Differentiation of Human Embryonic Stem Cell-Derived GABAergic Interneurons Using an Optimized Coculture System

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Table of Contents

Abstract .................................................................................................................. 1

Chapter 1: Introduction

Human embryonic stem cell-derived GABAergic interneurons—a hope for temporal lobe epilepsy? .......................................................... 3
The generation of forebrain derivatives from hESCs. ................................. 9
Specifying the GABAergic interneuron lineage in hESCs. ......................... 13
Unresolved questions concerning the in vitro differentiation and maturation of hESC-derived GABAergic interneurons. ........................... 19
Figures .................................................................................................................. 24

Chapter 2: Optimization of a coculture system for hESC-derived GABAergic interneuron generation

Abstract .............................................................................................................. 29
Introduction ......................................................................................................... 29
Materials and Methods ..................................................................................... 34
Results .................................................................................................................. 39
Discussion ........................................................................................................... 41
Figures .................................................................................................................. 48

Chapter 3: Astrocyte cocultures facilitate the maturation of hESNPs into GABAergic interneurons

Abstract .............................................................................................................. 54
Introduction ......................................................................................................... 55
Materials and Methods ..................................................................................... 57
Results .................................................................................................................. 59
Discussion ........................................................................................................... 63
Figures .................................................................................................................. 70

Chapter 4: Future Directions .............................................................................. 79

References .......................................................................................................... 89
# List of Figures

## Chapter 1

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CNS regional specification of hESCs</td>
<td>24</td>
</tr>
<tr>
<td>2</td>
<td>Forebrain patterning by signaling factors</td>
<td>26</td>
</tr>
<tr>
<td>3</td>
<td>Interneuron subtype origins</td>
<td>28</td>
</tr>
</tbody>
</table>

## Chapter 2

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>hESC neural differentiation protocol</td>
<td>48</td>
</tr>
<tr>
<td>2</td>
<td>Marker expression of ventralized hESNPs at differentiation day 30</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>MAP2 expression of hESNPs in different coculture conditions</td>
<td>52</td>
</tr>
<tr>
<td>4</td>
<td>GABA expression of hESNPs in different coculture conditions</td>
<td>53</td>
</tr>
</tbody>
</table>

## Chapter 3

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Live images of hESNP morphology and Nkx2.1 expression over time</td>
<td>70</td>
</tr>
<tr>
<td>2</td>
<td>Enhanced neuronal differentiation in cultures with astrocytes</td>
<td>72</td>
</tr>
<tr>
<td>3</td>
<td>Aggregation of non-neuronal clusters in cultures without astrocytes</td>
<td>74</td>
</tr>
<tr>
<td>4</td>
<td>Interneuron characterization at 14 weeks of differentiation</td>
<td>76</td>
</tr>
<tr>
<td>5</td>
<td>Electrophysiological maturation of hESC-derived neurons</td>
<td>78</td>
</tr>
</tbody>
</table>
Abstract

The differentiation of human embryonic stem cells (hESC) into GABAergic interneurons provides new options for the treatment of neurodegenerative diseases such as temporal lobe epilepsy (TLE). An hESC-derived interneuron therapy could assume the inhibitory activity of lost cells and reduce seizures. Using an hES3 Nkx2.1:GFP reporter cell line, we have optimized a protocol for generating hESC-derived neural progenitors (hESNPs) expressing Nkx2.1, a marker for the neurogenic region in which GABAergic interneurons arise.

This thesis focuses on efficiently maturing these hESNPs into GABAergic interneuron phenotypes to demonstrate their potential as a transplantable population. First, we tested a battery of culture conditions to optimize the generation of GABA-positive neurons. We find that coculturing hESNPs with astrocytes in a tapered serum medium condition treated with the Notch-inhibitor DAPT yields the highest percentage of neurons at 8 weeks of differentiation. In the optimized medium conditions, we examine the effect of coculturing by comparing hESNPs cultured alone to those cocultured with astrocytes. We find that the astrocytes promote hESNP survival, accelerate neuronal differentiation, and attenuate the formation of non-neural clusters that otherwise spread throughout hESNP cultures. Finally, hESNPs cocultured in the optimized condition for extended timelines exhibit expression of the interneuron subtype markers somatostatin (SST) and calbindin (CB). By 12 weeks of differentiation, neurons can fire trains of action potentials (APs), demonstrating their capacity for electrical function. Together, these data establish an astrocyte coculture system for the maturation
of hESNPs, confirm that the progenitors can produce high percentages of GABAergic interneurons, and show the electrophysiological function of derived neurons.
Chapter 1: Introduction

Human embryonic stem cell-derived GABAergic interneurons: a hope for temporal lobe epilepsy?

GABAergic interneurons in health and disease

A diverse hierarchy of neuron types exists throughout the brain. Each type modulates the electrical activity of other neurons by releasing neurotransmitters during action potentials (APs), electrical pulses induced by a depolarized membrane potential. The binding of neurotransmitters to transmembrane receptors leads to the opening of ion channels, increasing the receptive cell membrane’s conductance for specific positively or negatively charged ions. Most neurons of the mammalian cortex can be grouped into two broad categories. Glutamatergic projection neurons synapse both locally and distally. They communicate with neurons in the same cortical hemisphere, with neurons in the contralateral hemisphere across the corpus callosum, with subcortical neurons in lower brain regions, and with neurons of the spinal cord. These cells develop from progenitors in the developing cortex, and their postsynaptic effect is typically excitatory. In contrast, GABAergic interneurons arise from progenitors born in the ventral forebrain and are primarily inhibitory. They form synapses locally and release the neurotransmitter gamma-aminobutyric acid (GABA), which affects the postsynaptic cell through two types of receptors. GABA binds to ionotropic GABA_A receptors, which allow chloride ions to pass through the membrane, or to metabotropic GABA_B receptors, which activate G-proteins that open channels permeable to potassium ions (K^+). Various combinations
of GABA receptor subunits give rise to further receptor subtypes and diversify the postsynaptic effect of GABAergic input (Macdonald and Olsen 1994).

The net effect of GABA is usually inhibitory due to the hyperpolarizing action of K⁺ flowing out of the cell and/or Cl⁻ flowing into the cell. In special cases the effect can be excitatory, such as when receptive cells have either a particularly low resting membrane potential (Sauer et al. 2012) or a high intracellular Cl⁻ concentration as is the case during early periods of cortical development (Ben-Ari et al. 2004). Interneurons maintain a balance of excitation and inhibition in the cortex that is vital to cognitive function (Yizhar et al. 2011). In addition to preventing overexcitation, networks of interneurons connected by gap-junctions coordinate the temporal synchronization of neuronal circuits by imposing oscillations of characteristic frequencies on their target neurons (reviewed in Buzsaki and Chrobak 1995; Buzsaki and Draguhn 2004). Given that primates possess a higher proportion of cortical interneurons to projection neurons than mice (Hendry et al. 1987; Gabbot and Bacon 1996; Gabbot et al. 1997), it has been proposed that humans’ advanced cognitive abilities can be partially explained by an increased capacity for interneurons to modulate neuronal circuit activity (Clowry 2014).

The lack or dysfunction of GABAergic interneurons has been implicated in a number of neurological conditions. For example, the brains of patients with schizophrenia exhibit atypical neural oscillatory patterns (Uhlhaas and Singer 2010) and reduced inhibition (Yoon et al. 2010). Multiple lines of evidence suggest that deficiencies in the electrical activity of interneurons expressing the calcium-binding protein parvalbumin (PV) may contribute to this disorder (Marin 2012). Mouse models of other neuropsychiatric disorders including autism spectrum disorders and Fragile X syndrome
also exhibit impaired GABAergic activity. Mechanisms include a reduced production of the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD), low GABA receptor densities, and low numbers of interneurons (Marin 2012).

Epilepsy is a neurodegenerative disorder associated with a failure of GABAergic transmission. Temporal lobe epilepsy (TLE) is the most common form. Mouse models of the TLE exhibit a loss of PV and somatostatin (SST)-expressing GABAergic interneurons in the hilus of the dentate gyrus in the hippocampus (Kobayashi and Buckmaster 2003; Sloviter et al. 1987). The dentate gyrus acts as a gate for excitatory inputs to the hippocampus via the perforant pathway from the entorhinal cortex. Given a lack of inhibition, however, the hippocampus can easily become a seizure focus because it can form a positive feedback loop with the entorhinal cortex (Goldberg and Coulter 2013).

The promise of a hESC-derived treatment for TLE

Advances in the understanding of GABAergic interneuron function and dysfunction have opened up new possibilities for treating many of the abovementioned neurological conditions. Much research is currently underway investigating the possibility of developing an interneuron-replacement therapy to combat diseases such as TLE. To restore healthy electrical activity, replacement cells require the capacity to integrate into the appropriate host circuitry and must possess inhibitory functions characteristic of GABAergic interneurons. Some researchers have shown a seizure-reducing effect of transplanted cells engineered to produce inhibitory molecules such as GABA (Thomson 2005). However, most have focused on the possibility of transplanting
fetal-derived GABAergic interneuron precursors into the seizure focus, which would provide activity-dependent inhibition of excitatory cells. Cells of this type also possess a special capacity to migrate from their injection site and integrate into the host tissue, which may make them more amenable to transplantation than other types of neural progenitors (Tyson and Anderson 2014).

The success of studies transplanting mouse and human GABAergic interneuron progenitors shows the promise in this approach. The medial ganglionic eminence (MGE) is the developmental origin of most cortical interneurons. Fetal grafts of mouse MGE tissue into the cortex or hippocampus of mouse models of epilepsy differentiate into GABAergic interneurons, increase inhibitory activity, and reduce seizures (Baraban et al. 2009; Zipancic 2010). Injections of human fetal tissue into the bloodstream also significantly reduce seizures in a mouse model of epilepsy (Chu et al. 2004), although the mechanism of this reduction is ambiguous because only a quarter of the transplanted cells became GABAergic interneurons (Maisano et al. 2009). Transplantation studies using human cells are critical, since a GABAergic interneuron replacement therapy would need to be of human origin to avoid possible harmful immune responses and contamination of the host brain by animal products. However, the ethical roadblocks of using human fetal material has made it difficult to replicate mouse MGE transplant studies with human tissue and are not a realistic option for a widely applicable cell therapy.

The derivation of GABAergic interneuron progenitors from pluripotent stem cells (PSCs) provides a more promising source of transplantable cells. PSCs have the ability to generate cell types of all three primary germ layers, given the correct culture conditions. Since they also self-renew and can be maintained in the pluripotent state indefinitely in
vitro, PSCs can potentially generate many GABAergic interneuron precursors for transplantation. Induced pluripotent stem cells (iPSCs), for example, are generated from the somatic cells of individual patients by treatment with a cocktail of transcription factors. The patient-specific genotype of iPSC lines also reduces concerns of immune rejection of transplants and enables the in vitro study of gene-related diseases and their possible treatments (Takahashi et al. 2007). Despite these advantages, there are concerns that must be addressed before using iPSCs for human transplants. Most significantly, reprogramming requires the retroviral delivery of carcinogenic genes. These ectopic sequences remain inserted in the cell’s genome after reprogramming and can lead to tumor formation in iPSCs and their derivatives (Medvedev et al. 2010).

Recent investigation of GABAergic interneuron replacement therapies has used well-characterized embryonic stem cell (ESC) lines. ESCs are another type of PSC derived from the inner cell mass of the developing blastocyst. Initial studies transplanting ESC-derived neural progenitors (ESNPs) are promising. Mouse ESNPs (mESNPs) directed down the GABAergic interneuron lineage have been successfully transplanted into healthy mouse cortex (Maroof et al. 2010) and into the dentate gyrus of mice with TLE (Maisano et al. 2012). The transplanted cells were positive for markers of GABAergic interneuron subtypes and exhibited AP spiking patterns characteristic of interneurons (Maroof et al. 2010; Maisano et al. 2012). The hippocampal transplants also displayed excitatory post-synaptic potentials, indicating that they were electrically active and integrated into the host tissue (Maisano et al. 2012). In another study, human ESNPs (hESNPs) enriched for MGE- like GABAergic interneuron precursor identity that were transplanted into mice cortices also showed markers of GABAergic interneurons and were able to fire APs (Nicholas et al. 2013).
These studies reflect favorably on the prospect of a GABAergic interneuron cell therapy. However, there is still much to be learned about the subtype identity determination and maturation timeline of human ESC (hESC)-derived interneurons, which are features likely to determine the cells’ potential to affect the host circuitry post-transplant. Nicholas et al. showed that transplanted hESNPs could survive up to 7 months post-transplant and could fire trains of APs, and the same hESNPs differentiated with mouse cortical astrocytes \textit{in vitro} undergo a 30 week maturation period throughout which their electrophysiological properties mature (Nicholas et al. 2013). However, there was a near absence of the late interneuron marker PV in the cultures—only a low, transient expression was observed. In addition, only half of the surviving transplanted cells expressed GABA at 6 months post-transplant, which further increases uncertainty about the regulation of neuron subtype identity (Nicholas et al. 2013).

hESC-derived interneurons require study both \textit{in vitro} and post-transplant to further assess their potential as a cell therapy. The protracted period of maturation observed by Nicholas et al. would pose a significant challenge for the first clinical trials of PSC-derived interneurons, which would most likely involve very ill patients who would need the transplanted cells to mature quickly (Tyson and Anderson 2013). It may be the case that different culture protocols can accelerate hESC-derived interneuron differentiation and maturation. For example, Nicholas et al. observed a 7-month period of maturation of their hESC-derived interneurons and saw few or no PV-positive cells. In contrast, another group differentiated GABAergic interneurons from hESCs with “fairly mature” electrophysiological properties and a robust number of PV-positive cells after two months of differentiation by coculturing ESNPs with embryonic mouse cortical cells (Maroof et al. 2013). Assessing different differentiation protocols and coculture
techniques will be important for determining whether hESC-derived interneuron differentiation and maturation can be accelerated. Long-term cultures of putative MGE-like hESNPs can also supplement transplant studies by confirming their adoption of a GABAergic identity and revealing their interneuron subtype expression profile and capacities for electrophysiological function. In contrast to transplants, which are not observable or accessible until the animal is sacrificed, *in vitro* maturation allows examination of these characteristics of hESNPs in an observable and easily-controlled setting.

