CHARACTERIZATION OF ES CELL-DERIVED CORTICAL RADIAL PRECURSOR DIFFERENTIATION

by

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University of Toronto

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Abstract

Murine neural precursor cells have been a well studied model for neural cell fate determination and stem cell function both in vivo and in primary culture. However, factors such as cell number, the presence of multiple cell populations and of niche intrinsic factors made it difficult to dissect the mechanisms regulating cortical development. To overcome this issue, we have developed a culture system where mouse embryonic stem cells (ES) are differentiated to cortical radial precursors through retinoic acid treatment of embryoid bodies. One day after plating in neural differentiation conditions, ~70% of cells in the culture are cortical radial precursors (RPs) as indicated by the definitive cortical marker Emx1, and over 8 days in culture, these RPs differentiate to pyramidal glutamatergic neurons of the cortex mimicking in vivo development. Astrocyte differentiation can be observed later as the culture progresses, which again mimics the typical timed genesis of cells in the cortex. The stem cell properties and cell fate of these RPs can be manipulated with growth factors in culture as they are in vivo. In particular, FGF2 promotes proliferation and survival, while ciliary neurotrophic factor (CNTF) induces precocious astrocyte formation. Thus, our ES-derived cortical RP cultures can serve as an alternate and complementary in vitro model to examine neural precursor biology during early development.
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# Table of Contents

Acknowledgments ........................................................................................................ iii  
Table of Contents ......................................................................................................... iv  
List of Tables ............................................................................................................... vi  
List of Figures .............................................................................................................. vii  
Chapter 1 .................................................................................................................... 1  
  1 Introduction ............................................................................................................ 1  
    1.1 Cerebral cortex development ........................................................................... 1  
      1.1.1 Embryonic development ......................................................................... 1  
      1.1.2 Cortical stem and precursor cells ......................................................... 3  
    1.2 Molecular mechanisms regulating precursor behavior during cortical development ....... 9  
      1.2.1 Intrinsic factors regulating cortical precursors ........................................ 9  
      1.2.2 Extracellular signals regulating cortical precursors ............................... 13  
    1.3 Sources of cortical precursors ........................................................................ 19  
    1.4 Neurogenesis from embryonic stem cells .................................................... 20  
      1.4.1 Embryonic stem cells overview ............................................................ 20  
      1.4.2 ES cells as models of development and disease: advantages and disadvantages .. 21  
      1.4.3 Neural induction in ES cells ................................................................ 22  
      1.4.4 Types of neural lineages produced ....................................................... 22  
      1.4.5 Methods of generating neural precursors and neurons from ES cells ........ 24  
      1.4.6 Forebrain and cortical precursors ......................................................... 27  
Statement of Thesis Objective ..................................................................................... 35  
Chapter 2 .................................................................................................................... 36  
  2 Experimental Procedures ...................................................................................... 36  
    2.1 Cell culture and growth factor treatments .................................................... 36
List of Tables

Table 1.1: Neural differentiation from embryonic stem cells
List of Figures

Figure 1.1: Cortical development and cortical cell types.

Figure 1.2: Cortical stem and precursor cells

Figure 2.1: Embryonic stem cell differentiation to cortical radial precursors and neurons – culture progression.

Figure 3.1: Retinoic acid induces ES cells to adopt a neural radial precursor morphology

Figure 3.2: Dissociation of RA-treated EBs gives rise to cultures containing Pax-6 positive neural precursors at 1DIV that differentiate to βIII-tubulin positive neurons by 8DIV

Figure 3.3: Dissociation of RA-treated EBs cultured for 7 days instead of 8 days yields more precursors and less neurons at 1DIV

Figure 3.4: Dissociation of RA-treated EBs cultured for 7 days gives rise to cultures containing Pax-6 positive neural precursors at 1DIV that differentiate to βIII-tubulin positive neurons by 8DIV

Figure 3.5: Neural precursors derived from RA-treated EBs express cortical and forebrain radial precursor markers.

Figure 3.6: ES-derived precursors generate glutamatergic and GABAergic neurons

Figure 3.7: ES-derived precursors generate astrocytes

Figure 3.8: ES-derived precursors generate oligodendrocytes

Figure 3.9: ES-derived precursors generate Nkx2.1 expressing precursors

Figure 3.10: FGF2 affects the ES-derived culture by globally increasing cell numbers
**Figure 3.11**: FGF2 increases the proportion of Pax-6 positive precursors and decreases the proportion of neurons at 3DIV

**Figure 3.12**: FGF2 affects proliferation of Pax-6 positive precursors

**Figure 3.13**: FGF2 increases survival of ES-derived precursors at 1DIV

**Figure 3.14**: FGF2 increases survival of ES-derived neurons at 3DIV

**Figure 3.15**: CNTF induces precocious astrocyte formation

**Figure 3.16**: CNTF increases the number of astrocytes in ES-derived cortical radial precursors

**Figure 3.17**: CNTF increases the number of cycling cells and cycling astrocytes.

**Figure 3.18**: CNTF increases precocious astrocyte formation in the presence of FGF2 at 4DIV

**Figure 4.1**: ES-derived culture composition at 1 and 8DIV

**Figure 4.2**: ES-derived cortical radial precursor culture compared to in vivo and primary culture
Chapter 1

1 Introduction

1.1 Cerebral cortex development

1.1.1 Embryonic development

The cortex: the center of memory, learning and higher cognition in humans. The uniqueness of the human brain does not lie with the presence or absence of structures but the degree to which these structures are developed (Rakic, 2009). The high degree of evolutionary conservation in the general organization and patterning of the cortex has provided experimental access to model organisms for understanding of its development from which we have gained great insight not only into our own development but also into the root of developmental and neurodegenerative diseases.

The murine cortex has been a well studied model for understanding the molecular mechanisms that govern the generation, function and fate decisions of neural stem cells. Neural stem cells in the cortex are known to persist throughout the lifespan of the organism. Although the adult cytoarchitecture of the cortex is highly complex, consisting of heterogenous populations of neurons, astrocytes and oligodendrocytes, it arises from a small pool of stem cells and precursor cells during development. The three main neural cell types are specified in a defined temporal order where neurons are generated first from around embryonic day 10 (E10) to E18, astrocyte differentiation follows from E16 and lasts into the postnatal period while oligodendrocytes are generated postnatally (Figure 1.1).

Owing to the relatively late development of the cortex and its superficial location, neural stem cells and precursors are easily accessible for dissection and manipulation. Importantly, the ability to isolate early cortical stem and precursor cells revealed that they follow the same timed genesis of neural phenotypes in vitro indicating the presence of an intrinsic differentiation program (Qian et al., 2000). Transgenic, gene targeting and RNAi technologies have allowed us to ask and answer questions about development and disease in the mouse cortex that apply to other CNS regions as well as outside the CNS. More recently, advances in the embryonic stem cell field have added another tool for modeling corticogenesis allowing the derivation of cortical
neural stem cells, precursors as well as neurons, astrocytes and oligodendrocytes in an environment that can be controlled from start to finish (Bibel et al., 2004, 2007; Gaspard et al., 2008; Eiraku et al., 2008). Therefore, the purpose of the following sections is to introduce the reader to the current knowledge of the key mechanisms of corticogenesis in the murine model leading to recently developed protocols for embryonic stem cell derivation of cortical stem and precursor cells as alternate and complementary tools.

The neural lineages and the skin are derived from the ectoderm of the early embryo. At gastrulation, cells in the anterior region of the epiblast form the ectoderm. Classic experiments in Xenopus demonstrated that the neural fate, where neural induction converts ectoderm to neuroectoderm, is the “default” state of ectoderm (reviewed in Levine & Brivanlou, 2007). The absence or inhibition of signals (BMP, Wnt and activin/Nodal) that induce the generation of the other two germ layers generates neuroectoderm (Murry & Keller, 2008).

First to be specified is the prospective forebrain which further subdivides into the telencephalon and diencephalon (Stern, 2001). Morphogen gradients such as sonic hedgehog (Shh), BMPs and Wnts then signal to posteriorize the neural tube establishing the anterior-posterior axis and the rest of the CNS structures. Locally, dorsal-ventral Shh, BMP, FGF8 signals as well as differential expression of specific transcription factors specify telencephalic regionalization into dorsal and ventral structures (Campbell, 2003). These morphogens induce the expression of transcription factors to specify precursors to a dorsal or ventral identity. Dorsal precursors express the transcription factors Pax-6 and Emx1 while ventral precursors express Dlx1,2,5, Gsh2, Mash1, Lhx6 and Nkx2.1.

The dorsal part of the telencephalon forms the cortex (pallium), while the ventral telencephalon gives rise to the basal ganglia. The dorsal telencephalon generates neurons and astrocytes as well as myelinating oligodendrocytes that serve to insulate axons and promote efficient action potential conductance. Cortical neurons can be broadly divided into two classes: projection neurons and interneurons. The dorsal telencephalon generates exclusively projection neurons that utilize the neurotransmitter glutamate (Gorski et al., 2002). Cortical interneurons primarily utilize GABA as a neurotransmitter and are generated from precursors located in the ventral telencephalon that migrate to populate the cortical layers (Campbell, 2003).
At E8-9 in the mouse, the telencephalon is a single sheet of rapidly dividing neuroepithelial cells. This neuroepithelial layer constitutes the germinal zone where mitotic multipotent stem cells reside and from which the entire cortex will be eventually derived. Initially, neuroepithelial cells undergo proliferative symmetric divisions to increase the pool of precursors. At the onset of neurogenesis, a switch to asymmetric divisions allows the generation of neurons as well as other, more restricted progenitors. These two modes of division lead to the generation of two germinal zones, the ventricular and sub-ventricular zones (VZ and SVZ).

The adult cortex is comprised of six neuronal layers that are generated sequentially from the precursors in the VZ or SVZ. As cells exit the cell cycle and initiate neuronal differentiation programs, they migrate radially away from the germinal zones to their appropriate cortical layers in an inside-out fashion. The earliest-born neurons populate the deep layers and the later-born neurons migrate past them to form the superficial layers (Rakic, 1974). The first neurons to be generated migrate out of the VZ and form the pre-plate. The pre-plate is split into the marginal zone and the sub-plate by subsequent waves of neurons which migrate in to form the cortical plate (layers II-VI). The marginal zone becomes the most superficial layer, layer I. From each of these layers, projection neurons form connections with neurons in other cortical areas, subcortical areas that include the spinal cord or the opposite hemisphere of the cortex.

1.1.2 Cortical stem and precursor cells

All neural cells of the mature CNS are descended directly or indirectly from the ectoderm-derived neuroepithelial cell population. How this small pool of precursors generates the remarkable diversity of the adult cortex remains a matter of debate. A number of mechanisms, acting in concert are hypothesized to achieve this feat.

Firstly, the precursor pool is diversified by the generation of precursor subpopulations with different potentials. In vivo lineage tracing studies have identified several main types of neural stem and precursor cells that are directly or indirectly responsible for generating the full complement of neurons and glial cells in the cortex. They are the neuroepithelial stem cells,
radial precursors and basal precursors, all of which will be discussed individually below (lineage relationships between these precursors is outlined in Figure 1.2).

Initially, the neuropithelial cells rapidly and symmetrically divide to expand their numbers. As the germinal zone grows, the precursor cell population becomes heterogenous with the appearance of the other precursor types. Retroviral and transgenic based lineage tracing and time-lapse imaging studies of the cortex in vivo demonstrated that although neuroepithelial cells give rise to early born neurons, radial precursors are the main precursor type at the onset of neurogenesis and divide increasingly in a asymmetrical manner generating a neuron and another radial precursor (Malatesta et al., 2003; Noctor et al., 2001; 2004). Basal precursor cells originate from asymmetrically dividing neuroepithelial cells and radial precursors during neurogenesis (Haubensak et al., 2004; Miyata et al., 2001, 2004; Noctor et al., 2001; 2004). Basal precursors divide mostly symmetrically to produce two neurons.

Secondly, studies suggest that cortical precursors undergo fate restrictions in their differentiation potential with time. Precursors at early stages of cortical development can self-renew and are multipotent in that they are capable of generating neurons of all layers, astrocytes and oligodendrocytes. Later-stage precursors are restricted to forming only upper layer neurons (Desai & McConnell, 2002) or generate only glial cells (Qian et al., 2000). It has been suggested that neurogenesis does not need to occur for the fate restriction to occur but rather changes in extrinsic cues within the precursor environment affect precursor potential. Early cortical precursors blocked from undergoing neurogenesis by expression of a constitutively active Notch receptor could produce later born neurons when the differentiation block was removed by cre-based recombination (Mizutani & Saito, 2005). However, in vitro clonal analysis has shown that intrinsic fate decisions can occur in the absence of an instructive environment (Shen et al., 2006). Therefore, it is most likely that both intrinsic and extrinsic mechanisms converge on neural precursor cells to impose fate decisions.

Thirdly, altering modes of division in precursors as discussed above allows for the maintenance of the precursor pool while being able to generate differentiated phenotypes at the appropriate time and in the appropriate numbers (reviewed in Gotz & Huttner, 2005)
Neuroepithelial cells

Neuroepithelial cells are the only cells present in the neural tube before the onset of neurogenesis. Therefore they are directly or indirectly the origin of all cells of the CNS. Before neurogenesis is initiated, neuroepithelial stem cells (NESC) divide symmetrically generating two identical daughter cells to increase their numbers (Rakic, 1995). They are bipolar cells with processes contacting both apical and basal surfaces. As they divide, their soma migrates between the apical and basal surfaces in a process called interkinetic nuclear migration (reviewed in Malatesta et al., 2008). During mitosis, the soma is found near the apical surface, it then travels basally during G1 and completes S phase at the basal surface migrating back while in G2. Since NESC are not synchronized in their cell-cycle, the neuroepithelium appears to be stratified and it is referred to as pseudo-stratified.

Clonal analysis has shown that at least some NESC isolated from the E10 prospective cortex can generate both neurons and glial cells and are thus multipotent (Qian et al., 2000). Similar results were obtained by retrovirally labeling NESC in vitro (Williams & Price, 1995) and in vivo (McCarthy et al., 2001). The fact that not all NESC were able to generate all cortical cell types, suggests that even at this early stage there is heterogeneity amongst precursors and that perhaps fate restriction happens quite early. Neuronal and glial committed precursor cells have been found as early as E9.5 (McCarthy et al., 2001).

Thus, NESC are thought to generate the first neurons of the cortex, the remainder of neurons and astrocytes are generated by the NESC-derived radial precursors (reviewed in Pinto & Gotz, 2007 and discussed below).

Radial precursors

Shortly before the onset of neurogenesis, E9-10, NESC transition into a different type of precursor cell, the radial precursor. This transition may involve the expression of the transcription factor Pax-6 as overexpression of Pax-6 enhanced radial precursor production from Sox2 positive NESC (Suter et al., 2008). Another mechanism proposed to regulate this transition is Notch signaling. The loss of Notch effectors, the bHLH transcription factors Hes1
and Hes5 does not affect NESC5s at E8 but results in the loss of radial precursors by E9.5 as NESC5s undergo premature neuronal differentiation (Hatakeyama et al., 2004).

Some neuroepithelial characteristics such as bipolar morphology, contact with both the basal and apical surfaces, expression of the intermediate filament nestin, interkinetic nuclear migration, apical-basal polarity, adherence and gap junctions, remain in radial precursors (reviewed in Malatesta et al., 2008). Transition to the RP phenotype is marked by expression of astroglial genes such as BLBP, Vimentin, GLAST and the gradual loss of tight junctions (Mori et al., 2005). By mid-neurogenesis, RPs aquire other astroglial characteristics such as the presence of glycogen granules, expression of glutamine synthase (GS), the β subunit of calcium-binding protein S100 (S100β). The glial fibrillary acidic protein (GFAP) is only expressed in primate cortical radial precursors and not in the mouse (Mori et al., 2005). Due to these similarities shared with astrocytes, initial nomenclature designated this cell type to a glial lineage, when it was originally named radial glia. In addition, cortical RPs also express the regional marker Pax-6 (Gotz et al., 1998; Noctor et al., 2002), a characteristic not shared with either NESC5s or astrocytes.

Radial precursors were first thought to function as scaffolds for migration of newly-born neurons (Levitt & Rakic, 1980), however they are now considered the main source of neurons and glial cells in the cortex. Time-lapse fluorescence imaging and genetic fate-mapping studies have shown that radial precursors can generate not only astrocytes and oligodendrocytes but also neurons and neuron precursors (Noctor et al., 2004; Malatesta et al., 2003; Anthony et al., 2004). These experiments have suggested that radial precursors may very well be the source of most cortical cells. However, even the radial precursor population has been shown to be heterogeneous in both in vivo and in vitro studies where although RPs were able to generate neurons, astrocytes and oligodendrocytes, they very rarely gave rise to all three (Qian et al., 1998; Malatesta et al., 2000, 2003; Noctor et al., 2001, 2004).

Although no study has been conclusive in separating different radial precursor subpopulations, the expression analysis of specific proteins and cell surface epitopes such as RC2, GLAST, BLBP, JONES and A2B5, has illustrated differences in their expression profiles that correlated to different lineage potentials (Hartfuss et al., 2001; Maric et al., 2003, 2007).
Isolation of different RP subtypes by virtue of their levels of expression of GFP from the hGFAP promoter and subsequent transcriptome analysis revealed distinct differences in gene expression between these subpopulations co-existing at the same developmental time points reflecting their differentiation potential (Pinto et al., 2008). Neurogenic and non-neurogenic radial precursors could be distinguished at the peak of neurogenesis (E14) while RPs at the end of neurogenesis (E18) had transcription profiles that did not resemble any of the earlier RP populations.

At the onset of neurogenesis, RPs begin dividing asymmetrically and with each round of division produce more self-renewing RPs, a post-mitotic neuronal daughter cell or a more fate-restricted basal progenitor (Gotz & Huttner, 2005). At the end of neurogenesis, some RPs undergo terminal symmetric divisions yielding two neurons (Haydar et al., 2003) while others generate astrocytes. The proliferative zone where these precursors reside, the VZ, is reduced progressively during the post-natal period. However, a small proportion of RPs persist in the adult forebrain SVZ as adult neural stem cells (Merkle et al., 2004; Bofanti & Perreto, 2007).

