derived Interneurons
The schematic shows the subtypes of interneurons that were found to have originated at the early time points (left diagram) and later time points (right diagram). Overlapping boxes indicates interneuron populations which co-label for multiple markers. Yellow, orange, and red boxes indicate interneuron subtypes which originate from the MGE. Green, blue, and magenta boxes indicate interneuron subtypes which originate from the CGE. (Tricoire et al., 2011)

In order to direct the hESCs to differentiate into interneuron progenitors, we use some of the same molecular cues, which drive interneuron differentiation in vivo. We use a hESC GFP reporter line that is driven by the Nkx2.1 gene (Goulburn et al., 2011). Nkx2.1 is a transcription factor specific to the MGE during interneuron development (Wonders et al., 2006). The use of this reporter line enables us to visualize when the live cells are becoming MGE-like progenitors. During development, SHH is expressed along the rostral – caudal axis of the neural tube. Its presence induces the differentiation of ventral neural lineages (Ericson et al., 1995). A little later in development, when the ganglionic eminences have formed, SHH is expressed in the MGE and its presence is required for maintenance of the MGE-
specific transcription factor Nkx2.1. When SHH signaling is disrupted during this process, fewer Nkx2.1-positive progeny develop (Wonders, 2008). *In vitro* we are able to differentiate the hESCs along a neural lineage and, after treating the cultures with SHH and/or an agonist purmorphamine, which targets smoothened in the SHH pathway (Sinha et al., 2005), into Nkx2.1-positive progenitors (Germain et al., 2013).

Next, we confirmed that the hESC-derived Nkx2.1-positive interneuron progenitors are similar to endogenous MGE progenitors. To do this, we FACS isolated the differentiating hESNPs and obtained a 97% pure population of Nkx2.1-GFP-positive cells. In this sorted population, we used RT-PCR to look at the expression of a number of cell type specific markers and transcription factors. Figure 13 shows that the Nkx2.1-GFP-positive cells are indeed turning on the Nkx2.1 and Nkx6.2, which is specific to the dorsal portion of the MGE, genes. Importantly, they are not expressing genes that are specific to dorsal glutamatergic neural progenitors, such as Pax6 (Germain et al., 2013). Since one of the purposes of generating these Nkx2.1-positive hESNPs is to affect the neural circuitry *in vivo*, they need to be able to produce action potentials and functionally integrate into the neural circuitry. Other studies have shown that hESC derived Nkx2.1-positive progenitors are capable of firing action potentials (Goulburn et al., 2011). To test the firing properties of the cells generated in these experiments, we use patch clamp analysis and electrophysiology.

After differentiation *in vitro* and treatment with SHH and/or purmorphamine, the hESC-derived interneuron progenitors are transplanted into the hippocampus. The goal is to transplant these interneuron progenitors into a mouse with pilocarpine.
induced TLE in order to restore inhibitory inputs when they integrate into the circuitry, which could reduce seizure activity.

Figure 13. Nkx2.1-GFP-Positive Cells Have an MGE-Like Gene Expression Profile

RT-PCR data in the upper left compares expression levels of various genes between the GFP- and GFP+ populations. Higher intensity bands indicate higher expression levels in that cell population. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control. The schematic on the upper right outlines the regions of the developing brain where expression of these genes is localized.
PCR data showing up and down regulation of certain genes in the GFP+ population relative to the GFP- population (Germain et al., 2013)

Methods

Derivation of interneuron progenitors in vitro

hESC-derived interneuron progenitors were generated as described in Germain, et al., 2013. Briefly, hES3-Nkx2.1-GFP/Ubi-mCherry hESCs were passed into N2B27 medium for a day and then into terminal differentiation medium on laminin and poly-L-lysine coated culture dishes. Cultures were treated with human recombinant SHH to enrich the Nkx2.1-positive population. Once the progenitors were sufficiently abundant, FACS was used to isolate an Nkx2.1-GFP+ pure population for transplantation. For electrophysiology recordings, unsorted progenitors were passed onto glass coverslips coated with poly-L-lysine and laminin. Differentiating progenitors were cultured in a terminal differentiation medium consisting of neurobasal medium, non-essential amino acids, penicillin/streptomycin, L-glutamine, GDNF, BDNF, cyclic AMP, ascorbic acid, and B27. Laminin was added at 1 μL/mL to the medium for the progenitors on coverslips.

Electrophysiology

Patch clamp analysis was performed on cells from the differentiating cultures on coverslips at various time points. During the recordings, the coverslips were continuously perfused with carbon dioxide bubbled terminal differentiation medium, without the GDNF, BDNF, or B27 at 33°C. Cells were selected based on neuronal morphology. Glass electrodes with 7-11 MΩ resistance were used for patching. Electrodes were filled with IPSO solution containing biocytin. Once the electrode sealed with the membrane, the pressure on the electrode was released and the
electrode patched the membrane. Recordings were made using IGOR software during the administration of and response to a current injection of 20–100 pA in current clamp and with no current injection in voltage clamp in order to capture spontaneous activity. Analysis of data was made using IGOR software.