**The generation of forebrain derivatives from hESCs**

*Human embryonic stem cells*

hESCs are of interest because they are pluripotent and have the capacity to indefinitely self-renew (Thomson et al. 1998). They are derived from the inner cell mass (ICM) of the human blastocyst, the pluripotent cell population which gives rise to the three germ layers of the embryo during development. The resulting cell line can be expanded indefinitely with maintained pluripotency, or directed along specific lineages *in vitro* given the proper culture environment. hESCs provide an *in vitro* strategy for modeling disease and human development, and hold the possibilities of generating transplantable material to replace degenerated cells or deliver a drug. Even before hESCs were first derived, populations of donor cells, tissues, or organs could be transplanted into patients to combat diseases in which certain cell types are missing or damaged. However, this approach relies on the donors of the biological material and transplantation is not an option with many adult donor cell types, including neural tissue. hESCs have the
potential to provide a high number of transplantable cells if an appropriate differentiation protocol is developed.

A growing repertoire of specific cell types of all three germ layers have been derived from hESCs, including retinal pigment epithelium (Pan et al. 2013), red blood cells (Lu et al. 2010), and various neuronal subtypes (Kriks et al. 2011; Espuny-Camacho et al. 2013; Germain et al. 2013). With hESC-derived retinal pigment epithelium already being tested in clinical trials, and many other hESC-derived therapies at or approaching clinical trial-ready status (Schwartz et al. 2012), the therapeutic use of hESC derivatives is a rapidly progressing field, a highly promising clinical avenue, and a potent tool for studying human biology in vitro.

Neural induction and forebrain specification of hESCs

Forebrain cell types that have successfully been derived from hESCs include glutamatergic pyramidal neurons (Espuny-Camacho 2013) and GABAergic interneurons (Li et al. 2009; Maroof et al. 2013; Germain et al. 2013; Nicholas et al. 2013). Each protocol to generate forebrain cells contains steps to encourage increasingly differentiated classes of neural progenitors. The progression begins with neural induction from the pluripotent state, proceeds through neuroectodermal intermediates, and ends with hESNPs. The resulting cells possess regional identities corresponding to progenitors regionally patterned along the anterior-posterior and ventral-dorsal axes of the developing central nervous system. Culture conditions that encourage this succession of neural identities act on signaling pathways critical to neural development in vivo. While
different protocols vary in efficiency and reliability, the resulting cell populations mimic morphological and molecular aspects of normal neural development.

Methods for the neural differentiation of hESCs fall into three basic groups: those with an embryoid body intermediate, coculture with stromal cells, and adherent monolayer without coculture. Some techniques also use a combination of these methods (reviewed in Cai and Grabel 2007; Lupo et al. 2014). Common to many of them is a focus on minimizing signaling in the Activin/Nodal and bone morphogenetic protein (BMP) pathways during early differentiation, as hESCs will adopt a neuroectoderm fate under these conditions. This tendency is reminiscent of the early embryo, in which signaling centers secrete growth factor inhibitors that encourage neural differentiation (reviewed in Wurst and Bally-Cuif 2001). When hESCs are put in serum-free suspension culture, aggregates form clumps of epiblast-like cells surrounded by extraembryonic visceral endoderm, called embryoid bodies (EBs). Cell types of all three germ layers arise in this culture system. Neuroectoderm differentiation, which is induced in the epiblast core by cell-cell interactions with the extraembryonic visceral endoderm, can be enhanced by altering culture conditions (reviewed in Cai and Grabel 2007). hESCs can also be cultured on a monolayer with bone marrow stromal cells, which encourage neural induction through unknown signaling mechanisms.

Adherent monolayer protocols produce neural precursors from hESCs if treated with BMP antagonists such as noggin, which binds to BMPs 2, 4, and 7 (Gerrard et al. 2005; Germain et al. 2013). The efficiency of this induction is improved if the small molecule BMP-pathway antagonist SB-431542 is added to the cultures (Chambers et al. 2009) or noggin is replaced with LDN-193189, another BMP-pathway antagonist which
prevents the phosphorylation of BMP targets SMADs 1, 5, and 8 (Banda and Grabel 2014). Other protocols utilize inhibitors of other signaling pathways, such as Wnt or nodal inhibition by Dkk1 or lefty, respectively (Watanabe et al. 2005), but BMP antagonism by LDN-193189 alone is sufficient for generating high percentages of neural progenitors (Banda and Grabel 2014).

In addition to producing high percentages of neural precursors, adherent monolayer cultures form neural rosettes, *in vitro* structures that resemble morphological and molecular aspects of the neural tube (Germain et al. 2010). During development, neural progenitors adopt regional fates along the anterior-posterior axis by the time the neural tube has formed. At that stage, the presence of Wnt, TGF-beta, fibroblast growth factor, or retinoic acid specifies caudal fates such as hindbrain and spinal cord. In neuralized hESCs, the inhibition of these signals, or simply a lack thereof, can specify the development of forebrain progenitors (*Figure 1*, Lupo et al. 2014; Pankratz et al. 2007). This is consistent with the “activation-transformation” model of regional specification, in which all neural precursors are born with an anterior fate and only lose it if treated with caudalizing factors (Stern et al. 2006). Whether inhibitors of these factors are needed or their simple lack is sufficient to allow forebrain fate seems to vary between hESC lines (Kim et al. 2011).
Specifying the GABAergic interneuron lineage in hESCs

**GABAergic interneurons arise from the basal forebrain**

To understand the principles behind the specification of GABAergic interneurons from hESNPs, one must first understand how they arise *in vivo*. It was previously thought that all the neurons of the neocortex, including interneurons, were generated in the subventricular zone of the pallium and migrated radially to their final destination in the various layers of the cortex (Rakic 1971). While the progenitors of some neuron subtypes, including cortical projection neurons, do follow this path during development, a number of research groups in the 80’s and 90’s demonstrated that GABAergic interneuron progenitors arise from another location in the brain. In rodents, virtually all GABAergic interneuron progenitors arise in transient embryonic subpallial domains and migrate tangentially (perpendicular to the radial glia fibers of the pallium) to their final destinations in the neocortex (*Figure 2*, Van Eden et al. 1989, DeDiego et al. 1994, de Carlos et al. 1996). Some evidence has emerged suggesting that primates, including humans, differ from other mammals in that some interneurons may also arise from the ventricular and subventricular zones of the developing neocortex as well as in the subpallium (Letinic et al. 2002; Rakic and Zecevic 2003). However, doubt has been cast on this hypothesis by the suggestion that the expression of molecular markers used to identify interneuron progenitors in these studies was misinterpreted (Clowry et al. 2014). Subpallial domains have been firmly established as interneuron sources in primates (Hansen et al. 2013).

Subpallial interneuron progenitor-producing domains include the preoptic area (POa) and the ganglionic eminences. Around 10 percent of cortical GABAergic
interneurons derive from the POa (Gelman et al. 2009), which also is a source of striatal interneurons (Marin et al. 2000). The ganglionic eminences are comprised of three distinct domains: the lateral ganglionic eminence (LGE), the caudal ganglionic eminence (CGE), and the medial ganglionic eminence (MGE). The LGE is not generally thought to be a source of a large number of GABAergic interneurons and instead gives rise mainly to the projection neurons of the striatum and interneurons of the olfactory bulb (Stenman et al. 2003). LGE cells migrate into the cortex late in development, however, and some evidence suggests that a portion of these cells eventually express GABA (Anderson et al. 2001). The CGE is an established source of cortical GABAergic interneurons, especially for caudal-most portions of the cortex (Nery et al. 2002, Yozu et al. 2005). The MGE is the primary source of GABAergic interneurons, providing over 50% of cortical interneuron progenitors (Sussel et al. 1999, Pleasure et al. 2000) as well as serving as a source for striatal interneurons (Marin et al. 2000).

**GABAergic interneuron diversity**

One of the challenges to studying cortical GABAergic interneurons, and generating representative populations of them from hESCs, is the high number of subtypes. At least 20 functionally distinct basic cortical GABAergic interneuron subtypes have been identified, differing in location among the cortical layers, electrophysiological characteristics, molecular makeup, synapse location on the target cell, and laminar projections. Each interneuron subtype can also vary significantly across species and change in characteristics under different conditions or at different phases of the organism’s life. For example, the expression of the calcium-binding protein parvalbumin
(PV) is dependent on the electrical activity of the cell and intracellular neurotrophin levels (Marty et al. 1997). The challenge of characterizing such a broad array of interneuron phenotypes led a group of neuroscientists to meet in Petilla de Aragon, Spain (the hometown of Ramon y Cajal), to establish a standard nomenclature for discussing the various features of interneurons (Ascoli et al. 2008). Despite these efforts, there remain concerns that current nomenclature is not sufficient to encompass all interneuron types, and more sophisticated classification systems have been proposed (DeFelipe et al. 2013).

One may focus solely on molecular identity and site of origin for a simpler view of cortical GABAergic interneuron diversity (Figure 3). By these characteristics they can be grouped into three broad categories (Rudy et al. 2011). About 40 percent of all cortical GABAergic interneurons express the calcium-binding protein parvalbumin (PV) and are generated in the MGE (Xu et al. 2008). Also arising from the MGE, another 30 percent express the neuropeptide somatostatin (SST), about half of which co-express neuropeptide-Y (NPY) (Xu et al. 2008). The remaining 30 percent arise mainly from the CGE and express the 5HT3R-alpha serotonin receptor and many of these cells also express calretinin (CR) (Lee et al. 2010). Some of the 5HT3R-alpha receptor-positive interneurons may also arise from the POa, as the expression of the receptor extends from the CGE into the POa during development (Lee et al. 2010). These interneurons express NPY and/or the extracellular matrix glycoprotein reelin but not PV, SST, or CR (Gelman et al. 2009).
**Nkx2.1 in interneuron progenitors**

An understanding of the transcriptional regulation of interneuron identity can begin to account for the wide range of interneuron subtypes. In addition, the subpallial domains that give rise to GABAergic interneuron progenitors also give rise to other neuron types, and molecular characterization of these cells is important for distinguishing between the distinct subpopulations of progenitors that arise. The use of transgenic animals, genetic lineage tracing, and gene knockout experiments has helped establish the transcriptional profile of subpallial progenitors and their daughter neurons’ identities.

Cre-knockout and lineage tracing studies suggest that the distal-less homeobox (Dlx) genes 1 and 2, which are expressed in subpallial domains including the CGE and MGE, play a critical role in the development of GABAergic interneuron progenitors (Long et al. 2009). In these domains Dlx 1/2 induces the differentiation of cortical GABAergic neurons by regulating Notch signaling and promoting the production of glutamic acid decarboxylase 65 and 67, the enzymes which produce GABA (Anderson et al. 1999) and repressing the expression of the transcription factor Nkx2.1 in GABA interneuron progenitors as they migrate into the cortex (Long et al. 2009). Nkx2.1 is upregulated in ventral neural progenitors by a gradient of Sonic hedgehog (Shh) signaling originating from the underlying mesoendoderm (Ericson et al. 1995). Shh signaling acts in opposition to a dorsally-concentrated Wnt signaling gradient mediated by the activity of Gli3, and the two signaling pathways pattern the forebrain with ventral and dorsal neural identities (Figure 2, Gaspard et al. 2008, Goulburn et al. 2012). In the developing ventral forebrain, Nkx2.1 is expressed in nearly all of the cells of the MGE, POa, parts of
the septum, and the ventral-most edge of the CGE. There is also Nkx2.1 expression in the hypothalamic region of the developing midbrain (Xu et al. 2008).

Fate mapping in mice reveals that Nkx2.1-expressing cells contribute to dopaminergic, cholinergic, and GABAergic neurons of the neocortex, amygdala, olfactory bulb, striatum, globus pallidus, septum, and nucleus basalis (Xu et al. 2008). Most, if not all, of the interneurons of the neocortex are derived from Nkx2.1-positive progenitors, including at least 90 percent of the PV-expressing and 85 percent of the SST-expressing interneurons of the deep cortical layers and at least 70 and 60 percent, respectively, of the PV-positive and SST-positive interneurons in the superficial cortical layer (Xu et al. 2008). A similarly high percentage of hippocampal interneurons, striatal cholinergic interneurons, striatal GABAergic interneurons, pallidal projection neurons, and cholinergic projection neurons of the basal telencephalic nuclei are derived from Nkx2.1-positive progenitors (Xu et al. 2008). Interestingly, Nkx2.1-positive cells that migrate into the cortex downregulate the transcription factor as they mature, whereas those that become interneurons of the basal ganglia continue to express it (Marin et al. 2000).

Generating GABAergic interneuron progenitors from hESCs

In recent years, there has been a major focus on directing the differentiation of hESCs into GABAergic interneuron progenitors. In 2005, Watanabe and colleagues established that the expression of ventral telencephalic markers could be induced in ESC-derived telencephalic precursor cells by treatment with Shh, mirroring in vivo patterning by the morphogen during development (Watanabe et al. 2005). The group confirmed
ventral forebrain identity based on the upregulation of the markers Nkx2.1 and Islet 1/2. Subsequently, efficient protocols were developed for generating specific neural subtypes in hESCs, particularly ventral forebrain cell types (Watanabe et al. 2007, Aubry et al. 2008, Li et al. 2009, Germain et al. 2013). Ventral forebrain cell types can be generated from hESCs by either the application of Shh or the inhibition of Wnt signaling (Li et al. 2009). Most protocols that generate hESC-derived ventral forebrain progenitors rely on the activation of the Shh pathway by the ligand itself, the use of Purmorphamine (Pur), a small molecule hedgehog pathway agonist (Watanabe et al. 2007, Germain et al. 2013), and/or the combination of Shh pathway activation and Wnt inhibition (Aubry et al. 2008, Li et al. 2009, Nicholas et al. 2013, Maroof et al. 2013). Many of these protocols yield populations of cells that are over 50 percent Nkx2.1-positive.