**Basal progenitors**

The ventricular zone was considered for a long time to be the major site of neurogenesis in the cortex. Although mitotically active cells were detected in the SVZ it was not until recently that imaging of division of precursor cells showed that SVZ cells are derived from RPs and that the resulting cells further divide to produce neurons (Miyata et al., 2004; Noctor et al., 2004). Basal progenitors (BPs) appear after the onset of neurogenesis and represent another, more restricted cell type able to generate neurons, adding yet another level of precursor diversification in the cortex. Although some evidence suggests that neuroepithelial cells are also capable of producing BPs, the majority of BPs are generally thought to arise from RPs. As BPs differentiate, they lose contact with the apical and basal surfaces and migrate out of the VZ to form the secondary proliferative zone, the SVZ.

Unlike NESCs and RPs, basal progenitors do not undergo interkinetic nuclear migration and they divide symmetrically with a cleavage plane parallel to the VZ to produce two neuronal cells or two new BPs, thereby providing a way to exponentially increase the numbers of neurons
produced. Basal precursors also differ from RPs in the expression of the transcription factor Tbr2 (Englund et al., 2005), Cux1 and Cux2 (Nieto et al., 2004; Zimmer et al., 2004), the non-coding RNA Svt1 (Tarabykin et al., 2001) and Satb2 (Britanova et al., 2005) while losing expression of the cortical radial precursor marker Pax-6 (Englund et al., 2005). The loss of Pax-6 expression and up-regulation of Tbr2 indicates the transition of RPs to BPs (Englund et al., 2005) and Tbr2 is required for directing this transition (Sessa et al., 2008).

The molecular mechanisms regulating BP transition and/or differentiation remain unclear but evidence suggests that the absence of Hes gene expression directs neuronal differentiation, which normally promotes proliferation and prevents differentiation of precursors (reviewed in Kageyama et al., 2007). Basal precursors do not express Hes transcription factors while Tbr2 was also shown to direct neurogenesis from BPs (Arnold et al., 2008). Basal progenitors thus may represent a population of transit amplifying cells. They were initially shown to generate mostly upper-layer neurons (Tarabykin et al., 2001), however recent work has shown that they are capable of contributing neurons to all cortical layers (Kowalczyk et al., 2009).

Oligodendrocyte precursors

The origin of cortical oligodendrocytes is more complex than that of neurons and astrocytes. They are thought to originate from the dorsal and ventral telencephalon. Indeed, Cre-lox approaches in transgenic mice showed that an early wave of oligodendrocyte precursors expressing Nkx2.1 first appear around E12.5 and migrate to populate the cortex (Kessaris et al., 2006). A second wave of oligodendrocytes follows, also from ventrally derived precursors, but this time from Gsh2 expressing precursors. By E18 the majority of oligodendrocyte precursors in the cortex originate from the ventral telencephalon. Postnatally, a third wave of OLPs derived from cortical precursors constitute the main source of myelinating oligodendrocytes in the cortex while previous oligodendrocyte populations are eliminated (Kessaris et al., 2006).

In vitro, cortical precursor cells produce oligodendrocytes late in culture, after neurogenesis and astrogenesis (Qian et al., 2000). Oligodendrocyte precursor cells are typically specified from multipotent precursors, divide for a limited number of cycles and then terminally differentiate.
into oligodendrocytes. However, evidence suggests that the OPCs themselves are specialized bipotent or tripotent precursors dedicated to the generation of glial cell lineages. Differential integration of environmental cues is thought to direct OPCs towards one of the two glial lineages (Mabie et al., 1997 and reviewed in Miller, 2002). Glial restricted precursors (GRPs) are able to generate type-1 and type-2 astrocytes as well as oligodendrocytes (reviewed in Rao, 1999). Another type of glial precursor for astrocytes is the oligodendrocyte-type 2 astrocyte (O-2As) precursors that can only generate oligodendrocytes and type 2 astrocytes. However, there is no direct evidence that O-2A precursors differentiate into astrocytes in vivo. The differentiation potential of the O-2As is further complicated by studies showing that when certain cues are present, OPCs can be induced to exhibit characteristics of multipotential stem cells (Kondo & Raff, 2000).

1.2 Molecular mechanisms regulating precursor behavior during cortical development

The presence of heterogenous precursor pools that can generate neurons and glial cells at the appropriate time and in the appropriate numbers begs the question: How is this accomplished? What are the mechanisms that regulate the transition from one precursor type to another or their differentiation to neurons and glial cells? How is the switch from neurogenesis to astrogensis accomplished and coordinated? Efforts from many laboratories have shown that both intrinsic cellular programming and extrinsic signals from the environment are coordinated by precursors to generate the final, complex and functional, cortical architecture.

1.2.1 Intrinsic factors regulating cortical precursors

Intrinsic regulation of cortical precursor maintenance and differentiation include the interplay of transcription factors, cell polarity and adhesion molecules, cell cycle regulators and
regulators of precursor divisions. I will be focusing my discussion on a set of transcription factors important for neural and cortical specification: Sox proteins, Pax-6, Emx1 and Foxg1.

Other transcription factors like bHLH (Guillemot et al., 2007), CCAAT/Enhancer-binding proteins (Ramji & Foka, 2002; Miller & Gauthier, 2007), Notch effector Hes genes (Kageyama et al., 2007) and Id (inhibitor of differentiation) proteins (Tzeng, 2003) are also involved in precursor maintenance and differentiation and are reviewed as indicated.

Several classes of transcription factors have been shown to control the multiple steps of precursor generation and maintenance as well as fate determination through temporal and regional expression.

Sox proteins of the HMG-box transcription factor class regulate neural precursor maintenance, the neurogenic-gliogenic switch, astrogenesis and oligodendrogenesis (reviewed in Wegner & Stolt, 2005). Initially, the specification of neuroectoderm requires the expression of SoxB1 transcription factors, which include Sox1, 2 and 3 (Pevny et al., 1998; Zhao et al., 2004). They continue to be expressed in neural stem cells in both the embryo and the adult (D’Amour & Gage, 2003; Graham et al., 2003) and their similar functional properties allow them to compensate for each other, highlighting their important roles in neural stem cells and precursors. Their expression is thought to maintain precursors in an undifferentiated state by blocking neurogenesis without affecting precursor proliferation (Bylund et al., 2003). Loss of SoxB1 expression leads to premature neurogenesis during embryogenesis leading to neuronal loss in adulthood due to the depletion of the precursor pool (Ferr et al., 2004).

The action of SoxB1 proteins is only accomplished in cooperation with other transcription factors. Interactions with neurogenic bHLH proteins have been shown to balance precursor maintenance and differentiation. SoxB1 proteins can either promote maintenance by sequestering bHLH proteins or be suppressed by them allowing neurogenesis (Bylund et al., 2003). This balance is thought to regulate the appropriate onset of neurogenesis.

Pax-6 is another transcription factor that is highly conserved in vertebrates and has roles in multiple steps of CNS development, acting to pattern the neural tube and to regulate precursor proliferation and differentiation (reviewed in Osumi et al., 2008). It is expressed by most radial precursors at the onset of neurogenesis (Malasteza et al., 2000; Hartfuss et al., 2001) and in the
telencephalon it is specifically expressed in a gradient in dorsal precursors that will form the cortex (Stoykova et al., 2000).

The expression of a non-functional Pax-6 protein in small-eye mutant mice (Sey/Sey) results in major developmental defects in the cortex and eye, as well as in neuronal positioning due to axonal path-finding defects (Osumi et al., 2008). Pax-6 mutant brains show reductions in the thickness of the ventricular zone and cortical plate due to underproduction of neurons (Fukuda et al., 2000) suggesting defects in precursor maintenance and neurogenesis. Using chimera techniques, Quinn et al. (2007) showed that the loss of neurons in the Pax-6 mutant cortex was due to a loss of precursor cells though premature exit of the cell cycle and neuronal differentiation. In a different study, Pax-6 knockdown reduced generation of radial precursors from neuroepithelial cells while overexpression enhanced differentiation into radial precursors and neurons suggesting that it may function to regulate both the neuroectoderm to radial precursor transition and neurogenesis (Suter et al., 2008). The exact roles and mechanisms of action of Pax-6 in precursor maintenance are unclear. One way it might function would be through the regulation of cell adhesion between precursors (Tyas et al., 2003). Supporting this idea are studies showing the disruption of polarity and morphology of ventricular zone precursors (Gotz et al., 1998). Another way Pax-6 has been suggested to regulate precursor maintenance is by affecting proliferation of precursors. Direct regulation of the cell cycle regulator p27kip1 by Pax-6 is thought to be the cause of decreased numbers of dividing cells in the cortex (Duparc et al., 2007).

Lastly, regulation by Pax-6 of other transcription factors that promote precursor maintenance, like Sox2, has also been shown (Wen et al., 2008). Furthermore, some of its identified downstream effectors include another radial precursor marker, BLBP, also required for the maintenance of ventricular zone precursors as its knockdown resulted in increased neuronal differentiation and decreased proliferation (Feng et al., 2004; Arai et al., 2005). While Pax-6 may affect precursor proliferation through BLBP its regulation of Neurogenin 2 promotes neurogenesis. Precursors expressing BLBP, do not express Neurogenin 2 (Arai et al., 2005). This result is consistent with Neurogenin 2 being expressed in basal progenitors (Miyata et al., 2004) as they differentiate from Pax-6 positive radial precursors (Englund et al., 2005). Pax-6 downregulation also occurs during the transition to the neurogenic basal precursors (Englund et
These studies support earlier hypotheses regarding the role of Pax-6 in promoting neurogenesis. Cells from the Pax-6 mutant cortex exhibited low production of neurons, a defect rescued by ectopic expression of Pax-6 (Gotz et al., 1998; Heins et al., 2002). Taken together, these studies show that the mechanism of action of Pax-6 is context-dependent.

Expressed in an opposite gradient to Pax-6 in the developing cortex is the homeobox transcription factor Emx1 (Bishop et al., 2002, Zaki et al., 2003). Together with the other Emx family member in the mouse, Emx2, it is involved in the area specification of precursor cells of the cortex (Bishop et al., 2002). Emx1 expression is restricted to dorsal telencephalic precursors, and is often used as a definitive marker of this fate, while Emx2 expression extends to encompass the ventral telencephalic precursors as well. Emx1 is initially expressed in ventricular zone precursors but as corticogenesis progresses, it is also detected in differentiating and mature pyramidal neurons of the adult cortex (Chan et al., 2001), suggesting that Emx1 may be involved in multiple processes such as specification, proliferation and differentiation of cortical cells.

Emx1 knockout mice are viable and exhibit only mild defects in cortex development probably due to compensation by Emx2 (Yoshida et al., 1997). Emx1/Emx2 double knockouts are postnatal lethal with cortical defects where layer I and subplate neurons are completely absent, migration of cells from other forebrain areas is impaired resulting in a general reduction in the size of the cortex (Shinozaki et al., 2002). These observations indicate a role for the Emx genes in precursor fate specification.

The Emx1 precursor lineage has been shown to be the source of glutamatergic projection neurons, astrocytes and oligodendrocytes in the cortex but not interneurons, which are generally derived from the ventral telencephalon (Gorski et al., 2002). Furthermore, the Emx1 precursor lineage also seems to specify precursors that are initially generated in the cortical region but are destined to migrate and populate other forebrain areas such as the striatum (Gorski et al., 2002, Willaime-Morawek et al., 2006). Emx genes likely operate in complex networks that involve other transcription factors such as Pax6 (Muzio & Mallamaci, 2003) in response to patterning molecules such as those in the Shh pathway and Wnts. Expression of Emx1 and 2 is lost in the Gli3 extra toes mutants. Gli3 is a transcription factor involved in dorsal ventral patterning of the neural tube (Tole et al., 2000; Theil et al., 1999). Similarly, Wnt effector protein expression
patterns, known to be involved in proliferation of precursors, were drastically changed in Emx2 mutants (Muzio & Mallamaci, 2003).

The last transcription factor to be discussed, Foxg1 (also known as Bf1) is one of the earliest genes to be expressed in the neural tube where it serves to specify all forebrain precursors. Thus far evidence suggests that Foxg1 functions to arealize the forebrain into dorsal and ventral structures by regulation of signaling and morphogen molecules as well as by acting on precursor cells to prevent premature neurogenesis by affecting their cell cycle (Xuan et al., 1995, Hanashima et al., 2004). The Foxg1 knockout mice have loss or reduction of both dorsal and ventral telencephalic structures resulting from depletion of the precursor pool by the early exit from cell cycle (Xuan et al., 1995, Martynoga et al., 2005). Its function in specification of the ventral telencephalon is separable from its role on dorsal precursors. Foxg1 cooperates with Shh and FGF signaling pathways to specify the ventral telencephalon away from the dorsal (Hebert & Fishell et al., 2008).

Foxg1 also affects proliferation, differentiation and fate of dorsal precursors in part by regulating BMP and FGF signaling (Martynoga et al., 2005; Hanashima et al., 2004). Precursors lacking Foxg1 have increased cell cycle length, a characteristic associated with a committed neuronal fate (Takahashi et al., 1993, Caviness et al., 2003) and generate an excess of neurons (Martynoga et al., 2005). In addition Foxg1 affects neuronal cell fate by regulating the generation of layer I Cajal-Retzius neurons (Hanashima et al., 2004), the earliest born neurons in the cortex. Therefore, Foxg1 represents yet another intrinsic factor that cooperates with extrinsic factors to determine precursor fate.

1.2.2 Extracellular signals regulating cortical precursors

Below I will be discussing the growth factor FGF2 and the cytokine ciliary neurotrophic factor (CNTF) and their mechanisms of action in cortical precursors as they are relevant to this thesis. Other signaling molecules like Notch (Yoon & Gaiano, 2004; Kageyama et al., 2007), BMPs (Hall & Miller, 2004), EGF (Miller & Gauthier, 2007; Shi et al., 2008), neurotrophins
(Kaplan & Miller, 2000), PDGF (Tallquist & Kazlaukas, 2004), Wnts (Toledo et al., 2008; Shi et al., 2008) and Shh (Marti & Bobolenta, 2002) are also implicated in precursor maintenance and differentiation and are reviewed elsewhere as indicated.

**Extracellular signals regulating precursor maintenance and proliferation**

Fibroblast growth factors (FGFs) are a family of 22 secreted polypeptides with regulatory roles in many developmental processes and tissues. Their crucial role is especially evident during CNS development and particularly during corticogenesis. FGF signaling regulates multiple stages in neural induction, patterning and regional specification of the cortex as well as cell fate determination by regulation of proliferation, differentiation and survival of neural precursor cells (Mason, 2007).

FGFs activate downstream signaling cascades by binding and activating a family of four cell-surface tyrosine kinase FGF receptors (FGFRs 1-4). Receptor binding and activation is modulated by the heparan sulfate proteoglycans (HSPGs) which promote and stabilize ligand and receptor interactions (Eswarakumar & Schlessinger, 2005). Binding to an FGF receptor induces receptor dimerization and autophosphorylation initiating downstream signal transduction pathways that include the mitogen-activated protein kinase (MAPK) pathway which controls cell proliferation, the phosphatidylinositol-3 (PI3) kinase regulating cell survival and proliferation as well as the phospholipase Cγ (PCLγ) that leads to calcium signaling and eventual regulation of cell migration and adhesion (Eswarakumar & Schlessinger, 2005; Mason, 2007).

*In vivo* and *in vitro* studies have shown that the various functions of FGF signaling are accomplished in a context dependent manner in that is integrated with other extrinsic cues such as Notch, BMPs, Wnts and Shh and intrinsic cues (epigenetics, transcription factor expression etc) as they are present and available during a specific developmental stage or in a specific cell lineage (Mason, 2007). Further complexity to FGF signaling is given by the fact that ligands and receptors appear in different combinations and abundance in different areas of the developing cortex. (Dono et al., 2003; Mason, 2007). How ligands and receptors interact to activate FGF signaling in different contexts remains unclear. FGFRs 1-3 are expressed in the telencephalic
neuroepithelium and a requirement for FGF is indicated by studies where mice lacking all three FGFRs had both dorsal and ventral telencephalic domains ablated (Hebert & Fishell, 2008).

FGF2 (also known as basic fibroblast growth factor or bFGF), is perhaps the most studied FGF receptor ligand known to activate FGF signaling and is expressed throughout the developing cortex as well as in cultured precursor cells (Vaccarino, 1999; Raballo et al, 2000). FGF2 transcripts are detected in the neuroepithelium starting at E9 (Nurcombe et al., 1993) and it is expressed highly in the apical endfeet of precursors located in the VZ/SVZ (Vaccarino et al., 1999; Raballo et al., 2000). Its expression peaks between E14 and E18, dropping postnatally (Dono et al., 1998; Vaccarino et al, 1999, Raballo et al., 2000). FGF2 has early roles in neural induction and patterning of the neural tube and has been shown to be an important mitogen for neural precursor cells *in vivo* and *in vitro* (Temple & Qian, 1995; Qian et al., 1997).

Studies using FGF2 knockout mice reported a 50% reduction in cortical precursor cells before the onset of neurogenesis resulting in a comparative loss in neurons and glial cells in the adult (Vaccarino et al., 1999; Raballo et al., 2000; Korada et al., 2002) and a reduction in total cell numbers (Dono et al., 1998). Interestingly, FGF2 was shown not be required for the survival of precursors *in vitro* but not *in vivo* (Vaccarino et al., 1999; Raballo et al., 2000), although deletion of all three FGFR genes results in complete loss of the telencephalon due to cell death (Paek et al., 2009). These studies suggest that FGF family ligands in addition to FGF2 are required early for establishment of the telencephalic fate, with FGF2 specifically required before the onset of neurogenesis to maintain and increase the pool of precursors.

One way FGF2 could influence proliferation of neural precursors is by affecting cell cycle kinetics. The proposed “cell cycle length” hypothesis suggests that a lengthening of the G1 phase in precursors is indicative of a switch from a proliferative to a neurogenic state (Dehay and Kennedy, 2007). FGF2 was shown to shorten the length of G1 that was accompanied by an increase in proliferative divisions of E14-E16 cultured neural precursors (Lukaszewics et al., 2002).

Culture experiments also indicate that FGF2 affects the state of neural precursors based on its availability. High concentrations of FGF2 increased proliferation of precursors and differentiation towards an oligodendrocyte lineage while low FGF2 concentrations were
permissive to neuronal differentiation and astrocyte differentiation in the presence of IL-6 related cytokines (Qian et al., 1997). Furthermore, activation of FGFR 1 and 3 by high FGF2 concentrations in cultured precursors increased self-renewing symmetrical divisions (Maric et al., 2007). Therefore, FGF2 signaling controls not only precursor maintenance but also their differentiation to subpopulations of cortical cells. A recent study using conditional deletion of FGF receptors 1, 2 and 3 in all cortical precursors after the onset of neurogenesis, demonstrated the requirement for FGF signaling in preventing the transition of radial precursors to more restricted basal progenitors or neurons without affecting proliferation rates, cell cycle or survival (Kang et al, 2009). Thus FGF signaling is balanced to ensure that the correct numbers of precursors are available and that they do not prematurely differentiate. FGF2 is likely to act in concert with other FGF family members such as FGF 3, 8, 15 to achieve these functions in a time and concentration dependent manner (Dono, 2003).

