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Embryonic Stem Cell Neurogenesis and Neural Specification

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ABSTRACT

The prospect of using embryonic stem cell (ESC)-derived neural progenitors and neurons to treat neurological disorders has led to great interest in defining the conditions that guide the differentiation of ESCs, and more recently induced pluripotent stem cells (iPSCs), into neural stem cells (NSCs) and a variety of neuronal and glial subtypes. Over the past decade, researchers have looked to the embryo to guide these studies, applying what we know about the signaling events that direct neural specification during development. This has led to the design of a number of protocols that successfully promote ESC neurogenesis, terminating with the production of neurons and glia with diverse regional addresses and functional properties. These protocols demonstrate that ESCs undergo neural specification in two, three, and four dimensions, mimicking the cell–cell interactions, patterning, and timing that characterizes the in vivo process. We therefore propose that these in vitro systems can be used to examine the molecular regulation of neural specification. J. Cell. Biochem. 111: 535–542, 2010.

KEY WORDS: EMBRYONIC STEM CELLS; NEUROGENESIS; NEURAL ROSETTE; CORTICOGENESIS

Since the isolation of the first human ESCs, the potential for therapeutic applications, including drug design and delivery as well as cell replacement therapies, has driven the elucidation of conditions that promote differentiation down specific lineages. The area of ESC neurogenesis has been particularly active, due to prospects of treating a variety of neurological diseases and central nervous system (CNS) injuries. Several protocols for the production of NSCs, neurons, and glia from ESCs, as well as iPSCs, have been designed using what we have learned about neural specification during embryogenesis [Cai and Grabel, 2007].

These protocols rely upon inductive interactions, as required for neural specification in the embryo. These interactions can be established in vitro by having the appropriate cell types in culture, for example, extraembryonic endoderm combined with epiblast, or by directly modulating signal transduction cascades through the addition of pathway agonists or antagonists, for example, the addition of the BMP antagonist noggin or Wnt antagonist Dkk-1 [Gerrard et al., 2005; Watanabe et al., 2005]. ESCs cannot synchronously differentiate exclusively into viable NSCs without the transient presence of other cell types or the addition of exogenous factors.

During ESC neurogenesis, morphological structures comparable to those arising during embryogenesis have been observed in vitro. Numerous protocols for ESC neural differentiation start with an embryoid body stage. Embryoid bodies contain an outer layer of extraembryonic endoderm surrounding an epiblast-like core. The juxtaposition of these cell types is required for neural induction in the embryo.

Protocols based on adherent culture can lead to the formation of rosettes, NSCs arranged radially around a lumen. A number of studies, described below, point to the functional similarities of the rosette structure to the neural tube and of rosette NSCs to radial glia, the NSCs of the embryo. Recent reports, described below, document the formation of laminar structures in adherent culture that mimic the layers formed during corticogenesis in the developing forebrain. These data suggest that similar morphogenetic and patterning events direct regional specificity in vitro. In addition, numerous studies have demonstrated a temporal pattern to the emergence of neural cell types during ESC neurogenesis that mimics the timing observed during embryogenesis, for example, with neuronal derivatives emerging prior to glial derivatives.

These observations suggest that ESC neurogenesis can be used to study morphogenesis and patterning of the nervous system. We briefly describe neural induction during embryogenesis and how this information has been used to design protocols for ESC

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neurogenesis. We then focus on the morphogenesis and patterning events displayed by ESC-derivatives.

LESSONS FROM THE EMBRYO

Mechanisms that govern the in vivo development of the embryonic CNS also regulate the in vitro differentiation of ESCs into neural progenitors and various types of neurons. Development of the nervous system can be divided roughly into three processes; neural induction, neurulation, and regional specification. Cues taken from the embryo during each of these processes have been useful in establishing methods for in vitro neural differentiation.