In 2011, Goulburn et al. developed a hES3 hESC line using homologous recombination in which a GFP reporter is expressed downstream of the Nkx2.1 promoter (Goulburn et al. 2011). This live Nkx2.1:GFP readout facilitates the optimization of protocols for basal forebrain progenitors and allows for fluorescent-activated cell sorting (FACS) to isolate purified populations of Nkx2.1-positive cells. A number of groups use the hES3 Nkx2.1:GFP cell line to investigate the differentiation potential of Nkx2.1:GFP-positive ESNPs. The cell types generated are similar to those that arise from Nkx2.1-positive progenitors in vivo. hES3 Nkx2.1:GFP-positive hESNPs yield GABA-positive neurons expressing the interneuron subtype markers SST, PV, neuronal nitric oxide synthase (nNOS), calbindin (CB) and calretinin (CR), indicating GABAergic interneuron lineage commitments (Goulburn et al. 2011, Germain et al. 2013; Maroof et al. 2013; Nicholas et al. 2013). Other Nkx2.1-lineage cell types are also generated, such as tyrosine hydroxylase-expressing dopaminergic projection neurons, choline acetyltransferase-
positive cholinergic interneurons, or PDGFR-alpha-positive oligodendrocytes (Goulburn et al. 2011; Maroof et al. 2013).

Initiating the application of Shh and Pur at different points during hESNP differentiation leads to the emergence of discrete populations of Nkx2.1:GFP-positive progenitors that yield different proportions of neuron subtypes (Maroof et al. 2013). Applying Shh and Pur from day 2 to 18 of differentiation yields “hypothalamic-like” Nkx2.1-positive cell populations with low expression of the forebrain marker FoxG1, and high expression of the dopaminergic neuron marker tyrosine hydroxylase (TH) (Maroof et al. 2013). Application from day 6-18 results in an “striatal” population with low percentages of TH, SST, PV, and CB expression but high percentages of FoxG1 and choline acetyltransferase (ChAT), a marker for cholinergic neurons (Maroof et al. 2013). In contrast, applying Shh and Pur day 10 to 18 yields largely “MGE-like” FoxG1-positive progenitors that become largely SST, PV, nNOS, and CB-positive GABAergic interneurons (Maroof et al. 2013).

Unresolved questions concerning the in vitro differentiation and maturation of hESC-derived GABAergic interneurons

Characterization of GABAergic interneuron subtypes generated from the directed differentiation of mouse ESCs has involved a combinatorial analysis of molecular marker expression, electrophysiological properties, and morphology (Maisano et al. 2012). However, the study of these aspects of hESC-derived interneuron identity is limited by the ability to effectively differentiate and mature the progenitors. Because primate interneurons undergo a protracted period of development in vivo and may be dependent
on the contact of other neural cell types to mature (Anderson et al. 1995, Fertuzinhos et al. 2009), these needs may pose a problem for generating mature phenotypes from hESCs.

Two recent studies addressed this problem by coculturing enriched populations of Nkx2.1:GFP-positive hESC-derived GABAergic interneuron progenitors with either mouse cortical astrocytes (Nicholas et al. 2013) or mouse embryonic cortical cells, a mixture of excitatory neuron and glial cell progenitors (Maroof et al. 2013). While both groups achieved a high percentage of hESC-derived GABAergic interneurons that exhibited mature electrophysiological properties, their results differed in two important respects. First, the subtype identities of the two resulting populations of interneurons differed. The hESNPs cocultured with cortical cells produced neurons that exhibited markers characteristic of MGE-derived interneurons; SST, nNOS, and PV were each expressed in about 40 percent of the Nkx2.1-positive cells, and almost 80 percent stained positive for CB at about 2 months of differentiation. hESC-derived neurons in astrocyte cocultures also showed a robust expression of SST, about 40 percent staining positive after 30 weeks of differentiation. However, the neurons took much longer in culture to reach that percentage—only about 10-15 percent expressed SST at 20 weeks, and less than 5 percent expressed the marker at 10 weeks. In addition, the neurons showed a lower CB-positive percentage throughout the 30 week period (20-40 percent) and lacked PV expression altogether except for a low percentage (about 10 percent) at 15 weeks of culture.

It is unclear whether these differences in subtype expression are due to the difference in cell types used in cocultures or due to differential populations of
Nkx2.1:GFP-positive progenitors arising from their respective differentiation protocols. Maroof et al. used an adherent monolayer neural differentiation method. The small molecules LDN-193189 and SB431452 were used for SMAD inhibition, and the tankyrase inhibitor XAV939 was used to antagonize Wnt signaling. To ventralize the cells, Pur and Shh were added from days 10-18 (Maroof et al. 2013). In contrast, Nicholas et al. followed a neural differentiation protocol first forming suspended embryoid bodies, then adherent embryoid bodies, and finally an adherent monolayer. Five factors were used during neural differentiation: the ROCK inhibitor Y27632, the SMAD inhibitors SB431452 and BMPRIA, the Wnt inhibitor Dickkopf-related protein 1 (Dkk1), and the Shh pathway inhibitor Pur. The latter was used for ventralization from day 1 to 25 of differentiation. Further study is needed to parse out the differences in cell culture protocols that lead to the specification and maturation of particular interneuron subtypes.

The second way in which the experiments differed is the timeline of interneuron maturation. hESNPs cocultured with astrocytes followed a maturation timeline of over 7 months, whereas those cocultured with cortical cells were only cultured for about 2 months. It is possible that coculture with cortical cells accelerates interneuron differentiation and/or maturation. Alternately, interneuron development may follow a cell-intrinsic timeline regardless of culture conditions, and 7 month astrocyte cocultures generate a more mature phenotype than the 2 month cortical cell cocultures due to the extended period of culture. As the differentiation and maturation timelines of hESNPs in the two culture techniques have not been directly compared, it is difficult to assess which hypothesis is true. Further study of the effect of culture conditions on the differentiation and maturation of hESC-derived interneurons is warranted.
These recent studies demonstrating the maturation of hESC-derived GABAergic interneurons in vivo underscore the fact that we do not yet understand the molecular mechanisms guiding subtype identity, terminal differentiation, and electrophysiological maturation. A culture system optimized for the differentiation and maturation of hESC-derived GABAergic interneurons would help identify relevant parameters promoting these processes. This work would also supplement related efforts of groups at Wesleyan transplanting ESC-derived GABAergic interneuron progenitors into mouse brains (Carpentino et al. 2008; Hartman et al. 2010; Maisano et al. 2012; Germain et al. 2013) by demonstrating the developmental capacity of hESNPs in vitro.

This thesis examines how different cell culture systems can aid in promoting the differentiation and maturation of hESNPs into an enriched population of mature GABAergic interneurons. The experiments described below suggest a role for astrocytes in the efficient differentiation of GABAergic interneurons in vitro. First, we tested a combinatorial array of media and coculture conditions to optimize the generation of GABAergic interneurons from hESNPs. Next, we examined more closely the effect that the optimal coculture condition had on the hESNPs by comparing those cocultured to those cultured alone. We find that hESNPs cocultured with mouse cortical astrocytes in a tapered serum media condition treated with the Notch inhibitor DAPT yield a marginally higher percentage of MAP2-positive neurons than those cocultured in other media conditions or with embryonic mouse cortical cells. Of the resulting neurons, a high percentage express GABA. Our data also suggest that coculture with astrocytes encourages the differentiation of the hESNPs in long-term culture, and attenuates the
development of large, undifferentiated masses that often form in the absence of astrocytes. hESNPs cocultured with astrocytes produce trains of action potentials by 14 weeks of differentiation. None of the differentiated cells express PV and only a small percentage express SST, but over 25 percent express CB. Together, these data implicate a useful long-term culture system, suggest a role for astrocytes in hESNP differentiation, and demonstrate the in vitro differentiation and functional maturation of GABAergic interneurons from hESCs.
Figures
Figure 1. hESCs or mouse epiblast stem cells (EpiSCs) adopt regional neural identities according to the signaling pathways activated. Activin/Nodal and BMP inhibition promote neural induction. Upon neuroectoderm formation, low Wnt/β-Catenin and BMP signaling promote forebrain identity (Fb) as indicated by Foxg1 and Six3 expression. Activation of BMP, fibroblast growth factor (FGF), or moderate Wnt/β-Catenin signaling confers posterior forebrain or midbrain (Mb) fate. FGF, retinoic acid (RA), or Wnt/β-Catenin activation specifies hindbrain (Hb) and spinal cord (SC) fates (figure from Lupo et al. 2014).
Figure 2. Forebrain regionalization establishes distinct progenitor domains. Opposing gradients of Shh and Gli3 signaling pattern the developing forebrain on the ventral-dorsal axis. High Gli3 signaling specifies glutamatergic progenitors in the pallium, which express the markers Gli3, Ngn1/2, and Pax6. Glutamatergic progenitors undergo radial migration to populate the cortical layers and mature. Nkx2.1, Dlx1/2, and Lhx6 are induced in the MGE and POa, where primarily GABAergic and cholinergic neurons are generated, respectively. A different population of GABAergic interneurons are generated in the LGE, expressing the transcription factors Gsx2, Mash1, and Dlx1/2. Cortical and subcortical interneuron progenitors migrate tangentially from their origins in the subpallium to their final destinations in the cortex (Cx), hippocampus (H), striatum (Str) and globus pallidus (GP) (Goulburn et al. 2012).
Figure 3. Schematic diagram of interneuron subtype origins. The MGE gives rise to the progenitors of PV and SST-positive interneurons. MGE-derived SST-positive interneurons may coexpress CR or NPY. CR-positive progenitors arise from the dorsal CGE, a small subset of which coexpress SST. NPY-positive interneurons arise in both the ventral and dorsal CGE, and may also coexpress SST. The majority of 5HT3aR-positive interneuron progenitors are also generated in the CGE (not shown) (Wonders and Anderson 2006).
Chapter 2: Optimization of a coculture system for hESC-derived GABAergic interneuron generation

Abstract

Advances in hESC differentiation techniques have made it possible to generate enriched populations of neural progenitors positive for Nkx2.1, a transcription factor expressed in the developing ventral forebrain. Recently, populations of these progenitors have been differentiated into mature interneurons by coculturing them with either mouse cortical astrocytes or embryonic cortical cells. This work has raised questions as to which culture techniques are best suited for generating mature hESC-derived GABAergic interneuron phenotypes. Here we report the generation of GABAergic neurons from ventralized hESNPs under various culture conditions. Based on MAP2 expression, we observe that hESNPs cultured with astrocytes in media with a tapered concentration of serum and treated with the Notch inhibitor DAPT generate the highest proportion of mature neurons. In each of the culture conditions tested, a high percentage of the neurons generated express GABA. These data suggest an optimized maturation protocol for production of hESC-derived GABAergic interneurons.

Introduction

A neuronal replacement therapy must use cells that can become functional neurons of the desired type. Differentiating putative hESC-derived GABAergic interneuron progenitors in vitro allows confirmation of the progenitors’ ability to
generate mature interneuron phenotypes. An effective transplant of GABAergic interneuron progenitors would most likely consist of mitotic hESNPs, as passaging is toxic for postmitotic neurons. In addition to likely being more viable after transplant, a mitotic population might provide a continually replenishing population of interneuron progenitors. This may be useful in a therapy replacing cells that are lost to disease over long periods of time. Though a cell therapy would likely be mitotic, a population of cells consisting only of postmitotic neurons should be used for characterizing the fate of the hESNPs. Not only do interneurons require long periods of postmitotic maturation to properly characterize, but hESNPs can produce different cell types at early and late time points of culture (Gaspard et al. 2008). Analysis of neurons while a large pool of hESNPs still remains in the culture may bias the data towards early-born cell types.

Differentiating hESCs into specific cell types involves recapitulating relevant aspects of normal development in vitro. Protocols to differentiate GABAergic interneuron progenitors from hESCs first mimic the specification of neuroectoderm. Ligand or small molecule inhibitors are used to suppress factors such as BMPs which promote the formation of mesoderm and endoderm. The resulting neuroectoderm is further specified to a rostral fate by ensuring the absence of caudalizing factors like BMPs, Wnts, and RA. Finally, a ventral forebrain fate is induced by activation of the Shh signaling pathway and/or inhibition of dorsalizing Wnt activity (Lupo et al. 2014). These methods are effective at generating Nkx2.1-positive, ventral forebrain-like, GABAergic interneuron progenitors by paralleling patterning mechanisms at play in vitro. To generate mature interneuron phenotypes from the resulting hESNPs, it may be necessary to recapitulate aspects of the final steps of in vivo GABAergic interneuron development. Nkx2.1-positive neuroblasts arise in the developing ventral forebrain before migrating
tangentially into the cortex and undergoing brief radial migration to their final
destinations in the cortical layers (Wichterle et al. 2001). It is only after this journey
through cortical tissue that they synaptically and electrophysiologically mature (Bartolini,
Ciceri, and Marin 2013).

Recently, groups have sought to simulate this last phase of development by
coculturing Nkx2.1-positive hESNPs with mouse cortical astrocytes (Nicholas et al.
2013) or with mouse embryonic cortical cells (Maroof et al. 2013). It was hypothesized
that the interactions of the hESNPs with the mouse cells would simulate the interactions
of interneuron progenitors during their migration and integration into the cortex. Critical
signals from these culture environments may promote the differentiation and/or survival
of the hESNPs during their development into mature interneuron phenotypes. Excitatory
cells also provide a synaptic target for the hESC-derived interneurons. Although both
groups demonstrated the generation of mature GABAergic interneurons, differences in
their neural differentiation protocols obscure which of the two coculture systems was
more efficient at generating the mature phenotypes. A direct comparison of the effect of
different culture conditions on hESC-derived interneuron maturation is warranted.

