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DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS INTO CORTICOFUGAL PROJECTION NEURONS

A dissertation submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Molecular, Cell and Developmental Biology

by

Muriel Kmet

June 2013

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ABSTRACT

DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS INTO CORTICOFUGAL PROJECTION NEURONS

by Muriel Kmet

Understanding how neuronal diversity is achieved within the cerebral cortex remains a major challenge in neuroscience. The surge of human embryonic stem cells (hESCs) as a novel model system provides a unique opportunity to study human corticogenesis in vitro and identify the mechanisms that promote neuronal differentiation to achieve neuronal diversity in the human brain. Here, we demonstrate the derivation of corticofugal neurons from a genetically engineered Fezf2-YFP hESC reporter line, and uncover two distinct Fezf2 subpopulations that are reminiscent of the 2 Fezf2-expressing neuronal subtypes in the developing mouse brain. Fezf2 is a transcription factor that is both necessary and sufficient for the specification of subcerebral projection neurons in mouse. Its role in human corticogenesis is still unknown. However, the high conservation of FEZF2 protein between mouse and human suggest that Fezf2 is a specific marker of human cerebral neurons as well. Two hypotheses drove our research: 1) Extrinsic factors that modulate in vivo developmental signaling pathways play a critical role in the differentiation of hESCs to a corticofugal fate in vitro; 2) Human embryonic stem cell-derived neurons can survive in vivo and extend axonal projections to specific targets in the rodent brain. Our research shows that hESCs-derived corticofugal neurons are an effective model system to investigate the molecular pathways that
regulate human cortical differentiation, axon extension and survival, an endeavor that was until now proscribed due to the manipulation of human embryos.
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CHAPTER 1

INTRODUCTION
SECTION 1: The murine cerebral cortex

1.1 Projection neurons in the cortex

The cortex is a highly organized structure and the center of our highest cognitive functions including but not limited to our ability to make decisions, memories and is the seat of our perceptions and consciousness. The mammalian cortex is organized into six layers. Each layer contains neurons that are generated at the same time during neurogenesis and share common morphological features, transcription profiles, electrophysiological and hodological properties. Neurons from the deeper layers (layers 5, 6) of the cortex are generated first, followed by upper...
layers (layer 2-4) in a stereotypical “inside-out” pattern during neural development (Angevine and Sidman, 1961, Rakic, 1972, 1974). There are two classes of cortical neurons based on the axonal connections that neurons establish in the cortex. Interneurons are neurons that make local connections within the cortex while projection neurons project axons away from the cortex. Projection neurons are glutamatergic neurons that are classified according to their hodology. The three classes of projection neurons are: associative, commissural and corticofugal projection neurons. The associative neurons extend axons within a single hemisphere, while commissural neurons (also known as callosal neurons) send axons contralaterally through the corpus callosum to the other hemisphere.

*Figure 2. Corticofugal Projection Neurons.*
Schematic representing a sagittal view of the mousebrain showing corticothalamic neurons (blue), corticospinal motor neurons (green) and corticotectal neurons (red). Th: thalamus, sc: superior colliculus, Po: Pons, sp: spinal cord.
Corticofugal projection neurons (Figure 2) are a class of projection neurons that extend axons away from the cortex and include: corticothalamic and subcerebral projection neurons. Corticofugal neurons are located in layer 5 and 6 of the cortex and extend axons to the thalamus (corticothalamic neurons) or subcerebrally to the superior colliculus (corticotectal neurons), to the pons (corticopontine neurons) or to the spinal cord (corticospinal motor neurons) (Molyneaux et al., 2007).

Corticospinal motor neurons (CSMNs) are located in layer 5 and are involved in voluntary movement (Figure 3). They are also a clinically important class of neurons that degenerate in amyotrophic lateral sclerosis (ALS) and are under intense scrutiny for their role in this major neurodegenerative disease.

Figure 3. Corticospinal motor neurons (CSMNs).
Neurons are also classified upon the neurotransmitter they release into the synaptic cleft and include GABAergic ($\gamma$-aminobutyric acid) inhibitory neurons that are generated in the ventral telencephalon and migrate to their final location in the cortex (Figure 4) and include interneurons described above. Glutamatergic neurons, on the other hand, are excitatory neurons that are generated in the ventricular zone of the cortex and include projection neurons. My research was aimed at generating glutamatergic, excitatory neurons from hESCs that express the transcription factor *Fezf2* (Forebrain Embryonic Zinc-finger-2).
1.2 **Forebrain Embryonic Zinc Finger-2 (FEZF2) and its role in cortical fate specification**

Much progress has been made toward understanding the molecular mechanisms regulating the development of subcerebral projection neurons in mouse. Several transcription factors, including *Fezf2, Bcl11b, Tbr1, Sox5*, and *Bhlhb5* are part of an interconnected gene network that regulates cortical neuron fate specification (Bedogni et al., Han et al., McKenna et al., Arlotta et al., 2008, Chen et al., 2008, Joshi et al., 2008, Kwan et al., 2008). Among them, the zinc-finger transcription factor *Fezf2* (Forebrain Embryonic Zinc Finger 2) is both necessary and sufficient for the specification, differentiation and axon targeting of CSMNs in mouse (Chen et al., 2005a, Chen et al., 2005b, Molyneaux et al., 2005). During mouse brain development, *Fezf2* is expressed in progenitor cells as early as embryonic (E) day E8.5, and continues to be expressed at high level in subcerebral projection neurons. *Fezf2* null mice show a lack of subcerebral projection neurons and projections to the spinal cord (Chen et al., 2005a, Molyneaux et al., 2005). Strikingly, the mutant neurons adopt the identity of other cortical projection neuron subtypes (Chen et al., 2005a, Molyneaux et al., 2005, Chen et al., 2008, McKenna et al., 2011). Furthermore, misexpression of *Fezf2* in other neuron subtypes directs their axons to project toward the spinal cord (Franco and Muller, Rouaux and Arlotta, Chen et al., 2008). Ultimately, these studies demonstrate that in mouse, the identity and differentiation of subcerebral neurons is achieved through repression of alternate
neuronal subtype identities. Despite these advances, the molecular mechanisms regulating the development of human subcerebral neurons have not been directly investigated due to the lack of an appropriate human model system in which to study human cortical neuron differentiation. However, the high conservation of FEZF2 protein between mouse and human, and the similar expression patterns of Fezf2 during human fetal brain development (Zhu et al., Johnson et al., 2009) suggest that Fezf2 is a specific marker for human subcerebral neurons.

SECTION 2: Human embryonic stem cells

1.3 Human embryonic stem cells as a cellular model system in Neuroscience

Human embryonic stem cells (hESCs) are pluripotent cells derived from the inner cell mass (ICM) of day six blastocysts, which retain the potential to self-renew indefinitely and differentiate into the three germ layers. Owing to this potential, hESCs have been heralded as a potential source for cell-based therapies and regenerative medicine ever since their first isolation (Thomson et al., 1998). Neurological diseases such as amyotrophic lateral sclerosis (ALS), that have been notoriously difficult to model in rodents, are now being investigated in a human cellular system as hESCs are now becoming a bona fide research tool. This section exposes some of the reasons that make hESCs an ideal cellular system in the field of neuroscience, a field that has primarily used rodents as a research model system.
Animal models have contributed greatly to our understanding of basic neurobiology and neurological diseases. However, the rodent model system is less than perfect when studying uniquely human neuropathologies. Indeed, data acquired in rodent model systems need to be validated and extrapolated to humans before being translated to therapies. Thus, promising therapies tested in rodents have often failed to be replicated in human trials (Groeneveld et al., 2003, Rubin, 2008). This lack of data replication between rodent and human is undoubtedly due to species-specific cellular, anatomic and metabolic differences. These species differences are mostly unknown and a human cellular system would help deciphering the differing mechanisms between the two species. Transgenic mouse models often fail to display some of the cellular and behavioral changes present in human neuropathologies and as a result, cannot phenocopy many human diseases (Jakel et al., 2004). The transgenic rat model of Superoxide Dismutase 1 (SOD1), for example, while efficiently recapitulating motor neuron death, requires multiple copies of the mutant gene to replicate the human pathology, suggesting that rodent cells display a lower sensitivity to the mutant protein than human cells (Howland et al., 2002). Finally, rodent model systems cannot recapitulate the chronicity involved in most neurological diseases, possibly due to the rodent’s limited lifespan compared to human.

Anatomical differences between human and rodent cortices can also explain difficulties in replicating human neuropathologies in animal models. The human cortex contains an expanded outer subventricular zone (OSVZ) not present in the
mouse cortex (Hansen et al., 2010, LaMonica et al., 2012). This OSVZ contains OSVZ radial glia (oRG) cells that divide asymmetrically, thus self-renewing and producing neuronal precursor cells, hereby contributing to the increased complexity of the human cortex. OSVZ radial glia cells have also been identified in mice (Wang et al., 2011), but constitute a rare population of cells in the rodent brain. Conversely, oRG cells make up approximately 40% of all progenitor cells in the human OSVZ and could be responsible for the generation of a large portion of cortical neurons in the human neocortex (Lui et al., 2011). oRG cells have been estimated to contribute little to layer 6, more to layer 4 and 5 and importantly almost totally to layer 2-3 (Hansen et al., 2011). Even though more needs to be learned about this novel population of progenitor cells, this finding invites us to ponder on the impact of oRG cells on human neurodevelopmental diseases. Most specifically, diseases such as autism, schizophrenia, lissencephaly and microcephaly which involve the loss of neurons due to migration defect and/or a decrease in brain size might originate from oRG cells (LaMonica et al., 2012). Thus, the emerging field of pluripotent stem cells could play a major role in deciphering the impact of this new class of neural progenitors on specific human neuropathologies.

While neuronal cell replacement is a major goal of the field of the regenerative medicine albeit a long term one, stem cell-based therapies aiming at neural protection in neurological diseases are rapidly evolving. Neural protection is based on the observation that trophic factors such as Insulin-like factor 1 (IGF1) or Vascular endothelial growth factor (VEGF) when injected in the rodent brain induce
functional improvements in ALS mice models (Kaspar et al., 2003, Storkebaum et al., 2005). Such factors are thought to provide a favorable environment to support and protect neurons from undergoing degeneration. Ultimately, transplanted human embryonic stem cells genetically modified to express these growth and trophic factors, could provide a continuous release of these factors in vivo, and induce long-term improvement of neurological function (Behrstock et al., 2006).