The blastocyst stage mammalian embryo consists of an outer trophectoderm cell layer, contributing to the placenta, surrounding the inner cell mass of pluripotent cells. The inner cell mass gives rise to the epiblast, a layer from which the three primary germ layers—endoderm, mesoderm, and ectoderm—arise. Various signaling events are required to instruct the epiblast to become each of the three germ layers.

A wealth of studies have led to the idea of neural ectoderm as the default fate for epiblast cells as the earliest steps in neural induction are a result of the inhibition of mesoderm and endoderm promoting signals such as Wnts, Nodal, and BMPs [Stern, 2005]. The organizer region of the gastrulating embryo, first identified in amphibians, is responsible for the secretion of BMP antagonists such as chordin, noggin, and follistatin, the Wnt inhibitor Dkk1, and nodal inhibitors cerberus and lefty [reviewed in Levine and Brivanlou, 2007]. BMPs and Wnts are widely expressed in the epiblast but, due to local inhibition at the organizer, a band of neural ectoderm termed the neural plate develops along the anterior primitive streak. The anterior visceral endoderm is also implicated in induction of anterior neural ectoderm, due to its secretion of inhibitors of nodal, Wnt, and BMP [Levine and Brivanlou, 2007]. Studies in non-mammalian vertebrates suggest a positive role for FGF signaling in neural induction. Culture of *Xenopus* animal cap explants in the presence of FGF2, for example, can induce pan-neural markers and, in combination with noggin, generate both anterior and posterior CNS neurons [Lamb and Harland, 1995].

After neuroectoderm induction, the embryo undergoes neurulation in which the epithelial neural plate begins to furrow and the two neural ridges generated on either side of the neural groove move towards each other to form the neural tube. Closure of the neural tube progresses from the prospective midbrain region anteriorly to form the future telencephalon as well as posteriorly to generate the hindbrain and spinal cord. Following neural tube closure, an increase in neural progenitor proliferation anteriorly causes the telencephalon to balloon out generating the two brain hemispheres. The remaining neural tube begins to bulge at various points which will become the midbrain and the rhombomeres of the hindbrain (Fig. 1B) [Smith and Schoenwolf, 1997].

While many of the same signaling pathways are utilized for both dorsal-ventral (DV) and anterior–posterior (AP) patterning in the various domains of the CNS (discussed below), certain regions of the neural tube respond differently to the same signals [Shimamura et al., 1997]. This suggests that along the AP axis, individual fields

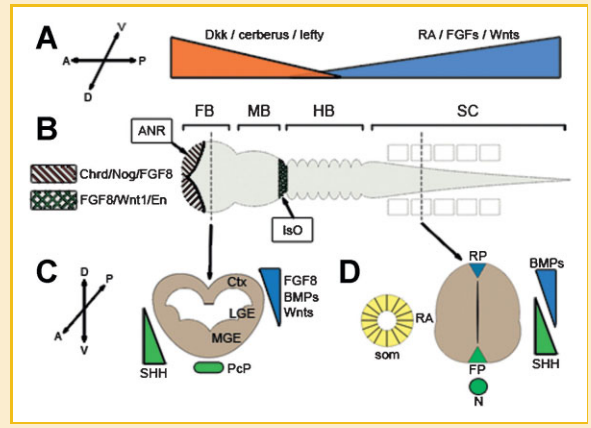

Fig. 1. Patterning the vertebrate embryonic CNS. A: Signals such as RA, FGFs, and Wnts are expressed in a concentration gradient from the posterior of the embryo to the anterior. Pathway antagonists such as Dkk, cerberus, and lefty, are produced in a gradient originating in the anterior most regions of the embryo. B: Two organizing centers, the anterior neural ridge (ANR) and isthmic organizer (IsO), secrete signals locally to pattern the forebrain and posterior midbrain/anterior hindbrain, respectively. C: The telencephalon is patterned along the dorsal–ventral axis by dorsally produced FGF8, BMPs, and Wnts and by a ventral gradient of SHH produced in the underlying prechordal plate (PcP). D: Dorsal–ventral patterning of the neural tube is a result of BMP signaling from the roofplate (RP) and SHH from the notochord (N) and floorplate (FP). RA, produced in the somites (som), also acts in patterning the neural tube. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

are established with varying potentials. Two main signaling centers have been identified that pattern the CNS along the AP axis, the anterior neural ridge (ANR) and the midbrain–hindbrain border (or isthmic organizer) [reviewed in Wurst and Bally-Cuif, 2001].