Transplantation

FACS isolated Nkx2.1-GFP-positive progenitors were transplanted using stereotaxic surgery, similar to the protocol used for transplanting the mESNPs in the previous chapter. The coordinates were altered in order to deposit the cells in the hilus, directly ventral to the SGZ and between the upper and lower blades of the dentate gyrus. The animal sacrificed 3 weeks after surgery received a bilateral transplant. The animal sacrificed 6 weeks after surgery received a transplant of cells in one hippocampus and a media injection in the contralateral hippocampus. The animals were given daily cyclosporine injections (Calbiochem, 100mg/L) (Lassiter, C. and Tagliatela, S.) and then sacrificed 3 and 6 weeks after transplantation (Tagliatela, S.) (case numbers 051012-01 and 051012-02, respectively). (Maisano et al., 2011)

Immunohistochemistry and Cell Counting

All immunohistochemical analysis used for the hESC-derived interneuron progenitor transplants followed the same protocol as described for the mESNP transplant analysis. When biotin was used, the protocol was altered in that the secondary antibody was replaced with animal specific biotin and incubated as a regular secondary antibody. After rinsing and washing with PbTr, the slice was incubated in streptavidin, which is conjugated to a fluorophore, for 30 minutes in a
humidified chamber. The rest of the protocol was followed as previously described.

The following additional primary antibodies were used in this analysis.

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<td>rb anti-NOS</td>
<td>Millipore</td>
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<td>rb anti-Chat</td>
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<td>ms anti-MAP2</td>
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<tr>
<td>rb anti-calretinin</td>
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Table 3. Primary Antibodies Used to Characterize hESNP Transplants

The table shows the specific antibodies used, the company that made them, and the concentration in blocking buffer at which they were used. The following abbreviations were used to denote the species in which the antibody was made: mouse (ms), rat (rt), and rabbit (rb).

Immunohistochemical data for each marker were collected by counting all RFP+ cells in the hilus and upper blade of the dentage gyrus (UDG) using NIS Elements AR software. Then, the number of transplanted cells positive for each marker was counted.

Results

*In Vitro Electrophysiology Characterization of Nkx2.1 hESNPs*

Whole cell patch clamp recording was used to assess the excitable capabilities of the hESC-derived interneuron progenitors *in vitro*. Once a cell was identified in culture as having processes and generally neuronal morphology, it was imaged in DIC and GFP. GFP+ cells were patched and their responses in current clamp to
500ms current injections ranging from 20 to 100 pA were monitored using IGOR software. At a membrane potential of -50 to -70mV, Nhx2.1-GFP-positive hESNPs were able to generate a single action potential in response to the current injection, but did not show mature firing patterns such as firing action potential trains. For example, after a current injection of 40 pA, one cell exhibited a 36mV threshold followed by an action potential with a 5.1mV peak and 9ms half width. While there was not a clear second action potential following this event, the membrane did hyperpolarize to -39mV before depolarizing to -14mV and finally resting at -24mV (Figure 14). No clear action potentials were observed during recordings of spontaneous electrical activity after multiple current injections. Over the various time points, 15 cells were successfully patched. These data suggest that Nhx2.1-GFP hESNPs have the ability to fire action potentials in response to a current injection and that their firing patterns are still immature.
Figure 14. Induced Excitability of Nkx2.1-GFP-Positive hESNPs
A shows a bright field (DIC) image of the cells in view during one recording. The box centers on two cells, the right cell was used for patch clamp analysis. The inset A’ shows a fluorescent image of the selected cell, which is GFP positive. The image was taken at 40X and scale bar = 64px. The graph shows membrane potential on the y axis and time in milliseconds (msec) on the x axis.
Characterization of Nkx2.1-GFP hESNPs 3 and 6 Weeks Post Transplantation

Since the ultimate goal of generating interneuron progenitors in vitro is to transplant them into an in vivo model for TLE, we need to understand how these hESC-derived interneuron progenitors behave in vivo. A population of these Nkx2.1-positive progenitors was enriched in vitro, and Nkx2.1-GFP-positive cells were isolated using FACS. This technique produces a population that is up to 99% GFP+ (Germain et al., 2013).

Using immunohistochemistry, we identified cells in both the 3 and 6 week transplants which were RFP+ and had healthy nuclei, based on their morphology. This tells us that the transplanted cells were able to survive at least 6 weeks in vivo. In order to characterize their differentiation, we examined the expression of a number of different cell and interneuron subtype specific markers including somatostatin (SST), nitric oxide synthetase (Nos), choline acetyltransferase (ChAT), and microtubule associated protein 2 (MAP2). During development, the SST+ and Nos+ interneuron subtypes originate from the MGE. The presence of ChAT indicates a cholinergic neuron which is also an MGE derivative. Both excitatory and inhibitory neuronal subtypes express ChAT. MAP2 is a pan-neuronal marker. Data are presented as percentages (Figure 15. Graph). The 3 week transplant was 58% MAP2/RFP+, 28% ChAT/RFP+, 0% Nos/RFP+, and 6% SST/RFP+. The 6 week transplant was 49% MAP2/RFP+, 20% ChAT/RFP+, 7.6% Nos/RFP+, and .42% SST/RFP+. These data suggest that the hESC-derived interneuron progenitors survive up to 6 weeks after transplantation in the mouse hippocampus, that many differentiate into neurons, and that some are able to differentiate into mature interneuron subtypes.
Figure 15. Characterization and Quantification of hESC-Derived MGE-like Progenitor Transplants