One of the most immediate applications for the development of human cell-based models is drug discovery. Drug screening and toxicity testing for neurological compounds is crucial, and hESCs represent a prime system in which to address drug testing. Using differentiated neurons from hESCs rather than transformed cell lines such as: the human teratocarcinoma cell line Ntera2, the human neuroblastoma cell line SH-SY5Y, or PC12 cells derived from the neuroendocrine tumor pheochromocytoma, would improve drug screening (Zeng et al., 2006). Indeed, these transformed cell lines greatly differ in their genetics and physiology from human neurons, but have been used to substitute for the lack of available human neurons. Furthermore, some drug trials that have used mouse models to test drugs for neurodegenerative conditions, have reported to show modest improvements to translate into viable ALS therapies in patients despite showing powerful effects in mouse models (Schnabel, 2008). Differentiated hESCs can play a significant role in the development of drugs compounds, specifically targeting human subjects and pathologies and translate more seamlessly to treatments.
Whether hESC derived neurons are used for drug therapy, cell replacement therapy or modeling human development, it is crucial to establish whether in vitro derived neurons are similar if not identical to in vivo neurons. Human embryonic stem cells are derived from the ICM of the blastocyst, and as such harbor the potential to differentiate into the 3 germ layers: endoderm, ectoderm and mesoderm. Naturally, this property makes hESCs a valuable source of cells in which to study early human development. Whether hESC derived neurons are indeed identical to in vivo neurons can be evaluated in vitro by determining whether hESCs have the capacity to undergo neural corticogenesis in a dish. Neural differentiation protocols have been able to generate hESC derived neurons that closely resemble a bona fide human neuron by recapitulating intrinsic features present in the developing neuron. Cell fate decisions during early development are regulated by transcription factors acting in a temporal and spatial manner. HESC-derived neurons have been shown to recapitulate in vivo neurogenesis by exhibiting the temporal generation of neurons followed by the generation of glia cells. The temporal and spatial generation of neurons within each layer of the mammalian cortex has been observed in vitro as well. Indeed, within the six-layered cortex deep layers neurons are generated first, followed by upper layer neurons resulting in an “inside-out” generation of the cortical layers (Angevine and Sidman, 1961, Rakic, 1972, 1974). Interestingly, the same sequential generation of neurons is observed in vitro. The generation of deep layer neurons occurs first in vitro; with the expression of deep layer markers such as CTIP2 and TBR1 expressed respectively in layers 5 and 6. The generation of upper layer neurons expressing the
transcription factor SATB2 has been reported to occur around 60-70 days of differentiation, well after the generation of deep layer neurons occurring between days 15-30 (Shi et al., 2012). These studies suggest that in vitro temporal pattern of expression strongly follows the temporal generation of neurons during in vivo neurogenesis.

Synaptogenesis is another feature of early neural development that is also observed in vitro. HESC derived neurons have been shown in vitro to display mature electrophysiological properties as well as functional excitatory synapse formation in culture (Yan et al., 2005, Shi et al., 2012, Espuny-Camacho et al., 2013). Excitatory synapse formation shows indeed that neurons communicate with each other and along with the electrophysiological properties displayed by neurons are hallmarks of functioning neurons. Another feature characteristic of neurogenesis is the acquisition of an apico-basal polarity from progenitor cells during the cell cycle also known as interkinetic nuclear migration (INM). This cellular polarity is displayed by neural progenitors, known as radia glia (RG) cells within the maturing cortex to generate newborn neurons. Interkinetic nuclear migration during neurogenesis is recapitulated in neural differentiation protocols in structures called neural rosettes (Eiraku et al., 2008, Shi et al., 2012). Neural rosettes are neuroepithelial stuctures formed after neural induction that exhibit this apico-basal polarity reminiscent of the polarity RG cells display in the ventricular zone of the cortex. Like in vivo conditions, neural rosettes express the proliferating marker Ki67, as well as the centrosomal protein CEP135 at the apical (luminal) pole of the rosette in much the same way proliferation
occurs in the cortical ventricular zone (Shi et al., 2012). Interestingly, interkinetic nuclear migration, a key process occurring in the ventricular zone of the nascent cortex, which results in the movement of progenitor cells between the apical and basal surface of the ventricular zone, is also observed within neural rosettes (Shi et al., 2012). These properties make hESC-derived neurons comparable, albeit not identical, to a human neuron and as such a valuable tool for studying the mechanisms of human neurogenesis and human cortical development in vitro. My research, presented in this dissertation, is aimed at deciphering the different signaling pathways that can generate homogeneous subpopulations of neurons from hESCs in an effort to reflect the diversity of the neocortex.

In order for hESCs derived neurons to be utilized for cell replacement therapy, one must determine whether these neurons are able to not only survive in vivo, but are able to display an hodological pattern similar to a bona fide neuron. Indeed, if hESC-derived neurons are to fulfill their potential to replace diseased neurons in neurological diseases, it is critical to study their ability to extend axonal connections to their appropriate targets. Therefore, the hodological properties of hESCs derived neurons upon engraftment in vivo represent an area of intense investigation. Studying axonal projections within such a complex network is more convenient considering the high organization that lies within the neocortex. Cortical neurons are organized into distinct layers within the cortex and extend their axons to specific targets within the brain to create a specific axonal network (Figure 1). Layer 6 neurons project their axons to the thalamus, while layer 5 neurons send their projections to the midbrain,
hindbrain and spinal cord. On the other hand, layer 2-4 neurons send their axons to the contralateral hemisphere through the corpus callosum. A recent study, aimed at addressing whether hESCs derived neurons are able to recreate this axonal circuitry in vivo, showed that upon engraftment in the mouse brain, differentiated pluripotent stem cells (hESCs and iPS) into neurons are able to survive in vivo and extend axons to specific layers within the mouse brain (Espuny-Camacho et al., 2013). More studies are sure to follow to address specific axonal targeting of differentiated hESCs transplanted in an areal specific fashion. Such studies will be able to access whether hESC-derived neurons can be cellularly specified in vitro and able to extend axons to their cortical target.

Another important feature that make hESCs an attractive research model system is their ability to be molecularly manipulated. Within the last five years, the stem cell field has vastly expanded its molecular toolbox by generating genetically modified stem cell lines that allow the experimental study of more sophisticated scientific questions. HESCs can now be easily targeted genetically by homologous recombination (Leavitt and Hamlett, 2011), lentiviral vectors (Dottori et al., 2011) or Zinc finger nucleases (Maury et al., 2011). These molecular tools can ultimately help establish model cellular systems of neurological diseases for example, but also help address some of the basic questions in neurobiology such as establishing the lineage tracing of human neurons in mouse in vivo.

While hESCs are an appealing emerging cellular model system it is unlikely to completely supplant in vivo animal model systems. Instead, both systems tightly
complement each other. Despite the many advantages ascribed to hESCs, it remains an *in vitro* system and as such, suffers from the limitations typical of all *in vitro* systems. It is not possible to collect behavioral data from *in vitro* studies, which is a major drawback considering that behavioral data are important in evaluating neurological function *in vivo*. Also, the absence of a systemic immune response from *in vitro* studies fails to provide an important dimension for the study of neurodegenerative diseases, in which the body’s immunological response might play an integral part in the disease etiology and progression.

Neural differentiation protocols need to be further refined to address the complexity of the cerebral cortex, a challenge for an *in vitro* system. The cerebral cortex is endowed with hundreds of different neuronal subtypes and while differentiation protocols have been devised to generate glutamatergic (Espuny-Camacho et al., 2013), GABAergic neurons or disease specific neurons such as dopaminergic neurons (Kriks et al., 2011) or motoneurons (Li et al., 2005), differentiation protocols need to be devised to create the diverse pool of neurons present in the mammalian neocortex. To this day, the heterogeneity of cell types arising in culture is a problem that plagues *in vitro* neural differentiation protocols. While neural differentiation protocols tend to generate heterogeneous culture of neurons, it is important to focus on generating large pure populations of neurons and to best target specific neurodegenerative diseases. Interestingly, some studies have reported an inherent bias towards the generation of deeper layer neurons compared to later born upper layer neurons under current protocols (Espuny-Camacho et al.,
It is still unknown whether this bias reflects a human characteristic or more likely results from differing culture conditions across laboratories. Also, neurons generated *in vitro* are often immature and have been shown to induce teratomas, which are tumors generated from pluripotent cells (Fong et al., 2010). Thus, it is clear that neural differentiation protocols currently need to: i) generate homogeneous subpopulations of neurons that reflect the diversity of the neocortex, ii) synchronize differentiated subpopulations, iii) improve consistency across neural protocols. While at this point the stem cell field has matured and shown that hESCs represent a good cellular system in which to address uniquely human pathologies, culture conditions need to be further refined to generate neural subpopulations of cells representative of the complexity of the neocortex. One *in vitro* neural protocol is unlikely to fit all.

1.4 **Neural differentiation of hESCs to cortical neuronal fate: knowledge learned from *in vivo* studies.**

Differentiation strategies aimed at generating neurons from hESCs have been inspired by knowledge acquired in early developmental biology. During gastrulation, ectodermal tissue undergoes epidermal or neural differentiation. Seminal transplantation studies performed in the 1930s unveiled that an unknown signal secreted by the Spemann’s organizer could induce neural fate in *Xenopus* non-neural ectoderm (Spemann, 1938). A long search ensued for the neural inducing agent
produced by the organizer. It was only six decades later, that cell dissociation experiments along with experiments conducted with a dominant negative Activin construct (which inhibits TGFβ signaling), that the search for the organizer’s neural inducting signal ended (Sato and Sargent, 1989, Hemmati-Brivanlou and Melton, 1994). It was then established that an inhibitory signal, not an inducing one, was secreted from the organizer to specify neural fate. Soon thereafter, many neural inductive molecules were successfully isolated including: Noggin (Smith and Harland, 1992), Chordin (Sasai et al., 1994), Cerberus (Bouwmeester et al., 1996), Follistatin (Hemmati-Brivanlou et al., 1994), all of which inhibit the bone morphogenetic protein (BMP) signaling pathway (Piccolo et al., 1996). BMP and Activin are both potent neural fate inhibitors, but only BMP induces epidermal fate (Wilson and Hemmati-Brivanlou, 1995). These studies established neural fate as a “default fate”, which is acquired cell autonomously while the epidermal fate is acquired through BMP4 induction. Building on the neural induction knowledge provided by these studies, many neural differentiation protocols now use the BMP inhibitor Noggin (Gerrard et al., 2005, Itsykson et al., 2005, Surmacz et al., 2012) or a small molecule BMP antagonist dorsomophin (Zhou et al., 2010, Morizane et al., 2011), as a staple of their protocol, all of which were derived from knowledge learned from early mammalian development.

The Nodal pathway, like the BMP pathway, acts through the activation of the Smad transcription factors (Miyazawa et al., 2002). Studies aiming at deciphering the role of the Nodal signaling pathway in early development describe that Nodal is
involved in mesendoderm induction during gastrulation (Gritsman et al., 2000), but Nodal also plays a role in neural patterning. Indeed Nodal/- mouse embryos show increased neuroectoderm, suggesting that Nodal plays a role in early neural specification by inhibiting acquisition of neural characteristics (Camus et al., 2006). Hence, in neural differentiation protocols in vitro the complete inhibition of Smads 2/3 (Activin-Nodal pathway) and Smads 1/5/8 (BMP pathway) have been shown to provide the conversion of more than 80% of hESCs into neurons (Chambers et al., 2009). Thus, dual-Smad inhibition has become a common approach utilized in neural stem cell protocols to convert hESCs to a dorsal neural fate.

The inhibition of BMP signal from the organizer is not the sole mechanism that can explain the complexity of neural induction in vertebrates. Indeed, more signaling pathways are at play in neural induction in the early embryo (Denham and Dottori, 2009). Fibroblast growth factor (FGF), which is a large family of secreted glycoproteins that bind to 4 known extracellular receptors FGFR 1 through 4, is also involved in neural specification during early neural development. Interestingly, while BMP induces a neural fate in vertebrates, FGF induces formation of the nervous system in the invertebrate chordate ascidian (Inazawa et al., 1998). The role of FGF in neural fate determination is still somewhat unresolved in vertebrates. FGF act as a neural inducer in amphibians (Lamb and Harland, 1995). In chicks, FGF can ectopically induce the expression of neural markers, whereas BMP antagonists cannot (Rodriguez-Gallardo et al., 1997). However in higher mammals, FGF’s role is still unknown. A study in mouse embryonic stem cells (mESCs) reported that the FGF
pathway is required for neural specification (Kunath et al., 2007), and that BMP inhibition is not able to rescue the loss of the neuroectodermal marker Sox1 from differentiation culture in presence of FGF inhibition (Ying et al., 2003). Studies in hESCs also show a role for FGF in promoting early neural fate specification in vitro, (Cohen et al., 2010). Interestingly, studies report that FGF acting through the mitogen activated protein kinase (MAPK) pathway downregulates BMP through the phosphorylation of Smad1 (a BMP effector), thereby inhibiting Smad1 and suggesting that FGF regulation of neural induction is dependent on the BMP pathway (Pera et al., 2003, Sapkota et al., 2007). It is not completely known whether FGF can induce neural fate determination independently from BMP inhibition, through BMP repression or even through both. From studies in hESCs, some evidence is surfacing that FGF might act independently from BMP signaling to specify neural fate (LaVaute et al., 2009). More studies in hESCs would be helpful in identifying whether FGF is required for the neural specification in vertebrates.