The technique of coculturing neural progenitors with mouse cortical astrocytes is
common. It is used to promote the survival of primary neuronal cultures (e. g. Jones et al.
2012). hESC-derived neural progenitors (Johnson et al. 2007) and human iPSC-derived
neural progenitors (Tang et al. 2013) have also been cocultured with mouse cortical
astrocytes in studies to examine how the interactions between the two cell types modulate
neuron maturation. These studies have implicated a role for astrocytes in neural
progenitor migration and differentiation, dendritic arborization, and synapse formation (Johnson et al. 2007; Tang et al. 2013).

Coculture with astrocytes is known to influence neuronal maturation. However, in theory, coculturing with embryonic mouse cortical cells more accurately mimics the in vivo development of interneurons. Mice cortices harvested at E13.5 are at a point in development just before migrating interneuron progenitors begin to enter the cortex (Anderson et al. 1997). The resulting population of cells consists mostly of glutamatergic projection neuronal and glial precursors and approximates the tissue into which interneuron progenitors migrate and integrate. Excitatory neuronal input from glutamatergic cells has diverse roles in interneuron maturation. Lack of excitatory input during interneuron development can decrease GABA (Marty et al. 1997) and PV (Cellerino et al. 1992) expression levels and inhibit interneuron differentiation and survival (Close et al. 2012). In addition, excitatory neurons have been implicated in guiding interneurons to their ultimate locations in the cortex (Lodato et al. 2011), and provide a synaptic target for the cells. Astrocytes developing in cortical cell cultures might also confer the astrocyte-specific developmental benefits mentioned above on the hESNPs.

Another approach that may accelerate the differentiation of hESNPs into GABAergic interneurons is the inhibition of Notch signaling. Notch is a transmembrane protein which is activated when bound to an extracellular ligand. When Notch is activated, its intracellular domain is cleaved by gamma-secretase, enters the cell nucleus, and modifies gene expression to promote proliferation and prevent differentiation in neural stem cells (Selkoe and Kopan 2003). The small molecule N-(N-[3,5-
difluorophenacetyl-l-alanyl-S-phenylglycine t-buty1 ester (DAPT) inhibits the cleavage activity of gamma-secretase and thereby downregulates Notch signaling. Addition of DAPT to the media of neural progenitors, including those derived from hESCs, accelerates neuronal differentiation (Crawford and Roelink 2007; Borghese et al. 2010; Banda and Grabel 2014).

Coculturing hESNPs with astrocytes or mixed cortical cells poses the challenge of providing a culture medium with components that will promote the survival of both cell types used without discouraging neural differentiation. Fetal bovine serum (FBS) contains a diverse array of vitamins, growth factors, hormones, and other proteins. The supplement is often added to the media of primary neural cultures including cortical cell cultures (Xu et al. 2004) and astrocyte cultures (Pfrieger and Barres 1997; Kawano et al. 2012) to promote proliferation and survival. However, serum-free culture conditions promote the neuronal differentiation of ESNPs and inhibit the growth of non-neural cell types (e.g. Shulz et al. 2003). One way to strike a balance between the attributes of serum is to maintain cocultures in media with an initially moderate percentage of serum that is tapered off over time (Xu et al. 2004; Maroof et al. 2013).

Here we report the generation of high percentages of GABAergic neurons from ventralized hESNPs under various culture conditions. Eight conditions were tested, varying (1) the cell type cocultured with hESNPs between embryonic cortical cells or astrocytes, (2) whether serum was maintained in the media or tapered off, and (3) whether DAPT or a vehicle control was added. Cocultures on astrocytes, treated with DAPT, and kept in the tapered serum media condition produced the highest percentage of
neurons from the hESNPs. Astrocyte cocultures in the tapered serum condition produced a higher proportion of neurons than those in the maintained serum medium condition—a difference which was not evident among the cortical cell coculture conditions. A high proportion of the human neurons in each culture expressed GABA. These data suggest that coculturing hESNPs on astrocytes can increase the percentage of neurons in the culture, but only in the tapered serum medium condition. Astrocyte cocultures treated with DAPT in the tapered serum media condition represents an optimized culture system for generating hESC-derived GABAergic interneurons from hESNPs.

**Materials and Methods**

*hESC culture, neural differentiation, and ventralization*

hES3 Nkx2.1:GFP Ubi:mCherry cells were used for all experiments (Goulburn et al. 2011). hESCs were cultured on a feeder layer of mouse embryonic fibroblasts (MEFs) in hESC media containing Dulbecco’s Modified Eagle’s Media: F12 (DMEM:F12, Sigma), KnockOut serum replacement (KSR, Gibco), L-glutamine (Gibco), Penicillin/Streptomycin (Gibco), non-essential amino acids (NEAA, Gibco), basic fibroblast growth factor (bFGF, ConnStem, Inc), and β-mercaptoethanol (BME, Sigma). hESC media was changed every day. Cells were mechanically passaged whenever dishes became confluent (Germain et al. 2013).

Neural differentiation was conducted using an adherent monolayer protocol (Germain et al. 2013). To promote neural induction, hESCs were passaged into a nutrient-poor neural differentiation medium containing neurobasal medium (Gibco), N2
(Gibco), B27 (Gibco), L-glutamine (Gibco), insulin-transferrin-selenium (ITS, Gibco) and penicillin/streptomycin (Gibco). From day 1 to day 12 of differentiation, the medium was supplemented with the BMP antagonist LDN-193189 (100nM, Millipore) to promote neural differentiation. To ventralize the cells, the medium was supplemented with recombinant human sonic hedgehog (rhShh, 125ng/mL, R & D Systems) and the sonic hedgehog pathway agonist purmorphamine (1uM, Calbiochem) at days 10-18 of differentiation. Frozen stocks of these ventralized hESNPs were produced at day 18 of differentiation and thawed as needed. hESC culture, neural differentiation, and hESNP freezes were conducted by Nichesha Anderson.

**Cortical cell preparation**

Primary cortical cells were prepared from the cerebral cortex of E13.5 C57 mice embryos. Timed pregnant dams were sacrificed by cervical dislocation on embryonic day 13.5. Embryos were removed from the dam and put in ice-cold Hank’s Balanced Salt Solution (HBSS). To isolate the developing cortex the head was removed from the body and an incision was made at the back of the skull. The skull was cut on the midline of the anterior-posterior axis up to the forehead. After peeling back the skull laterally, the brain could be scooped out of the head. The cerebral cortex hemispheres were then separated from the rest of the brain. To prevent contamination by meningeal cell types, the meninges were carefully peeled away from cortex hemispheres. The ganglionic eminences were also removed from the cortices to prevent the growth of interneurons in the final cortical cell feeder layer. The cortices could then be cut into pieces, transferred into Eppendorf tubes, and enzymatically dissociated with TrypLE Express (Gibco) with
DNase I at 37°C for 30 minutes. After quenching the suspension with serum-containing medium, the cells were then resuspended in a cortical cell medium containing DMEM with 10% fetal bovine serum (FBS, Atlas) and penicillin/streptomycin (Gibco). The cells were counted and plated at a density of 500,000 cells per well on poly-D-lysine and laminin-coated 8-well glass chamberslides (Labtek). Cells were cultured alone for one day before adding hESNPs.

*Astrocyte preparation*

Astrocytes were prepared from the cerebral cortex of P0-4 C57 mouse pups. Cortices from four pups were isolated, their meninges removed, and dissociated with 2.5% Trypsin and DNase I before being put through a cell strainer and plated into 75cm² tissue culture flasks coated with poly-L-lysine. Cultures were kept in glial cell medium consisting of DMEM with 10% FBS and penicillin/streptomycin. Glial cell medium was exchanged every 2-4 days.

To enrich the cortical cell cultures for astrocytes, tissue culture flasks were slapped against a hand 15-20 times before each media change. This helped to dislodge contaminating cell types such as microglia and neurons, which are less adherent to the flask than astrocytes. When astrocyte layers reached confluence, cells were passaged using TrypLE and split into two tissue culture flasks. Before passaging, the flasks were also placed on an orbital shaker in an incubator at 34°C for 4-6 hours to dislodge additional contaminating cell types including oligodendrocyte precursor cells.
After 18-23 days of culture, when astrocyte layer was confluent, cells were passaged using TrypLE onto chamberslides (Labtek) coated with poly-D-lysine and laminin at a density of approximately 160,000-200,000 cells/cm². Astrocytes were cultured alone for one day before hESNPs were added.

*Coculture with cortical cells or astrocytes and DAPT treatment*

One day after mouse embryonic cortical cells or astrocytes were plated into chamberslides, hESNPs were thawed onto the cultures at approximately 185,000 cells/cm². Each coculture was kept in one of two medium conditions. In the first condition, termed the tapered serum medium condition, cultures contained half neural differentiation medium and half glial cell medium at the time of hESNP thaw. As the glial cell medium contains 10% FBS, the starting serum concentration of the cocultures was 5%. Every 1-2 days afterwards, half of the medium was exchanged for neural differentiation medium, quickly tapering off the serum by reducing its concentration by half at each media exchange. In the second medium condition, termed the maintained serum medium condition, cocultures were kept in neural differentiation medium containing 5% FBS, and this concentration was not reduced throughout the period of culture. Half the volume of medium was exchanged for fresh neural differentiation medium with serum every 1-2 days. All coculture media were supplemented with 1µL/mL laminin to promote cell adhesion.

Some cultures were treated with the gamma-secretase inhibitor DAPT (5µM) on days 10-28 of coculture. To ensure a stable concentration of DAPT, full exchanges of medium were conducted every 1-2 days during DAPT treatment. After the treatment,
medium exchanges were once again conducted by half exchanges. hESNPs were cultured for 56 days of total differentiation (38 days of coculture) before being fixed for immunocytochemistry.

**Immunocytochemistry, Imaging, and Quantification**

After the desired period of culture, cells in chamberslides were fixed in 3.7% formaldehyde for 10 minutes. They were then permeabilized with 0.5% Triton-X in PBS for 10 minutes before being blocked with 10% neutral goat serum in 0.1% Triton-X in PBS at room temperature for 45-60 minutes. Primary antibodies diluted in the blocking buffer were then applied and incubated at 4°C overnight in a humidified container. Primary antibodies used were mouse anti-Oct4 (Santa Cruz Biotech, 1:1000), rabbit anti-Musashi-1 (Abcam, 1:400), mouse anti-human nestin (Chemicon, 1:1000), chicken anti-GFP (Aves, 1:1000), mouse anti-human nuclear antigen (Chemicon, 1:100), chicken anti-MAP2 (Aves, 1:1000), and rabbit anti-GABA (Sigma, 1:500). Secondary antibodies (Life technologies, 1:1000) diluted in blocking buffer were applied in a light blocked container at room temperature for 60 minutes, followed by Hoechst staining for 6 minutes. The chamber walls were then removed and coverslips applied with gelvitol. Fluorescent images of cells were obtained using NIS Elements software through a Retiga 2000R camera mounted on a Nikon Ti microscope. Four random 20x fields in each well were counted and averaged to quantify marker expression. ANOVA tests were conducted to determine statistical significance between the results under different culture conditions.
Results

*Neural differentiation and generation of Nkx2.1:GFP-positive hESNPs*

hESNP differentiation from hES3 Nkx2.1-GFP Ubi-mCherry hESCs was achieved using an adherent monolayer protocol. hESCs immunoreactive for the pluripotency marker Oct4 were cultured in a nutrient-poor medium with the BMP inhibitor LDN to induce neural differentiation (*Figure 1B and 1A*). After 12 days of differentiation, LDN was removed and the culture was continued. At 18 days of differentiation, the cells were positive for the neural stem cell markers Musashi-1 and Nestin (*Figure 1C*; hESC culture, neural differentiation and ventralization, and hESNP freezes conducted by Nichesha Anderson).

To ventralize the cells, Shh and Purmorphamine were added to the cultures from days 10 to 18 of differentiation (*Figure 1A*). After 30 days of differentiation, between 90 and 100 percent of the cells expressed Musashi and/or Nestin and over 50 percent of the cells were positive for Nkx2.1-GFP, indicating that most of the cells had adopted the neural lineage and more than half had become ventrally specified neural progenitors (*Figures 2A,B,C,E*). About 25 percent of the cells also expressed the neuronal marker microtubule-associated protein 2 (MAP2), suggesting that neuronal differentiation had begun by 30 days of culture (*Figure 2D,E*). Day 18 populations of ventralized hESNPs fluorescent-activated cell sorted (FACS) for Nkx2.1:GFP expression also consistently exhibit about 50 percent GFP expression (Nichesha Anderson, data not shown).
Astrocyte cocultures in the tapered serum condition treated with DAPT produce the most neurons

To determine whether different culture conditions could accelerate neuronal maturation in cultures of ventralized hESNPs, frozen hESNP stocks were thawed into chamberslides containing either mouse cortical astrocytes or embryonic mouse cortical cells. These two conditions were further subdivided by adding either the Notch inhibitor DAPT or a vehicle control to the cultures. The media of each of the four culture conditions contained either a starting concentration of 5 percent FBS that was quickly tapered off or a maintained concentration of 5 percent FBS for the duration of the coculture period. These serum conditions were evaluated because primary cultures did not survive well in initial coculture experiments without the supplement (data not shown). With three variables being examined, eight total coculture conditions were tested for their ability to encourage neuronal maturation.