FGF signaling has also been shown to cooperate with other signaling pathways to enact the different cellular functions of proliferation, survival and differentiation of precursors at the appropriate times. For example, Notch signaling, known to promote radial precursor maintenance and inhibit neurogenesis in early precursors, seems to increase responsiveness of cultured precursors to FGF signaling in terms of the efficiency of neurosphere formation (Yoon et al., 2004). During neurogenesis, the effects of FGF signaling are modulated by neurotrophin signaling. As examples, the neurotrophin NT-3 could antagonize the mitogenic effects of FGF2 in vitro (Ghosh & Greenberg, 1995) while BDNF increased proliferation and neurogenesis in vivo (Bartkowska et al., 2007). At the onset of gliogenesis, FGF signaling is most likely affected by gliogenic signals like EGF signaling, BMPs and JAK-STAT (discussed below) activation. These extrinsic cues are thought to contribute to generation of diverse precursor populations, progressive fate restrictions in precursors and the production of appropriate numbers of cells of each type.

**Extracellular signals regulating gliogenesis**

Cortical precursors are initially biased to first make neurons while astrocytes are generated subsequently, and this timed cell genesis can be recapitulated *in vitro* in primary cortical culture
(Qian et al., 2000). The mechanisms that drive the change in precursor competency have been shown to include intrinsic factors such as dilution of neurogenic factors, expression of new growth factor receptors such as EGFR or epigenetic modification of astrocyte-specific genes (Barnabe-Heider et al., 2005). However, a large body of work has implicated extracellular factors that can induce precocious astrocyte generation demonstrating that cortical precursors cells are in fact competent to make astrocytes at any time point provided that the appropriate gliogenic factor is present in their environment (Morrow et al., 2001; Barnabe-Heider et al., 2005). One of the first lines of evidence supporting this latter hypothesis showed that when early precursor cells were cultured over slices of the early cortex, they mostly generated neurons while when cultured over late or post-natal cortical slices they primarily generated astrocytes (Morrow et al., 2001).

What are the extracellular factors promoting the astrocytic fate? Several such factors have been identified and include cytokine, BMP and Notch signaling (reviewed in Sauvageot & Stiles, 2002). Cytokines are small proteins initially identified as regulators of the immune and hematopoietic systems, but were subsequently found to have a broader and essential role in many other systems including the central nervous system (reviewed in Deverman & Patterson, 2009).

In particular, one subgroup of structurally related cytokines has been shown to act as extracellular cues modulating fate choice decisions of cortical precursor cells. Members of the IL-6 cytokine family includes ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1) and leukemia inhibitory factor (LIF), neuropoietin (NP) and cardiotrophin-like-cytokine (CLC), all of which signal through a ubiquitously expressed gp130 receptor complex that activates the Janus-activated kinase-signal transducer and activator of transcription (JAK-STAT) and mitogen-activated protein kinase (MAPK) signal transduction pathways (Bonni et al., 1997; Rajan & McKay, 1998; reviewed in Deverman and Patterson, 2009). CNTF, LIF, CT-1, OsM, CLC and NP all bind the LIFR which then heterodimerizes with gp130 to initiate these signaling cascades (CNTF, CLC and NP bind the CNTFR receptor first before binding LIFR).

Activation of the LIFR/gp130-JAK-STAT signaling pathway is required for astrocyte generation in cortical precursors. Knockout animals lacking the common receptors gp130 or LIFR have severe deficits in astrocyte formation in the CNS (Nakashima et al., 1999; Koblar et al., 1998) and acute knockdown of gp130 and STAT3 results in astrocyte reduction both in vitro
and in vivo (Barnabe-Heider et al., 2005). These studies suggest that cytokine signaling through the LIFR/gp130-JAK-STAT pathway is central to astrocyte generation. In vitro stimulation with the cytokines that signal through this receptor complex induces early astrocyte generation while inhibiting neurogenesis (Barnabe-Heider et al., 2005). Individual knockouts for CNTF and LIF show no obvious defects in astrocyte formation (Masu et al., 1993; Bugga et al., 1998) indicating that despite their ability to induce astrogenesis in cultured embryonic cortical precursors (Bonni et al., 1997; Johe et al., 1996; Rajan & McKay, 1998; Barnabe-Heider et al., 2005) they are not key factors in this process. Consistent with this is their lack of expression in the embryonic brain (Stockli et al., 1991; Barnabe-Heider et al., 2005). Instead another member of this family, CT-1, was shown to be the relevant cytokine regulating the switch to gliogenesis during cortical development (Barnabe-Heider et al., 2005). CT-1 is synthesized and secreted by newly-born neurons in the developing cortex, thereby providing a feedback mechanism where neurons instruct the parent precursors to make the switch from neurogenesis to astrogenesis. In support of this hypothesis, CT-1 knockout mice show significant reductions in cortical astrogenesis (Barnabe-Heider et al., 2005).

The coupling of these various intrinsic and extrinsic mechanisms is hypothesized to regulate the neurogenic to gliogenic switch. Thus, cortical precursor fate choice is influenced by extracellular factors which initiate signaling pathways resulting in the downstream modulation of gene transcription. Although not expressed until early postnatal life (Stockli et al., 1991; Barnabe-Heider et al., 2005) and shown not to be the principal factors involved in astrogliogenesis in the developing cortex in vivo, CNTF and LIF have been the two most widely used gp130 cytokines for in vitro gliogenesis studies. Although undetectable in the embryonic brain they have been suggested to have compensatory roles (Stocki et al., 1991). CNTF can induce the expression of astrocyte specific genes in late and not early cortical precursors, even though JAK-STAT pathway members are present at early time points (Barnabe-Heider et al., 2005). This ability is modulated by FGF2, which was shown to increase responsiveness of precursors to CNTF by allowing binding of STAT to the astrocytic gene, GFAP (Song & Ghosh, 2004). This represents yet another way precursor competence is regulated during development.

CNTF has also been shown to promote self-renewal and expansion of subpopulations of neural stem cells in vitro through the CNTF/LIF/gp130 receptor complex (Shimazaki et al. 2001;
Gregg & Weiss, 2005). Similarly, ventricular LIF injections at E15 caused an increase in proliferating cells and neurons generated in the cortex (Hatta et al., 2002). Furthermore, CNTF and LIF promote secondary sphere formation in neurosphere assays (Gregg & Weiss, 2005). Interestingly, this cytokine/ gp130-JAK-STAT mediated precursor maintenance is reminiscent of pluripotency maintaining signaling pathways in embryonic stem cells (Williams et al., 1988).

1.3 Sources of cortical precursors

It is evident from the information in the previous sections that in vivo, cortical precursors are regulated by a multitude of intrinsic and extrinsic factors converging to balance their maintenance, survival and differentiation. Several model systems have been developed to study the molecular mechanisms underlying these processes. Early studies of the cortex involved retroviral based gene expression delivered in utero or in cortical explants (for example Burrows et al., 1997). With the development of transgenic technologies, mice were generated carrying reporter constructs allowing fate mapping experiments and visualization of precursors and their progeny. Knockout mice and mice carrying specific mutations have allowed the dissection of molecular pathways of corticogenesis. Another powerful method to study corticogenesis in vivo uses in utero electroporation of expression plasmids directly into the E13-15 embryonic cortex allowing direct access to cortical precursors for loss and gain of function studies (Barnabe-Heider & Miller, 2003, Paquin et al., 2005; Gauthier et al., 2007). This method is less time consuming and less costly than transgenic methods and allows the observation of direct gene effects without the possible compensatory mechanisms that may obscure effects. This technique also allows the targeting of specific cell populations as the uptake of expression plasmids can be directed.

In order to control the environment of precursors, many groups have developed in vitro cortical precursor culture systems (Gosh & Greenberg, 1995; Barnabe-Heider & Miller., 2003, Paquin et al., 2005; Gauthier et al., 2007). These cortical precursors cells isolated at the onset of neurogenesis (E12-13) and cultured in serum-free conditions survive and differentiate in the same temporal fashion as observed in vivo. Dividing nestin positive precursors produce neurons for the first 5 days in vitro, astrocytes are first seen at 6 days in vitro followed by
oligodendrocytes a few days later. Importantly, these precursors respond to mitogenic and differentiating cues as seen in vivo (Ghosh & Greenberg, 1995; Qian et al., 1997; Menard et al., 2003; Barnabe-Heider & Miller, 2003). The neurosphere culture is yet another in vitro method offering a different set of advantages (reviewed in Gottlieb, 2002). Precursors can be propagated and expanded without differentiation using growth factors. Although this is not typical of precursors in vivo, it allows the manipulation of undifferentiated precursors as well as access to more material. Upon withdrawal of growth factors, they can be induced to differentiate into all three neural lineages.

Development of embryonic stem cell technologies has provided a new platform for studying development ex vivo. Neural differentiations from embryonic stem cells will be discussed in the sections below highlighting this source of cortical precursors as an alternate and attractive model for studying corticogenesis.

1.4 Neurogenesis from embryonic stem cells

1.4.1 Embryonic stem cells overview

Mouse embryonic stem cells were first derived from the inner mass of the blastocyst over 20 years ago (Evans & Kauffman, 1981). They are pluripotent in that they have the ability to differentiate along all cell lineages in vitro and participate in normal development upon re-introduction into the blastocyst. They can be maintained in vitro without losing their pluripotent potential in long-term culture using media containing leukemia inhibitory factor (LIF). The directed differentiation of ES cells involves their removal from pluripotency maintaining media containing LIF and their growth in suspension as 3D cell aggregates termed embryoid bodies (EBs) (Doetschman et al., 1985) although other differentiation methods using adherent culture have been developed (Ying et al., 2003). Differentiation of ES cells in embryoid bodies in vitro recapitulates in vivo events such as gastrulation where the primary germ layers – ectoderm, endoderm and mesoderm- are specified (Murry & Keller, 2008). Enrichment of specific lineages
is achieved by the manipulation of EBs with growth factors and signaling molecules or expression of lineage specific genes. \textit{In vitro} differentiation of embryonic stem cells has become a powerful experimental system for studying developmental and disease pathways and mechanisms.

1.4.2 ES cells as models of development and disease: advantages and disadvantages

The goal in the development of ES based differentiation systems has been to create an easily manipulated and observable \textit{in vitro} system, that mimics \textit{in vivo} development, and that can be used to dissect out mechanisms of normal and abnormal development and also offer a potentially unlimited source of cells for cell replacement therapies. Embryonic stem cells can potentially differentiate into any cell type and previous knowledge of developmental pathways has lead to the development of two major strategies for directing their differentiation: lineage selection and targeted differentiation (Murry & Keller, 2008; Fritsch & Singer, 2008). These strategies have lead to differentiation of ES cells into many cell types: neural, cardiomyocytes, insulin producing cells, hematopoietic progenitors (Murry & Keller, 2008).

An important requirement for the application of ES cell derived cells is the ability to produce pure cultures of the desired cell type. It remains a challenge in the stem cell field to eliminate unwanted cell types including undifferentiated ES cells, that can give rise to teratomas and teratocarcinomas upon transplantation (Fritsch & Singer, 2008). This has led to the refinement of methods either by more specific culturing methods (chemically defined media, use of inhibitors and growth factors), by genetic manipulation of ES cells, by isolating cell lineages with the use of transgenic ES reporter lines or cell-surface epitopes (for examples see references in Table1.1). From this body of work, we have learned much about molecular mechanisms - transcription factors and extracellular signals - that instruct cell lineage and have helped transition these methods from mouse ES models to the more relevant differentiation of human ES cells (reviewed in Odorico et al., 2001 and Erceg et al., 2008), de-differentiation iPS technologies (reviewed in Amabile & Meissner, 2009) and most recently trans-differentiation strategies (Vierbuchen et al., 2010). In the following sections I will be presenting the reader with
a review of such efforts and what we have learned from them with a focus on mouse ES neural differentiation.

1.4.3 Neural induction in ES cells

How do ES cells generate neural phenotypes? Many methods have been developed with the purpose of finding the appropriate culture techniques to generate specific lineages and increase the purity of the resultant populations. During differentiation towards a neural fate, ES cells undergo progressive lineage restrictions similar to those seen *in vivo*. Neuralization of ES cells is thought to involve the induction of Wnt signaling inhibitors such as the secreted frizzled-related protein 2 (Sfrp2) and dickkopf homolog 1 (Dkk1) (Aubert et al., 2002, Watanabe et al., 2005) and specification to neuroectoderm in ES cells by expression of SoxB transcription factors as it occurs *in vivo* (Wegner & Stolt, 2005). Forced expression of Sox1 and 2 selectively generated neuroectoderm at the expense of mesoderm and endoderm (Zhao et al., 2004). The neural specification “default” model seen *in vivo* seems to also apply to ES cells where the absence of signals specifying mesoderm or endoderm formation (such as Wnts) leads to neuroectoderm induction (Murry & Keller, 2008). In fact ES cells cultured in PBS alone without addition of any growth factors underwent neural specification as indicated by expression of Sox2 and Nestin (Smuckler et al., 2006) and then underwent massive cell death due to lack of survival factors.

Therefore ES neural differentiation protocols give access to a large range of neural precursor populations that are difficult to access *in vivo* and represent an important tool to study the molecular and cellular events that are involved in neural precursor function and differentiation of specific neuronal types.

1.4.4 Types of neural lineages produced

All three major neural cell types – neurons, astrocytes and oligodendrocytes – have been differentiated from mouse ES cells (reviewed in Keller, 2005). Neuronal differentiations include a wide variety of neuronal phenotypes like dopaminergic (Kawasaki et al., 2000; Kim et al., 2002, 2006, 2007, Sonntag et al., 2007), motor (Wichterle et al., 2002), glutamatergic (Reyes et
al., 2008), GABAergic (Chatzi et al., 2009), midbrain and hindbrain (Chung et al., 2002), cerebellar (Salero & Hatten, 2007) and cortical (Bibel et al., 2004, 2007; Watanabe et al., 2005; Gaspard et al., 2008; Eiraku et al., 2008) (see Table 1 for select examples).

Perhaps the most successful neuron derivations have been those towards a dopaminergic and motor neuron phenotype. Success is measured not only in being able to direct differentiation of ES cells into highly efficient and homogenous cultures of the cell type desired but also functional and transplantation studies. After transplantation into animal models of Parkinson’s disease and spinal injury, precursors promoted phenotypic recovery (Kim et al., 2002; Wichterle et al., 2002). Efforts have also succeeded in the generation of highly pure cultures of GABAergic neurons (Chatzi et al., 2009) that have been postulated to be useful in treatment of neurological conditions such as Huntington’s disease, brain and spinal cord injuries, schizophrenia and others.

The generation of so many neural phenotypes implies the progressive lineage restrictions of ES cells similar to that seen in vivo resulting in the generation of a wide range of precursor populations. The generation of lineage-specific neural precursor cells, not just terminally differentiated cells is important for the success of transplantation studies. Precursors that maintain a limited differentiation potential but are able to migrate to their niches are relevant to the study of regulatory molecular mechanisms of differentiation and can be used as sources of replacement. A great deal of work has been dedicated to identifying and generating specific populations of neural precursors. Indeed, analysis has revealed the presence of neural precursors exhibiting the molecular characteristics of the neuronal lineages obtained in ES differentiations. These cells exhibit the hallmarks of neuroepithelial stem cells and radial precursors from which most neurons are derived in the CNS during development along with specific regional identity markers.

Are ES-derived neurons and precursors identical to their somatic counterparts? Firstly, in a comparison between ES-derived neural precursors and neurospheres generated from fetal neural precursors, ES-derived neural precursor cells proliferated more and preferred adherent conditions versus suspension culture (Colombo et al., 2006). This difference in proliferation ability is consistent with studies suggesting that most ES-derived cells are not fully mature, but need to be put into an in vivo environment to become mature as they have a broader differentiation potential.
(Wichterle et al., 2002; Colombo et al., 2006). Somatic precursor cells have a restricted potential reflecting region of origin (Temple, 2001). ES cells on the other hand first produce precursors characteristic of early neuroepithelial cells that express broad precursor markers like Sox2, nestin and Pax-6 or broad regional markers of the brain, forebrain and spinal cord like Foxg1 and Olig1/2 (Wicherle et al., 2002, Ying et al., 2003, Plachta et al., 2007). Furthermore, these “naïve” neural precursors can then be regionalized in vitro by morphogens like Wnt (Wantanabe et al., 2005) and Shh (Gaspard et al., 2008) as they would be in vivo. In terms of gene expression profiles, studies have shown that somatic and ES-derived neural precursors have either very similar or limited gene expression in common (Colombo et al., 2006; Shin et al., 2007).

1.4.5 Methods of generating neural precursors and neurons from ES cells

Differentiation of neural phenotypes, especially neurons, has been at the forefront of studying ES differentiation, generating a large number of protocols. Although producing similar end products, they often involve strikingly different methods. This stresses the complexities of development and the need for identifying the molecular pathways involved. The different methods themselves give clues as to how neural lineage commitment is determined in ES cells.

Early work on ES cell differentiation in vitro, in serum-supplemented media found that the aggregation of cells into embryoid bodies and exposure to retinoic acid enhanced conversion of ES cells to a neural phenotype producing both neurons and astrocytes (Bain et al., 1995; Fraichard et al., 1995; Strubing et al., 1995). Embryoid body formation in the presence of serum alone favors differentiation into mesoderm and endoderm derivatives like cardiac cells (Bain et al., 1996). Since then, variations on this initial method, and the development of alternate methods has advanced progress in this field. Methods generating neural precursors and differentiated neural cell lineages can be categorized based on media components, differentiation substrates (stromal feeder layer, adherent monolayer or floating aggregates) and the types of cells produced (see Table 1.1). The overall goals have been to direct differentiation to a cell type of interest while obtaining high yields of pure cultures of that cell type.
Neural differentiation of ES cells was initially achieved by induction of neuroectoderm in embryoid bodies followed by enrichment of neural precursors using a variety of factors. Retinoic acid treatment of EBs is the earliest and most widely used factor for neural differentiation of ES cells generating spinal cord interneurons and motor neurons (Wichterle et al., 2002, Kim et al., 2002, Barberi et al., 2003, Maden, 2007) as well as forebrain and cortical precursors and neurons (Bain et al., 1995; Liour et al., 2003, 2006; Bibel et al., 2004, 2007). Explaining the varied results obtained with retinoic acid is its involvement as a morphogen at multiple stages and in many aspects of neural development in vivo (reviewed in Maden, 2002). A retinoic acid gradient regulates patterning of the antero-posterior axis and specification of caudal structures. RA signaling has also been implicated in induction of neuronal gene expression (Kim et al., 2007).