Wnt signaling pathway is another candidate pathway that can be modulated in vitro to induce a cortical fate specification in hESCs. Wnt genes encode a highly conserved family of secreted glycoproteins that play an essential role in the formation of the vertebrate nervous system (Ciani and Salinas, 2005), but whether Wnt inhibition or activation plays a role in neural specification of hESCs to a cortical neuron fate is still unclear. Prior to blastula, when the dorso-ventral polarity is established and the neural fate is acquired in frog embryos, Wnt is required for the acquisition of neural tissue from the ectoderm. The mechanism by which Wnt induces
neural fate is thought to be through BMP4 inhibition (Baker et al., 1999). Later, during blastula/early gastrula on the other hand, it is thought that Wnt needs to be downregulated in order to induce neural cells. Indeed, in Xenopus, Wnt overexpression ventralizes embryos and prevents neural tissue formation (Christian and Moon, 1993). In hESCs, the role of the Wnt/β catenin signaling pathway in the generation of cortical neurons is controversial. Conflicting reports in mESCs either claim that Wnt enhances neural differentiation (Hirabayashi et al., 2004, Otero et al., 2004), or that it inhibits neural fate (Aubert et al., 2002, Verani et al., 2007). Activating Wnt in mESCs has also been associated with self-renewal (ten Berge et al.). In hESCs, the inhibition of Wnt was reported to convert hESCs derived dorsal telencephalic progenitors to ventral progenitors (Li et al., 2009), while its activation through GSK3β inhibition has also been reported to maintain hESC pluripotency (Sato et al., 2004). In an effort to reconcile these conflicting results, I attempted to address the following question in my research: what role do extrinsic Wnt ligands play in the generation of Fezf2 subcerebral neurons from hESCs and in the acquisition of the human cortical neural fate?

Another pathway that plays a crucial role in the formation of the nervous system is the sonic hedgehog (Shh) pathway. Shh, a vertebrate homolog of the Hedgehog gene in Drosophila, induces the expression of ventral neurons in vivo (Ericson et al., 1995). Whether the role of Shh pathway is conserved in humans has not been addressed. In early development, the notochord and the floor plate of the embryonic neural tube secrete Shh. Shh is a morphogen and forms a gradient along
the ventral neural tube to induce different neural progenitor cell types of ventral origin (Briscoe et al., 2000). Applying this knowledge, hESC neural differentiation protocols have successfully converted dorsal progenitors expressing PAX6, to ventral progenitors expressing the ganglionic eminence progenitor marker NKX2.1 upon Shh treatment (Li et al., 2009). This study shows that the complete elimination of PAX6+ progenitors while NKX2.1 population was increased to 84%, thereby showing Shh as a ventralizing factor in human in vitro context. In the prospective midbrain, Shh is involved in the generation of dopaminergic neurons, which are the neurons that degenerate in Parkinson’s disease (Hynes et al., 1995). Other studies involved FGF8, an extracellular protein secreted by the anterior neural ridge (ANR), which along with Shh play a role in the specification of midbrain dopaminergic neurons. Interestingly, the convergence of these FGF8 and Shh signals in the midbrain results in the generation of dopaminergic neurons (DA neurons) (Ye et al., 1998). Knowledge acquired from such studies, using a combination of Shh and FGF8, have been applied in deriving hESCs to dopaminergic neurons (Perrier et al., 2004, Kriks et al., 2011). HESC-derived dopaminergic neurons are expressing tyroxine hydroxylase (TH), an enzyme necessary for the synthesis of dopamine. Moreover, these hESC-derived DA neurons display electrophysiological properties characteristic of in vivo dopaminergic neurons, making these hESC-derived neurons attractive for transplant studies in pre-clinical model of Parkinson disease. Since the cerebral cortex is generated from the dorsal portion of the anterior neural tube, the signaling pathways that promote anterior neural fate and dorsal cell identities are thus likely to promote the
differentiation of hESCs into cortical neurons. Shh acting as a ventralizing morphogen is inhibited in hESCs differentiation protocols aimed at cortical neuron fate. The Shh inhibitor cyclopamine (Taipale et al., 2000, Chen et al., 2002) has been shown in mouse embryonic stem cells (mESCs) to increase dorsal while repressing ventral identities (Gaspard et al., 2008). In human cells, however, whether cyclopamine has the same effect is still unresolved. Some clue that Shh inhibition does indeed induce a dorsal fate in human cells, is a report that suggests that the inhibition of Shh through the expression of the repressive form of GLI3, a Shh signaling mediator, dorsalizes neural progenitors (Li et al., 2009). However, a recent report contradicts this finding and instead reports that the inhibition of Shh through cyclopamine treatment does not significantly increases the expression of dorsal progenitor markers (Espuny-Camacho et al., 2013). However, according to my own research, hESC differentiation supplemented with cyclopamine and dual-Smad inhibition treatment is sufficient to induce a significant 2.2 fold increase in efficiency in generating Fezf2-expressing neurons at day 30 (see Chapter 2). These discrepancies are still unresolved but could stem from the difference in cyclopamine concentrations used in the different experiments. Espuny-Camacho et al failed to mention the concentration and the time at which the quantitative RT-PCR was performed (day 19 versus day 30 for my experiment). Also, surprisingly Espuny-Camcho et al reported in the control experiment that the Shh treatment condition failed to show an increase of ventral markers such as GSX2 and DLX2 as one would expect.
In order for hESC protocols to truly recapitulate \textit{in vivo} differentiation, hESC-derived neurons need to be able to survive \textit{in vivo} without the formation of tumors, and extend axons to their appropriate targets within the brain. Many studies have shown the derivation of important classes of neurons needed for regenerative medicine. Experiments are now starting to focus on assessing the capacity of these hESC-derived neurons to function as \textit{bona fide} neurons following transplantation in animal models of Parkinson’s disease (Kriks et al., 2011), amyotrophic lateral sclerosis, spinal cord injuries etc. To date, only a few reports have been successful at transplanting hESC-derived neurons. Most notably, the study from Vanderhaeghen’s group (Espuny-Camacho et al., 2013) showed that cortical neurons generated \textit{in vitro} from hESCs and induced pluripotent stem cells (iPSC) can stably integrate \textit{in vivo} and extend their axonal projections according to their hodological properties. This study represents the first report that addresses the fate of the hESC-derived neurons axonal projections \textit{in vivo} by testing the projections of pyramidal neurons to their expected targets. According to their report, hESC-derived neurons project to area-specific targets. As expected, retrograde axon targeting identified 70\% of neurons labeled with the corticothalamic marker TBR1 in the thalamus, while less than 30\% were labeled with the corticospinal marker CTIP2. The axonal projections originating from the cortex did extend to expected targets and did not extend to the substantia nigra, a structure found in the midbrain or in the cerebellum, where cortical subcerebralaxons are not found \textit{in vivo}. Vanderhaeghen’s careful characterization of hESCs-iPSCs derived grafts in rodents is a first step in understanding the hodological
characteristics of hESC-derived cortical projection neurons \textit{in vivo}. More studies are likely to follow.

1.5 \textbf{Are induced pluripotent stem cells (iPSCs) supplanting hESCs?}

For the purpose of regenerative medicine, induced pluripotent stem cells (iPSC) (Takahashi et al., 2007, Yu et al., 2007) have benefited from the expansion of knowledge of hESCs differentiation protocols and are now favored for transplant studies. Indeed, iPSCs provide a major advantage over hESCs as they are derived from the patient’s own somatic cells and are not bereft with potential immunogenic responses from the host, even though there have been claims to the contrary (Okita et al., 2011, Zhao et al., 2011). While iPSC technology holds a tremendous potential in the regenerative field, the technology is to this day highly inefficient. The reprogramming efficiency is reported to stand between 1-10\%, depending on the laboratory, the reprogramming method and the starting cell type (Stadtfeld et al., 2010, Yamanaka, 2012). Also, the long-term safety of human iPSC remains uncertain. New transcription factors have been tested to replace the oncogene c-myc from the initial transcription factor cocktail. Thereby, attenuating the safety concern of having the c-myc transgene expressed in the long-term (Nakagawa et al., 2008). The integration methods to induce reprogramming have been a cause for safety concerns. Integration of the transcription factors in the somatic cells has improved to avoid vector integration in the host genome and favored episomal vectors instead.
The generation of iPSCs has now been reported from many transgene-free methods such as Sendai virus (SeV) infections (Fusaki et al., 2009), synthesized modified RNAs (Warren et al., 2010), or proteins (Kim et al., 2009). Still, an important question remains: Are iPSC equivalent to ESCs? Indeed, while ESCs are derived from embryos, iPSCs are “in vitro constructs” (Chin et al., 2009). Also, if there are differences between these two pluripotent cells types are they relevant and cause for concern? Many research groups are actively studying these questions and whether iPSCs are molecularly equivalent to embryonic-derived ES cells still needs to be fully elucidated. A report comparing hESC and iPSC lines by genome wide methods identified a distinct gene expression profile characteristic of iPSC lines and different from hESC lines profile, suggesting that iPSCs are a subtype of pluripotent cells (Chin et al., 2009). According to their report, Chin et al identified hundreds of genes differentially expressed between iPSC and hESCs at early passages, while 97% of the methylation patterns identified were identical. Others reported a decrease in efficiency and increase variability in differentiation propensities between hESCs and iPSCs (Hu et al., 2010, Lister et al., 2011). In one report, iPSCs were reported to convert into PAX6+ cells at an efficiency of 10-50%, while more than 90% of the hESCs converted to PAX6+ in a side-by-side study of 5 hESCs and 12 iPSC cell lines (Hu et al., 2010). Other studies reported changes in DNA methylation with the identification of aberrant genomic hotspots in a whole genome profiling of the DNA methylomes of iPSCs (Lister et al., 2011). Yet, we can still marvel at how similar iPSCs and hESCs are from each other considering their different origin. It is likely
that today’s advances in the differentiation of hESCs will be made using hESCs and hiPSCs in tandem, before we can really establish whether iPSCs are safe and can be used *in vivo*. However, hESCs are still a valuable asset in devising differentiation protocols due to their undeniably close relationship to the human embryo.