The ANR, which is located at the boundary between the anterior non-neural ectoderm and the prosenchephalon, is largely responsible for forebrain patterning (Fig. 1B). FGF8 is produced in the ANR and induces expression of BF-1, a forebrain specific transcription factor required for proper specification of anterior progenitors [Shimamura et al., 1997]. In addition, the ANR also expresses chordin and noggin to antagonize BMP signals emanating from surrounding non-neural ectoderm and mesendoderm [Anderson et al., 2002]. This local BMP antagonism may also be responsible for supporting FGF8 expression in the ANR as chordin/noggin mutants display reduced FGF8 in the ANR [Anderson et al., 2002]. There is some evidence that retinoic acid (RA) signaling from the posterior of the embryo plays a role in regulating the boundaries of the forebrain, as increased RA shifts the limit of forebrain specific Otx2 expression more anteriorly (Fig. 1B) [Ang et al., 1994].

The isthmic organizer forms at the junction between the prospective midbrain and the first rhombomere of the hindbrain and produces FGF8, Wnt-1 and engrailed, which pattern the midbrain and anterior hindbrain (Fig. 1B) [reviewed in Liu and Joyner, 2001]. FGF8 is necessary for the establishment and maintenance of this border [Martinez et al., 1999]. The two homeobox transcription factors Otx2 and Gbx2 are expressed in reciprocal gradients that abut each other at the midbrain–hindbrain border. Proper regulation of the expression boundaries of these two

genes is required to establish the midbrain–hindbrain border [Millet et al., 1999].

RA is also a critical signaling factor in the AP patterning of CNS domains. Expressed in the paraxial mesoderm in a concentration gradient from posterior to anterior, RA enters the neural tube and regulates expression of Hox genes required for hindbrain and spinal cord development [Maden, 2002]. Disturbances in the tight regulation of the generation and degradation of RA along the AP axis lead to alterations in the establishment of CNS domains, with the loss of posterior rhombomeres observed under reduced RA signaling and truncation of the forebrain with RA overexpression [Maden, 2002]. Additionally, RA acts with FGFs in a concentration dependent manner in the developing spinal cord to induce domains of Hox gene expression, which are necessary for the correct patterning of the various spinal neurons (Fig. 1A) [Liu et al., 2001].

In the telencephalon, patterning along the DV axis is established by opposing gradients of sonic hedgehog (SHH), secreted by the underlying prechordal mesendoderm, and FGF8 and BMP signaling from the roofplate (Fig. 1C) [Hoch et al., 2009]. The dorsal domain of the telencephalon largely produces cortical projection neurons while the ventral domains, comprised of the medial and caudal ganglionic eminences, generate cortical interneurons, and striatal neurons in the midbrain. A similar system of FGF, BMP, and SHH signaling acts in the spinal cord to specify motor neurons ventrally and interneurons dorsally (Fig. 1D) [Maden, 2002; Helms and Johnson, 2003].

IN VITRO NEUROGENESIS OF ESCs

The differentiation of both mouse and human ESCs, as well as iPSCs, into neural derivatives is accomplished by a handful of basic protocols which have been reviewed extensively [Cai and Grabel, 2007]. These approaches fall into three major categories; embryoid body-based, direct monolayer, or co-culture.

Embryoid bodies are generated by placing ESCs into suspension culture. Over several days they develop an outer layer of extraembryonic endoderm surrounding an epiblast core, mimicking the two cell layers observed in the egg cylinder stage mouse embryo or the bi-laminar germ disc of the human embryo. Interactions between these cell layers facilitate neural ectoderm specification [Gilbert, 2010]. The epiblast layer can generate derivatives of all three primary germ layers, including ectoderm, depending upon conditions.