Furthermore, RA is a known teratogen and has been shown to affect neural precursor proliferation (Wohl & Weiss, 1998, Maden, 2002). More recently, RA activity secreted from the meninges layer covering the cortex was shown to regulate cortical precursor differentiation (Siegenthaler et al., 2009). Given its roles in vivo, it is not surprising that the effects of RA on ES neural differentiation have been shown to be both time and dose-dependent. Various groups have taken advantage of this fact to direct their differentiations towards specific lineages. For example, a higher concentration of RA is required to produce GABAergic neurons (Chatzi et al., 2009) than radial precursors and astrocytes (Bain et al., 1995; Bibel et al., 2004). Retinoic acid has also been used to define anterior-posterior neuronal identity (Okada et al., 2004). The window of application of RA to EBs also influences neural phenotype obtained (Bibel et al., 2004, 2007). Remarkably, despite the wide range of RA action, highly homogenous cultures can be obtained using RA (Bibel et al., 2004, 2007, Chatzi et al., 2009).

As discussed earlier, the “default” model of neural induction implies that the lack of inductive signals generates neuroectoderm (Munoz-Sanjuan & Brivanlou, 2002). The presence of serum exposes ES cells to a large number of factors that can influence differentiation. For example BMP4 was shown to inhibit neural differentiation in a system where ES cells formed nestin positive neurospheres in the absence of serum and the presence of LIF that could be expanded in FGF2 and induced to differentiate into neurons, astrocytes and oligodendrocytes (Tropepe et al., 2001). Using this same paradigm, Ying et al., 2003 efficiently generated high yields of neuroepithelial cells using adherent monoculture. Based on these observations, various other methods have since been adapted to include the use of growth factors, expression of
lineage specific genes and inhibitors that mimic developmental inductive events to produce specific precursor and neuron populations (Wicherle et al., 2002; Barberi et al., 2003). Defined media for ES neural differentiations usually involve the use of supplements like N2 and B27 or similar cocktails originally designed for primary neuron cultures, in combination with growth factors and inhibitors. Wnt and Shh inhibitors have successfully been used to generate telencephalic and cortical precursors and neurons respectively (Barberi et al., 2003, Wantanabe et al., 2005, Gaspard et al., 2008), effects consistent with their role in vivo as dorso-ventral morphogens. The caudalizing effects of RA and Shh were used together to direct spinal cord neuron specification (Wichterle et al., 2002). Similarly, treatment of ES cells with FGF8, a growth factor known to pattern the mid/hindbrain, resulted in differentiation towards that lineage (Lee et al., 2000; Barberi et al., 2003).

Lineage selection by expression of specific genes in ES cells has also proved efficient in inducing desired phenotypes. For example constitutive expression of Nurr1 in ES cells, a transcription factor required for the differentiation of midbrain dopaminergic neurons, efficiently increases the production of these neurons (Chung et al., 2002). In another example, recapitulation of the sequence of growth factors and transcription factors - Neurogenin1, BNDF and GDNF- resulted in the generation of glutamatergic spiral ganglion neuronal differentiation (Reyes et al., 2008).

Another method of generating dopaminergic neurons from ES cells uses monolayer co-culture of ES cells with bone marrow-derived stromal cells (Kawasaki et al., 2000) without the use of RA or any other factors. It is unclear what factor stromal cells produce but serum addition or BMP4 (a factor present in serum) blocks neural differentiation. Consistent with this, a comparison between ES neural differentiations on stromal layers versus feeder free conditions revealed that stromal cells produced caudalizing factors leading to the generation of precursor and neuron identities of the fore-, mid and hindbrain as well as spinal cord (Watanabe et al., 2005). Telencephalic specification increased when the stromal layer was removed.

Regardless of the method used or cell type generated, it is clear that ES cells use similar molecular pathways of differentiation seen with primary cultures and in vivo. Furthermore, many
of the methods above have been successfully translated to human ES cell differentiations (reviewed in Erceg et al., 2008).

1.4.6 **Forebrain and cortical precursors**

Early work proposed that precursors in the CNS are initially specified to a forebrain identity, and then caudalized to generate posterior areas (reviewed in Levine & Brivanlou, 2007). This idea has been supported by studies of ES neural differentiations (Tropepe et al., 2001, Wantanabe et al., 2005, Gaspard et al., 2008). The derivation of forebrain-like precursors has been reported previously and most interestingly, it was found that ES cells depend on cell-intrinsic mechanisms to recapitulate the complexities of corticogenesis in minimal media conditions. The challenge has been to stop the further regionalization of these precursors at this stage. Most likely, endogenously produced factors or media components contributing to the rapid re-specification of precursors to more caudal fates have made these differentiations difficult. Several published protocols employing significantly different methods, do however report the production of cortical precursor and neuronal phenotypes (Bibel et al., 2004, 2007; Watanabe et al., 2005; Eiraku et al., 2008, Gaspard et al., 2008). In these studies, forebrain identity is generally defined by Foxg1 expression while cortical radial precursors have generally been identified by Pax-6, Emx1, nestin, BLBP and GLAST, as discussed earlier in this chapter.

One of the first attempts at directed differentiation of telencephalic/cortical precursors and neurons used treatment of ES cells with the Wnt antagonist, Dkk1, to increase efficiency of generation of telencephalic precursors in serum free conditions suggesting that unknown caudalizing factors must be suppressed to promote anterior neural fates (Watanabe et al., 2005). Although this protocol enriched for Foxg1/Emx1 forebrain/cortical precursors, the numbers obtained remained low and neuronal identity remained uncertain. More recently, another study reported the generation of up to 80% cortical precursors (Gaspard et al., 2008). In minimal media, Shh inhibition was used to endow precursors with a cortical identity, consistent with its role *in vivo* in dorsal-ventral patterning of the telencephalon (Campbell, 2005, Gaspard et al., 2008). Glutamatergic neurons of virtually every layer were generated in a temporal order according to their birthdates *in vivo*. However, an under-representation of upper layer neurons was observed. Upon transplantation, these precursors integrated appropriately and the resultant
neurons formed connections typical of cortical neurons. Interestingly, Gaspard et al., 2008 observed that the majority of neuronal projections were typical of visual and limbic deep layer neurons regardless of transplantation site. This raises interesting questions about cortical neuron specification. Is it intrinsic or extrinsic? Is it that only the early events are recapitulated whereas later events require cues that are generated by the cortical niche?

Similar results were also obtained by another group using a different approach. Eiraku et al., 2009 demonstrated the efficient generation of cortical precursors, with up to 90% of cells expressing Emx1 and Foxg1. The authors show that the polarized organization seen in the developing cortex can be recapitulated in vitro by ES cells. Precursors organized themselves radially around empty spaces reminiscent of the neural tube while differentiated neuronal progeny migrated to the periphery of the neural tube-like structures. The same sequential generation of cortical neurons is also observed here, however the “inside-out” pattern of cortical neuron generation is not seen, suggesting that environmental cues may be responsible for this in vivo.

While the previous studies used defined media, the last study to be discussed reports the generation of radial precursors and neurons of the cortex using neural induction of EBs in serum with RA treatment. As previously discussed, RA induction protocols have yielded a variety of neural phenotypes. The protocol by Bibel et al. (2004, 2007) is the only one using RA treatment of EB culture in serum and stopping further regionalization of precursors from a cortical identity stage, without the use of inhibitors and growth factors, while generating homogenously high yields of precursors and neurons. Up to 97% of the initial population of precursors express markers of radial precursors: Pax-6, BLBP, Nestin, RC2 and GLAST (Bibel et al., 2004). The authors attribute this ability to the selection of highly proliferative ES cells, the differentiation state of the starting population of ES cells, the window of addition and concentration of RA in the presence of serum. However, due to the widespread expression of Pax-6 and other radial precursor markers during CNS development (Walther & Gruss, 1991) as well as the presence of glutamatergic neurons in many other CNS areas, further experiments were required to determine the identity of these cells. In a subsequent study these cells were shown to express Emx2, a marker encompassing precursors in both the dorsal and ventral telencephalon (Nikoletopoulou et al., 2007).
In vitro, these precursors then rapidly differentiate to functional glutamatergic neurons while hardly any astrocytes or oligodendrocytes are produced. Suppression of glial lineages is likely due to RA treatment, which was suggested to promote neuronal phenotype and the expression of genes associated with the mature state (such as those associated with neurite outgrowth) and thus speeding up differentiation (Kim et al., 2009). Furthermore, the modified B27 cocktail was designed specifically to suppress glial lineages as stated by the authors (Bibel et al., 2007).

Nonetheless, this ES derived system was successfully used as an in vitro model to investigate mechanisms of neural precursor differentiation (Nikoletopoulou et al., 2007; Mohn et al., 2008). The method used in this thesis to derive cortical precursors and neurons is based on the Bibel et al. 2007 RA induction protocol.
Figure 1.1: Cortical development and cortical cell types.

(a) Diagram adapted from Sheen & Walsh (2003) showing a cross section through the embryonic brain and the location of the neocortex (box). Higher magnification of the box shows precursors in the ventricular zone, and migration of differentiated cells into the cortical plate. (b) Temporal genesis of neurons, astrocytes and oligodendrocytes in vivo and in vitro.
Figure 1.2: Cortical stem and precursor cells

Diagram showing the lineage relationships between the earliest neural stem cells, the NSCs to radial precursors, basal precursors and differentiated neurons, astrocytes and oligodendrocytes and adult neural stem cells.
Table 1.1: Neural differentiations from embryonic stem cells

Select examples of ES neural differentiations illustrating a variety of methods, with and without retinoic acid, with and without serum, and the multitude of neural phenotypes generated. Efficiency of differentiation and heterogeneity of culture in terms of neural and non-neural phenotypes is noted.

<table>
<thead>
<tr>
<th>Type of neuron</th>
<th>Type of precursor</th>
<th>Method</th>
<th>Heterogeneity</th>
<th>Functionality</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortical</td>
<td>Radial precursors (Pax6, Nestin, BLBP)</td>
<td>RA induction in serum</td>
<td>90% precursors Astrocyes and oliges</td>
<td>Electrophysiology Transplantation</td>
<td>Bibel et al., 2004, 2007; Plachta et al., 2007</td>
</tr>
<tr>
<td>Cortical</td>
<td>Cortical radial precursors (Emx1, Foxg1, Pax6)</td>
<td>Serum free adherent monoculture; Shh inhibition</td>
<td>80% precursors; astrocytes + other</td>
<td>Electrophysiology Transplantation</td>
<td>Gaspard et al., 2008</td>
</tr>
<tr>
<td>Cortical</td>
<td>Cortical radial precursors (Emx1, Foxg1, Pax6)</td>
<td>Serum free suspension</td>
<td>96% precursors; heterogeneous precursor marker expression</td>
<td>Transplantation Calcium imaging</td>
<td>Eiraku et al., 2008</td>
</tr>
<tr>
<td>IsH-tubulin</td>
<td>Forebrain (Emx2), Hindbrain (Hoxa11) &amp; Nestin</td>
<td>Low density clonal neurosphere; serum free +LIF+FGF2</td>
<td>Neurons and glia</td>
<td>NA</td>
<td>Smukler et al., 2006</td>
</tr>
<tr>
<td>Spinal cord motor</td>
<td>Spinal cord Hoxc6; Hoxb8 Pax6; Nossx.1; Olig2</td>
<td>RA induction in serum</td>
<td>NA - FACS sorted precursors</td>
<td>Transplantation</td>
<td>Wichtere et al., 2002</td>
</tr>
<tr>
<td>Spinal cord motor</td>
<td>Spinal cord (Pax2 Pax6, RC2, Nestin)</td>
<td>RA induction</td>
<td>Neurons and astrocytes</td>
<td>Electrophysiology</td>
<td>Kim et al., 2007</td>
</tr>
<tr>
<td>Dorsaline mixed</td>
<td>Mixed (Pax2, Pax6, Math1)</td>
<td>Serum free EB culture</td>
<td>Neurons, astrocytes and oligodendrocytes</td>
<td>Electrophysiology</td>
<td>Li et al., 2003</td>
</tr>
<tr>
<td>Midbrain dopaminergic</td>
<td>Nestin; RC2</td>
<td>Serum free stromal cell co-culture</td>
<td>Neurons and astrocytes</td>
<td>Transplantation</td>
<td>Kawasaki et al., 2000</td>
</tr>
<tr>
<td>Type of neuron</td>
<td>Type of precursor</td>
<td>Method</td>
<td>Heterogeneity</td>
<td>Functionality</td>
<td>Reference</td>
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<tr>
<td>GABA-RA vOut+RA</td>
<td>Pax6, RC2, Nestin, Telo-, Dc- Mesencephalon precursors without RA; spinal cord with RA</td>
<td>Serum free +/-RA</td>
<td>Neurons, astrocytes</td>
<td>Electrophysiology</td>
<td>Kim et al., 2009</td>
</tr>
<tr>
<td>Dorsaminergic, Serotonin, motor, GABA; astrocytes, oligodendrocytes (depending on GF addition)</td>
<td>Region specific</td>
<td>Stromal cell co-culture of fertilization and nuclear transfer ES cells in serum replacement; individual GF cocktails for each neuronal type</td>
<td>50-70% of cells of the appropriate lineage; other neural lineages</td>
<td>Electrophysiology</td>
<td>Barberi et al., 2003</td>
</tr>
<tr>
<td>Glutamatergic, spiral ganglion</td>
<td>Transient expression of lineage transcription factor Nrg1 induces lineage specific precursor markers</td>
<td>Serum free DCX induction of Nrg1 and treatment with BDNF and GDNF</td>
<td>80-70% desired lineage; astrocytes and non-neural</td>
<td>Transplantation</td>
<td>Reyes et al., 2008</td>
</tr>
<tr>
<td>GABAergic immature and not regionalized</td>
<td>Nestin+ radial precursors</td>
<td>RA induction in EBs Serum free -bFGF/EGF differentiation</td>
<td>90% cells in culture GABAergic neurons, RA concentration increased GABAergic neuronal phenotype; astrocytes</td>
<td>Electrophysiology</td>
<td>Chazin et al., 2009</td>
</tr>
<tr>
<td>GABA, TH</td>
<td>Sox1, Nestin</td>
<td>Serum free adherent monoculture</td>
<td>75% neural; neurons, astrocytes and oligodendrocytes + non neural</td>
<td>NA</td>
<td>Ying et al., 2003</td>
</tr>
</tbody>
</table>
Statement of Thesis Objective

Recent advances in the embryonic stem cell field have shown that ES cells can be differentiated into numerous cell types, particularly cortical precursor cells and cortical neurons. Therefore, I asked if ES cells can be induced to generate cortical radial precursors that can be used to model precursor cells in vivo, thereby providing us with an alternate source of cells and a system to complement our in vivo and primary culture work. Potentially, this model can be adapted to systems where primary cells are not available such as human models of development and disease and also providing us large quantities of cells for use in proteomic approaches.

Specifically, I set out to differentiate mouse ES cells to a cortical radial precursor lineage and determine if these cells exhibit characteristic of cortical precursor cells and that they differentiated to cells typically found in the cortex. The approach used was to adapt a recently published method for generating ES derived neural precursors using retinoic acid. I used immunocytochemical analysis with markers expressed by cortical radial precursors in vivo to determine that the ES-cell derived precursors a cortical radial precursor identity and that they differentiated to cortical neural cell types.

Secondly, most ES neural differentiation efforts and specifically differentiation towards a cortical lineage to date have been focused on developing efficient protocols to generate high yield cultures with high efficiency. There have been no efforts to determine if these cells respond to the same extracellular cues that regulate them during cortical development. The approach used was to examine the effects of growth factors that typically regulate the proliferation, differentiation and apoptosis of cortical radial precursors in the ES cell-derived cultures. I exposed the ES cell-derived precursors to fibroblast growth factor 2 and ciliary neurotrophic factor and assessed proliferation, differentiation and apoptosis by marker analysis and cell counting techniques.
Chapter 2

2 Experimental Procedures

2.1 Cell culture and growth factor treatments

2.1.1 Embryonic stem cell culture

Cell culture and propagation methods used for the R1 ES cell line were initially established and
described by Nagy et al. (1993). Initially, 1 stock vial of passage 11 R1 ES cells isolated from
E3.5 mouse blastocysts was thawed and expanded for an additional 2 passages, to establish a
common cell stock for all experiments. For each experiment, 1 vial containing $3 \times 10^6$ cell
was thawed and plated on a 60 mm dish on mytomycin inactivated feeders in media containing 2mM
GlutaMAX (Invitrogen), 0.1mM 2-mercaptoethanol (Sigma), 0.1mM MEM Non-essential
Amino-acids (Invitrogen), 1mM Sodium pyruvate (Invitrogen), 50 U (µg)/ml each
Penicillin/Streptomycin (Invitrogen), 1000 U/ml LIF (Chemicon), 15% ES qualified FBS
(Hyclone), high glucose, 4500 mg/liter DMEM (Gibco). Cells were grown in an incubator with a
constant 37°C and 5% CO₂. The reproducibility and quality of differentiations strongly
depended on the quality of ES cells and the culture method used. Therefore, highly proliferative
ES cells were kept at optimal densities to prevent differentiation (70-80% density at passaging).
Plates for passaging were selected on the basis of optimal ES cell colony morphology – oblong or
circular, refractive colonies with tight borders that did not touch. Plates were generally
subcultured every other day and media changed daily. Cells were passaged by treatment with
0.05% Trypsin, 0.53 mM EDTA (Invitrogen) to obtain a single cell suspension. Since the
presence of feeders prevents differentiation to a neural lineage (Bibel et al., 2007), cells were first
cultured on feeders for 3 passages and then cultured on dishes coated with 0.1% gelatin (Sigma)
for an additional 3 passages to remove the feeders. The passage ratio was maintained between 1:3
and 1:6 based on the growth patterns so that an average of 20-30 million cells on feeders or 15-25
million on gelatin could be collected from plates at the time of passaging.
2.1.2 Embryoid body culture

EB culture methods were adapted from Bibel et al. (2007) with some modifications. Two days after the third passage on gelatin, ES cells were trypsinized as described above and 4x10^6 cells were seeded on 10cm low-adhesion Corning dishes in 15ml media containing 2mM GlutaMAX (Invitrogen), 0.1mM 2-mercaptoethanol (Sigma), 0.1mM MEM Non-essential Amino-acids (Invitrogen), 50 U (µg)/ml each Penicillin/Streptomycin (Invitrogen), 10% ES qualified FBS (Hyclone), high glucose, 4500 mg/liter DMEM (Gibco). Cells were grown in an incubator at a constant 37°C and 5% CO₂. Media was changed every two days. EBs were treated with retinoic acid at days 4 and 6 of culture unless otherwise stated in the results section of this thesis.