### 1.6 Hypotheses

The following hypotheses are developed in my research:

1. Extrinsic factors that modulate *in vivo* developmental signaling pathways play a critical role in the differentiation of hESCs to a corticofugal fate *in vitro*.
2. Human embryonic stem cell derived neurons can survive *in vivo* and extend axonal projections to specific targets in the rodent brain.
CHAPTER 2

DIRECTED DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS INTO CORTICOFUGAL NEURONS UNCOVERS HETEROGENEOUS FEZF2-EXPRESSING SUB-Populations
2.1 ABSTRACT

Understanding how neuronal diversity is achieved within the cerebral cortex remains a major challenge in neuroscience. The advent of human embryonic stem cells (hESCs) as a model system provides a unique opportunity to study human corticogenesis in vitro and identify the mechanisms that promote neuronal differentiation to achieve neuronal diversity in the human brain. The transcription factor Fezf2 is necessary and sufficient for the specification of subcerebral projection neurons in mouse. However, its function during human corticogenesis is poorly understood. This study reports the differentiation of an hFezf2-YFP hESC reporter line into corticofugal projection neurons capable of extending axons toward the spinal cord upon transplantation into neonatal mouse brain. Additionally, we show that triple inhibition of the TGFβ/BMP/Wnt-Shh pathway promotes the generation of hFezf2-expressing cells in vitro. Finally, this study unveils the isolation of two novel and distinct populations of hFezf2-YFP expressing cells reminiscent of the distinct Fezf2-expressing neuronal subtypes in the developing mouse brain. Overall our data suggests that the directed differentiation of hESCs into corticofugal neurons provides a useful model to identify the molecular mechanisms regulating human corticofugal differentiation and survival.
2.2 INTRODUCTION

Hundreds of different neuronal subtypes are generated in the central nervous system during development. Among these, cortical projection neurons are essential for high order cognitive and sensory functions in the human brain. Within the six-layered cerebral cortex, subcerebral projection neurons are located in layer 5 and extend their axons to the midbrain (corticotectal projection neurons), brain stem (corticobulbar neurons) or spinal cord (corticospinal motor neurons, or CSMNs). CSMNs are clinically important, as their degeneration has been implicated in upper motor neuron diseases such as Amyotrophic Lateral Sclerosis (ALS) (Bruijn et al., 2004, Pasinelli and Brown, 2006). Neurons from layer 6 project their axons to the thalamus (corticothalamic neurons), and together with CSMNs are referred to as corticofugal projection neurons. Due to their high relevance in human diseases, the molecular mechanisms that underlie the specification and differentiation of subcerebral projection neurons have been the subject of intense investigation.

Overall, the specific signaling pathways promoting the generation of human subcerebral neurons including CSMNs are largely unclear. In this study, we utilized a genetically modified hESC line in which a YFP reporter was targeted into the endogenous hFezf2 locus to investigate the differentiation of hESCs into cortical projection neurons (Ruby and Zheng, 2009). We demonstrate that hESCs can differentiate into corticofugal neurons, including CSMNs and corticothalamic neurons in vitro. Upon transplantation into neonatal mouse brains, differentiated
human cells can extend axons toward the spinal cord. The \textit{in vitro} hESC differentiation presented in this chapter reveals two distinct hFezf2-YFP$^+$ subpopulations, similar to the Fezf2-expressing populations in the developing mouse cortex. Interestingly, hFezf2$^{Hi}$ and hFezf2$^{Low}$ cell populations are molecularly similar to mouse corticothalamic and subcerebral neurons. Overall, our study show that hESCs-derived cortical projection neurons can be used as an effective model system to investigate the molecular pathways that regulate human CSMN differentiation, axon extension and survival.

\section*{2.3 RESULTS}

\subsection*{2.3.1 Differentiation of human embryonic stem cells (hESCs) into cortical projection neurons}

To generate cortical neurons from hESCs, we developed a differentiation protocol based on both current knowledge of early cortical development in mice and established hESC neural differentiation approaches (Reubinoff et al., 2001, Chambers et al., 2009). A schematic of our neural differentiation strategy is shown in Figure 5A, and involves three steps spanning 30 days. First, neural induction was initiated by the generation of embryoid bodies (EBs), in which undifferentiated H9 or HUES5 cells were allowed to aggregate in suspension culture, mimicking the spatial organization of the morula stage of development. Then, neural rosette formation was initiated by
plating down EBs onto matrigel, supplemented with SB431542 and Noggin (Lamb et al., 1993, Chambers et al., 2009, Patani et al., 2009) for the first 12 days of culture. In the final neural differentiation stage, cells were grown in a culture medium that promoted cell survival and allowed neuronal differentiation.

Expression of forebrain progenitor markers Pax6, Emx2, Nestin, Otx2, Foxg1, Tbr2 and Fezf2 (Miyoshi and Fishell, Gotz et al., 1998, Cecchi, 2002, Sessa et al., 2008) was confirmed using reverse transcriptase-polymerase chain reaction (RT-PCR) (Figure 5B) and immunohistochemical staining (Figure 5 C-E). Pax6, Emx2 and Foxg1 showed a progressive increase in expression throughout differentiation until day 32 as assayed by RT-PCR (Figure 5B). The intermediate progenitor marker Tbr2 was expressed concurrently with the immature neuronal marker βIII tubulin (Tuj1) as determined by immunohistochemistry staining (Figure 5E). Subcortical neuron markers Fezf2 and Nfib (Plachez et al., 2008), corticothalamic neuron marker Tbr1, and CSMN marker Bcl11b were robustly expressed at day 32 (Fig 5B, F-H). Our neural differentiation protocol did not solely generate cortical neurons, as the expression of ventral cell marker Ascl1 (Casarosa et al., 1999) was detected at day 32 (Figure 5B). Expression of the pluripotency marker Nanog (Chambers et al., 2003) was also maintained throughout the neuronal differentiation procedure, suggesting that some cells retained their stem cell characteristics throughout differentiation (Figure 5B). However, another pluripotency gene, Pou5F1 (Nichols et al., 1998, Niwa et al., 2000) (also known as Oct4) showed steady down-regulation upon differentiation (Figure 5B).
Fezf2 is a specific marker for corticofugal neurons in mouse and human (Zhu et al., Johnson et al., 2009). To further investigate the hESC-derived subcerebral neurons, we differentiated a genetically modified hESC line (Fezf2-YFP HUES9) in which the YFP reporter was inserted into the endogenous human Fezf2 gene locus (Ruby and Zheng, 2009). Following our neuronal differentiation protocol, many Fezf2-YFP-expressing cells extended long neuronal processes by day 40 (Figure 5 I, J and Figure 6). In addition to H9 (Figure 1B-H) and Fezf2-YFP HUES9 (Figure 5 I, J) lines, our neuronal differentiation protocol also successfully generated cortical projection neurons, including CSMNs, from HUES 5 cells (Supplementary Figure 1).
Figure 5 (A) Schematic representation of human embryonic stem cell neural differentiation, displaying culture and treatment conditions.
Figure 5 (B) Reverse transcriptase-PCR (rt-PCR) of differentiated cells. H9 cells show differential expression of pluripotent markers (Pou5F1, Nanog), dorsal progenitors (Pax6, Emx2, Otx2, Foxg1), cortical neurons (Fezf2, Nfib, Tbr1), and ventral marker (Ascl1) at neural differentiation day-6, 12 and 32.
Figure 5 (C-E) Immunofluorescence staining of H9 differentiated cells yields neural progenitor markers Nestin, Pax6, and Tbr2 co-expressed with immature neuronal marker TUJ1. (F-H) Immunofluorescence staining of H9 cells yields cortical neurons of layer 5 Bcl11b, Nfib (F,H), layer 6 marker Tbr1 (G). Scale bars=Nestin, Pax6: 50µm, Tbr2: 25µm, Bcl11b, Nfib, Tbr1: 100µm.
Figure 5 (I) Immunofluorescence staining of differentiated *Fezf2*-YFP-HUES9 reporter line on day 40 stained with GFP antibody, scale bar 200µm. (J) Differentiated *Fezf2*-YFP reporter line expressing GFP and the human specific HuNu antibody, scale bar: 25µm. Abbreviations: DAPI, 4',6'-diamidino-2-phenylindole; YFP, yellow fluorescent protein; GFs, growth factors;
2.3.2 Transplanted hESCs-derived *Fezf2*-YFP-expressing neurons extend axons in the mouse brain

To determine whether the *in vitro* differentiated corticofugal neurons could integrate and project their axons to appropriate subcortical targets, we tested their ability to extend axons subcortically upon transplantation into neonatal mouse cortices. We dissociated neural rosettes from day 12 cultures and injected them using ultra-sound guided imaging into the P0 cortex of wild-type mice. Ninety days after transplantation, the recipient mice were sacrificed and their brains analyzed with GFP and human nuclei (HuNu) antibodies by immunohistochemistry (Figure 6). The expression of the human nuclear antigen confirmed the human origin of the transplanted cells (Figure 6B). Consistent with corticofugal identity, *Fezf2*-YFP$^+$ cells were observed extending axons from the cell injection site in the cortex to subcortical targets (Figure 6A). *Fezf2*-YFP$^+$ cells were observed in the white matter (Figure 6C), and turning into the internal capsule (Figure 6D). YFP$^+$ axons were also observed at the base of the ventral forebrain (Figure 6E), and in the pyramidal decussation on their way to the spinal cord (Figure 6F). Injected *Fezf2*-YFP cells in the cortex expressed the CSMN marker BCL11B (Figure 6G). These experiments demonstrated that transplanted hCSMNs integrated into the host brains and extended axons toward subcortical targets, similar to endogenous *Fezf2*-expressing projection neurons.
Figure 6. Human ESCs-derived Fezf2-YFP-expressing neurons extend axonal projections in the mouse brain at P90.

(A) Schematic of sagittal mouse brain indicates the location of panels B-F. YFP⁺HuNu⁺ cells were present in the injection site in the cortex (B), some YFP⁺ axons were present in the white matter (C, D), at the base of the forebrain (E), and pyramidal decussation (F). Scale bars: 100 µm (B, C, F); 200 µm (D, E)
Figure 6 (G) Transplanted hFezf2-YFP cells at the injection site in the deep layer of the cortex expressed the CSMN marker BCL11B. Mouse layer 5 neurons expressing BCL11B are indicated by white arrow. Scale bar: 100 µm.
2.3.3 Differentiated hESCs contain two distinct Fezf2-YFP+ subpopulations

To identify the culture conditions that efficiently promote hESC differentiation into CSMNs, we differentiated Fezf2-YFP HUES9 cells under different growth factor conditions. We subsequently performed Fluorescence Activated Cell Sorting (FACS) analysis at day 30 of differentiation to quantify the percentage of Fezf2-expressing cells. Our goal was to identify the culture conditions most efficient at promoting the generation of Fezf2-YFP-expressing neurons. Treatment with SB431542, Noggin, DKK1 and cyclopamine, as described in Figure 1, resulted in an increase in the percentage of Fezf2-expressing cells as compared to the no growth factor control (Figure 7C). Interestingly, under these different differentiation conditions, in addition to a Fezf2-YFP population, we consistently observed two distinct Fezf2-YFP+ cell populations (Figure 7A,C): cells expressing high levels of Fezf2-YFP (YFP\textsuperscript{Hi}) and cells expressing low levels of Fezf2-YFP (YFP\textsuperscript{Low}) (Figure 7A). To confirm the presence of these Fezf2-YFP\textsuperscript{+} populations, we performed qRT-PCR on each sorted population to test the expression levels of the human Fezf2 transcript. As expected, Fezf2 was highly expressed in the YFP\textsuperscript{Hi} sorted subpopulation compared to the Fezf2-YFP\textsuperscript{-} cell population (F= 8.655; p=0.0171; n=3) (Figure 7B), whereas expression of Fezf2 in the YFP\textsuperscript{Low} population was intermediate between Fezf2-YFP\textsuperscript{-} and Fezf2-YFP\textsuperscript{Hi} populations (Figure 7B). Interestingly, both YFP\textsuperscript{Hi} and YFP\textsuperscript{Low} populations were observed even when differentiation occurred in the absence of growth factors (Figure 7C, first panel). Thus, our Fezf2-YFP HUES9...
neural differentiation protocol generated two novel and distinct \textit{Fezf2-YFP}$^{+}$ populations (Figure 7A,C), suggesting they might exhibit different molecular characteristics.
Figure 7. Differentiated hESCs contain two distinct hFezf2-YFP$^+$ subpopulations detected by FACS under different cell culture conditions.