After eight weeks of hESNP differentiation (about 5.5 weeks of coculture), cells were fixed and assayed for neuronal differentiation. Immunocytochemical analysis was conducted using an anti-MAP2 antibody to mark mature neurons, an anti-human nuclear antigen (HuNu) antibody to mark human cells, and a nuclear counterstain. Neuronal differentiation efficiency was quantified by counting the number of cells that coexpressed MAP2 and HuNu. (Figure 3). Six of the eight culture conditions brought about a similar percentage of MAP2-positive cells from the hESNPs (all between 27.2 and 35.3 percent). Astrocyte cocultures in the tapered serum medium condition, however, yielded higher percentages (51.5 and 59.3 percent). At 59.3 percent, hESNPs cocultured with astrocytes
in the tapered serum medium condition treated with DAPT result in the highest percentage of MAP2-expressing cells out of those expressing HuNu at eight weeks of differentiation. Overall, hESNPs cocultured with astrocytes produced a significantly higher percentage of neurons than those cocultured with cortical cells (Figure 3).

**GABA is expressed in high percentages of neurons differentiated in each of the coculture conditions**

To establish whether the ventralized hESNPs had adopted a GABAergic interneuron identity, we conducted immunocytochemistry with an anti-GABA antibody on neurons fixed at 8 weeks in each coculture condition. This also allowed us to determine whether the eight coculture conditions yielded different percentages of GABAergic neurons among the total sum of neurons that were generated. Although there were modest differences among the culture conditions, between 65.9 and 86.6 percent of the MAP2-positive, HuNu-positive cells were also positive for GABA. (Figure 4).

**Discussion**

*An optimized culture condition for neuronal differentiation*

Maroof et al. and Nicholas et al. produced hESC-derived GABAergic interneurons using coculture systems with embryonic cortical cells and postnatal astrocytes, respectively (Maroof et al. 2013; Nicholas et al. 2013). They demonstrated the generation of mature interneurons on very different timelines by quantifying mature
subtype markers. Cortical cell cocultures showed high expression of mature interneuron subtype markers such as SST and PV among Nkx2.1-positive cells by 8 weeks of differentiation. Astrocyte cocultures did not exhibit high percentages of SST (out of β-III-tubulin-positive neurons) until 30 weeks of differentiation, and never showed high PV percentages. Neither indicated how many of the total human cells were neurons at the time of analysis. Since subtype marker expression was only calculated as a proportion of Nkx2.1:GFP or β-III-tubulin-positive cells, the extent of neuronal differentiation in the cultures is unclear (Maroof et al. 2013; Nicholas et al. 2013).

Our data indicate that in cocultures with both cortical cells and astrocytes, over 25 percent of the ventralized hESNPs become neurons by 8 weeks of differentiation. This amount can be increased to 51.5 percent if astrocyte cocultures are in the tapered serum medium condition, and increased even further to 59.3 percent if also treated with DAPT. The same medium conditions do not increase the percentage of MAP2-positive cells generated in cortical cell cocultures. This may indicate that the lowered serum concentration modulates astrocyte function in such a way that promotes neuronal differentiation, whereas the reduced serum does not modulate the function of cortical cells and their derivatives.

We acknowledge that these data are averages taken from a single set of experiments whose goal was to establish an efficient protocol for generating mature neuronal phenotypes from our hESNPs. In additional experiments we have reproducibly generated high percentages of GABAergic interneurons in the optimized astrocyte coculture condition, but any conclusions drawn about the other culture conditions are tentative and limited by lack of replication.
With that caveat in mind, it is unexpected that the cortical cell coculture exhibited a trend towards a lower percentage of mature neurons than the astrocyte coculture in the tapered serum medium condition since cortical progenitors produce both astrocytes and excitatory neurons. Both cell types have been shown to have roles in interneuron development (Johnson et al. 2007; Close et al. 2012; Tang et al. 2013). The low yield of mature neurons may be partially explained by the fact that embryonic cortical cell cultures exhibit stages of neural differentiation similar to the developing cortex in vivo, producing first neurons and then astrocytes (Shen et al. 2006). A high number of astrocytes may not have been present in the cortical cell cocultures for much of the experiment, limiting their effect on maturation. In addition, the cells in the astrocyte coculture were enriched for astrocytes and cultured for a three week-long maturation period before being replated for experiments with hESNPs. Perhaps a more mature astrocyte phenotype is required to effectively promote the differentiation of hESNPs into neurons. Finally, MAP2 stabilizes the microtubules of mature, postmitotic neurons (Harada et al. 2002), but cells are specified as neurons before the protein is expressed in high amounts. It is possible that, for example, cocultures with astrocytes in the tapered serum medium condition contain the same amount of differentiated neurons as the other culture conditions but accelerate their progression to the MAP2-expressing stage. Future experiments should examine earlier markers, such as β-III-tubulin, which is expressed in immature neurons (Menezes et al. 1995). With MAP2 and beta-III-tubulin percentages, one could differentiate between the effects different culture conditions have on neuronal differentiation from their effects on the maturation of cells that have already adopted a neuronal identity.
Notch inhibition further boosts the percentage of neurons generated by 6-8 percent in astrocyte cocultures. Another study using DAPT to enhance neuronal differentiation from hESCs describes a similar increase in the percentage of cells becoming neurons, though it may not be comparable as it examines an earlier phase of differentiation (Borghese et al. 2010). A DAPT-dependent increase in neuron percentage was not seen in cortical cell cocultures. One possible reason for this is the selective cell death of neurons in cortical cell cocultures. DMSO, the delivery for DAPT, functions by dissolving holes in the cell membrane to allow the passage of DAPT. The concentration may have been high enough that the DMSO was toxic to the extra neurons generated in the DAPT condition.

Efficient generation of GABAergic interneurons from a heterogeneous progenitor population

The generation of high percentages of GABAergic neurons supports the hypothesis that the Nkx2.1-positive hESNPs in the cell population present after neural differentiation and ventralization were MGE-like interneuron progenitors. Nkx2.1 is downregulated over time in the hESNPs, which is characteristic of a cortical interneuron phenotype (Chapter 3, Figures 1 and 4). However, since about half of the hESNPs were Nkx2.1-negative at differentiation day 30 and not all interneuron progenitor domains express the transcription factor, it is possible that at least some of the GABA-positive neurons were generated from Nkx2.1-negative progenitors. For example, CGE-like progenitors would be Nkx2.1-negative but generate GABA-positive neurons. As there are other fates of Nkx2.1-positive progenitors, such as dopaminergic and cholinergic
neurons, some of these types might also make up the GABA-negative cells.

Characterizing a population of cells purified for Nkx2.1-positive hESNPs by fluorescence-activated cell sorting (FACS) would confirm that the GABAergic neurons were indeed generated from the Nkx2.1-positive progenitors.

Our data show that a high percentage of the hESC-derived neurons generated express GABA regardless of the culture conditions. This is consistent with evidence that GABAergic interneuron lineage commitment is already set in neural progenitors of the developing ventral forebrain (Inan et al. 2012). If the progenitor populations are already committed to the GABAergic lineage, changes to the culture conditions may not affect their fate.

**Electrophysiological maturation**

The experiments described above use MAP2 expression as a readout for mature neurons to compare hESC-derived interneuron derivation efficiencies in different culture conditions. The optimized coculture condition provides an *in vitro* system for assessing our hESNPs as a potential cell therapy. To truly assess the cells’ potential to modulate neural circuit function post-transplant, however, one must examine the maturation of electrophysiological function. Given that primary astrocytes can modify the electrophysiological properties of developing neurons *in vitro* (Tang et al. 2013), it would be interesting to determine whether one of the culture conditions tested above accelerates electrophysiological maturation. The recent work of Nicholas et al. and Maroof et al. has prompted interesting questions about the effects of coculture conditions on interneuron maturation. hESNPs cocultured on astrocytes up to 30 weeks of
differentiation generate neurons that exhibit similar resting membrane potentials (RMPs), afterhyperpolarization (AHP) amplitudes, and firing frequencies as those observed in rodent SST-expressing regular spiking non-pyramidal (RSNP) neurons (Nicholas et al. 2013; Halabisky et al. 2006; Kawaguchi & Kubota 1996). On the other hand, progenitors cultured on cortical cells generate neurons with comparable RMPs, AP half-widths, and AHP amplitudes after only 8 weeks of differentiation. The AP frequencies of these cells, however, are low and resemble those of Nicholas et al.’s neurons at 8 weeks. The two groups use different differentiation protocols and generated different interneuron subtypes, which prevents a direct comparison of the two cell populations. Maroof et al. uses a monolayer protocol, inhibits SMADs with LDN and SB431452, inhibits Wnt signaling with XAV939, and agonizes the Shh pathway starting on day 10 of differentiation with both the ligand and Pur (Maroof et al. 2013). On the other hand, Nicholas et al. uses a protocol which begins as EBs and ends in a monolayer, inhibits SMADs with SB431452 and BMPRIA, inhibits Wnt signaling with Dkk1, and agonizes the Shh pathway with only Pur starting on day one of differentiation (Nicholas et al. 2013). In Maroof et al.’s experiments, starting Shh agonism on day one leads to neurons with reduced APs (Maroof et al. 2013). It would be interesting to directly compare electrophysiological measures in interneurons derived from hESNPs differentiated under the same protocol but matured in different coculture conditions.

This chapter describes the optimization of a culture protocol for the generation of mature GABAergic neurons from hESNPs. Astrocyte cocultures in the tapered serum medium condition treated with DAPT produce the highest percentage of these cells.
There are many GABAergic interneuron subtypes that may make up this population. Also, it is important to confirm the ability of these cells to display mature electrophysiological function if they are to be used in transplants. Chapter 3 more closely examines the effects of coculturing hESNPs with astrocytes, and investigates the molecular identity and electrophysiological function of the GABAergic cells produced.
Figures
Figure 1. Neural differentiation protocol to generate ventralized human embryonic stem cell-derived neural progenitors (hESNPs). hESCs are positive for the pluripotency marker Oct4 before differentiation protocol begins (B). For neural differentiation, hESCs are put into the nutrient-poor medium N2B27 supplemented with the BMP pathway antagonist LDN from day 1 to 12 (A). To ventralize emerging neural progenitors, recombinant human sonic hedgehog (Shh) and the hedgehog pathway agonist purmorphamine (Pur) are applied from days 10-18 (A). By day 18, this protocol generates hESNPs which are positive for the neural stem cell markers Musashi-1 (Msi) and human nestin (C) Figure and data from Nickesha Anderson. Scale bars are 100 micrometers.
Generation of Nkx2.1:GFP + Neural Progenitors from Human Embryonic Stem Cells

<table>
<thead>
<tr>
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<th>Percent Positive (Differentiation Day 30)</th>
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<tr>
<td>Nkx2.1:GFP</td>
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<tr>
<td>Msi/GFP</td>
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<td>Nestin/GFP</td>
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<tr>
<td>Map2/GFP</td>
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Figure 2. Characterization of ventralized hESNPs at day 30 of differentiation. A majority of the cells have adopted a ventral neural fate, as approximately 55 percent of the cells are positive for Nkx2.1:GFP (A, E). Out of the Nkx2.1:GFP-positive cells, between 90 and 100 percent are positive for the neural stem cell markers Musashi-1 (Msi) and Nestin, indicating that the cultures are mostly immature neural phenotypes (B, C, E). Neurons have begun to emerge by this time, however, as demonstrated by the quarter of the Nkx2.1:GFP-positive cells expressing the mature neuronal marker MAP2 (D, E) Figure and data from Nickesha Anderson. Scale bars represent 10 micrometers.
MAP2 Expression in HuNu+ Cells

![Graph showing MAP2 expression in HuNu+ cells with bars for different conditions: DAPT Tapered Serum, Vehicle Tapered Serum, DAPT Maintained Serum, Vehicle Maintained Serum.](image)

**Astrocytes**
- HuNu+/MAP2
- Tapered Serum
- Maintained Serum
- DAPT
- Vehicle

**Cortical Cells**
- Tapered Serum
- Maintained Serum
- DAPT
- Vehicle

* p<0.05
Figure 3. Neuronal differentiation of ventralized hESNPs in different culture conditions at 8 weeks of differentiation. All of the cocultures with cortical cells and those with astrocytes in the maintained serum medium condition display a similar percentage of MAP2-positive neurons out of all human cells (all between 27.2-35.3 percent). Cocultures with astrocytes in the tapered serum medium condition display higher percentages (51.5 and 59.3 percent). hESNPs cocultured with astrocytes in the tapered serum medium condition treated with DAPT result in the highest percentage of neurons (59.3 percent). Overall, cells cocultured with astrocytes display a significantly higher percentage of MAP2-positive cells differentiated at 8 weeks than cells cocultured with cortical cells (ANOVA test, p=0.0299). Scale bar is 50 micrometers.
Figure 4. GABA expression among hESC-derived neurons in each of the culture conditions. Similar percentages of MAP2, HuNu-positive cells express GABA in each condition. Each value falls between 65.9 and 86.6 percent. The difference between values is not significant as determined by an ANOVA test. Percentage of MAP-positive cells that are GABA positive was found to be a variable independent of the MAP2 percentage of HuNu-positive cells by MANOVA test (Roy’s Max Root=0.0322). Scale bar is 50 micrometers.
Chapter 3: Astrocyte cocultures facilitate the maturation of hESNPs into GABAergic interneurons

Abstract

The therapeutic potential of hESC-derived GABAergic interneurons for treating epilepsy lies in the hope that transplanted populations will provide activity-dependent inhibitory input in an overexcited brain. Coaxing hESNPs to adopt mature interneuron phenotypes in vitro would confirm that the correct cell types are being made that could realize that potential. In addition, the study of culture conditions that promote interneuron maturation may provide indications of how to best achieve maturity in transplants. We have developed an optimized protocol for generating GABAergic interneurons from hESNPs through coculture with mouse cortical astrocytes. Using the medium conditions from the optimized protocol, we compare hESNPs alone to cocultures with astrocytes to examine the effects the astrocytes have on hESNP maturation. We observe that coculture with astrocytes accelerates hESNP maturation into neurons, promotes hESNP survival, and attenuates the formation of large clusters of non-neural cells in cultures. Long-term cocultures give rise to high percentages of GABAergic neurons. By 12 weeks of differentiation, neurons in culture can fire trains of APs and express the interneuron subtype markers SST and CB. These data suggest that long-term astrocyte coculture facilitates the generation of functional GABAergic interneurons from hESNPs.
**Introduction**

A hESC-derived replacement therapy is a promising approach for treating TLE. An appropriately prepared transplant of hESNsPs could assume the inhibitory function of lost interneurons and help suppress seizures. Although GABAergic interneurons have been successfully derived from hESCs (Germain et al. 2013; Maroof et al. 2013; Nicholas et al. 2013), many questions remain about the ability of these cells to mature into adult phenotypes. Evidence suggests that GABAergic interneurons mature *in vivo*, at least in part, through a cell-intrinsic process that lasts well into adolescence (Le Magueresse et al. 2011; Le Magueresse and Monyer 2013). However, results have differed on the developmental timeline of their hESC-derived counterparts (Maroof et al. 2013; Nicholas et al. 2013). hESNsPs expressing Nkx2.1 develop into “relatively mature” interneuron phenotypes by two months of differentiation when cocultured with mouse cortical cells (Maroof et al. 2013). On the other hand, Nkx2.1-positive hESNsPs differentiated under a different protocol and cocultured with mouse cortical astrocytes display a maturation of electrophysiological properties and interneuron subtype markers over a period of over 7 months (Nicholas et al. 2013). Further study of hESC-derived GABAergic interneuron development *in vitro* is required to determine which culture techniques and timelines are necessary to mature the cells.