Retinoic acid (Sigma) was made at 5mM in DMSO and stored in the dark for no longer than 2 weeks at -80°C. It was added to a final concentration of 5uM to EB media before EB resuspension.

2.1.3 ES-derived neural precursor culture

The differentiation procedure for neural precursors was adapted from Bibel et al. (2007) with several modifications. EBs were collected after 7 or 8 days of culture as stated in the results section, dissociated with 0.05% Trypsin in 0.05% EDTA-PBS (Sigma) and plated in 1:1 DMEM/F12 media containing 1% N2 supplement (Gibco), 2mM GlutaMAX (Invitrogen), 50 U (µg)/ml each Penicillin/Streptomycin (Invitrogen). Cells were seeded at a density of 3x10^5 cells/cm² on chamber slides coated with Poly-DL-ornithine hydrobromide (Sigma) and laminin (Roche). Media was changed at 2hrs and then again at 24hrs. At 48 hrs post-dissociation, media was changed to DMEM media containing 1% B27 supplement (Gibco), 2mM GlutaMAX (Invitrogen) and 50U (µg)/ml each Penicillin/Streptomycin (Invitrogen). Cells were subsequently maintained in this media with replacement every two days.

Media was supplemented with FGF2 (BD Biosciences) at 40ng/ml and/or CNTF (Cedarlane Laboratories) at 50ng/ml wherever stated in the results section.
2.2 Immunocytochemistry and quantitation

Cells were washed with PBS and fixed for 5 minutes with 4% paraformaldehyde. Cells were washed again with PBS, permeated for 5 minutes with 0.2% NP-40 in PBS and then blocked for 2 hours at room temperature with buffer containing 6% goat serum and 0.5% bovine serum albumin. Cells were incubated overnight at 4°C with primary antibodies in PBS containing 3% goat serum and 0.25% BSA. Primary antibodies were then removed with three washes with PBS. Cells were then incubated at room temperature for 45-60 minutes with secondary antibodies diluted in PBS containing 3% goat serum and 0.25% BSA. Secondary antibodies were removed with three washes in PBS and then cells were counterstained for 2 minutes with Hoechst 33258 (1:2000, Sigma). Samples were then mounted and examined by fluorescence microscopy. Digital image acquisition was performed using Northern Eclipse software (Empix Inc.) with a Sony XC-75CE CCD video camera. For quantification 4-16 randomly selected fields across wells of ES-derived precursor cultures were counted for every condition and for each experiment. All nuclei per field were counted in most experiments, unless cell density was too high in which case 200 cells per field were counted for every condition in each experiment. Images were analyzed using ImageJ 1.40g software (NIH, public domain). For all graphs, statistical analysis was performed using Student’s t-test and error bars indicate Standard Error of the Mean (SEM).

Primary antibodies

Immunocytochemistry was performed using rabbit anti-Pax-6 (1:2500, Covance), mouse anti-Pax-6 (1:200, Developmental Studies Hybridoma Bank), mouse and rabbit anti-βIII-tubulin (1:1000, Covance), mouse anti-Nestin (1:200 Chemicon), rabbit anti-Foxg1, rabbit anti-Emx1 (1:500, Santa Cruz, a gift from Dr. van der Kooy), rabbit anti-BLB (1:500, Abcam), mouse anti-KI67 (1:200, BD Bioscience), rabbit anti-GFAP (1:1000, Chemicon), rabbit anti-cleaved caspase 3 (1:500, Cell Signaling), rabbit anti-vGlut (1:500, Synaptic Systems), mouse anti-Gad67 (1:200, Invitrogen).

Secondary antibodies

Primary antibody binding was detected using the following secondary antibodies: Alexa Fluor 488 or 555 goat-anti-mouse or anti-rabbit (1:1000, Molecular probes).
Figure 2.1: Embryonic stem cell differentiation to cortical radial precursors and neurons – culture progression.

Embryonic stem cells are cultured for 3 passages on feeders and then for 3 passages without feeders. Embryoid bodies are established after 12 days of ES culture. Embryoid bodies are cultured for 8 days with media change every two days. Retinoic acid was added as indicated after 4 and 6 days of EB culture. EBs are then dissociated and plated in neural differentiation media.
Chapter 3

3 Results

Retinoic acid treatment of EBs induces differentiation of neural precursors and neurons.

Bibel and colleagues (2004) recently reported that treatment with retinoic acid of mouse ES cells leads to the generation of a uniform population of neural progenitors characteristic of radial precursors found in the developing dorsal telencephalon. Specifically, 90-99% of cells in their culture expressed the radial precursor markers nestin, RC2, BLBP and Pax-6 (Bibel et al., 2004). In a subsequent report, EBs prior to dissociation were shown to largely express the dorso-ventral marker Emx2 (Plachta et al., 2007). Thus, these radial precursors exhibited characteristics of forebrain lineages while the expression of more posterior neural tube markers was very low. Furthermore, these precursors differentiated to functional glutamatergic projection neurons, typically found in the cortex. Astrocytes and oligodendrocytes were also detected at late stages of culture, comprising less than 2% of cells. The purpose of these studies was to generate large quantities of highly homogenous neuronal cultures that could be used in biochemical approaches looking at neuronal biology, and thus a full characterization of precursors that generated these neurons was not a primary focus. These findings prompted us to ask whether this protocol can be adapted as an additional model to study the characteristics and differentiation potential of cortical precursors, a focus in our lab.

Initially, I established EBs from pluripotent mES cells and cultured them with and without RA treatment. After 8 days in culture, the EBs were dissociated, plated in neural differentiation medium and cultured further for another 8 days. As controls, RA untreated EBs were also dissociated and cultured identically. As early as two hours after plating in neural differentiation medium, cells derived from RA treated EBs had a spindle-like morphology typical of neural precursors while cells derived from untreated EBs displayed a large array of morphologies (Figure 3.1 a,b). To ask whether these cells were radial neural precursors that generated neurons as previously reported (Bibel et al., 2004; Plachta et al., 2004;
Nikoletopoulous et al., 2007), I first performed immunocytochemical analysis for the transcription factor Pax-6, a marker expressed by radial precursors in the cortex (Gotz et al., 1998) and the neuronal marker βIII-tubulin. At 1 day post-plating, almost all of the cells derived from RA treated EBs expressed either Pax-6 or βIII-tubulin (Figure 3.2 a). In contrast, cells derived from untreated EBs expressed Pax-6 in patches, while a few cells could be found expressing βIII-tubulin (Figure 3.2 b). Quantification at 1 DIV showed that approximately 30-40% of cells expressed Pax-6 in both conditions (Figure 3.2 e). However, ~60% of cells expressed the neuronal marker βIII-tubulin with RA treatment in contrast to less than 10% without RA, and while only ~4% of RA treated cells were negative for Pax-6 or βIII-tubulin, the majority of untreated cells did not express either of these two markers (Figure 3.2 e).

To determine the differentiation potential of these cells more long-term, immunocytochemical and quantitative analysis was performed, culturing the cells in neural differentiation conditions for 8 days rather than 1 day. Cultures derived from RA treated EBs consisted of mostly neuronal cells expressing βIII-tubulin (~90%) that had developed dense neurite networks (Figure 3.2 c, f). The proportion of Pax-6 expressing cells was found to be reduced by the same amount that βIII-tubulin expressing cells was increased (Figure 3.2 f), suggesting that the Pax-6 expressing cells differentiated into neurons over 8 days in culture. In contrast, cultures derived from untreated EBs consisted mostly (~80%) of cells that did not express either Pax-6 or βIII-tubulin (Figure 3.2 d, f). These results demonstrate that unmanipulated ES cells can generate a small proportion of neural precursors and neurons, but that, under these culture conditions, RA treatment pushes ES cells towards a neural lineage with high efficiency as previously reported.

As I was interested in developing an alternate model for cortical precursor differentiation, a starting population of ~40% Pax-6 expressing cells with remaining cells already terminally differentiated into neurons was inadequate (Figure 3.2 e). I therefore asked if the proportion of precursors could be increased if I dissociated RA-treated EBs at shorter timepoints. In this regard, my initial experiments (see Figure 3.2 e) indicated that neuronal differentiation had already occurred within the EBs prior to their dissociation. I therefore dissociated EBs 1 day earlier, at Day 7 of EB culture, and examined the proportion of precursors and neurons at 1 day post-dissociation and plating in neural differentiation media. Immunocytochemistry for the
precursor marker Pax-6 and the neuronal marker βIII-tubulin and quantitative analysis showed that in cultures derived from Day 7 EBs, the proportion of Pax-6 expressing cells was increased to ~70% while the proportion of neurons decreased to ~20% compared to the Day 8 EB dissociations (Figure 3.3 a,b,c). Thus all subsequent analysis of the ES-derived neural precursor culture was done on EBs dissociated at Day 7.

To determine the differentiation potential of these younger Day 7 EB cultures, I directly compared EB dissociations cultured with and without RA treatment and analyzed them by immunocytochemistry for Pax-6 and βIII-tubulin expression at 1 day (Figure 3.4 a, b) and 8 days (Figure 3.4 c, d) in neural differentiation media. Quantitative analysis revealed that, when EBs were not treated with RA, they contained only a low percentage of cells that were Pax-6 or βIII-tubulin positive and that this was similar whether they were dissociated at 7 or 8 days as EBs. Specifically, at 1DIV Pax-6 expressing cells were ~30-40%, 3-8% expressed βIII-tubulin, and ~50% did not express either marker (Figure 3.4 b, e) while at 8DIV 10-30% expressed Pax-6, 5-8% expressed βIII-tubulin and the large majority, 60-80% did not express either marker (Figure 3.4 d, f) results similar to 8 day dissociated EBs (Figure 3.2 e, f). In contrast, the RA treated cultures consisted of ~70% Pax-6 positive precursors, 20% βIII-tubulin positive neurons and ~10% of cells not expressing either marker at day 1 (Figure 3.4 e). At 8 days, the proportion of βIII-tubulin expressing cells increased to ~70%, 20% of cells continued to express Pax-6 and ~8% did not express either marker (Figure 3.4 f). These results indicate that dissociation of Day 7 RA treated EBs for neural precursor and neuronal differentiation was as efficient in terms of neural induction as Day 8 EB dissociation but had a greater proportion of precursors at early time points.

Analysis of culture composition

**ES-derived neural precursors have cortical radial precursor identity**

These results are consistent with the interpretation that RA induces the differentiation of ES cells into neural radial precursors. The transcription factor Pax-6 as well as other markers like nestin, BLBP, GLAST and RC2 are expressed by radial precursors throughout the developing CNS (reviewed in Jessell, 2000; Guillemot, 2007) but Pax-6 was the only marker used by Bibel
et al., (2004, 2007) to definitively establish these cultures as cortical. To more definitively ask whether these were forebrain radial precursors, I examined the expression of specific proteins such as Foxg1, which is a transcription factor specifying forebrain precursors (Martynoga et al., 2005). Furthermore, I also examined the expression of the transcription factor Emx1, which is specifically expressed in radial precursors of the dorsal forebrain (Cecchi & Boncinelly, 2000).

To determine the identity of the Pax-6 expressing ES-derived precursors, I performed immunocytochemical analysis for these general and specific radial precursor markers at 1 DIV. The ES-derived precursors were found to express nestin, Foxg1, Emx1 and BLBP (Figure 3.5 a, b, c, d). Quantitative analysis revealed that ~80% of cells expressed Foxg1, ~81% expressed Emx1, 71% expressed nestin and 65% expressed BLBP individually (Figure 3.5 f). Furthermore, over 80% of the Pax-6 expressing cells also expressed nestin, Foxg1 and Emx1 (Figure 3.5 g). Cortical precursor cells display heterogeneity in that specific markers are not expressed by all of the cells all of the time indicating the presence of distinct precursor populations (Hartfuss et al., 2001). Moreover, regional markers like Pax-6 and Emx1 are expressed in gradients, a characteristic that serves to delineate boundaries between the prospective cortex and other structures (Puelles et al., 2000). Thus, while the large majority of the ES-derived precursors display a phenotype similar to cortical radial precursors, there appears to be some heterogeneity that may simply reflect the normal diversity seen within cortical precursors in vivo.

Since the transcription factor Pax-6 is also expressed by pancreatic islet cells during development (St-Onge et al., 1997), I also performed immunocytochemical analysis for Pax-6 and the pancreatic islet cell specific marker Pdx1 (Figure 3.5 e). None of the cells in culture expressed Pdx1 excluding the possibility that the Pax-6 expressing cells may be of this non-neural lineage. These experiments demonstrate that the ES-derived precursors have characteristics of radial precursors of the cortex.
**ES-derived precursors generate glutamatergic and GABAergic neurons**

The cortex is composed of two major neuronal subtypes, excitatory glutamatergic pyramidal neurons and inhibitory GABAergic interneurons. Cortical radial precursors are the source of all glutamatergic neurons of the cortex (Malatesta et al., 2003). The majority of GABAergic neurons are derived from the ventral telencephalon and migrate to populate the cortex while a small number of interneurons are generated in the cortex itself (Campbell, 2003).

To examine the neuronal sub-types generated by the ES-derived precursors, I performed immunocytochemistry with antibodies that identify glutamatergic neurons and GABAergic neurons, vGlut1 and Gad67, respectively. Antibody staining showed that both neuron types were present in the culture and that expression of these two markers did not overlap (Figure 3.6a). Furthermore, analysis showed that although the majority of neurons expressed the glutamatergic marker vGlut, about 13% expressed the interneuron marker Gad67 (Figure 3.6b). Therefore, the ES-derived precursors generate neurons with characteristics of cortical neurons.

**ES-derived precursors generate astrocytes**

Multipotent cortical precursors *in vivo* and *in vitro* generate neurons first and then glial cells. Bibel et al. (2004) reported that the RA treated EBs generate a uniform population of mostly neurogenic precursors and glial cells that appeared only after long culture periods and in negligible proportions. I therefore examined the potential of the ES-derived precursors to generate astrocytes. Immunocytochemical analysis for the astrocyte specific marker GFAP showed that astrocytes are generated in this culture. The earliest time point at which expression of GFAP could be detected was 6 days post-EB dissociation (6 DIV) where expression was sporadic and could not be quantified. However, quantitative analysis at 8 DIV showed that although the majority of the culture was comprised of neurons, about 2% of the cells now expressed GFAP (Figure 3.7a, b). These results demonstrate that these ES-derived precursors are competent to generate astrocytes and do so in temporal order, after most of neurogenesis is complete, an observation consistent with corticogenesis *in vivo* and in primary culture.
**ES-derived precursor culture includes oligodendrocytes and Nkx2.1 expressing cells**

Multipotent cortical radial precursors also generate oligodendrocytes. I therefore set out to examine the ability of the ES-derived precursors to generate oligodendrocytes. Since there are two major waves of oligodendrogenesis in the cortex, early and late (Kessaris et al., 2006), I first examined the culture by immunocytochemistry for the oligodendrocyte marker O4 at an early time point, 1 DIV (Figure 3.8a). Quantitative analysis revealed that ~2% of cells expressed O4, and that these cells did not express the precursor marker Pax-6 (Figure 3.8b). Late time points remain to be examined in this culture. In this case, the appearance of oligodendrocytes is out of temporal order compared with cortical oligodendrocytes in vivo, which happens after the neurogenic period is complete. However, oligodendrocytes are specified as early as E12 in the ventral telencephalon and their appearance early in this culture may indicate the presence of ventrally specified precursors.

In the cortex, oligodendrocytes are first derived from Nkx2.1 expressing precursors that either migrate from the ventral telencephalon during the early wave of oligodendrogenesis (Kessaris et al., 2006) or are generated in small numbers in the cortex itself and may also be the source of some cortically derived interneurons. I therefore asked if Nkx2.1 expressing precursors are present in the ES-derived culture. Immunocytochemical analysis using both Pax-6 and Nkx2.1 precursor markers showed that both were expressed at 1DIV (Figure 3.9a). Quantification revealed that about 2% of cells expressed Nkx2.1 only and ~1% expressed both Pax-6 and Nkx2.1 (Figure 3.9b). Typically in vivo, Pax-6 and Nkx2.1 have distinct expression patterns that generally do not overlap allowing distinction between dorsal and ventral telencephalic structures. However, although no specific studies have shown this, cells in the border regions between their respective structures have the potential to overlap. (Puelles et al, 2001). Taken together, these results suggest precursors with a ventral identity are present in this culture, an observation made in other reports of ES-differentiation of cortical precursors (Wantanabe et al., 2005). However, the regional identity of the Nkx2.1 expressing cells is still unclear. Analysis of Nkx2.1 along with other ventral markers at this and later time points remains to be performed.
These results demonstrate that the ES-derived precursors are capable of making all three neural cell types and that the precursor pool is more heterogenous than previously reported but perhaps typical of what is seen in vivo (Bibel et al., 2004).

**Growth factor analysis**

Growth factor signaling affects all aspects of corticogenesis both in vivo and in vitro. The growth factor FGF2 and the cytokine CNTF are two extrinsic cues that regulate cortical precursor development. To model corticogenesis in an ES based system, it was necessary to ask if the same cues can induce similar responses as in vivo or in primary culture.

**FGF2 affects proliferation and survival of ES-derived precursors and neurons**

The growth factor FGF2 has been implicated in the proliferation, survival, self-renewal and differentiation of neural precursors and it is a key factor for generating and maintaining our primary cortical cultures. Since FGF2 has been shown to be a proliferative and survival factor in vivo (Vaccarino et al., 1999; Raballo et al., 2000) and in vitro (Ghosh & Greenberg, 1995), I asked if it could also regulate these processes in the ES-derived precursor cultures. To address this question, EBs were dissociated and plated in media with and without FGF2, which was replaced fresh at each media change. Initially, I looked at the global effect FGF2 might be having on the ES-derived culture by counting total cell numbers by Hoechst nuclei staining without and with FGF2 treatment at 1DIV (Figure 3.10 a,b) and 3DIV (Figure 3.10 c,d). This analysis showed a small but significant increase, from 95 to 125 cells per field as early as 1DIV in the presence of FGF2. By 3DIV, FGF2 had caused a significant 2 fold increase in the total number of cells, averaging 260 cells per field compared with 115 cells per field without FGF2. These results indicated that FGF2 had affected proliferation and/or survival of cells in this culture.