(A) Representative FACS pseudo-color plot and FACS histogram of differentiated Fezf2-YFP cells on day 30, showing three subpopulations based on hFezf2-YFP expression: YFP$^{\text{Neg}}$, YFP$^{\text{Low}}$, and YFP$^{\text{Hi}}$. 


Figure 7 (B) Quantitative RT-PCR of hFezf2 mRNA from the three sorted cell subpopulations (*p<0.05; F= 8.655; p=0.0171; n=3).
Fig 7 (C) Representative FACS histograms of differentiated hFezf2-YFP cells
hFezf2-YFP cells from the same experiment were either non treated (no growth
factors) or treated with SB+NG+DKK1+cyclopamine. (p and F values were assessed
by one-way ANOVA with Tukey’s post-test.) Abbreviations: SB, SB431542; NG,
Noggin; DKK1, Dickkopf-1.
2.3.4 Inhibiting Wnt signaling promotes the generation of Fezf2-YFP-expressing cells

Next we sought to determine whether activating or inhibiting specific signaling pathways during the first 12 days of the neural differentiation protocol affected the efficiency of generating Fezf2-YFP$^+$ cells. Interestingly, the frequency of Fezf2-YFP$^{\text{Low}}$ cells (Figure 8B) on day 30 was consistently higher (range: 23-26.7%, n=4 per differentiation condition) than the frequency of Fezf2-YFP$^{\text{Hi}}$ cells (range: 5-10.8%, n=4 per condition) (Figure 8A). In addition, Fezf2-YFP$^{\text{Hi}}$ and Fezf2-YFP$^{\text{Low}}$ subpopulations responded differently to the 5 treatments conditions tested, which included 1) no growth factor, 2) dual SMAD inhibition, 3) dual SMAD inhibition plus Dickkopf-1 (DKK1, inhibiting Wnt signaling pathway), 4) dual SMAD inhibition plus cyclopamine (inhibiting Shh pathway), and 5) dual SMAD inhibition plus DKK1 and cyclopamine.

The generation of Fezf2-YFP$^{\text{Hi}}$ cells was more sensitive to inhibition from the TGFß/BMP/Wnt-Shh signaling pathways as compared to the Fezf2-YFP$^{\text{Low}}$ population (Figure 8A). Inhibition of the Wnt pathway using the secreted protein DKK1 induced a 2-fold ($p=0.0235$) increase in the Fezf2-YFP$^{\text{Hi}}$ subpopulation over the no growth factor control (Figure 8A). A similar increase in induction of the Fezf2-YFP$^{\text{Hi}}$ subpopulation was observed upon inhibition of Shh signaling with cyclopamine. However, we did not observe further enhanced induction of the Fezf2-YFP$^{\text{Hi}}$ population when using both DKK1 and cyclopamine, suggesting a lack of synergism between inhibiting these two pathways. In contrast to the YFP$^{\text{Hi}}$
population, generation of YFP\textsuperscript{Low} cells was not significantly influenced by inhibition of TGF\textbeta/BMP, Shh or Wnt signaling pathways (Figure 8B). Despite the ability to induce YFP\textsuperscript{Hi} cells by growth factor treatment, the percentage of YFP\textsuperscript{Hi} expressing cells was consistently lower compared to the YFP\textsuperscript{Low} cells. The difference in response to inhibition of signaling pathways, as well as the difference in population sizes suggested that \textit{Fezf2}-YFP\textsuperscript{Low} and \textit{Fezf2}-YFP\textsuperscript{Hi} cells represented two distinct populations.

To further determine the effects of activating and inhibiting Wnt signaling on generating \textit{Fezf2}-YFP-expressing cells from hESCs, we next assessed the effect of Wnt activation by addition of Wnt3a to the media. We observed an almost complete loss of both \textit{Fezf2}-YFP\textsuperscript{Hi} and \textit{Fezf2}-YFP\textsuperscript{Low} populations upon addition of Wnt3a (Figure 8C, D). Compared to inhibition of Wnt signaling with DKK1, the percentage of \textit{Fezf2}-YFP\textsuperscript{Hi} cells at day 30 showed a 39.8 fold decrease (n=3) and the percentage of \textit{Fezf2}-YFP\textsuperscript{Low} cells showed a 10.65 fold decrease (n=3) when Wnt3a was added to the media (Figure 8C,D). Moreover, when we substituted Wnt3a with the small molecule Wnt agonist CHIR99021, the generation of \textit{Fezf2}-YFP-expressing cells was completely inhibited (Figure 8E). Wnt activation therefore had strong inhibitory effect on the generation of both h\textit{Fezf2}\textsuperscript{Hi} and h\textit{Fezf2}\textsuperscript{Low} populations. Collectively, these experiments demonstrate that the differentiation of h\textit{Fezf2}-YFP-expressing populations and \textit{Fezf2} expression was robustly activated by the triple inhibition of TGF\beta/BMP/Wnt-Shh signaling.
Figure 8. TGFβ/BMP/Wnt-Shh triple inhibition activates Fezf2 expression in hESCs

(A) Quantification by FACS of differentiated hFezf2-YFP cells under different conditions shows a 2.2 fold increase in efficiencies in generating hFezf2-YFP^Hi subpopulation using SB+NG+DKK1 and SB+NG+Cyclopa mine treatments compared to a no growth factor control (*p<0.05. p= 0.0235; F= 13.74); Error bars represent standard error of mean, sem (n=4). p values were assessed by one-way ANOVA with Tukey’s post-hoc test). The efficiency of generating Fezf2-GFP^Hi cells was in the range: 5-10.8%, n=4 per condition, quantified by FACS. (B) FACS analysis of differentiated hFezf2-YFP cells shows that the efficiencies to generate hFezf2-YFP^Low subpopulation were similar under different conditions (range: 23-26.7%, n=4) but yielded an overall higher percentage than the Fezf2-GFP^Hi population.
Figure 8. Wnt inhibition activates Fezf2 expression while Wnt activation represses Fezf2 in hESCs

(C) FACS analysis of differentiated hFezf2-YFP cells shows that the efficiencies to generate hFezf2-YFP^Low subpopulation were similar under different conditions (range: 23-26.7%, n=4) but yielded an overall higher percentage than the Fezf2-GFP^Hi population. Representative FACS histograms and FACS dot plots showing differentiated hFezf2-YFP cells under Wnt activation (Wnt3a) and Wnt inhibition (DKK1). (D) Quantification of the efficiencies of generating hFezf2-YFP^Hi and hFezf2-YFP^Low subpopulations under Wnt activation and Wnt inhibition conditions. Student t-test, unpaired, two-tailed, Welch’s corrected (t=3.91; p= 0.0298 for low) and (t= 6.329; p=0.0080 for high). (E) FACS histogram showing inhibition of both hFezf2-YFP^Hi and hFezf2-YFP^Low populations under treatment with the Wnt agonist CHIR99021 at day 30.
2.3.5 Differentiated hFezf2-YFP\textsuperscript{hi} and hFezf2-YFP\textsuperscript{low} populations recapitulate Fezf2 expression in the mouse cortical layers

To determine whether the hFezf2-YFP\textsuperscript{hi} and hFezf2-YFP\textsuperscript{low} populations arose from a cell culture artifact or were biologically relevant, we analyzed Fezf2 expression in the developing mouse cortex to determine if these two populations were present \textit{in vivo}. Since a FEZF2 antibody suitable for immunohistochemistry is not available, we performed \textit{in situ} hybridization to detect Fezf2 expression at the postnatal day 0 (P0) in the mouse cortex (Figure 9A). As previously reported (Molyneaux et al., 2005), Fezf2 mRNA expression was observed in two different cortical neuron layers: a higher level of Fezf2 expression was detected in layer 5, while a lower Fezf2 expression level was detected in layer 6 (Figure 9A). To further investigate whether there were two distinct Fezf2\textsuperscript{Hi} and Fezf2\textsuperscript{Low} populations in the developing mouse cerebral cortex, we analyzed cell populations in the Fezf2-GFP BAC transgenic mouse containing a modified bacterial artificial chromosome in which an EGFP open reading frame was inserted at the start codon of the mouse Fezf2 gene.

We dissected cortices from P0 Fezf2-GFP BAC mice, dissociated the cells, and analyzed GFP-expressing cells by FACS (Figure 9B). Dissociated cells from the cerebellum of Fezf2-GFP BAC mice, which do not express Fezf2, were used as the negative control. We found that two distinct Fezf2-GFP-expressing populations existed in the P0 mouse cortex (Figure 9B). To directly examine the expression levels
of endogenous *Fezf2* mRNA in these populations, we sorted *Fezf2*-GFP\textsuperscript{Neg}, *Fezf2*-GFP\textsuperscript{Hi}, and *Fezf2*-GFP\textsuperscript{low} populations, and performed qRT-PCR (Figure 9C). *Fezf2* was not expressed in *Fezf2*-GFP\textsuperscript{Neg}, but was highly expressed in *Fezf2*-GFP\textsuperscript{Hi} population with a 33-fold increase (n=3) over the *Fezf2*-GFP\textsuperscript{low} population (Figure 9C). These results demonstrate that two *Fezf2*-expressing populations exist in the developing mouse cortex and can be successfully isolated by FACS, therefore suggesting that the *Fezf2*-YFP\textsuperscript{Hi} and *Fezf2*-YFP\textsuperscript{Low} cells generated from hESCs are biologically relevant, and likely resemble the two populations of *Fezf2*-expressing neurons present during mammalian cortical development.
Figure 9. *Fezf2*-GFP BAC transgenic mice contain two distinct *Fezf2*-GFP^+ populations.

(A) *In situ* hybridization shows that a P0 wild-type mouse cortex contains layer 5 neurons expressing *Fezf2* at a high level and layer 6 neurons expressing *Fezf2* at a low level. Scale bar 100 µm.
Figure 9 (B) *Fezf2*-GFP BAC transgenic mice contain two distinct *Fezf2*-GFP$^+$ populations. Representative FACS histograms and dot plots of P0 *Fezf2*-GFP BAC transgenic mice show that the cerebellum contains a m*Fezf2*-GFP$^{\text{Neg}}$ population, and the cerebral cortex contains m*Fezf2*-GFP$^{\text{Hi}}$ and m*Fezf2*-GFP$^{\text{Low}}$ populations.
Figure 9 (C) Quantitative RT-PCR analysis of mFezf2 mRNA expression
Quantitative RT-PCR analysis was performed in sorted mFezf2-GFP^Hi, mFezf2-GFP^Low and mFezf2-GFP^Neg sub-populations.
2.3.6 Differentiated $\text{Fezf2-YFP}^{\text{Hi}}$ and $\text{Fezf2-YFP}^{\text{Low}}$ cells express corticofugal neuron markers

Next, we sought to determine the molecular identities of the differentiated $\text{Fezf2-YFP}^{\text{Hi}}$ and $\text{Fezf2-YFP}^{\text{Low}}$ cell populations by comparing their gene expression. We first investigated whether these populations differed in their progenitor versus post-mitotic neuronal states by performing qRT-PCR analysis on the hFezf2-YFP-expressing populations at day 30. Indeed, $\text{Fezf2}$ is a gene expressed during early mouse development (E8.5) and continues to be expressed in post-mitotic neurons in subcortical projection neurons (Chen et al., 2005a). Expression levels of neural progenitor markers $\text{Pax6}$ and $\text{Nestin}$ were not significantly different between $\text{Fezf2-YFP}^{\text{Hi}}$ and $\text{Fezf2-YFP}^{\text{Low}}$ populations at day 30 (Figure 10A). Cell cycle analysis based on DNA content by FACS confirmed that $\text{Fezf2-YFP}^{\text{Hi}}$ and $\text{Fezf2-YFP}^{\text{Low}}$ cell populations shared similar cell cycle kinetics and that approximately 20% of the cells were proliferating in both populations (Figure 10B).