Astrocytes have diverse effects on the maturation of neural progenitors. During development, these cells emerge from radial glial cells after the cortical layers have been formed (Zhang 2006), and ultimately outnumber neurons 9 to 1. They have been postulated to have myriad functions in neuronal development and function. *In vitro*, astrocytes play a role in the survival (Banker et al. 1980), differentiation (Song et al.
2002; Tang et al. 2013), dendritic growth (Tang et al. 2013), and synaptic formation (Johnson et al. 2007) of developing neurons. Nicholas et al. used astrocyte coculture to mature hESC-derived GABAergic interneurons in vitro. However, their published paper did not examine the effects of the coculture system by comparing hESNPs cultured with and without astrocytes (Nicholas et al. 2013). Determining the aspects of interneuron development that astrocytes facilitate in hESNPs may be important for ensuring the maturation of cells post-transplant.

Chapter 2 describes an optimized protocol for generating GABAergic interneurons from hESNPs by 8 weeks of differentiation. The progenitors are cocultured with astrocytes, kept in a tapered serum medium condition, and treated with DAPT. This chapter examines the effect that the astrocytes have on the hESNPs more closely by comparing cocultures to hESNPs cultured alone. In addition, hESNPs are cultured in the optimized condition for longer periods of time—up to 14 weeks of differentiation—to examine the interneuron subtype specification and electrophysiological properties of the resulting neurons. We find that coculturing hESNPs with astrocytes enhances neuronal survival and differentiation, and prevents the formation of large clusters of other primary germ lineages that otherwise spread throughout cultures. Cells differentiated for 14 weeks express interneuron subtype markers and can fire trains of APs, indicating a functional phenotype. These data implicate diverse roles for astrocytes in hESNP maturation into GABAergic interneurons and demonstrate the ability of hESNPs to form functional GABAergic interneurons.
Materials and Methods

*hESC culture, neural differentiation, and ventralization*

All hESC culture, differentiation, and ventralization was conducted by Nickesha Anderson under the protocol described in Chapter 2.

*Astrocyte preparation*

Astrocytes were prepared and plated as described in Chapter 2, except that enriched astrocytes were plated both into chamber slides and onto plastic cover slips.

*Long-term culture with or without astrocytes and DAPT treatment*

One day after mouse astrocytes were plated into chamberslides or cover slips, hESNPs were thawed onto the astrocyte cultures or empty chamberslides at approximately 1.85 x 10^5 cells/cm^2. Cocultures and hESNPs-only cultures were kept in the optimal media condition described in Chapter 2 (tapered serum treated with DAPT). hESNPs were cultured for 8, 10, 12, or 14 weeks of total differentiation before being whole-cell patch clamped or fixed for immunocytochemistry.

*Immunocytochemistry, Imaging, and Quantification*

Immunocytochemistry, imaging, and quantification were conducted as described in Chapter 2. Primary antibodies used were chicken anti-MAP2 (Aves, 1:1000), mouse
anti-human nuclear antigen (Chemicon, 1:100), rabbit anti-GABA (Sigma, 1:500), mouse anti-smooth muscle actin (SMA, Sigma, 1:1000), rat anti-Troma-1 (Developmental Studies Hybridoma Bank, 1:10), mouse anti-Oct4 (Santa Cruz Biotech, 1:1000), mouse anti-human nestin (Chemicon, 1:1000), rat anti-SST (Chemicon, 1:100), rabbit anti-CB (Swant, 1:1000), and chicken anti-GFP (Aves, 1:1000).

*Whole-cell patch clamping*

To whole cell patch clamp cells, plastic cover slips were removed from culture medium and bathed in artificial cerebrospinal fluid (ACSF) bubbled with 45% O₂ and 5% CO₂. ACSF contained 125 mM NaCl, 25 mM NaHCO₃, 1.25 mM NaH₂PO₄, and 2.5 mM KCl. Pulled glass electrodes were filled with a solution containing 130 mM KMeSO₄, 10 mM K-HEPES, 10 mM KCl, 5 mM NaCl, 2.5 mM Mg-ATP, 0.3 mM Na-GTP, and 0.3-0.5% biocytin. Electrode resistance was 6-12 MΩ at time of patching. Current injections and recordings were made with a Model 2400 Patch Clamp Amplifier from A.M. Systems, Inc. connected to an interface using IGOR software. Whole cell patch clamping was conducted by Gloster Aaron.
Results

*hESNPs cultured with astrocytes generate a higher percentage of neurons, produce neurons with slightly longer dendrites, and survive better than those cultured without astrocytes*

To examine the effect coculturing hESNPs with mouse cortical astrocytes has on neuronal differentiation, cocultures were compared to hESNPs cultured without astrocytes. Frozen stocks of ventralized hESNPs (differentiated for 18 days using the ventralization protocol described and frozen by Nickesha Anderson) were thawed into chamber slides containing either astrocytes or only a coat of PDL and LN, then cultured up to 12 weeks of differentiation. Throughout the time course of the experiments, Nkx2.1:GFP expression appeared to diminish in cultures both with and without astrocytes (Figure 1). Indeed, over 50 percent of hESNPs fixed at day 30 of differentiation are immunoreactive for Nkx2.1:GFP (Chapter 2, Figure 2), whereas only 6.6 percent of HuNu-positive cells in cocultures express the marker at week 14 (Figure 4). Cultures without astrocytes also seemed to generate fewer cells with neuron-like morphologies, exhibit more cell death, and form large clusters of cells with diverse morphologies (Figure 1, arrows).

These qualitative data were confirmed by immunocytostaining cultures fixed at week 8 and 12 of differentiation (Figure 2). In cultures without astrocytes, only 2 percent of HuNu-positive cells expressed MAP2 at 8 weeks of differentiation, which was significantly lower than the 59.3 percent of cells observed in cultures with astrocytes at determined by a t-tailed t-test (Figure 2A, B). At 12 weeks of differentiation, 15.9 percent
of HuNu-positive cells in cultures without astrocytes and 59.4 percent of HuNu-positive cells in cultures with astrocytes express MAP2 (Figure 2A, B).

We also tested whether coculture with astrocytes increases the dendrite length of neurons produced from ventralized hESNPs. Cocultures and hESNPs cultured without astrocytes fixed at week 8 of differentiation were immunocytostained for HuNu and MAP2. As MAP2 is found in dendrites but not axons, we then measured the total length of MAP2-positive dendrites contained in each field of view. By dividing the resulting value by the number of HuNu-positive nuclei in the same view coexpressing MAP2, we obtained average dendrite lengths per neuron. We found that neurons in cocultures had an average dendrite length of 111.7 micrometers (µm) and those in cultures without astrocytes had an average length of 88.5 µm, although this difference was not significant (Figure 2C).

Cultures without astrocytes appeared to have more cell death than those with astrocytes (Figure 1). Indeed, in two out of the total eight runs of the experiment, human cells survived in cultures with astrocytes but not in cultures without astrocytes. In another two runs, hESNPs did not survive under either condition. Four runs of the experiment resulted in viable human cells under both conditions (data not shown).

*Cultures without astrocytes develop large clusters of non-neural cells, typically absent in cultures with astrocytes, and express non-neural markers*

Within the first few weeks after thaw, we observed that hESNPs cultured without astrocytes would form phase-dark areas of densely packed cells with diverse
morphologies (*Figure 3A, arrows*). By 12 weeks of differentiation, these areas were extremely dense and would occupy much of the area of the well (*Figure 3A, arrows*). Clusters developed in every culture without astrocytes that still had cells surviving at the time of analysis (all four independent runs with surviving cells). In contrast, cultures with astrocytes typically exhibited cells with neuron-like morphologies distributed relatively evenly at a moderate density throughout the well (*Figure 3A*). In two of the six coculture experiments in which hESNPs survived, dense clusters of cells began to arise by 12 weeks of differentiation. While the clusters were similar in morphology to those seen in the cultures without astrocytes, they were limited to small areas of the well and arose only at late time points (data not shown).

To investigate the identity of the cells forming clusters, we conducted immunocytochemical analysis on cultures without astrocytes at week 12 of differentiation. This experiment revealed the expression of markers for all three germ layers. Cells were immunoreactive for the mesoderm lineage marker smooth muscle actin (SMA), the endoderm marker Troma-1, and the neural stem cell marker nestin (*Figure 3B*), indicating that both ectodermal and non-ectodermal lineages were generated in the cultures. Some cells also expressed Octamer-binding transcription factor 4 (Oct4), a pluripotency marker (*Figure 3B*). This suggests that undifferentiated embryonic stem cells also remained in the culture.
GABAergic neurons produced in long-term astrocyte cocultures express interneuron subtype markers and are electrophysiologically functional

To evaluate whether long-term culture of ventralized hESNPs in the optimized coculture condition can generate mature interneuron phenotypes, immunocytochemical analysis was conducted at week 14 of differentiation. At this time point, 85.3 percent of the cells immunostained positive for HuNu also expressed MAP2 (Figure 4). Of the HuNu-positive, MAP2-positive cells, 67.7 percent also expressed GABA, indicating that 57.7 percent of the total human cells were GABA-expressing neurons (Figure 4). Rare HuNu-positive cells expressed the interneuron subtype marker SST (under 1 percent), and 29 percent expressed calbindin (CB). We did not detect PV, another interneuron subtype marker (data not shown). Finally, 6.6 percent of the HuNu-positive cells were Nkx2.1:GFP-positive. Low Nkx2.1:GFP expression at late time points is consistent with a cortical interneuron phenotype, in which Nkx2.1 is downregulated as the cells mature.

The electrophysiological function of the neurons was evaluated by whole-cell patch clamping (conducted by Gloster Aaron). In this method, a giga-ohm seal is first established between a microelectrode and the surface of a neuron. Then, a small patch of the cell membrane is ruptured in the electrode, making the inside of the electrode continuous with the inside of the cell. A ground electrode in the ACSF allows for the measurement of the membrane potential. The neuron’s ability to fire action potentials (APs) can then be determined by injecting current into the cell to see if spikes in membrane potential are generated. At 8-10 weeks of differentiation, cells exhibit only very small APs with amplitudes of about 5 mV upon 40-70 pA current injection, indicating a high input resistance and an immature phenotype (n=2 at 8 weeks; n=2 at 10
weeks). At 12-14 weeks, however, trains of APs can be observed in neurons (n=2 at 12 weeks, one of which fired APs; n=1 at 14 weeks). The AP trains display an accommodating phenotype, individual APs within a train having progressively lower amplitudes and wider widths. Later recordings of cells at 16 weeks (n=3) and 17 weeks (n=1) of differentiation display a single AP upon current injection. These data suggest that neurons are electrophysiologically functional by 12 weeks of differentiation, although some neurons still cannot fire AP trains at that point and later (Figure 5). While there are mature interneuron and principle neuron subtypes with an accommodating phenotypes such as those observed, the accommodation could also be a sign of immaturity due to lack of voltage-gated ion channels. Therefore, further analysis of the electrophysiological and molecular parameters of the cells is needed to evaluate cellular identity and maturity.

Cellular recordings obtained during a voltage clamp of many of the patched cells at various time points revealed no PSC production (not shown). This indicates that the neurons that we patched are not receiving synaptic input.

Discussion

*Long-term astrocyte cocultures enable the efficient generation of GABAergic interneurons from ventralized hESNPs*

This chapter details how astrocytes support the maturation of hESNPs into GABAergic interneurons in long-term culture. Cells in these cocultures express MAP2, GABA, SST, and CB at week 14 of differentiation. By week 12, they are
electrophysiologically functional. Compared to culturing hESNPs without astrocytes, coculturing with astrocytes accelerates neuronal differentiation, slightly increases dendrite length, improves hESNP survival, and attenuates the accumulation of non-neural cells. hESNPs cultured without astrocytes express markers of all three primary germ layers and the pluripotency marker Oct4.