To ask how the proportions of precursor and neuronal cell populations had changed in the presence of FGF2, I performed immunocytochemical analysis for the precursor marker Pax-6
and the neuronal marker βIII-tubulin at 1 and 3DIV (Figure 3.11 a-h). Quantitative analysis revealed that at 1DIV the respective proportions of Pax-6 precursors and βIII-tubulin neurons were not significantly affected (Figure 3.11 i). However, at 3DIV the proportion of Pax-6 positive cells was significantly increased from 14% without FGF2 to nearly 50% in the presence of FGF2 (Figure 3.11 i). In contrast, the proportion of βIII-tubulin positive neurons was significantly decreased from about 80% without FGF2 to about 50%, a change matching the increase in the number of Pax-6 positive precursors (Figure 3.11 i). These results suggested that FGF2 maintains the pool of precursors and prevents their differentiation into neurons, as has been observed for other assays of ES neural differentiation (Tropepe et al., 2001, Li et al., 2009).

To ask whether FGF2 affects the proliferation of precursors, I then performed immunocytochemical analysis for the precursor marker Pax-6 and Ki67 to identify dividing cells at both 1DIV (Figure 3.12 a, c) and 3DIV (Figure 3.12 b, d). Quantification of double-positive cells revealed that while at 1DIV there was no detectable change in cycling precursors with or without FGF2, by 3DIV FGF2 treatment increased the proportion of cycling precursors 4 fold from 8% without FGF2 to 34% with FGF2 (Figure 3.12 e). Furthermore, while the proportion of Pax-6 cells that were Ki67 positive remained at ~80% at 1DIV, it was significantly increased by FGF2 at 3DIV from 55 to 75% (Figure 3.12 f). This effect suggests that FGF2 promotes proliferation of precursors and maintains the pool of proliferating precursors. This does not rule out the possibility that it also prevents differentiation and/or increases survival of precursors. However, either with or without FGF2, Pax-6 positive precursors can still differentiate. Both the Pax-6 and proliferating Pax-6 precursor pools are significantly depleted between 1 and 3DIV regardless of the treatment (Figure 3.11 i; 3.12 e), indicating that other intrinsic or extrinsic signals are present in this culture which promote differentiation.

This analysis also demonstrated a small but significant increase in the proportion of Ki67 positive cells that did not express Pax-6 at 1DIV from 1-2% without FGF2 to 3-4% with FGF2 (Figure 3.12, g). The nature of these other proliferating cells remains to be determined.

FGF2 increases survival of both precursors and neurons in vitro and the increases in cell numbers seen in this culture with FGF2 may in part be due to survival effects. In order to
evaluate this possibility, I independently measured survival by immunostaining with the apoptosis marker cleaved-caspase 3 in combination with precursor specific marker, Pax-6 at 1DIV (Figure 3.13 a, b). I focused on the precursor proportion since it was the dominant cell population at this time point (Figure 3.11 i). I first quantified the total cells in this culture that were positive for cleaved-caspase 3 (Figure 3.13 c) as well as cells with fragmented and pyknotic nuclei by Hoechst (Figure 3.13 e). In cultures without FGF2, about 7% of cells were apoptotic as assessed by cleaved caspase 3. FGF2 reduced this significantly to about 5%. Similarly, in cultures without FGF2 fragmented and pyknotic nuclei amounted to about 11%, and this was reduced to about 8% in the presence of FGF2. Furthermore, when I analyzed the Pax-6 positive population for cleaved caspase 3 expression, this analysis showed that in the absence of FGF2, ~6% of Pax-6 positive cell population was apoptotic while ~2% of the Pax-6 negative cell population expressed cleaved caspase 3. FGF2 significantly decreased the proportion of Pax-6 positive precursors positive for this marker at this time point from ~6% to 3%, while not affecting other cell populations (Figure 3.13 d, f). Thus, FGF2 enhances survival of precursors at this time point.

A similar analysis was performed at 3DIV using the neuronal specific marker βIII-tubulin and the apoptosis marker cleaved-caspase 3 (Figure 3.14 a,b) to ask whether FGF2 also promoted survival of newly-born neurons. I first quantified total cells positive for cleaved-caspase 3 (Figure 3.14 c) as well as cells with fragmented and pyknotic nuclei by Hoechst (Figure 3.14 e). In cultures without FGF2, about 24% of cells were apoptotic as indicated by cleaved caspase 3. FGF2 reduced this significantly to about 6%. Similarly, in cultures without FGF2 fragmented and pyknotic nuclei amounted to about 29%, a proportion reduced to about 6% in the presence of FGF2. Neuronal marker analysis in combination with the apoptosis marker cleaved caspase 3 showed that in the absence FGF2, ~14% of the βIII-tubulin positive cell population was apoptotic (Figure 3.14 d). This was a significantly higher proportion than the 4% of βIII-tubulin positive cells dying by apoptosis in the presence of FGF2 at this time point. A smaller but significant change in cells dying by apoptosis was observed in the cell fraction not expressing βIII-tubulin, presumably neural precursors. Similar results were obtained when counting fragmented and pyknotic nuclei in combination with the neuronal specific marker βIII-tubulin.
Thus, FGF2 has a major survival effect in these cultures promoting survival of radial precursors and of newly-born neurons.

**CNTF induces ES-cell derived radial precursors to generate astrocytes versus neurons**

Another well characterized cue that regulates cortical precursors is the cytokine CNTF. CNTF has been shown to induce premature astrocyte generation and can increase the number of astrocytes and inhibit neurogenesis of cortical precursors both in vivo and in vitro (Bonni et al., 1997; Rajan & McKay 1998; Barnabe-Heider, 2005). In addition, another cytokine, CT-1, was shown to be the key factor inducing the neurogenic-to-gliogenic switch in cortical precursors (Barnabe-Heider et al., 2005). Both CNTF and CT-1 utilize similar receptors and signal astrogenesis by similar mechanisms (Rajan & McKay, 1998; Barnabe-Heider et al., 2005).

I therefore asked whether gliogenic cytokines also induce astrocyte formation from my ES-derived cortical precursors. I initially showed in a previous section that upon dissociation, the RA treated EBs give rise to cortical radial precursors that first produced neurons, with astrocytes first being detected by GFAP expression at 6 DIV post-plating in neural differentiation medium. I therefore dissociated EBs, plated the cells in media with and without CNTF, which was replaced fresh at each media change, and performed immunocytochemistry at various time points. This analysis of ES-derived precursor cultures revealed that in cultures treated with CNTF and not in control cultures, the astrocyte marker GFAP could be detected as early as 4 DIV (Figure 3.15 a,b) when it was expressed in approximately 1% of the cells (Figure 3.15 c).

I then performed a more long term analysis, culturing cells with and without CNTF for 8 days, a time point well characterized in terms of culture composition (see previous sections). These cultures were analyzed by immunocytochemistry for GFAP (Figure 3.16 a, b). Quantification at 8 DIV revealed that CNTF caused a significant increase in the percent of GFAP positive cells present in the culture (Figure 3.16 c) from 2 to 7%. Furthermore, when total astrocytes per field were counted, CNTF increased the average number of astrocytes 3 fold over the control (Figure 3.16 d).
Since both CNTF and CT-1 can activate the JAK-STAT signaling pathway, which was shown to enhance gliogenesis and inhibit neurogenesis (Bonni et al., 1997; Barnabe-Heider et al., 2005), I then asked whether CNTF also affected the genesis of neurons in this culture. Quantification of βIII-tubulin positive cells revealed that CNTF caused a small but significant decrease in the number of neurons generated in the presence of CNTF (Figure 3.16 e). This decrease in the proportion of neurons (~10%) is roughly similar to the increase in astrocytes (~6%). Whether this reflects astrocytes being made at the expense of neurons could be determined by performing clonal analysis.

To ask if CNTF also promoted proliferation of astrocytes, I analyzed cultures by immunocytochemistry for the cell cycle marker Ki67 and the astrocyte marker GFAP. Quantification showed that CNTF caused a significant increase of approximately two-fold in the total number of cells expressing Ki67 (Figure 3.17 a). A significant increase in Ki67 positive cells that expressed GFAP was also observed with CNTF compared to controls, from ~60% to 80% (Figure 3.17 b). These data suggest that CNTF causes an overall increase in astrocyte genesis, part of which is likely due to its proliferative effects on astrocytes.

**FGF2 potentiates the effects of CNTF on ES-derived cortical radial precursors**

In our lab, primary cortical precursors are cultured in the presence of FGF2, largely because, as seen here, in the absence of FGF2, a significant proportion of neurons and precursors die. I therefore performed similar CNTF experiments in the presence of FGF2 to more closely mimic these primary culture studies. In addition, FGF2 has been shown to positively regulate the competence of precursors to respond to gliogenic cues such as CNTF (Song & Ghosh, 2004). To do these studies, at the time of EB dissociation, the ES-derived precursors were plated in media with FGF2 or FGF2 and CNTF, with growth factors added fresh at each media change. The mitogenic effects of FGF2 increased cell density such that the latest time point that could be quantified was 4 DIV. I therefore performed immunocytochemical analysis of ES-derived precursor cultures at this time point. In contrast to what was seen at 4 DIV in the absence of FGF2, the astrocyte marker GFAP could be detected at this time point both with and without CNTF (Figure 3.19 a, b). However, quantitative analysis revealed that although GFAP could be
detected at 4 DIV in cultures treated with FGF2 alone, it was expressed in only ~0.35% of cells compared to ~2% with FGF2 and CNTF (Figure 3.19 c) or to ~0.7% with CNTF alone (Figure 3.15 c). This significant increase in GFAP expression is consistent with previous studies showing that FGF2 enhances astrogenesis in response to gliogenic cytokines like CNTF (Song & Ghosh, 2004). The increase in number of cells expressing GFAP in the presence of FGF2 and CNTF at 4 DIV was comparable to that obtained with CNTF alone after 8 DIV (Figure 3.16 c).

Quantification of the total number of GFAP positive cells per field confirmed this increase in astrogenesis showing that CNTF increased their average number 8 fold in the presence of FGF2 compared to FGF2 alone at 4 DIV (Figure 3.18 d).

Having demonstrated that FGF2 enhances CNTF’s ability to promote astrogenesis, I then asked whether this growth factor combination also resulted in changes in the proportion of neurons. To address this question, I quantified the proportion of βIII-tubulin positive cells at 4 DIV. Interestingly, no significant change in the proportion of neurons was detected at this time point (Figure 3.18 e). Therefore, I then asked if the proportion of other cell populations, namely precursors, was affected. Interestingly, quantitative analysis of immunocytochemically stained cultures for the radial precursor marker Pax-6 and the neuronal marker βIII-tubulin revealed that the proportion of cells expressing Pax-6 was decreased from ~40% to ~25% in the presence of both FGF2 and CNTF (Figure 3.18 e). This decrease in Pax-6 positive precursors was accompanied by an almost 10% increase in the proportion of cells that did not express either Pax-6 or βIII- tubulin (Figure 2.18 e). While ~2% of these are undoubtedly astrocytes (Figure 2.18 c), the nature of the remainder of these cells remains to be determined.
Figure 3.1: Retinoic acid induces ES cells to adopt a neural radial precursor morphology

(a,b) Phase microscopy images of ES-derived cultures at 1DIV post-EB dissociation. Arrows denote cells with radial morphology and two processes present in cultures derived from RA treated EBs. (a). Arrows denote cells with various morphologies present in cultures derived from untreated EBs. Scale bar = 50µm.
Figure 3.2: Dissociation of RA-treated EBs give rise to cultures containing Pax-6 positive neural precursors at 1DIV that differentiate to βIII-tubulin positive neurons by 8DIV

(a-d) Immunocytochemical analysis for Pax-6 (red) and βIII-tubulin (green) in cultures derived from RA treated EBs at 1DIV (a) and 8DIV (c), and from untreated EBs at 1DIV (b) and 8DIV (d) post-EB dissociation. Cells were counterstained with Hoechst 33258 (blue) to show all nuclei in the field. Scale bar = 50µm.

(e) Quantification of the percentage of cells expressing Pax-6 and βIII-tubulin with and without RA at 1DIV from one experiment. (f) Quantification of the percentage of cells expressing Pax-6 and βIII-tubulin with and without RA at 8DIV from one experiment. Eight random fields were counted per condition per time point with at least 200 cells counted per field. Error bars indicate SEM.
Figure 3.3: Dissociation of RA treated EBs cultured for 7 days instead of 8 days in yields more precursors and less neurons at 1DIV

(a,b) Immunocytochemical analysis for Pax-6 (red) and βIII-tubulin (green) of cultures at 1DIV from RA treated EBs for 8 days (a) or 7 days (b). Cells were counterstained with Hoechst 33258 (blue) to show all nuclei in the field. Scale bar = 50um (c) Quantification of the percentage of cells expressing Pax-6 and βIII-tubulin in 1DIV cultures derived from RA treated EBs cultured for 8 versus 7 days. One experiment is shown. Eight random fields were counted per condition with at least 200 cells counted per field. Error bars indicate SEM.
Figure 3.4: Dissociation of EBs cultured for 7 days in RA give rise to cultures containing Pax-6 positive neural precursors at 1DIV that differentiate to βIII-tubulin positive neurons by 8DIV.

(a-d) Immunocytochemical analysis for Pax-6 (red) and βIII-tubulin (green) in cultures derived from RA treated EBs at 1DIV (a) and 8DIV (c), and from untreated EBs at 1DIV (b) and 8DIV (d) post-EB dissociation. Cells were counterstained with Hoechst 33258 (blue) to show all nuclei in the field. Scale bar = 50µm.

(e) Quantification of the percentage of cells expressing Pax-6 and βIII-tubulin with and without RA at 1DIV. (f) Quantification of the percentage of cells expressing Pax-6 and βIII-tubulin with and without RA at 8DIV. Eight random fields were counted per condition per time point in one experiment with 200 or more cells were counted per field. Error bars indicate SEM.
Figure 3.5: Neural precursors derived from RA-treated EBs express cortical and forebrain radial precursor markers.

(a) Immunocytochemical analysis for Pax-6 (red) and Nestin (green) at 1DIV post-EB dissociation. (b) Immunocytochemical analysis for Pax-6 (green) and FoxG1 (red) at 1DIV post-EB dissociation. (c) Immunocytochemical analysis for Pax-6 (green) and Emx1 (red) at 1DIV post-EB dissociation. (d) Immunocytochemical analysis for BLBP (red) and nestin (green) at 1DIV post-EB dissociation (e) Immunocytochemical analysis for Pdx1 (red) and nestin (green) at 1DIV post-EB dissociation. Cells were counterstained with Hoechst 33258 (blue) to show all nuclei in the field. Scale bar = 50um. (f) Quantitative analysis of the percentages of cells expressing Pax-6, Foxg1, Emx1, nestin and BLBP individually. Four to eight random fields were counted for each marker. Data is representative of 3 independent experiments for Pax-6, 1 experiment for Foxg1, 2 independent experiments for Emx1, 2 independent experiments for nestin and 1 experiment for BLBP. (g) Quantitative analysis of the percentages of Pax-6 positive cells that also expressed nestin, Foxg1 or Emx1. Four to eight random fields were counted per experiment per marker with at least 200 cells per field counted. Results are representative of one experiment per marker. Error bars indicate SEM.
**Figure 3.6: ES-derived precursors generate glutamatergic and GABAergic neurons**

(a) Immunocytochemical analysis of GAD67 (green) and vGlut (red) at 8DIV. Cells were counterstained with Hoechst 33258 (blue) to show all nuclei in the field. Scale bar = 50µm. (b) Quantitative analysis of the percentages of cells expressing GAD67 and βIII-tubulin 8DIV. Results represent one experiment where 4 random fields were counted and at least 200 cells counted per field. Error bars indicate SEM.
**Figure 3.7: ES-derived precursors generate astrocytes**

(a) Immunocytochemical analysis for GFAP (red) and βIII-tubulin (green) at 8DIV. Cells were counterstained with Hoechst 33258 (blue) to show all nuclei in the field. Scale bar = 50µm. (b) Quantitative analysis of cells expressing either GFAP or βIII-tubulin at 8DIV. Results were pooled from two independent experiments with 8 random fields counted per experiment and at least 200 cells counted per field. Error bars indicate SEM.
Figure 3.8: ES-derived precursors generate oligodendrocytes

(a) Immunocytochemical analysis of Pax-6 (green) and O4 (red) at 1DIV. Cells were counterstained with Hoechst 33258 (blue) to show all nuclei in the field. Scale bar = 50µm. (b) Quantitative analysis of the percentage of cells expressing either Pax-6 or O4 positive cells at 1DIV. Results were pooled from two independent experiments with 4 random fields counted for each experiment and at least 200 cells per field. Error bars indicate SEM.
**Figure 3.9: ES-derived precursors generate Nkx2.1 expressing precursors**

(a) Immunocytochemical analysis of Pax-6 (green) and Nkx2.1 (red) at 1DIV. Cells were counterstained with Hoechst 33258 (blue) to show all nuclei in the field. Short arrows indicate cells positive for Nkx2.1. Long arrows indicate double positive cells. Scale bar = 50µm. (b) Quantitative analysis of cells expressing Nkx2.1 and Nkx2.1 positive cells expressing Pax-6 both at 1DIV. Results are representative of 1 experiment where 4 random fields were counted with 200 cells counted per field. Error bars indicate SEM.
Figure 3.10: FGF2 affects the ES-derived culture by globally increasing cell numbers

(a-d) Hoechst 33258 staining showing all nuclei at 1 DIV without FGF2 (a) and with FGF2 (c) and at 3DIV without FGF2 (b) and with FGF2 (d). Scale bar = 50µm.
(e) Quantification of the average number of cells per field with and without FGF2 at 1 and 3DIV. Results were pooled from three independent experiments where 4 random fields were counted per condition per time point per experiment. Error bars indicate SEM. **p<0.01 and ***p<0.001 (Student’s t test)
Figure 3.11: FGF2 increases the proportion of Pax-6 positive precursors and decreases the proportion of neurons at 3DIV

(a-d) Immunocytochemical analysis for the precursor marker Pax-6 (red) without FGF2 (a) and with FGF2 (b) at 1DIV. Immunocytochemical analysis for the neuronal marker βIII-tubulin (green) without FGF2 (c) and with FGF2 (d) at 1DIV.