We next investigated whether differentiated hFezf2-YFP$^{\text{Hi}}$ and hFezf2-YFP$^{\text{Low}}$ populations preferentially expressed markers for cortical projection neurons of layer 5 and/or 6, since in situ hybridization revealed that $\text{Fezf2}$ was expressed in layers 5 and 6 of the mouse cortex, where subcerebral and corticothalamic neurons are respectively located (Figure 9A).
Figure 10 (A) Differentiated hFezf2-YFP cell subpopulations have similar progenitor marker identities. Quantitative RT-PCR analysis of progenitor markers, *Pax6* and *Nestin*, on differentiated and sorted cells at day 30
Figure 10 (B) Differentiated hFezf2-YFP cells show similar cell cycle kinetics. Flow cytometry analysis of cell cycle properties of differentiated and sorted hFezf2-YFP cells. The cells were stained with propidium iodide to reveal DNA content. Abbreviation: PI, propidium iodide.
Cortical neuron markers expressed at a high level in layer 5 subcerebral neurons, and low level in layer 6 corticothalamic neurons in mice include $N_{fib}$, $Bcl11b$, and $Sox5$ (Supplementary Fig 2), in addition to $Fezf2$ (Figure 9A). When $hFezf2$-$YFP^{Neg}$, $hFezf2$-$YFP^{Low}$ and $hFezf2$-$YFP^{Hi}$ populations were tested for these markers by qRT-PCR, $N_{fib}$, $Bcl11b$ and $Sox5$ showed the general trend of being more highly expressed in $hFezf2$-$YFP^{Hi}$ compared to $hFezf2$-$YFP^{Low}$ cells (Figure 11A, C, I). Conversely, genes expressed at high levels in layer 6 corticothalamic neurons in mice, such as $Nfia$ (Shu et al., 2003) showed elevated levels of expression in $Fezf2$-$YFP^{Low}$ cells as compared to $Fezf2$-$YFP^{Hi}$ cells (Figure 11G). These results suggested that $hFezf2^{Hi}$ cells were more similar to layer 5 subcerebral neurons whereas the $hFezf2^{Low}$ population was more similar to layer 6 corticothalamic neurons. However, expression of another layer 6 corticothalamic marker, $Tbr1$ (Supplementary Figure 3), showed a highly elevated expression in the $Fezf2$-$YFP^{Hi}$ population, with a 2 fold increase over $Fezf2$-$YFP^{Low}$ (Figure 11E). In addition, expression levels of $Darpp32$ (supplementary Figure 3) were comparable between the $Fezf2$-$YFP^{Low}$ and the $Fezf2$-$YFP^{Hi}$ cells (Figure 11J). These results suggested that $hFezf2$-$YFP^{Hi}$ population showed both layer 5 and 6 (corticospinal and corticothalamic neurons) molecular characteristics. The ventral marker $Dlx5$ (Panganiban and Rubenstein, 2002) was not expressed in either $Fezf2$-$YFP^{Hi}$ or $Fezf2$-$YFP^{Low}$ populations, confirming the enrichment of corticofugal neurons in $hFezf2^{+}$ cell populations (Figure 11K). Immunohistochemical analysis of differentiated $hFezf2$-$YFP$ cells further confirmed that $hFezf2$-$YFP^{+}$ cells expressed...
NFIB, BCL11B, TBR1, and NFIA proteins (Figure 11B, D, F, H). These results suggest that $Fezf2$-YFP$^{Hi}$ is most similar to layer 5-6 projection neurons and $Fezf2$-YFP$^{Low}$ cells more similar to layer 6. Taken together, these data support the idea that h$Fezf2$-YFP$^+$ cells represent deep-layer corticofugal projection neurons.
Figure 11. Differentiated hFezf2-YFP\textsuperscript{hi} and hFezf2-YFP\textsuperscript{low} cells express corticofugal neuron markers.

(A) Quantitative RT-PCR analysis for Nfib mRNA expression on sorted hFezf2-YFP subpopulations (*p<0.05; F=7.405; p=0.0240). (B) Immunofluorescence staining showing NFIB expression in differentiated hFezf2-YFP\textsuperscript{+} cells at day 35. (C) Quantitative RT-PCR analysis of Bcl11b mRNA expression on sorted hFezf2-YFP subpopulations. (D) Immunofluorescence staining showing BCL11B expression in differentiated hFezf2-YFP\textsuperscript{+} cells at day 40. (E) Quantitative RT-PCR analysis of Tbr1 mRNA expression on sorted hFezf2-YFP subpopulations. (**)p<0.01; F=15.63; p=0.0042). (F) Immunofluorescence staining showing TBR1 expression in differentiated hFezf2-YFP cells at day 40. (G) Quantitative RT-PCR expression of Nfia mRNA expression on sorted hFezf2-YFP subpopulations. (*p<0.05; F=7.316; p=0.0246). (H) Immunofluorescence staining showing NFIA expression in differentiated hFezf2-YFP cells at day 40. (I) Quantitative RT-PCR analysis of Sox5 mRNA on sorted hFezf2-YFP subpopulations. (**)p<0.01; F=11.87; p=0.0082). (J) Quantitative RT-PCR analysis of Darpp32 mRNA on sorted hFezf2-YFP subpopulations. (**)p<0.01; F=14.80; p=0.0048). (K) Quantitative RT-PCR analysis of Dlx5 mRNA on sorted hFezf2-YFP subpopulations. (**)p<0.001; F=58.90; p=0.001). Statistical analysis: ANOVA with Tukey’s post-hoc test.
2.4 DISCUSSION

Human embryonic stem cells allow the manipulation of cellular signaling pathways thus providing researchers with an in vitro tool to assess human cortical cell fate specification. While well studied in mouse models (Bedogni et al., Han et al., Hevner et al., 2001, Chen et al., 2005a, Molyneaux et al., 2005, Chen et al., 2008, Kwan et al., 2008, Lai et al., 2008, McKenna et al., 2011), modulation of signaling pathways that regulate projection neuron fate is challenging in human cells largely due to the lack of relevant models. Here we report the successful generation of corticofugal neurons, including corticospinal and corticothalamic neurons from human ESCs that are able to integrate upon transplantation in a manner suggesting corticofugal identity.

Our study identified two distinct Fezf2 subpopulations in differentiated hESCs that are reminiscent of Fezf2-expressing subpopulations in the mouse cortex. The two Fezf2-expressing cell populations isolated in this study demonstrate that Fezf2-expressing cells exist in phenotypically distinct states, with one subpopulation expressing layer 5, 6 markers, and the other expressing layer 6 markers. While the Fezf2\textsuperscript{Hi} population, predominantly expressed layer 5 markers, the corticothalamic marker (layer 6) Tbr1 was also highly expressed in this population. In addition, Darpp\textsuperscript{32}, a gene highly expressed in layer 6 neurons in mouse (Supplemental Fig 3), showed comparable expression levels between the human Fezf2-YFP\textsuperscript{Low} and Fezf2-YFP\textsuperscript{Hi} populations. These results indicate that the Fezf2-YFP\textsuperscript{Hi} population was not
strictly analogous to CSMN (layer 5), but instead exhibited molecular characteristics of deep layer projection neurons in general. It is also possible that human subcortical neurons are different from the mouse subcortical neurons, and that the different gene expression levels observed between the mouse subcerebral and corticothalamic neurons may not be identical in human subcortical projection neurons. A recently published study reports the expression of the transcription factor FoxA2 usually restricted to floor plate neurons in mouse, in ventral forebrain progenitors derived from hESCs. They confirmed this surprising finding in human cortical tissue (Maroof, 2013). It is clear from this and other examples that uniquely human gene expression can be derived from studies performed with hESCs derived neurons and that this trend is probably just beginning.

We also investigated the formation of two Fezf2-YFP+ populations after day 30 and observed whether a potential conversion of Fezf2-YFPLow to a Fezf2-YFPHi population was occurring. Our data showed that Fezf2 subpopulations were still clearly distinct after day 100 (data not shown), suggesting that Fezf2-YFPLow is not a precursor of Fezf2-YFPHi but instead a bona fide population. However, since human ES cell differentiation procedures cannot fully recapitulate in vivo brain development conditions, it is possible that even though we were able to generate neurons with subcortical neuron features, the chromatin state and gene expression levels in the differentiated neurons were not identical to endogenous human subcortical neurons. Our findings highlight the use of the hESC system not only to dissect signaling pathways affecting neuronal differentiation, but also to identify cell heterogeneity
within a population of differentiated neurons. Further delineation of the characteristics of these two populations by transplant experiments, which have thus far been difficult to execute due to low live cell recovery rate after FACS, would provide more information on their heterogeneity and distinct functions.

Using hFezf2-YFP as a marker for subcerebral cortical neurons, we investigated the effects of different signaling pathways on generating corticofugal neurons. Previous studies have established a role for TGFβ/BMP inhibition using the small molecule SB431542 and the BMP inhibitor Noggin in the differentiation of hESCs towards a neuroectodermal fate (Itsykson et al., 2005, Chambers et al., 2009). Motor neurons have been differentiated from induced pluripotent stem cells (iPS) or hESCs using Shh agonists and retinoic acid (Li et al., 2005, Singh Roy et al., 2005, Dimos et al., 2008, Li et al., 2008). Recently, the retinoid-signaling pathway induced by vitamin A treatment was shown to efficiently generate cortical neurons from hESCs and iPS cells (Shi et al., 2012). Our study shows that the inhibition of both Wnt and Shh pathways enriches for hFezf2-YFP-expressing neurons. Landmark studies in zebrafish and mouse model systems have established that Wnt antagonism is required for proper telencephalic patterning early in development (Glinka et al., 1998, Mukhopadhyay et al., 2001, Houart et al., 2002, Niehrs, 2004). Fezf2 is expressed from E8.5 in early mouse development and functions in the rostro-caudal (anterio-posterior or A/P) patterning of the forebrain (Shimizu and Hibi, 2009). The expression of Fezf2 early in development and its function in A/P axis places this gene at the crossroads of important patterning events involving the Wnt pathway, and
could explain the complete repression of hFezf2-YFP by Wnt in vitro. Incidentally, Fezf2 was first identified in a DKK1 overexpression screen in zebrafish, and showed low or no expression in embryos overexpressing Wnt8b (Hashimoto et al., 2000). This study further demonstrated that Fezf2 overexpression in zebrafish inhibited Wnt1 expression. A separate study showed that Fezf2 was able to rescue a headless (hdl/tcf3) mutant embryo, encoding a Wnt transcriptional repressor (Jeong et al., 2007). Overall, these studies suggest that Wnt and Fezf2 act in a common pathway and that Wnt signaling might act upstream of Fezf2 (Shimizu and Hibi, 2009).