A) shows 3 week RFP+ transplanted Nkx2.1-GFP hESNPs in red in the UDG, hilus, and lower blade of the dentate gyrus. Nuclei are marked with Hoechst in blue. A’) shows the same view as A with localization of the marker Map2 in green. Scalebars=200um. B) shows RFP+ transplanted cells in red and nuclei labeled with Hoechst in blue. C) shows the same nuclei as B with the marker Map2 in green. D) is a merge of the RFP and Map2 from B and C, respectively. Yellow denotes RFP+ transplanted cells which are also Map2+. E) shows a red RFP+ transplanted cell (arrow) and its nucleus labeled with Hoechst in blue and an endogenous RFP- nucleus (arrow head). F) shows the same nuclei as E, with a SST+ endogenous cell (arrow head) and a SOM+ transplanted cell (arrow). G) shows a merge of the RFP and SOM from E and F, respectively. Scalebars=25um. Graph) The marker localization for 3 and 6 week Nkx2.1-GFP hESNPs are shown as a percent of the RFP+ transplanted population. The 3 week transplant was 58% Map2/RFP+, 28% ChAT/RFP+, 0% Nos/RFP+, and 6% SOM/RFP+. The 6 week transplant was 49% Map2/RFP+, 20% ChAT/RFP+, 7.6% Nos/RFP+, and .42% SST/RFP+.

Discussion

These results analyzing the Nkx2.1-GFP hESNP populations demonstrate that the Nkx2.1-GFP-positive hESNPs are excitable in vitro, can survive when transplanted in vivo for 3 and 6 weeks, and can differentiate into mature neurons and some interneuron subtypes in vivo. The Nkx2.1-GFP-positive population is particularly relevant for those neurodegenerative diseases that result in the loss of inhibition in the neural circuitry, including TLE.

As we continue to characterize these progenitors, we are working to enhance the percent of the population that turns on Nkx2.1-GFP following treatment with various combinations of ventralizing factors (i.e. SHH and purmorphamine) in vitro. We are also working to create an environment in which the differentiating progenitors...
develop mature electrical signaling capabilities. We are able to induce single action potentials, but do not yet see mature firing patterns, such as action potential trains. It is important that these progenitors are able to connect, respond to signaling, and generate inhibitory inputs to the neural circuitry in vivo, if they are going to effectively restore the inhibition to the diseased neural circuitry in TLE, for example. To possibly promote maturation of their electrochemical excitability, we are using a co-culture system in which the Nkx2.1-GFP hESNPs are cultured in monolayer with mouse fetal cortical cells during the differentiation process. This more closely resembles the signaling environment during endogenous neural development, in which excitatory and inhibitory neurons develop in close proximity to form the neural circuitry. The co-culture system may promote better survival of the hESNPs as well as earlier maturation of ion channels, which are responsible for producing mature firing patterns (Song et al., 2002).

In the future, more transplants will be made into both naïve and disease model animals. One goal of future transplantation experiments with these Nkx2.1-GFP hESNPs is to generate material for electrophysiological analysis on live hippocampal slices containing transplanted cells. Data showing that a transplanted cell was able to synapse onto an endogenous neuron and produce an inhibitory response would confirm that the hESNPs were able to connect with and influence the inhibitory/excitatory balance of the neural circuitry (as observed for mESNPs: Maisano, 2012). Ultimately, these Nkx2.1-GFP hESNPs will be transplanted into animals with pilocarpine induced TLE. The pilocarpine model is characterized in part by recurring seizures. Since others have found that transplanting fetal MGE-derived
interneuron progenitors into a mouse model of TLE reduced seizure threshold and convulsive activity (Calcagnotto et al., 2010). Replicating these findings using hESC derivatives would bring us significantly closer to implementing this type of cell base therapy for patients who suffer from recurring seizures, especially those who do not currently respond to traditional pharmacological treatments.
Conclusions

The *in vitro* and *in vivo* characterization of ESNPs allows us to better understand the process of neural differentiation, model complex diseases, and even direct the differentiation of specific neuronal subtypes. We show that both mESNPs and hESC-derived interneuron progenitors survive and continue to mature over time along their neural lineages when transplanted into the adult mouse hippocampus. Future research will continue to characterize the differentiation of the transplanted m and hESNPs. Using whole cell patch clamp recordings, we demonstrate that *in vitro* hESNPs are capable of firing an action potential in response to 500ms current injection. This suggests that they may have the ability to form functional synapses with neurons *in vivo*. Future experiments *in vivo* will investigate this type of functional interaction between the transplanted cells and the endogenous circuitry and subsequently the ability of these hESC-derived interneuron progenitor transplants to suppress seizure activity in the pilocarpine model of TLE.
References