(e-h) Immunocytochemical analysis for the precursor marker Pax-6 (red) without FGF2 (e) and with FGF2 (f) at 3DIV. Immunocytochemical analysis for the neuronal marker βIII-tubulin (green) without FGF2 (g) and with FGF2 (h) at 3DIV. Cells were counterstained with Hoechst 33258 (blue) to show all nuclei in the field. Scale bar = 50µm. (e) Quantification of the percentage of cells positive for Pax-6 and βIII-tubulin with and without FGF2 at 1 and 3DIV. Results were pooled from three independent experiments where 4 fields were counted per condition per time point per experiment with 200 cells counted per field. Error bars indicate SEM.

***p<0.001 (Student’s t test)
Figure 3.12: FGF2 affects proliferation of Pax-6 positive precursors

(a,c) Immunocytochemical analysis for Pax-6 (red) and Ki67 (green) at 1 DIV without FGF2 (a) and with FGF2 (c). (b,d) Immunocytochemical analysis for Pax-6 (red) and Ki67 (green) at 3DIV without FGF2 (b) and with FGF2 (d). Cells were counterstained with Hoechst 33258 (blue) to show all nuclei in the field. Scale bar = 50µm. (e) Quantitative analysis of the percentage of Pax-6 and Ki67 double positive cells with and without FGF2 at 1 and 3DIV. (f) Quantitative analysis of the percentage of cells that are double positive for Pax-6 and Ki67 out of the proportion of Pax-6 positive cells with and without FGF2 at 1 and 3DIV. (g) Quantitative analysis of the percentage of cells positive for Ki67 that do not express Pax-6 with and without FGF2 at 1DIV. Results were pooled from three independent experiments with 4 random fields counted per condition per time point per experiment and 200 cells counted per field. Error bars indicate SEM. *p<0.05, **p<0.01 and ***p<0.001 (Student’s t test)
Figure 3.13: FGF2 increases survival of ES-derived precursors at 1DIV

(a-b) Immunocytochemical analysis for Pax-6 (red) and cleaved-caspase 3 (green) at 1 DIV without FGF2 (a) and with FGF2 (b). Cells were counterstained with Hoechst 33258 (blue) to show all nuclei in the field. Scale bar = 50µm. (c) Quantification of the percentage of cells positive for cleaved-caspase 3 with and without FGF2 at 1DIV. (d) Quantitative analysis of the percentage of cells positive for cleaved-caspase 3 and Pax-6 with and without FGF2 at 1DIV. (e) Quantification of the percentage of dead cells (fragmented and pyknotic nuclei) by Hoechst staining with and without FGF2 at 1DIV. (f) Quantification of the percentage of dead cells (fragmented and pyknotic nuclei) by Hoechst staining that are Pax-6 positive with and without FGF2 at 1DIV. Results were pooled from three independent experiments with 4 random fields counted per condition per experiment with 200 cells counted on each field. Error bars indicate SEM. *p<0.05 ***p<0.001 (Student’s t test)
Figure 3.14: FGF2 increases survival of ES-derived neurons at 3DIV

(a-b) Immunocytochemical analysis for βIII-tubulin (green) and cleaved-caspase 3 (red) at 3DIV without FGF2 (a) and with FGF2 (b). Cells were counterstained with Hoechst 33258 (blue) to show all nuclei in the field. Scale bar = 50µm. (c) Quantification of the percentage of cells positive for cleaved-caspase 3 with and without FGF2 at 3DIV. (d) Quantitative analysis of the percentage of cells positive for cleaved-caspase 3 and βIII-tubulin with and without FGF2 at 3DIV. (e) Quantification of the percentage of dead cells (fragmented and pyknotic nuclei) by Hoechst staining with and without FGF2 at 1DIV. (f) Quantification of the percentage of dead cells (fragmented and pyknotic nuclei) by Hoechst staining and βIII-tubulin positive cells with and without FGF2 at 1DIV. Results were pooled from three independent experiments with 4 random fields counted per condition per experiment with 200 cells counted on each field. Error bars indicate SEM. *p<0.05 **p<0.01 ***p<0.001 (Student’s t-test)
Figure 3.15: CNTF induces precocious astrocyte formation

(a,b) Immunocytochemical analysis for GFAP (green) in ES-derived cortical radial precursors without CNTF (a) and with CNTF (b). Cells were cultured for 4 DIV. Cultures were counterstained with Hoechst 33258 (blue) to show all nuclei in the field. Scale bar = 50μm. (c) Quantitative analysis of the percentage of GFAP positive cells with and without CNTF. Results were pooled from two independent experiments with 4 random fields counted per condition per experiment with over 200 cells counted on each field. Error bars indicate SEM.
Figure 3.16: CNTF increases the number of astrocytes in ES-derived cortical radial precursor cultures

(a,b) Immunocytochemical analysis for GFAP (green) in ES-derived cortical radial precursors without CNTF (a) and with CNTF (b). Cells were cultured for 8 DIV. Cultures were counterstained with Hoechst 33258 (blue) to show all nuclei in the field. Scale bar = 50µm.

(c) Quantitative analysis of the percentage of GFAP positive cells in cultures with and without CNTF at 8DIV. Results represent two pooled experiments where 4 random fields were counted per condition per experiment with 200-700 cells counted per field. (d) Quantitative analysis of the average numbers of GFAP positive cells per field with and without CNTF. Results represent two pooled experiments where over 16-20 random fields were counted. (e) Quantitative analysis of the percentage of cells expressing βIII-tubulin in cultures with and without CNTF at 8DIV. Results represent two pooled experiments where 4 random fields were counted per condition per experiment with 200-400 cells counted per field. Error bars indicate SEM. *p<0.05 **p<0.01 and ***p<0.001 (Student’s t test)
Figure 3.17: CNTF increases the number of cycling cells and cycling astrocytes. 

(a) Quantification of the percentage of cells expressing Ki67 in cultures with and without CNTF at 8DIV. (b) Quantification of the percentage of GFAP-positive cells expressing Ki67 in cultures with and without CNTF at 8DIV. Results represent two pooled experiments where 4 random fields were counted per condition per experiment with at least 200 cells counted per field. Error bars indicate SEM. 
* p<0.05 and ** p<0.01 (Student’s t test)
Figure 3.18: CNTF increases precocious astrocyte formation in the presence of FGF2 at 4DIV

(a,b) Immunocytochemical analysis for GFAP (green) in ES-derived cortical radial precursors with FGF2 (a) and with FGF2+CNTF (b). Cells were cultured for 4 DIV. Cultures were counterstained with Hoechst 33258 (blue) to show all nuclei in the field. Scale bar = 50 µm. (c) Quantification of the percentage of cells expressing GFAP in cultures with FGF2 or with FGF2+CNTF at 4DIV. (d) Quantification of average numbers of GFAP positive cells per field with FGF2 or FGF2+CNTF. Results are representative of one experiment where 16-20 random fields were counted per condition. (e) Quantification of the percentage of cells expressing Pax-6 or βIII-tubulin in cultures with FGF2 or FGF2+CNTF at 4DIV. Results are representative of one experiment where 4 random fields were counted per experiment per condition with at least 200 cells counted per field. Error bars indicate SEM. *p<0.05 **p<0.01 and ***p<0.001 (Student’s t test)
Chapter 4

4 Discussion

The major aim of the experiments presented in this thesis was to develop an alternate source of cortical radial precursors that closely mimic and can complement existing in vivo and in vitro models of cortical development in our lab. Specifically, the results describe a method of generating cortical radial precursors from embryonic stem cells by directed lineage differentiation using retinoic acid. The results demonstrate efficient differentiation of a heterogeneous population of Pax-6, Emx1, Foxg1, BLBP and Nestin positive radial precursors typical of cortical precursors that can then go on to differentiate to neurons with cortical identity, as well as astrocytes following the temporal order seen in vivo (Figure 4.1).

The starting culture resembles cortical development at approximately E13.5, during neurogenesis, and these cultures then progress to making astrocytes as do primary cortical precursor culture established from E12.5-13.5 cortices. Figure 4.2 describes the content of the culture at early and late time points, delineating typical cell populations that have been identified but also cell populations of unknown identity, although their presence is minimal.

As with all ES differentiations, this method generates a culture displaying cellular heterogeneity in terms of precursor populations and possibly presence of cells from non-neural lineages as well as the appearance of oligodendrocytes out of typical temporal order. The majority of neurons generated by these precursors express markers of glutamatergic projection neurons, while a small proportion express GABAergic interneuron markers, characteristics typical of neurons found in the cortex.

Data further indicate that growth factor responses of these ES derived precursors mimic those seen in vivo or in primary cultures demonstrating that although there is an intrinsic mechanism that guides corticogenesis in a dish, precursor fate is still subject to extrinsic cues and will conform to its environment. The growth factor FGF2 affected proliferation, survival and differentiation of ES-derived precursors while the cytokine CNTF induced expression of
astrocytic genes in these precursors, affecting the timing of astrogenesis as well as the numbers of astrocytes generated. FGF2 increased the ability of CNTF to induce astrocyte gene expression.

Embryonic stem cell directed differentiation to cortical precursors and neurons has previously been reported using methods with and without retinoic acid (Bibel et al., 2004, 2007; Wantanabe et al., 2005; Eiraku et al., 2008; Gaspard et al., 2008). Studies using EB aggregation in serum without retinoic acid generally adopt mesendodermal characteristics (Bain et al., 1996) and are mostly devoid of neural cell types (Bain et al., 1996). However, Bibel et al. (2004, 2007) developed media conditions specifically designed for neural differentiation but did not show that identically cultured untreated EBs, cannot also generate similar results under the same culture conditions. Even in transplantation studies, control EBs (RA untreated) were used from an earlier stage of differentiation to illustrate differentiation potential of ES cells (called naïve ES cells probably because they were at a pre-gastrulation stage) (Plachta et al., 2007). I therefore first asked if the retinoic acid treatment of differentiating ES cells described by Bibel et al., 2007, can indeed preferentially and efficiently direct ES cells towards a neural fate while repressing lineages of endoderm and mesoderm. Treatment of EBs with RA and subsequent dissociation and plating in neural differentiation media resulted in over 90% of the cells in culture expressing either the neural precursor marker Pax-6 or the neuronal marker βIII tubulin in contrast to RA untreated-EBs. This initial data suggested that, in this system, retinoic acid is able to efficiently convert ES cells to neural precursors and neurons. The efficiency of generation of Pax-6 expressing cells and subsequent neuronal differentiation was higher than other published reports using RA for neural differentiation (Bain et al., 1995) and consistent with Bibel et al., (2004, 2007).

One of the main challenges of differentiation to any lineage is the presence of unwanted cell types, particularly undifferentiated ES cells or cells that have differentiated towards non-neural lineages. Consistent with data by Bibel et al. (2004), by 8 days in neural differentiation media cultures derived from RA treated EBs contained less than 2% of cells that were not precursors or neurons by Pax-6 or βIII tubulin staining. With no addition of growth factors, the culture was entirely dependent on endogenously produced factors for differentiation and survival. This was evident from the fact that the successful propagation of cells was highly
dependent on plating density. Cell death occurred rapidly at non-optimal densities, an observation also noted by Bibel et al. (2004, 2007). The fact that this method does not use growth factors, inhibitors, selection of EB size or shape, aspects that can increase the technical complexity and potential variability between experiments, confers an important advantage to this model. Moreover, my substitution of the specific neural differentiation media components used by Bibel et al., (2007) with the commonly used and standardized N2 and B27 supplements, decreased culturing complexity and made conditions similar to those used for primary culture of cortical precursors as well as various other neural cell types used in our lab, while obtaining similar quality differentiations as Bibel and colleagues (2007).

One difference between the data generated by Bibel et al., (2004, 2007) and my own data was that more neurons than precursors were found at the initial stages of culture in neural differentiation media indicating that my ES differentiation progressed at a more rapid rate. This likely can be attributed to differences in ES cell culture techniques and media components (i.e. different serum lots). Indeed, differentiations using EBs cultured for 7 instead of 8 days generated cultures containing precursor numbers comparable to those from Bibel et al. (2004), as well as to Pax-6 positive precursor numbers in the E13.5 cortex (Gauthier-Fisher et al., 2009). Differentiations using EBs from an even earlier time point (Day 6 EBs) resulted in massive cell death due to the inability of cells to attach to the substrate. This may be because RA can induce Pax-6 expression in ES cells (Gajovic et al., 1997) and although cells in these EBs already expressed Pax-6 (data not shown) they had not acquired all the necessary characteristics of neural precursor cells.

Thus differentiations from Day 7 EBs met our subsequent experimental needs. However, differentiation from Day 7 EBs also resulted in an increase in the number of cells that could not be identified with the markers used (Pax-6 or βIII tubulin) at this stage. At early time points these cells could represent populations of neural precursors that are not yet neurogenic (Pax-6+ve), neural precursors of various CNS identities, or cells of a non-neural lineage altogether.

My data argues that the majority of cells present in this culture at early time points are cortical radial precursors by expression analysis of lineage specific markers. The proportions in
which these markers are expressed by these precursors are reminiscent of the heterogeneity observed in radial precursor populations in the cortex (Hartfuss et al., 2001). Firstly, the transcription factor Foxg1, one of the earliest genes to participate in forebrain specification, is expressed in a large proportion of the cells (Martynoga et al., 2005). Secondly, there is expression of Pax-6 and Emx1, also consistent with their concerted action in specifying the dorsal telencephalon (Muzio & Mallamaci, 2003). Thirdly, general radial precursor markers nestin and BLBP are also expressed in this culture consistent with cortical radial precursors in vivo and in primary culture (Hartfuss et al., 2001; Gauthier-Fisher et al., 2009). This supports a model where ES cells can intrinsically recapitulate the early stages of neural induction where the initial precursor specification is to the forebrain lineage.

It is not known what signals induce Pax-6 expression in the early neuroepithelium although it is thought that it may involve factors from the surrounding mesoderm (Osumi, 2001). At E8 it is one of the earliest genes expressed in the neural tube and is involved in proliferation and cell-cycle regulation of proliferating neuroepithelial cells (Quinn et al., 2007). Later in development, Pax-6 expression is likely modulated by Shh, FGF, BMP, Wnt and retinoid signaling gradients and functions to pattern the neural tube by controlling a network of downstream genes (Osumi et al., 2008). In ES neural differentiations Pax-6 is likely to be induced by retinoic acid and, in combination with Wnt and BMP activities in the serum, may serve to specify cells to forebrain, then cortical lineages (Glaser & Brustle, 2005).

Although Pax-6 is a marker of radial precursors in the cortex, on its own it is not definitive of any particular cell lineage. ES neural differentiations often report the initial precursor population exhibiting radial precursor markers like Pax-6, BLBP and nestin (Bain et al., 1995, Liour et al., 2003, 2006). Pax6, Blbp and Nestin are expressed in radial precursors of the cortex (Malatesta 2003; Hartfuss 2001) but are found in other, non-cortical radial precursor populations such as the spinal cord. Moreover retinoic acid differentiations have been reported to give rise to precursor cells typical of more posterior regions of the CNS consistent with a developmental role of RA in vivo. While in other systems further regionalization of precursors from a cortical fate has been prevented by blocking neural tube patterning morphogens like Wnt (Wantanabe et al., 2005) and Shh (Gaspard et al., 2008), the method used by Bibel et al. (2004, 2007) and adapted in this work is likely to involve effects of RA at multiple stages of differentiation in combination with serum and specific culture conditions.
So, how is it that the resultant cells are mostly cortical? Perhaps their rapid commitment and differentiation to neurons prevents further changes in area specification. Retinoic acid is a neurogenic factor that induces neuronal genes (Siegenthaler et al., 2009; Maden, 2007) and promotes cell cycle exit in ES neural differentiations (Kim et al., 2009). Cortical cells in vivo are also under retinoid signaling influence from the meninges layer, where radial precursors contact the pial side of the prospective cortex (Siegenthaler et al., 2009). Furthermore, precursors derived from ES cells have the ability to be molded by their environment in transplantation studies. However, precursors generated by the method described in this thesis display some in vitro acquired fate restrictions that upon transplantation into the chick neural tube allowed appropriate differentiation in some areas but not others compared to naïve ES cells (Plachta et al., 2007). Taken together, these studies suggest that the ES derived radial precursors in this thesis and Bibel et al. (2004, 2007), are unique in that the culture medium/techniques impose certain restrictions on differentiating cells capturing them at a specific stage while other ES cortical precursor differentiations guide lineage commitment by promoting or inhibiting signaling pathways known to pattern the neural tube.

Thus, one of the major conclusions reported here is that, under these culture conditions, the neural precursors generated resemble those of the cortical ventricular zone shortly after neurogenesis has begun – E12.5-13.5 (Figure 4.2). Further analysis to define these populations using different marker combinations will be necessary in the future to further delineate commonalities with radial precursors in vivo and to also exclude the presence of precursors and neuronal phenotypes of other CNS regions.

In this regard, immunocytochemical analysis of marker expression in these cells can be used to characterize their identity, it could be further complemented by using ES cell lines carrying reporter genes as Sox2-GFP, which have been used to study early neural induction of ES cells (Ying et al., 2003). ES lines that would induce GFP expression upon Emx1 induction are presently unavailable, however this could be circumvented by stably transfecting ES cells with plasmids where reporter gene expression is activated upon cortical precursor lineage commitment. Reporter lines for telencephalic differentiation such as Foxg1-venus have been used for this purpose (Eiraku et al., 2008). Similarly, FACS sorting of lineage specific precursors can be used to purify specific cell populations for culturing and analysis (Aubert et al., 2002; Wicheterle et al., 2002).
Radial precursors generate neurons through a more restricted precursor type, the basal precursor. In the cortex, basal precursors appear at mid-neurogenesis and are characterized by the downregulation of Pax-6 and upregulation of the transcription factor Tbr2 (Miyata et al., 2004; Englund et al., 2005). Analysis of the ES derived culture at 1DIV showed that basal precursors were not present at this early time point. This suggests that either these precursors represent cortical precursors at the onset of neurogenesis, before the generation of basal precursors, or that the ES derived precursors cannot recapitulate this aspect of corticogenesis. However, this last point is not likely the case as other ES-derived cortical precursor differentiations did detect the presence of basal precursors by Tbr2 immunostaining (Gaspard et al., 2008, Eiraku et al., 2008). Analysis of the culture at a later time point will be necessary in future experiments.