While all available data indicate that a much higher percentage of neurons arises in cocultures than in cultures without astrocytes, the fate of hESNPs cultured without astrocytes varied from experiment to experiment. In most runs, very few neurons developed or the cells did not survive. It was observed that 15.9 percent of hESNPs cultured without astrocytes express MAP2 at 12 weeks of differentiation. However, this may be an overestimation because the 12 week culture quantified for those data appeared to contain more cells with neuron-like morphologies than any other trial without astrocytes. Part of the reason for the low percentage of neurons in cultures without astrocytes may be that developing neurons were more susceptible to cell death. There often appeared to be considerable cell death in live images of hESNPs cultured alone, and there were experiments in which hESNPs died within the first few weeks in cultures without astrocytes, but survived in cultures with astrocytes. As astrocytes facilitate the survival of developing neurons in vitro through the actions of media-born trophic factors (Banker 1980), perhaps they are needed to reliably produce high numbers of neurons in long-term cultures of hESNPs.

Even if the 12 week count of neurons in cultures without astrocytes was unusually high, the percentage of human cells expressing MAP2 in that experiment was still about 40 percent lower than in cultures with astrocytes. Cocultures consistently produced high
levels of neurons from hESNPs. In addition to supporting survival, astrocytes may encourage a high neuron percentage by increasing the rate of neuronal differentiation. Astrocytes also enhance neurogenesis of both primary cultures of neural stem cells (Song et al. 2002) and hPSC-derived neural progenitors (Tang et al. 2013). Experiments using astrocyte-conditioned media have revealed that this effect is at least partially through secreted factors (Song et al. 2002; Tang et al. 2013). Without astrocytes, perhaps hESNPs lacked the necessary environmental signals to efficiently differentiate.

Only 2 percent of cells in hESNP cultures without astrocytes are neurons at week 8 of differentiation, whereas cultures fixed at day 30 of differentiation contain about 25 percent neurons (Chapter 2, Figure 2). These data are unexpected since hESNPs cultured for an extended period have more time to mature. Perhaps the preferential death of neurons in long-term cultures without astrocytes contributed to why there were so few of them at week 8. Alternately, the transient concentration of serum in the long-term cultures may have hindered the cells’ neuronal differentiation. Comparisons of the two cultures are challenging as the latter population was not frozen and was plated at an undefined density. Additional experiments should answer the question of whether freezing and thawing significantly affects hESNP survival and/or differentiation.

Astrocytes suppress formation of non-neural clusters in hESNP culture

hESNP culture without astrocytes also resulted in the formation of dense areas of cells which often grew to fill the wells of the chamber slides. Cells in these clusters expressed markers for all three primary germ layers and a pluripotency marker. Non-neural lineages were likely generated from cells that remained pluripotent after neural
differentiation. Previous research by our laboratory and others has shown that cells of pluripotent and heterogeneous lineages can remain in hESC-derived cell populations, even after an efficient differentiation protocol is followed (Fujikawa et al. 2005; Brederlau et al. 2006; Germain et al. 2012). These cells can form teratomas when the hESNP population is transplanted into the brain (Germain et al. 2012).

While hESNPs cultured alone formed dense clusters in all four experiments in which the cells survived, this was only observed in two of the six viable astrocyte cocultures. In addition, clusters that were observed in the coculture conditions only arose at late time points of culture and were limited to small areas of the well. These data are only qualitative, but they suggest that astrocytes may play a role in suppressing or delaying the formation of non-neural lineages. A detailed study of pluripotent marker expression over time in hESNP cultures with and without astrocytes is needed to confirm this effect. A major concern in transplanting hESC-derived material into patients is the possibility of residual stem cells in the transplanted population forming tumors. If astrocytes do confer a pluripotency-inhibiting effect on hESNP cultures, investigating the mechanism of the inhibition could uncover new possibilities for suppressing pluripotency and making hESC-derived material safer for transplant.

*Cultures may require further maturation to induce robust subtype marker expression and adult electrophysiological properties*

The experiments described above show that long-term coculture of hESNPs with astrocytes results in a high percentage of neurons, most of which express GABA. The percentage of HuNu-positive cells expressing MAP2 remains just below 60 percent at 8
and 12 weeks of differentiation, but increases to just over 85 percent by week 14. This late increase may be due to the depletion of a pool of proliferating progenitors that had maintained a constant ratio of neurons to progenitors between weeks 8 and 12 of differentiation. At each time point, the percentage of neurons that are GABA-positive is greater than 65 percent.

Some of the HuNu-positive cells also expressed interneuron subtype markers. While no PV expression was evident, we observed a low percentage of SST-positive cells and a moderate percentage of CB-positive cells. PV is expressed in about 40 percent of cortical interneurons, which develop from Nkx2.1-expressing progenitors in the MGE (Xu et al. 2008). The lack of PV expression may be explained by the fact that excitatory input is required for the marker to be expressed (Marty et al. 1997). One group coculturing hESC-derived GABAergic interneurons with excitatory neurons observed about 40 percent of Nkx2.1:GFP-expressing cells in their cultures immunoreactive for PV at 8 weeks of differentiation (Maroof et al. 2013). Another group cocultured Nkx2.1:GFP-positive hESNPs with astrocytes and observed PV-positive neurons only rarely throughout a 30 week differentiation period (Nicholas et al. 2013).

It is more difficult to explain the low percentage of SST expression in our cultures. About 30 percent of cortical interneurons express SST, and progenitors of this population also arise in the Nkx2.1-positive MGE (Xu et al. 2008). Maroof et al. observed about 40 percent SST expression among Nkx2.1:GFP-expressing cells at 8 weeks of differentiation in cortical cell cocultures (Maroof et al. 2013), and Nicholas et al. saw a similar percentage among neurons in astrocyte cocultures (Nicholas et al. 2013). However, Nicholas et al. did not observe such a high SST percentage until week 30 of
differentiation—at week 10, less than 5 percent of neurons expressed the marker. It is possible that many of the neurons in our cultures are still too immature to express SST, but would express the marker if cultured for longer periods of time.

The cells that are immunoreactive for CB may represent PV and/or SST-lineage neurons. Most CB-expressing interneurons are generated from Nkx2.1-positive progenitors (Sussel et al. 1999). The calcium-binding protein is expressed in subsets of both SST-containing interneurons (Gonchar and Burkhalter 1997) and, some evidence suggests, cells that will become PV-positive interneurons (Alcantara et al. 1996). Therefore, CB-positive cells found in culture may represent immature phenotypes of interneurons that will express SST or PV at a later stage.

Cortical interneurons downregulate Nkx2.1 expression as they migrate from ventral progenitor domains into the cortex. This is in contrast to striatal interneurons, which maintain Nkx2.1 expression into adulthood (Marin et al. 2000). hESNPs in our cultures appear to downregulate Nkx2.1:GFP over time. Live images of both culture conditions show high Nkx2.1:GFP expression one day after thaw, but by 8 weeks of differentiation there is very little expression. Over 50 percent of hESNPs fixed at day 30 of differentiation are immunoreactive for Nkx2.1:GFP (Chapter 2, Figure 2), whereas only 6.6 percent of HuNu-positive cells in cocultures fixed at 14 weeks of differentiation express the marker. These data suggest that Nkx2.1 is downregulated over time in our hESNPs, which indicates maturation to a cortical interneuron identity. However, it is possible that the lowered expression is due to selective death of Nkx2.1:GFP-positive cells or selective proliferation of cells negative for the marker. Monitoring a population
FACS-sorted for Nkx2.1:GFP-positive cells will be necessary to confirm this downregulation.

By 14 weeks of differentiation, cells are also able to fire trains of APs. Although we were unable to confirm that the cell that fired APs was GABA-positive, immunocytochemical data show that 67.7 percent of neurons at week 14 of differentiation express the neurotransmitter (Figure 4). The neuron exhibited accommodating APs, which may indicate that it did not yet possess the sodium and potassium channels necessary for mature membrane oscillations. Nicholas et al. observed immature, accommodating spiking patterns upon current injection at 8 weeks of differentiation, but not afterwards (Nicholas et al. 2013, Figure S6). This may indicate that our cells are maturing more slowly than those derived by Nicholas et al. Alternately, we may have patch clamped a cell that had arisen from the progenitor pool recently in culture, and thus it may not be a good representative for the population as a whole. Further whole-cell patch clamping is needed to replicate this finding and determine whether other spike patterns are also present at 14 weeks.

The overall health of neurons cultured on plastic cover slips, required for patch clamp analysis, was generally poor compared to those cultured in glass chamberslides. There were often few neurons to be found at late time points, and sometimes the astrocyte layer was unhealthy-looking. The death of mature neurons may account in part for the generally immature electrophysiological phenotypes. Further optimization of protocols to coculture astrocytes and hESNPs on cover slips may be necessary to reliably generate mature interneurons for patch clamping.
As discussed above, long-term coculture of our ventralized hESNPs with mouse cortical astrocytes leads to the efficient generation of GABAergic interneurons. Longer culture periods or additional culture factors may be necessary to achieve fully mature interneuron phenotypes. However, the confirmation of GABAergic identities and an ability to fire APs suggests that these cells are good candidates for a transplant when the goal is inhibition of host circuitry. The immature phenotype we observe even at 14 weeks in vitro suggests that hESNPs transplanted into the mouse brain may require a long period of maturation or additional factors in culture to have an effect on host circuitry. Additional study of hESNP maturation in vitro may provide further clues for generating an adult phenotype and thereby inform transplant experiments.
Figure 1. Live images of the typical progression of hESNP morphology in cultures with or without astrocytes. At one day post-thaw (DPT) of day 18 ventralized hESNPs, hESNP cultures with or without astrocytes express high amounts of Nkx2.1:GFP and display morphologies characteristic of neural progenitors. Some of the GFP-positive cells already have branched processes and display small somas, indicating neuronal differentiation, but most possess flat, neural stem cell (NSC)-like morphologies. At 14 DPT, more of the GFP-positive cells in the cultures with astrocytes display a neuronal morphology than those in cultures without astrocytes, and both populations still contain cells with NSC-like morphologies. There appears to be more cell death in cultures without astrocytes, and cells appear to be clumping together. By 56 DPT, few cells in each condition express GFP. In cultures without astrocytes, large clusters of cells have formed throughout the cultures and few neurons are evident. Cultures with astrocytes exhibit cells with neuronal morphologies spread fairly evenly throughout the well. Arrows point to clusters, which are outlined in black. Scale bar is 200 micrometers.
Figure 2. Differentiation of GABAergic neurons in cultures of hESNPs with or without astrocytes. At 8 weeks of differentiation, very few MAP2-positive cells are seen in cultures of hESNPs without astrocytes, whereas almost 60 percent of human cells in cultures with astrocytes are MAP2 positive (A, B). By 12 weeks of differentiation, a moderate percentage of neurons emerge in cultures without astrocytes, while cultures with astrocytes maintain a high percentage (A, B). A high proportion of the MAP2-positive cells in each condition express GABA (A). The average length of the MAP2-positive dendrites extending from cells also tends to be longer in cultures with astrocytes, although this difference is not significant (C). Scale bar is 100 micrometers.
Figure 3. Non-neural cells in cultures without astrocytes. Phase contrast images show large clusters of cells spreading throughout wells in hESNP cultures without astrocytes, in contrast to cultures with astrocytes (A). Cultures with astrocytes appear to maintain a moderate density of putative neurons distributed fairly evenly throughout the well (A, phase-bright cells in images of cultures with astrocytes). Fluorescent images of immunocytostained cells reveal that markers of mesoderm (SMA) endoderm (Troma-1) and ectoderm (Nestin) are expressed in hESNP cultures without astrocytes at 12 weeks of differentiation, as well as the pluripotency marker Oct4 (B). Arrows point to clusters, which are outlined in black. Scale bar represents 200 micrometers.
Figure 4. Marker expression of hESNP cocultures with astrocytes fixed at 14 weeks of differentiation. Over 85 percent of HuNu-positive cells coexpress MAP2, about 67 percent of which also express GABA. While less than one percent of the HuNu-positive cells express the interneuron subtype marker SST, almost 30 percent express CB. Only about 7 percent of HuNu-positive cells express Nkx2.1:GFP. Scale bar represents 50 micrometers.
Figure 5. Whole-cell patch clamping shows the emergence of electrophysiological function in hESC-derived neurons over time. At 8 and 10 weeks of differentiation, current injection elicits only a slight depolarization of the membrane potential (n=2 at 8 weeks, n=2 at 10 weeks). However, by 12 weeks, a train of APs can be elicited (n=1). Trains of APs at 12 and 14 weeks (n=2, one of which fired APs) exhibit an accommodating phenotype, each AP having a diminished amplitude and greater width than the last in the train. This may indicate the membrane properties of the cell are not yet mature, or may indicate certain neuron subtypes that exhibit this phenotype.
Chapter 4: Future Directions

Maturing hESC-derived GABAergic interneurons in vitro

Generating mature interneuron phenotypes from hESNs in vitro would affirm the potential of these cells to provide inhibitory inputs post-transplant. This thesis describes the generation of high percentages of GABAergic neurons through molecular markers. Electrophysiological analysis suggests that neurons in these cultures are electrically functional, but relatively immature. As GABAergic transmission from interneurons is dependent on the development of electrophysiological properties, further studies should optimize the functional maturation of the cells.

In vivo, a combination of cell-intrinsic and environmentally-controlled mechanisms mediates interneuron maturation (Le Magueresse and Monyer 2013). Genetic programs unaffected by the local environment mediate the development of the electrophysiological properties of interneurons. Embryonic interneuron precursors ectopically grafted into the postnatal cortex undergo the same timeline of maturation as their non-grafted counterparts, displaying a protracted schedule of maturation for input resistance, capacitance, AP half-width, and maximum firing frequency (Le Magueresse 2011). Synaptic transmission and connectivity, on the other hand, are modulated by environmental changes. For example, dendrite morphology, synaptic wiring, and GABA transmission strength are altered if NKCC1 channels, a group of Cl channels that modulate GABAergic activity, are blocked (Wang and Kreigstein 2008; Wang and Kreigstein 2011).