Interestingly, in Xenopus, the co-expression of BMP and Wnt inhibitors cooperate to induce ectopic head structures (Glinka et al., 1998), suggesting a “two-inhibitor model” of anterior-posterior (A/P) neural induction, in which BMP inhibition alone induces posterior neural patterning while BMP-Wnt inhibition anteriorizes neural structures (Glinka et al., 1997, Niehrs, 1999). This model could extend to our finding that triple inhibition of TGFβ/BMP/Wnt-Shh signaling induces hFezf2 expression in hESCs.
2.5 CONCLUSION

This study demonstrates that hESCs are a useful model system to dissect signaling pathways functioning during neuronal differentiation towards corticofugal projection neurons. We demonstrate that triple inhibition of TGFβ/BMP/Wnt-Shh enhances the differentiation of hFezf2-expressing cells greater than 2-fold over the no growth factor control condition. Furthermore, we uncover the presence of two novel and distinct hFezf2-YFP-expressing subpopulations suggesting that Fezf2-expressing cells are heterogenous in vivo. Knowledge derived from such studies can be used for in vitro differentiation of patient-derived induced pluripotent stem cells (iPSCs) into corticospinal motor neurons, to elucidate disease mechanisms underlying upper motor neuron diseases, as well as provide a platform to discover potential targets for drug development.
2.6 MATERIAL AND METHODS

All the human embryonic stem cell experiments and animal studies were performed in accordance with protocols approved by the IRB committees and IACUC at the University of California, Santa Cruz and performed in accordance with institutional and federal guidelines.

2.6.1 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Quantitative Polymerase Chain Reaction (qRT-PCR)

RNA was extracted using RNeasy (Qiagen) kit. RNA quality was evaluated using Agilent 2100 Bio Analyzer (Agilent technologies). The RNA samples were reverse-transcribed into cDNA using SuperScript III first strand synthesis system (Life Technologies). Quantitative PCR (qPCR) was performed using the ViiA 7 real-time PCR (Applied Biosystems). Expression values (CT values) were normalized with two ubiquitously expressed endogenous reference genes, Beta-2-microglobulin (B2M) and Beta-actin. The normalized expression levels of the chemically tested samples were compared to those of an untreated sample according to the ΔΔCT method (Schmittgen and Livak, 2008). Each sample was run in triplicate and at least three biological replicates were performed for each gene. The primers for RT-PCR and qRT-PCR are listed in Supplementary table 4 and Supplementary table 5.
2.6.2 Stem Cell Culture

H9 (WA-09, passages 28 to 50) (Wicell Research Institute) cells were maintained on a feeder layer of mitotically inactivated mouse embryonic fibroblasts (MEFs) as described (Chiao et al., 2008). MEFs were plated onto coated growth factor reduced Matrigel plates (BD Biosciences) diluted 1:20 into DMEM-F12 medium (Life Technologies). H9 were cultured in Dulbecco’s modified Eagle’s medium-F12 (DMEM-F12) supplemented with 20% Knockout serum replacement, 0.1mM MEM nonessential amino acids (MEM NEAA), 2mM GlutaMAX, 0.55 mM, 2-mercaptopethanol (all from Life Technologies) and 8ng/ml of human recombinant basic fibroblast growth factor (Fgf2) (Peprotech). Cells were fed daily and passaged every 4 days with 200 units/ml of collagenase IV (Life Technologies).

The hFezf2-YFP HUES-9 cells were generously provided by Drs. Binhai Zheng from the University of California at San Diego, and used between 46-52 passages (Ruby and Zheng, 2009). Both the hFezf2-YFP HUES-9 cells and the HUES-5 cells (passages 19 to 30) (Harvard University) were cultured in knockout Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) supplemented with 10% Knockout Serum Replacement, 10% Plasmanate (Bayer HealthCare), 0.1 mM MEM nonessential amino acids, 2 mM GlutaMAX, 0.55 mM 2-mercaptopethanol (all from Life Technologies) and 15 ng/ml human basic Fgf added fresh to the medium daily. Cells were passaged every 4 days by trypsinization with 0.5%
trypsin/EDTA (Life Technologies) or digestion with collagenase IV at 200 units/ml (Life Technologies) for embryonic body (EB) formation.

2.6.3 Neural Differentiation from hESCs

Embryoid bodies (EBs) were generated by gentle dissociation of undifferentiated hESCs from mouse feeder layer using 200ug/ml of collagenase IV (Life Technologies). Dissociated cells were washed three times with hESC media and were allowed to settle by gravity to form EBs in N2 medium in an ultra low attachment plate overnight (Day 0). The next day (Day 1) different growth factors or their inhibitors: SB431542 (10um) (Sigma-Aldrich), recombinant Human Noggin (500ng/ml), recombinant human Wnt3a (200ng/ml) (both from R&D Systems), recombinant human DKK1 (250ng/ml) (Peprotech), Cyclopamine (4uM) (Biomol) were added to the N2 media. Feeding with growth factors was performed daily with a no growth factor condition used as a control. N2 media was composed of Dulbecco’s modified Eagle’s medium-F12 (DMEM-F12) medium supplemented with 0.1mM MEM nonessential amino acids (MEM NEAA), 2mM GlutaMAX, 1X N2 supplement (all from Life Technologies), Heparin (1mg/ml)(Sigma-Aldrich) and human basic Fgf (10ng/ml)(Peprotech). EBs remained for 6 days in suspension and plated down on day 7 onto a growth factor reduced Matrigel coated plate (BD Biosciences). Feedings continued every day or every other day for 12 days. On day 12 cells were scraped from their support, resuspended in N2 media and plated down onto a new growth
factor reduced Matrigel (BD Biosciences) coated plate. The next day, N2 media was replaced with freshly made differentiation media. The differentiation media was composed of Neurobasal medium supplemented with 2mM GlutaMAX, 1X N2 supplement, 1X B-27 supplement minus vitamin A (all from Life Technologies), cAMP (1uM), L-ascorbic acid (100uM) (both from Sigma-Aldrich), and Human Recombinant Brain-Derived Neurotrophic Factor (BDNF) (10ng/ml), Human Recombinant Glial-Derived Neurotrophic Factor (GDNF) (10ng/ml) and Insulin-like Growth Factor-I (IGF-I) (10ng/ml) (all from Peprotech). From day 13-32, cells were fed daily or every other day with differentiation medium to induce differentiation. Differentiated cells were passaged on day 21 onto a growth factor reduced Matrigel (BD Biosciences) coated plates at 1:20 dilution and fed as described above.

2.6.4 Flow Cytometry

Cells were gently dissociated using 0.5% Trypsin/EDTA (Invitrogen) followed by a neutralization step with hESC culture media containing serum as described above. Cells were then resuspended into 1XPBS, 0.5M EDTA and 2% FBS (all from Life Technologies). Prior to sorting, propidium iodide (1ug/ml, Molecular Probes, Invitrogen) was added to each sample at 1:5,000 dilution to distinguish between live and dead cells. Cells were sorted on a fluorescence-activated cell sorter FACS ARIA II (BD Biosciences) with a 100 µm nozzle at 22 PSA referred to as “gentle FACS” (Pruszak et al., 2007). Sorted cells were collected in 500ul of HBSS
media supplemented with 20mM glucose, 10% FBS, 1X penicillin/streptomycin (Invitrogen). Gating was performed using unstained, or negative controls that were not expected to express Fezf2. Data Analysis was performed using the FlowJo software (Tree Star, Inc). All experiments, including analysis and sorting were repeated at least three times.

2.6.5 Immunohistochemistry

Differentiated Fezf2-YFP cells and H9 cells were fixed with 4% paraformaldehyde in PBS for 15 minutes, washed twice in PBS, permeabilized with 0.1% Triton X-100 in PBS for 20 minutes, and then blocked in 5% horse serum for 1 hour. Cells were incubated overnight at 4°C with primary antibodies. After three PBS washes, cells were incubated in the appropriate secondary antibodies for 1 hour at room temperature protected from light. Cells were washed three times in 1XPBS and stained with DAPI. The primary antibodies used in this study for mouse and human cells (Supplementary table 6) are the following: chicken anti-GFP (Life Technologies), rat anti-BCL11B (Abcam), rabbit anti-TBR1 (Abcam), rabbit anti-NFIA (Active Motif), rabbit anti-NFIB (Active Motif), mouse anti-HuNu (Millipore), mouse Tuj1 antibody (Covance), rabbit anti-TBR2 (Abcam), rabbit anti-PAX6 (Covance), mouse anti-Nestin (Millipore). Secondary antibodies were as follows: Alexafluor 488, 594 and 647 (Life Technologies).
2.6.6 **Analysis of Fezf2-GFP mouse cells**

Fezf2-GFP BAC transgenic mice brains (GENSAT) were dissected at P0 (n=3) and immediately dissociated using Papain dissociation system (Worthington Biochemical Corporation). GFP positive and negative populations were sorted using a FACS ARIA II (BD Biosciences). 50,000-75,000 cells were sorted and processed for RNA isolation using RNeasy Plus mini kit (Qiagen) and the quality assessed using a Bioanalyzer 2100 (Agilent Technologies). cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen).

2.6.7 **Fezf2 In situ Hybridization**

Fezf2 anti-sense probe was generated by PCR amplifying cDNA templates and *in vitro* transcription. Tissue sections were hybridized overnight with digoxigenin-labeled RNA probes at 60°C, washed in 2× SSC at 65°C, incubated with RNase, washed in 0.2× SSC at 65°C, blocked in PBT with 10% horse sera, and incubated in alkaline phosphatase labeled anti-DIG antibody (Roche) (1:2000, 10% sera) overnight. Sections were developed with NBT (nitroblue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate).
2.6.8 Transplants and Animals

Rosettes from day 12-20 were mechanically isolated from *in vitro* differentiated *Fezf2*-YFP cells, dissociated using 0.5% Trypsin/EDTA (Invitrogen) and resuspended in serum containing media. Dissociated cells were transplanted (500-2,000 cells) using ultrasound guided imaging (VisualSonics) targeting the deep layers of the mouse motor cortex. Transplants were performed using postnatal day 0 (P0) CD-1 wild-type mice (n=40) (Charles River Laboratories, Hollister, CA). Mice were sacrificed at P90, intracardiac perfusion were performed with fresh 4% PFA and embedded in O.C.T compound (Sakura Finetek) and kept at -80°C until sectioning was performed using Microm (ThermoScientific) floating sections of 50μm.
Reverse Transcriptase PCR (RT-PCR) shows expression of cortical cell markers in differentiated HUES 5 cells.