Neuronal subtype

The majority of the ES derived radial precursor cells characterized here differentiated rapidly into neurons. Consistent with the main neuronal subtype generated by cortical precursors in vivo, excitatory glutamatergic neurons, the majority of the ES derived neurons expressed the glutamate vesicular transporter 1 (Malatesta et al., 2003). A small proportion of all the neurons were positive for the GABAergic interneuron marker Gad67 whose origins in vivo can be traced to precursors both in the dorsal and ventral telencephalon, with the main source being the ventral precursors (Campbell, 2003). Given that the majority of precursors present in culture at early time points are Emx1 and Pax-6 positive and that these two transcription factors were found to be sufficient to specify the cortex while suppressing adjacent structures (Muzio et al., 2002; Bishop et al., 2002), it is likely that the interneurons found in this culture are derived from them. However, this does not exclude the possibility that the small proportion of these precursors that are not expressing these markers have a ventral identity, and therefore could also contribute to the generation of interneurons. Ventral telencephalic precursors do not express Pax-6 and give rise to interneurons (Campbell, 2003) and Pax-6 mutant ES cells switch their neurotransmitter phenotype to give rise to mostly GABAergic neurons (Nikoletopoulou et al., 2007). This interpretation would also be consistent with expression of the ventral transcription factor Nkx2.1 as reported and discussed in more detail below. A more detailed analysis of interneuron
subclasses and precursors will be required to clarify this issue. It will be important to exclude glutamatergic neurons from non-cortical areas and exclude interneurons from other areas where Pax-6 is expressed in radial precursors.

In the context of neuronal types generated, it will also be interesting to determine if neurons of the different cortical layers are present and if they are generated in typical temporal order. Both Gaspard et al., (2008) and Eiraku et al., (2008) demonstrated that layer specific neurons were born according to temporal sequences seen in vivo and in primary in vitro culture.

Moreover, it would be interesting to determine if my method also shows an under-representation of later born neurons, an observation made in other cortical ES differentiations, demonstrating that although ES cells can recapitulate early corticogenesis, cells require the cortical environment for later instructive signals.

Astrogenesis

Astrocytes are produced in this culture in accordance with the temporal sequence of cell genesis seen in vivo and in primary culture (Qian 2000; Barnabe-Heider et al., 2005; Gauthier et al., 2007). Nearly all ES differentiations that are described produce astrocytes in this manner (Bain et al., 1995; Fraichard et al., 1995; Ying et al., 2003; Smuckler et al., 2006). However, very little attention has been dedicated to this issue with regard to identifying the relevant precursors, timing of astrogenesis and/or concrete quantification of astrocyte numbers. This probably has been due to a greater interest in generating pure populations of neurons and eliminating non-neuronal phenotypes. In this ES derived precursor culture, precursors generate astrocytes at late time points suggesting that as in vivo and in primary culture, precursors change in competency over time from neurogenic to gliogenic. A more detailed analysis will be required to further delineate culture composition (Figure 4.1).

These data argue that a subpopulation of ES derived radial precursors generate glial cells, perhaps in addition to neurons. Clonal analysis of embryo derived precursor cultures indicate that early precursors (E10) can generate all three neural phenotypes in the temporal order seen in vivo (Qian et al., 2001). These stem/precursor cells usually generate clones containing either neurons or glial cells and a small proportion can generate both. In our clonal analysis we quite frequently
see both types. At the onset of neurogenesis the majority of radial precursors are exclusively neurogenic, becoming gliogenic as the environment changes. Radial precursors are competent to differentiate to astrocytes as early as the onset of neurogenesis but they do not do so due to limiting environmental cues, such as cytokines, and intrinsic factors that suppress the astrocytic fate until the appropriate time (Miller & Gauthier, 2007). In many ES differentiations of neural precursors, including the one presented in this thesis, astrocytes are generated after the main neurogenic period in culture, an observation consistent with \textit{in vivo} and primary models. The mechanisms governing the neurogenic to gliogenic switch have been shown to involve a complex interplay of intrinsic and extrinsic determinants that work to both suppress neurogenesis and promote astrogenesis. Gliogenic cytokines, Notch, BMP, EGF, signaling proteins like SHP2, epigenetic mechanisms and dilution of neurogenic factors determine the switch from neurogenesis to astrogenesis of precursors (reviewed in Miller & Gauthier, 2007).

Although it is clear that ES neural differentiations can intrinsically recapitulate astrogenesis, it is not known how or if the same players are present here. For example, the cytokine cardiotrophin-1 has been shown to be secreted from newly-born cortical neurons and feeding back to their parent precursors to start making astrocytes by activating the gp130-JAK-STAT pathway (Barnabe-Heider et al., 2005). It would be interesting to determine if a similar mechanism operates here.

Oligodendrocytes and Nkx2.1 expressing precursors

Radial precursor cells in the cortex can also generate oligodendrocytes, however the origin of the final complement of oligodendrocytes in the cortex is still under investigation (Miller, 2002; Kessaris et al., 2001, 2006; Gorski et al., 2002). The current model describes three waves of oligodendrogenesis distinguished by time of appearance and precursor origin. The early wave of oligodendrocytes is derived from the ventral telencephalon Nkx2.1 expressing precursors in MGE, which are first detected at E11.5 and migrate, entering the cortex at around E16 (Kessaris et al., 2006). A second wave of oligodendrocytes, also from ventrally derived Gsh2 expressing precursors of the LGE and CGE migrate to overtake the first wave postnatally. Lastly, cortical Emx1 expressing cells give rise to oligodendrocytes in the last wave (Kessaris, 2006). Cultured primary cortical precursors give rise to oligodendrocytes in temporal sequence
after neurons and astrocytes at about 5-6 days in culture and are representative of oligodendrocytes that would be made locally within the cortex (Barnabe-Heider et al., 2005; Gauthier et al., 2007).

The finding that oligodendrocytes are generated by the ES-derived precursors at early time points raises questions as to their origin, and suggests several interpretations. First, these oligodendrocytes could be derived from Emx1 expressing precursors and would be consistent with studies using Emx1-Cre transgenic mouse lines suggesting that many oligodendrocytes are generated locally within the cortex although this study also detected Emx1 expression outside the dorsal telencephalon (Gorski et al., 2002). Emx1 expressing cells can migrate to populate the ventrally derived striatum (Willaime-Morawek et al., 2006). However, such an early differentiation would imply the presence of erroneous fate determining programs since even though oligodendrocyte precursors are specified early, they do not differentiate until most of neurogenesis and astrogenesis is complete (Tekki-Kessaris et al., 2006). Secondly, they could be derived from ventralized precursors, such as those not expressing Pax-6 or Emx1 but expressing Nkx2.1. Both possibilities would require the endogenous production of Shh. Sonic hedgehog signaling patterns the ventral telencephalon by controlling expression of transcription factors like Pax-6 and Nkx2.2 (Tekki-Kessaris et al., 2001). In vivo, Shh is not expressed by cortical precursors but it is expressed by cortical precursors in vitro (Tekki-Kessaris et al., 2001). Sonic hedgehog is sufficient to induce oligodendrogenesis in cortical precursors in culture while its inhibition prevented production of oligodendrocyte precursors (Tekki-Kessaris et al., 2001, Kessaris et al. 2004).

The expression of Shh by ES-derived precursors has been noted in ES cortical differentiation by several groups and was thought to contribute to the generation of ventral precursor fates, however the presence of oligodendrocytes was not analyzed in these cultures (Gaspard et al., 2008). The inhibition of Shh resulted in an increase in the number of cells expressing dorsal precursor markers like Emx1 (Wantanabe et al., 2005; Gaspard et al., 2008). A more detailed analysis of oligodendrocytes and oligodendrocyte precursors at both early and late time points in culture remains to be assessed.

The finding that there are Nkx2.1 expressing cells in this culture has implications as to the regional identity of precursors that seem to be able to generate oligodendrocytes early in
culture as well as GABAergic neurons as will be discussed below. Nkx2.1 expression is typically restricted to the ventral telencephalon structures (Puelles et al, 2000; Sussel et al, 1999). However, our lab has found evidence that the cortex itself can generate precursors expressing Nkx2.1, suggesting that although found in small proportions, they would be able to generate cell lineages normally specified by this transcription factor directly in the cortex itself. Nkx2.1 normally functions to specify the ventral telencephalon and is repressed by Pax-6 to separate ventral from dorsal identities (Quinn et al., 2007). The finding here of co-expression of Nkx2.1 with Pax-6 in a subset of Nkx2.1 positive cells would seem to argue that precursors that are not immediately neurogenic, are responsive to endogenously produced factors like Shh that ventralize them and may be representative of a subset of precursors in the transitional region that delineates the border between the dorsal and ventral telencephalon, where the two markers could overlap (Puelles et al., 2000). Marker analysis of ventral precursor populations that intersect those expressing Nkx2.1, like the Dlx and Lhx transcription factors as well as other Nkx family members, will be required to clarify the regional specification of these cells.

ES-derived cortical radial precursor growth factor responses

During development, radial cortical precursors are exposed to a variety of different cues that instruct them to proliferate, die or differentiate so that the appropriate cell numbers and cell types are generated at the appropriate times. Neural differentiations from ES cell have been based on information obtained from in vivo and primary culture studies to direct the differentiation of ES cells to specific cell lineages. Due to the challenges of developing just the right culturing conditions most studies have concentrated on refining methods to generate pure populations of any given lineage using marker analysis of precursor populations and their competence to differentiate to the desired end product as the main read-outs. In some transplantation studies, it has been shown that neural precursors obtained from ES cells can be instructed by the local environment to differentiate appropriately even when the precursor type belonged to a different lineage (Wichterle et al., 2002). Other studies have shown in vitro imposed restrictions in precursor fates that did not allow appropriate differentiation unless the precursor was transplanted in the appropriate place (Plachta et al., 2007). It is unclear then what
cues ES derived precursors are exposed to in the culture they come from and whether these cues are relevant in vivo.

ES derived cortical precursor studies show, at least early on, an intrinsic ability to recapitulate events in corticogenesis but it is not known whether this in vitro niche compares with the in vivo niche or conditions in which primary cortical cells are cultured and differentiated. No efforts have been made to show that ES derived precursor populations can respond to the same extracellular cues or that the same signaling pathways are activated in response to these signals with similar outcomes to what has previously been reported in in vivo studies or primary culture.

My data supports the idea that FGF2 signaling, in combination with unknown factors present in the culture, determines precursor proliferation, survival and differentiation into neurons, or astrocytes in the presence of CNTF, in a manner observed with primary cortical precursor culture and in vivo.

FGF2 is an important mitogen for neural precursor cells and is required for the expansion of neural stem cells in culture (Qian et al., 1997; reviewed in Rao, 1999). During development FGF2 is required for the proliferation of cortical precursors both in vivo and in primary culture (Raballo et al., 2000; Qian et al., 1997). Studies of FGF2 knockout cortices show impaired proliferation of precursors resulting in reduced neuronal and glial cell numbers (Vaccarino et al., 1999; Raballo et al., 2000). Furthermore, FGF2 knockout mice also show decreased cell numbers in the adult (Ortega et al., 1998; Vaccarino et al., 1999). In contrast, the exogenous introduction of FGF2 in the cortex by injection significantly increases the number of neuronal and glial cells (Vaccarino et al., 1999). Consistent with these studies, my data suggests that FGF2 promotes the proliferation of precursors, thereby increasing the proportion of proliferating Pax-6 positive precursors. In the future, clonal analysis on these precursors will be required to support the conclusion that FGF2 promotes proliferation by examining the precursor composition of individual clones.

FGF2 has been used in ES neural differentiations to maintain the precursor pool without differentiation (Tropepe et al., 2001, Li et al., 2009). While these studies reflect specific aspects of FGF2 signaling, they do not necessarily reflect what happens in vivo. Importantly, neuroepithelial cells in vivo do not expand indefinitely. Prior to neurogenesis and FGF2 is
required for their proliferation to expand the precursor pool. At the onset of neurogenesis, they receive instructive cues to change modes of division and begin generating differentiated cell types (Gotz & Huttner, 2005). The results presented in this thesis are consistent with FGF2 acting on precursors during the neurogenic period where it promotes proliferation of precursors and prevents differentiation.

The requirement of FGF2 for cell survival in cultured cortical precursors is well documented, although a similar requirement has not been documented found in vivo (Vaccarino et al., 1999; Raballo et al., 2000) potentially due to the presence of other survival factors like neurotrophins. FGF2 and neurotrophin signaling through the PI3-K/Akt pathway support precursor cell survival and neuronal survival (Kaplan & Miller, 2000) and similar mechanisms may operate in this culture as exogenous FGF2 increases survival of ES-derived precursors and neurons.

How does FGF2 contribute to so many different aspects of precursor biology? The heterogeneity of precursors in the prospective cortex in terms of sensitivity to growth factors, cell fate and cell cycle kinetics has also been noted in the literature (Acklin & van der Kooy, 1993; Vaccarino et al., 1999; Raballo et al., 2000) and it reflects a complex niche where cells adapt to their extrinsic environment. For example, FGF2 was demonstrated to be required for proliferation and differentiation by only subpopulation of cortical precursors (Raballo et al., 2000). FGF2 directly affects the cell cycle of cortical precursors by upregulating cyclin D2 and downregulating p27 (Lukaszewics et al., 2002). On the same note, precursor sensitivity to growth factors switches from FGF2 to EGF, with FGF2 inducing EGFR expression, a change coincident with the switch from neurogenesis to astrogenesis (Tropepe et al., 1999; Lillien & Raphael, 2000). Differential expression of FGF2 and its receptors in combination with other FGF family members as well as other intrinsic and extrinsic cues make FGF2 effects cell-context dependent, potentially due to differential activation of downstream signaling pathways.

The changing cortical environment signals the onset of astrogenesis with the upregulation of cytokines and JAK-STAT signaling components. As discussed earlier, the cytokine CT-1 is enriched in cortical neurons and participates in the precursor switch from neurogenesis to astrogenesis. Although not expressed until postnatally in the mouse brain, the cytokine CNTF, from the same family as CT-1, is known to have a similar mechanism of action
CNTF activates the JAK-STAT signaling pathway to promote astrogenesis (Bonni et al., 1997; Barnabe-Heider et al., 2005). CNTF has been implicated in astrocytic differentiation in many studies (Johe et al. 1996, Bonni et al., 1997, Barnabe-Heider et al., 2005) and those previous studies are consistent with data presented here where we show precocious GFAP expression and overall increase in GFAP expressing cells.

As with cortical precursors in vivo and primary culture, the ES-derived culture seems to have mechanisms in place to prevent the early differentiation of precursors into astrocytes. For example, CNTF activation of JAK-STAT signaling is inhibited during the neurogenic period by the growth factor-regulated phosphatase SHP-2 (Gauthier et al., 2007). As CNTF overcomes normal restrictions on gliogenesis, it also causes a small reduction in the number of neurons generated as precursors become more responsive to astrogenic cues (Pearson & Doe, 2004; Barnabe-Heider, 2005; Guillemot, 2006). The ES-derived precursors were continuously exposed to CNTF from the time of EB dissociation and plating in neural differentiation media however GFAP expression was delayed. This is typical of CNTF effects on cortical precursor cultures at the peak of neurogenesis, where precursors are mostly neurogenic, and GFAP expression is somewhat delayed relative to later precursors (Bonni et al., 1997, Nakashima et al., 1999).

Regardless of the key players involved in cell fate determination in these ES-derived precursors, they show a time dependent change in competency seen in vivo and in vitro. FGF2 signaling converges on CNTF mediated astrogenesis by allowing the STAT activation complex, access to the GFAP promoter by inducing local chromatin modifications (Song & Ghosh, 2004). While FGF2 enhances the effects of CNTF it also functions to promote proliferation of precursors and survival, likely working in combination with neurotrophins secreted from both precursors and neurons. My data regarding neurogenesis in the presence of CNTF and FGF2 suggests similar signaling interactions are present in my ES-derived cultures a possibility that remains to be explored.

The data presented here indicate that FGF2 and CNTF have similar effects on primary cortical precursors and the ES-derived precursors I have characterized here. Importantly, this means that 1) the ES-derived cultures can recapitulate corticogenesis events in response to a changing environment and, 2) these cultures can be manipulated in vitro to complement and validate existing in vivo and in vitro work. Furthermore, this culture system can serve as
discovery tool, to complement the primary culture taking advantage of the many genetically modified ES lines, the availability of human ES cells, and the opportunity to generate large quantities of ES-derived cells.

The aim of this study was to develop an alternate source of cortical radial precursors that can be used to complement existing *in vivo* and *in vitro* models of cortical development in our lab. The work described in this thesis suggests that ES cells can be induced to efficiently differentiate into cortical radial precursors that mimic differentiation patterns and have characteristics typical of their counterparts *in vivo* and in primary culture, although more investigations will be required.

The ES derived cortical radial precursor *in vitro* system offers not only the potential to validate the existing models but to also allow comparisons between the different systems in order to define characteristics in terms of gene expression, growth factors and signaling pathways that define the environment of cortical precursor cells during development. Selective isolation of different precursor populations from this culture can allow study of cell fate and differentiation potential of the different precursor types serving to understand the heterogeneity of precursors *in vivo*.

Importantly, the ability to generate large numbers of cells will allow study of signaling pathways where delivery of inhibitors, gene silencing constructs and pharmacological agents is straightforward and providing enough material for biochemical studies. Furthermore, the use of genetically modified ES lines can overcome the difficulties inherent to transgenic and mutant mouse lines and investigations can be adapted using more relevant *in vitro* systems such as human ES cell and iPS lines.
Figure 4.1: ES-derived culture composition at 1 and 8DIV

Neurons and astrocytes are generated in temporal sequence seen in vivo and in primary culture. At 1DIV ES-derived cortical precursors resemble cortical precursor primary culture generated from the E12.5-13.5 cortex.
Figure 4.2: ES-derived cortical radial precursor culture compared to in vivo and primary culture

(a) Chart showing estimations of the different cell populations at 1 DIV. While the majority of cells express indicated markers, a small proportion remain to be characterized. (b) Chart showing estimations of the different cell populations at 8 DIV. The proportion of cells not expressing indicated markers is higher at this time point. They may represent early astrocytes and oligodendrocyte and their precursors, cell populations that remain to be characterized at this time point.
References


Pax6 is required to regulate the cell cycle and the rate of progression from symmetrical to asymmetrical division in mammalian cortical progenitors. *Development* **129**, 455-466.