We observe the generation of immature, accommodating trains of APs upon current injection in hESNP-derived neurons by 12 weeks of differentiation. This suggests
that neurons are functional but there is not an adequate number of Na$^+$ and K$^+$ channels in the cell membrane to produce a mature AP phenotype. Nicholas et al. observe similar phenotypes in some cells at 8 weeks of differentiation followed by more mature phenotypes at later time points. By 12 weeks of differentiation, they observe trains of non-accommodating APs. Over the next 18 weeks, APs become more frequent and neurons display increasingly mature properties such as shorter AP half-widths, deeper after-hyperpolarizations (AHPs), lower resting membrane potentials (RMPs), and an ability to fire repetitively upon superthreshold current injection (Nicholas et al. 2013). The development of functional properties is accompanied by an emergence of interneuron subtype markers that roughly corresponds to the timeline of subtype generation in the human brain (Fertuzinhos et al. 2009; Nicholas et al. 2013). These experiments suggest that the hESNPs described in this thesis may require additional time in culture to fully mature.

Although hESNPs seem to mature according to an intrinsic timeline when cocultured with astrocytes, another study suggests that aspects of interneuron maturation can be accelerated in vitro (Maroof et al. 2013). hESNPs cocultured with mouse embryonic cortical cells or with astrocytes produce neurons that fire non-accommodating trains of APs of similar amplitudes and frequencies by 8 weeks of differentiation. However, those cocultured with cortical cells have shorter AP half-widths and deeper AHPs than cells cocultured with astrocytes, indicating more mature membrane properties. Their average RMP at 8 weeks is particularly low, even lower than that observed at 30 weeks of differentiation in astrocyte cocultures. Cells in cortical cell cocultures also expressed the interneuron subtype marker PV, which was nearly absent in astrocyte cocultures (Maroof et al. 2013; Nicholas et al. 2013). These results suggest that aspects of
interneuron development that are intrinsically regulated during development can be accelerated by culture conditions.

Another possible explanation for the divergences in electrophysiological characteristics and PV expression between coculture conditions is that the starting population of ventralized hESNPs had significant differences. Maroof et al. cocultured hESNPs with cortical cells and observed indications of accelerated interneuron maturation. They used an adherent monolayer neural differentiation method using the molecular factors LDN, SB431452, XAV939, Pur, and Shh. On the other hand, Nicholas et al. did not observe an accelerated phenotype in astrocyte cocultures. They used hESNPs differentiated under a protocol forming first embryoid bodies and then a monolayer using Y27632, SB431452, BMPRIA, DKK1, and Pur. We did not observe acceleration and used an astrocyte coculture protocol using LDN, Shh, and Pur. The different factors used in hESNP differentiation protocols may have influenced the rate of their maturation. Examination of astrocyte and cortical cocultures with hESNPs derived under identical protocols is needed to determine the effects of the different cocultures on hESC-derived interneuron maturation.

There are a number of different approaches that might encourage faster maturation of hESNPs in future experiments. Cortical cell coculture warrants further study, given that it can accelerate the maturation of some electrophysiological characteristics and induce PV expression. One challenge that remains to be overcome is maturing PV-positive interneurons \textit{in vitro} to a point at which their characteristic fast-spiking oscillations are observable. In rodents, these phenotypes arise at postnatal stages (Goldberg et al. 2011). Long-term cortical cell coculture may enable the generation of
such a phenotype. However, since we observed during coculture optimization experiments that primary cortical cells usually die in culture within one month after plating (data not shown), conditions would have to be optimized for their survival. Given that astrocytes promote the survival and neuronal differentiation of both primary neural progenitors and hESNPs, a triple-culture experiment with embryonic cortical cells, astrocytes, and hESNPs would likely be an effective system for maturing hESC-derived interneurons *in vitro*. This system would provide excitatory inputs and synaptic targets for the generated interneurons as well as trophic support from astrocytes.

Other aspects of maturation may be enhanced by soluble factors. Brain derived neurotrophic factor (BDNF) is a protein secreted by excitatory neurons in an activity-dependent manner. It plays a role in the development of GABAergic innervation and transmission in interneurons (Huang et al. 1999). Preliminary experiments suggest that BDNF may contribute to more cells expressing SST in hESC-derived interneurons. Additional trials are underway to evaluate the effects of BDNF on *in vitro* interneuron maturation.

_Demonstrating the potential of hESC-derived interneurons_

While molecular and electrophysiological properties can measure relative maturation, it is difficult to determine when an interneuron has reached an adult phenotype. Most knowledge of interneuron electrophysiological properties comes from recordings of rodent brain slices isolated before the fourth postnatal week, a time during which interneurons are not fully mature (Le Magueresse and Monyer 2013). In addition, different interneuron subtypes display distinct periods of maturation and firing properties
(Kepecs and Fishell 2014). Characteristics such as AHP shape, RMP, and AP frequency suggest maturation, and ESC-derived interneuron phenotypes can be classified into groups based on these properties as a stand-in for definitive identification (Maisano et al. 2012; Nicholas et al. 2013). However, there currently are no clear-cut criteria for adult interneuron phenotypes.

The primary requirement for an hESC-derived interneuron therapy is not a set of phenotypes identical to those found in vivo but one that can provide inhibitory input. Nicholas et al. and Maroof et al. investigated the inhibitory capacity of hESC-derived interneurons in vitro by monitoring spontaneous post-synaptic currents (sPSCs) with and without the GABA\textsubscript{A} receptor antagonist bicuculline. sPSCs were increased upon addition of bicuculline to the media, indicating that GABAergic inputs modulated neuronal activity in both of the coculture systems (Maroof et al. 2013; Nicholas et al. 2013). Nicholas et al. also used optogenetics to transfect some of the cells in culture with channelrhodopsin2-EYFP downstream of the neuron-specific synapsin promoter. Patch clamping neurons targeted by EYFP-positive cells showed the production of PSCs resembling GABAergic currents upon blue light stimulation (Nicholas et al. 2013). These methods demonstrate the hESC-derived neurons’ potential for GABAergic transmission, which suggests their value for inhibitory input post-transplant.

Getting more specific: hESC-derived GABAergic interneuron subtype generation

We observe that astrocyte cocultures can produce high percentages of GABA-expressing neurons from hESNPs by 8 weeks of differentiation. An hESC-derived interneuron cell therapy, however, may require the generation of specific interneuron
subtypes for treating different diseases. For example, mouse models of TLE exhibit a preferential loss of SST and PV-expressing interneurons (Kobayashi and Buckmaster 2003; Sloviter et al. 1987). Interneuron subtypes confer different electrophysiological functions, which may play specific roles in repairing damaged circuitry.

A high percentage of the hESNPs used in the above experiments express Nkx2.1:GFP (Chapter 2, Figure 2). As this transcription factor marks precursors for a number of neural fates, future experiments should assay for other lineages that may be present in culture. Nkx2.1-positive progenitors give rise to GABAergic, cholinergic, and dopaminergic neurons as well as oligodendrocytes (Xu et al. 2008). The GABAergic fates include cortical, striatal, and hypothalamic interneurons. To specify the cells as forebrain (cortical or striatal) GABAergic neurons, we should look for expression of the forebrain marker FoxG1. This is particularly important since the experiments in this thesis use LDN for neural induction, which can reduce the amount of hESNPs that express FoxG1 (Maroof et al. 2013). FoxG1 expression would distinguish forebrain GABAergic neurons from more caudal Nkx2.1 lineages, such as the GABAergic interneurons of the arcuate nucleus in the hypothalamus (Yee et al. 2009). During development, Nkx2.1 is downregulated in cortical neurons as they mature but expression remains in striatal interneurons (Marin et al. 2000). Therefore, apparent downregulation of Nkx2.1:GFP in our cultures suggests a cortical interneuron phenotype. Demonstrating the expression of the cortical interneuron marker Lhx6 would confirm this identity.

Cortical interneurons have a diverse array of subtypes. The Nkx2.1-lineage cells can be grouped broadly into SST- and PV-expressing populations. Within the non-overlapping PV- and SST-expressing populations, coexpression with the calcium-binding
proteins CB and CR, the neuropeptide NPY, and the enzyme nNOS establishes further subpopulations (Kepecs and Fishell 2014). At 14 weeks, a low percentage of our hESC-derived interneurons express SST, 30 percent express CB, and none are immunopositive for PV. These data suggest that GABAergic interneurons are generated, but the subtypes produced are largely unspecified. Practically all MGE-derived interneurons express either SST or PV (Xu et al. 2008), so one would expect MGE-like hESNPs to give rise to large numbers of these subtypes. CB-positive interneurons also arise from Nkx2.1-positive progenitors (Sussel et al. 1999) and CB protein can be expressed in both SST and PV-lineage cells (Alcantara et al. 1996; Gonchar and Burkhalter 1997). Since 57.7 percent of the human cells in our cultures were GABAergic neurons, as many as half of the GABA-positive cells may be coexpressing CB.

The work of others suggests that further maturation may encourage SST and PV expression in our cells. In cocultures of Nkx2.1-positive hESNPs with astrocytes, only about 5 percent of neurons express SST at week 10 of differentiation. By week 20, this increases to about 15 percent, and by week 30, about 40 percent of neurons express SST (Nicholas et al. 2013). Culturing our neurons later than 14 weeks may lead to higher SST expressions.

Eliciting PV expression, on the other hand, may require the presence of excitatory cells. Coculture of Nkx2.1:GFP-positive hESNPs with astrocytes for 30 weeks leads only to occasional PV-positive neurons (Nicholas et al. 2013). In contrast, about 40 percent of Nkx2.1:GFP-positive cells in cortical cell cocultures express the marker at 2 months of differentiation (Maroof et al. 2013). The presence of excitatory input is likely responsible for the PV expression, which is activity-dependent (Marty et al. 1997). Cortical cell
coculture also promotes high levels of SST at 2 months. However, this measure is difficult to compare to the astrocyte coculture experiments because only Nkx2.1:GFP-positive cells were quantified (Maroof et al. 2013). Cortical cell input may be necessary to identify PV-lineage interneurons in culture. Alternatively, these cells may be characterized in future experiments by examining expression of synaptotagmin 2, which is selectively expressed in PV-positive synapses (Sommeijer and Levelt 2012).

It may ultimately be possible to preferentially derive certain interneuron subtypes from hESCs. In vivo, SST-positive interneurons are generated in the dorsal MGE and PV-positive interneurons are generated in the ventral MGE (Wonders et al. 2008). Experiments using mosaic elimination of the Shh pathway effector smoothened demonstrate that this subtype specification is mediated by differential levels of Shh signaling in the dorsal and ventral MGE (Xu et al. 2010). In addition to spatial origin, MGE-derived interneuron subtypes differ in their time of generation. PV interneuron progenitors arise in constant numbers in mice from E9.5-E15.5. In contrast, SST progenitors are generated in high numbers at E9.5-E12.5, but few are generated at E15.5. Almost no CR interneurons are made E9.5-E12.5, but many arise around E15.5 (Miyoshi et al. 2007).

Maroof et al. has demonstrated that stimulating the Shh pathway at different times during neural differentiation can modulate the neuronal fate of Nkx2.1:GFP-positive hESNPs (Maroof et al. 2013). Shh treatment from day 2-18 of differentiation leads to high expression of markers for dopaminergic neurons and the interneuron subtype nNOS. Day 6-18 treatment specifies mostly cholinergic precursors. A high percentage of SST and PV interneurons are generated when treatment is applied day 10-18 (Maroof et al.)
Our laboratory has observed higher percentages of Nkx2.1:GFP and GABA when hESNPs are treated with Shh and Pur under this time line versus other timelines (Nickesha Anderson, unpublished data). Perhaps further experiments examining the interneuron subtypes generated by modulating signaling pathways in a time-dependent manner will elucidate methods to efficiently generate those subtypes.

An in vitro system to supplement hESNP transplant studies

This thesis describes the optimization of a protocol to generate GABAergic neurons from hESCs. Coculturing ventralized hESNPs with mouse cortical astrocytes in the tapered serum medium condition treated with DAPT generates the highest percentage of neurons. The derived neurons express markers for interneuron subtypes and can fire trains of APs by 12 weeks of differentiation. The astrocytes also aid in cell survival and prevent the formation of dense clusters of non-neural cells that form when hESNPs are cultured alone. These data suggest that coculturing hESNPs with astrocytes encourages neuronal differentiation and/or survival. They also suggest that ventralized hESNPs become functional GABAergic interneurons under a protracted timeline, supporting the findings of other groups (Nicholas et al. 2013).

Maturing hESC-derived GABAergic interneurons in vitro is a valuable method for addressing questions concerning their potential as a cell replacement therapy. For example, our experiments show that hESNPs are difficult to mature into GABAergic interneurons. Their maturation will likely require an extended period of time post-transplant unless additional maturation-promoting measures are developed and implemented pre-transplant. In vitro maturation also allows the evaluation of specific
protocols for their ability to generate particular interneuron subtypes. This is crucial given that the treatment of neurodegenerative diseases may require the replacement of a certain subtype. Finally, an *in vitro* model system may provide a control setting to help investigate problems that might arise concerning transplant effectiveness and viability.

Seizures induce changes in the brain that alter the activity of interneurons and may affect the survival of transplanted cells (reviewed in Naegele et al. 2012). Comparing transplanted hESNPs to parallel populations *in vitro* may, for example, indicate whether the emergence of a particular phenotype in the transplanted population is caused by characteristics of the *in vivo* environment or the original hESNP population. In sum, this thesis provides a basis for the study of hESC-derived GABAergic interneurons *in vitro* alongside transplants into mice. *In vitro* hESNP maturation represents another tool to guide the continued push towards effective cell transplant therapies for neurological diseases such as TLE.
References


Banda, E., & Grabel, L. (2014). Directed Differentiation of Human Embryonic Stem Cells into


Neuroscience, 51(4), 749-753.


Uhlhaas, P. J., & Singer, W. (2010). Abnormal neural oscillations and synchrony in
schizophrenia. *Nat Rev Neurosci, 11*(2), 100-113. doi: 10.1038/nrn2774