Pluripotent markers *Pou5F1* and *Nanog* are expressed in undifferentiated cells, their expression persists throughout differentiation albeit at lower levels. Radial glia marker, *Pax6* is strongly expressed at day 6 and can still be detected at day 60. The neural progenitor marker *Emx2* is detected starting at day 6 and its expression increases throughout differentiation until day 60. The corticospinal motor neuron *Fezf2* is first detected at day 6 and is strongly expressed at day 60. Corticofugal neuron markers *Bcl11b, Nfib* and *Tbr1* are all robustly expressed at day 60. None of the markers show expression in mouse embryonic fibroblasts (MEFs) control.
Immunohistochemistry of wild-type mouse cortex showing layer 5 markers expression at P0

(A) Subcortical neuron marker BCL11B (green) is expressed strongly in layer 5 and weakly in layer 6 of the mouse cortex. DAPI indicates nuclear staining (blue) in all sections. (B) Corticofugal neuron marker SOX5 (green) is expressed in layer 5-6 of the mouse cortex. (C) Subcortical neuron marker NFIB (green) is expressed in layer 5-6 of the mouse cortex. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; Scale bars: 150 µm.
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SUPPLEMENTAL FIGURE 3

Immunohistochemistry of wild-type mouse cortex showing expression of layer 6 markers at P0

(A) NFIA (green) is expressed strongly in layer 6 of the mouse cortex. DAPI indicates nuclear staining (blue) in all sections. (B) DARPP32 (green) is strongly expressed in layer 6 of the mouse cortex. (C) Corticothalamic marker TBR1 (green) is expressed strongly in layer 6. Abbreviations: DAPI, 4’,6-diamidino-2-phenylindole; Scale bars: 150 μm.
### SUPPLEMENTAL TABLE 1

Human and mouse RT-PCR primers

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SUPPLEMENTAL TABLE 2

A. Human qPCR primers

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<td>TCGTGTCATAATTATCCCGAAATCC</td>
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<td>GTTGGATCCGGAAGCTCC</td>
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<td>hNestin</td>
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<td>hDlx5</td>
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<tr>
<td>hBcl11b</td>
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<td>GACTCAGGGTGAGGTGCAG</td>
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B. Mouse qPCR primers

<table>
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<th>GENES</th>
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<th>PRIMERS REVERSE</th>
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<tr>
<td>mFezf2</td>
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<td>TTGCACACAAACGGTCTAGC</td>
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<tr>
<td>mActinb</td>
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**SUPPLEMENTAL TABLE 3**

Primary antibodies used in this study

<table>
<thead>
<tr>
<th>MARKERS/GENES</th>
<th>SPECIES</th>
<th>MANUFACTURER</th>
<th>DESCRIPTION</th>
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<tbody>
<tr>
<td>NFIA</td>
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<td>Active Motif</td>
<td>Layer 6</td>
</tr>
<tr>
<td>Nestin</td>
<td>Mouse</td>
<td>Millipore</td>
<td>Neural progenitor</td>
</tr>
<tr>
<td>Tuj1</td>
<td>Mouse</td>
<td>Covance</td>
<td>Immature neurons</td>
</tr>
<tr>
<td>HuNu</td>
<td>Mouse</td>
<td>Millipore</td>
<td>Identifying cells of human origin</td>
</tr>
<tr>
<td>GFP</td>
<td>Chicken</td>
<td>Life Technologies</td>
<td>Identifying transplanted hFezf2 cells</td>
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<tr>
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<td>Rabbit</td>
<td>Active Motif</td>
<td>Layer 5-6</td>
</tr>
<tr>
<td>TBR2</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>Neural progenitor</td>
</tr>
<tr>
<td>PAX6</td>
<td>Rabbit</td>
<td>Covance</td>
<td>Neural progenitor</td>
</tr>
<tr>
<td>CTIP2/BCL11B</td>
<td>Rat</td>
<td>Abcam</td>
<td>Layer 5-6 cortical neurons</td>
</tr>
<tr>
<td>TBR1</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>Layer 5-6 cortical neurons</td>
</tr>
</tbody>
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I would like to thank Drs. David Feldheim, Camilla Forsberg and Amy Ralston for helpful scientific discussions and serving on my thesis committee. Further thanks to the Chen lab members as well as Drs. Fernando Ugarte, Anna Beaudin for helpful comments on the manuscript. Thank you to Mrs. Bari Holm Nazario at the Institute for the Biology of Stem Cells (IBSC) Flow Cytometry Facility at UCSC for technical expertise. This work was supported by a SEED grant from the California Institute of Regenerative Medicine (CIRM) RS1-00170 to B.C. and M.K. was a CIRM training grant TG2-01157 scholar.
CHAPTER 3

CONCLUSION AND FUTURE DIRECTIONS
In vitro culture of neurons from fetal or postmortem cortical tissues has been the sole way of procurement of neural cells for decades. Whether neural progenitors derived from fetal tissue are able to generate all cortical subtypes is however unclear. The recent upsurge of human embryonic stem cells (hESCs) as a source of pluripotent cells able to generate a wide array of neural cells provides new hopes for the therapeutic use of these cells and a new resource to study human corticogenesis in a dish. The subsequent advent of induced pluripotent stem cells (iPSCs) propelled the field of stem cell research to a new high. Indeed, the discovery that transcription factors could erase the epigenetic signature of a somatic cell and return it to a naïve pluripotent state (Nakagawa et al., 2008) has been a breakthrough that has evidently raised doubts about the use of hESCs in research. Are hESCs obsolete and iPSCs becoming the ideal research tool? I contend in this dissertation and my research, that hESCs are a valuable cellular system in which to study basic human neurobiology and develop differentiation protocols to generate a diverse pool of human neuronal subtypes. It likely that iPSCs will be preferred for transplantation studies in vivo because the transplanted cells won’t be rejected by the immune system, but iPSCs are still under scrutiny for safety concerns which will need to be addressed before human iPSCs can be used as therapeutics.

My research was aimed at deciphering the signaling pathways and growth factors that promotes the generation of a homogeneous population of cortical cells. Using Fezf2 as a marker for cortical cell fate, I showed that Fezf2+ cells are not a homogeneous cell population but instead comprised of 2 distinct subpopulations in
vitro. While it is the first report demonstrating that Fezf2-expressing cells are a heterogeneous population, studies done in mice have previously reported a differential RNA pattern of Fezf2 expression by in situ hybridization (Figure 9A). In vitro, the Fezf2\textsuperscript{HI} and Fezf2\textsuperscript{Low} subpopulations are varying in the way they respond to growth factors, their transcription factor expression pattern as well as their flow cytometry pattern. As a whole it is clear that these Fezf2+ subpopulations are distinct.

What are these two populations? A first hypothesis is that Fezf2 subpopulations represent different cell subtypes. Indeed, Fezf2 is expressed in a highly dynamic pattern in the rodent cortex during early development. Fezf2 is first detected at E8.5 in the dorsal telencephalic wall of the mouse cortex and in the ventricular zone between E12.5 and E14.5 at a time when deep layer neurons are generated (Hirata et al., 2004). In situ hybridization at E18.5 shows that Fezf2 is highly expressed in layer 5, weakly in layer 6 and expression subsides in the ventricular zone where progenitors are generated. Fezf2 is also expressed outside the cortex in CA1 of the hippocampus (Chen et al., 2005b), as well the vomeronasal organ (VNO), which is part of the olfactory system in mice (Hirata et al., 2004, Eckler et al., 2011), as well as the thalamic eminence at E12.5 (Hirata et al., 2004). In light of Fezf2 dynamic expression pattern both regionally and temporally; it is clear that Fezf2 expression is a heterogeneous population in vivo. However, while determining whether Fezf2 was present in the mouse cortex of Fezf2-GFP mice, we also isolated 2 distinct populations by FACS, suggesting that these Fezf2
subpopulations are probably cortical in origin and not the results of Fezf2 expression across brain structures.

To further determine the identity of the differentiated Fezf2+ subpopulations, we hypothesized that the Fezf2 subpopulations were temporarily distinct and represented progenitor versus post-mitotic in origin. Quantitative PCR and cell cycle analysis revealed instead that these subpopulations were similar in their progenitor markers Pax6 and Nestin expression as well as their cell cycle kinetics (Figure 10A, B). We then considered the possibility that these distinct Fezf2 subpopulations could represent the differential expression of Fezf2 in layer 5 versus layer 6 cortical layers which are clearly visualized from mouse in situ hybridization (Figure 9A). According to our data, we concluded that Fezf2Hi cells were more similar to layer 5-6 whereas Fezf2Low cells were more similar to layer 6. To refine the identity of these Fezf2 subpopulations, it would be beneficial to perform gene profiling on the two-sorted populations to determine any differential gene expression between the subpopulations. Also to demarcate layer 5 from layer 6 markers, it would be interesting to transplant the sorted Fezf2+ populations and assess if the axonal projection pattern differs between the subpopulations. Indeed, layer 6 neurons project to the thalamus, while layer 5 neurons extend their axonal projections to the spinal cord. It would also be interesting to test whether the Fezf2 subpopulations identified in our study express upper layer neurons as was suggested by a study conducted by another laboratory (Hirata et al., 2004).

The default anterior neural fate, would suggest that differentiation protocols
to the cortical fate are trivial. However, many caudalizing agents, which induce midbrain-hindbrain specification rather than a forebrain-anterior specification, are included in media formulations. Caudalizing signals are also endogenously present in the feeder support during culturing and can derail differentiation attempts to heterogeneous cell populations instead of homogeneous ones. A major impetus to differentiate pluripotent cells to specific neural subtypes that are relevant to neurological diseases is a prominent goal in the stem cell field. The isolation of the 2 distinct Fezf2 subpopulations may provide insights about the molecular characteristics and growth conditions that induce specific subpopulations. In the case of Fezf2, insight could be gained in understanding the dual role of Fezf2 in the specification of progenitors versus postmitotic neurons during early development (for example). Another important question derived from such studies is whether a cell has an innate differentiation program that drives it to a specific fate or, instead whether extrinsic factors are needed to guide a cell to a specific fate. Intriguingly in our study, Fezf2Low subpopulation was not affected by growth factor treatments, whereas the Fezf2Hi subpopulation was enriched by TGFβ/BMP/Wnt-shh inhibition. This property of Fezf2Low cells suggests an intrinsically distinct differentiation pathway adopted by these cells from the Fezf2Hi cells. Also, it would be important to find out whether generating neurons with regional specificity before transplanting them is necessary or instead the regional specificity is acquired in vivo by the location of the graft in the brain. Conflicting reports either claim that in vitro, cells have an inherent plasticity and acquire the areal identity of the transplant location (Ideguchi et al., 2010) while
others claim that cells have an inherent areal identity “imprinted” within them and will project to the target typical of their cell identity no matter the location where they were grafted (Gaspard et al., 2008). While both of the above studies were performed in mouse ES cells, it would be interesting to address this issue in human cells. Also, it would be interesting to learn whether the commitment of in vitro cells to a specific fate is acquired with time, as it is the case in vivo. Whether human cells are intrinsically plastic prior to transplantation is still unknown. And if human cells are plastic, knowing when the loss of cellular plasticity occurs would be helpful to direct differentiation efforts as well as transplants studies. Finally, the molecular mechanisms involved in this process will need to be understood for human replacement therapies to take place safely. Major questions need to be addressed before transplantation studies are underway.

Differentiating pluripotent cells to layer specific cortical neurons is a challenge that needs to be addressed in order to recapitulate in vitro human corticogenesis. Human neural differentiation protocols have been able to readily generate deep layer neurons, while the generation of upper layer neurons has only been reported recently. Indeed, recent reports exposed a possible bias toward the generation of deep layer neurons from in vitro differentiation cultures. The reasons of this bias and whether this bias is due to in vitro conditions are unknown. In vivo, radial glia cells (RGCs) divide asymmetrically to give rise to an RGC and an intermediate progenitor cell (IP) (Noctor et al., 2001). The mechanisms that give rise to a specific subtype of cortical neuron produced by RGCs during neurogenesis are
not well understood. *In vitro*, arresting RGCs in a time specific manner would allow RGCs to produce a specific neuronal subtype thereby creating a homogeneous subpopulation of committed daughter cells. A similar approach has been undertaken using the notch inhibitor DAPT that promotes the differentiation of progenitors. In that case, depending on the timing of DAPT treatment, the authors of the study obtained a pure population of layer 1 (*Reelin*), a mixed population of layer 1 and 6 neurons or mixed population of layers 1, 5, 6 (Eiraku et al., 2008). The idea behind such experiments is to generate a population of neurons that is pure and synchronized thereby decreasing the incidence of immature cells within populations of differentiated neurons that could induce tumors. An area of intense investigation in stem cell differentiations to indeed maximize cell homogeneity *in vitro* is to synchronize or “arrest” cells in a timely fashion thereby producing the same daughter cells.

The stem cell differentiation field is living an exciting time. The advent of iPSCs as well as an overall maturation of the knowledge to drive hESCs to distinct neuronal subtypes is allowing the field to foresee the direct use of pluripotent cells for regeneration within a not so distant future.


Franco SJ, Muller U Shaping our minds: stem and progenitor cell diversity in the Mammalian neocortex. Neuron 77:19-34.