Other protocols, such as the direct monolayer approach, take advantage of the ''default'' state of development by limiting cell–cell interaction and growth-factor influence by culturing the ESCs at low densities in nutrient poor media [Ying et al., 2003]. These methods can lead to the generation of NSC rosettes, described in more detail below. Lastly, co-culture with bone marrow stromal cells, or conditioned medium derived from these cells, can also promote neurogenesis due to the production of signals that have not yet been identified [Kawasaki et al., 2000]. Aspects of these different protocols can be combined, for example embryoid bodies treated with RA can be plated on adherent substrates in the presence of serum-free defined media, to maximize neural differentiation [Okabe et al., 1996].

Several groups have taken advantage of inductive signals found in the embryonic organizer to increase the efficiency of generating neuroectoderm cells in vitro and reduce the presence of non-neural cell types. Treating mouse ESCs with Dkk1 or lefty, to inhibit Wnt and nodal signaling, increases generation of early neuroectoderm, based on Sox1 expression [Watanabe et al., 2005]. Treatment of human ESCs with noggin significantly reduces the presence of extraembryonic endoderm and biases differentiation towards neural progenitors [Gerrard et al., 2005]. In addition, it has recently been shown that inhibition of SMAD signaling with a synthetic TGF-β antagonist can effectively produce neural progenitors at high percentages [Chambers et al., 2009]. Use of these signals has led to a lessened dependence on embryoid body-mediated differentiation, which may result in purer neural progenitor populations.

Forebrain specific neural progenitors and neurons have been generated in vitro mainly by growth in serum-free media at very low densities, although initially at low yields [Tropepe et al., 2001]. The low percentage of forebrain progenitors is likely due to the caudalizing factors, such as FGFs and RA, used in these protocols. Following initial aggregation, growth in serum-free monolayer culture, supplemented with Dkk1 and Lefty, increases the number of cells expressing forebrain specific markers such as BF-1 [Watanabe et al., 2005]. It has recently been demonstrated that fine adjustments in the levels of SHH and Wnt signaling generate forebrain specific neurons with a range of dorsal and ventral phenotypes [Li et al., 2009]. A combination of Dkk1 and SHH peptide are effective in biasing neural progenitors towards a ventral Nkx2.1-positive fate, while treatment with Wnt3a conditioned medium directs the formation of dorsal progenitors [Li et al., 2009].

GABAergic interneurons of the hippocampus and cerebral cortex are generated in vitro by mimicking the conditions of the ventral ganglionic eminences in which they develop in vivo. The observation that SHH signaling is active in many culture systems for generating neural progenitors may explain the production of GABA-positive ventral neurons. For example, a modest percentage of GABA and Gad67 (the GABA synthesizing enzyme)-positive neurons are generated in embryoid body-mediated differentiation followed by serum-free, RA-free selection protocols [Westmoreland et al., 2001]. The efficiency of GABA-positive neuron production is improved by treating the neural progenitors with SHH and FGF8 [Barberi et al., 2003] or, as mentioned earlier, combined treatment with Dkk1 and SHH [Li et al., 2009]. In the developing medial ganglionic eminences, high levels of SHH bias progenitors towards the somatostatin expressing interneuron subtype, while lower levels result in parvalbumin expression [Xu et al., 2010]. Therefore, it may be necessary to modify SHH concentration in culture, depending on the desired interneuron subtype.

The generation of cortical neurons has markedly been improved by treatment of ESCs with the SHH antagonist cyclopamine during serum-free monolayer culture [Gaspard et al., 2008]. Under these conditions, roughly 70% of resulting neurons express vesicular glutatmate transporters, indicative of cortical pyramidal neurons. A modification of the serum-free embryoid body differentiation system increases the efficiency of telencephalic neural progenitor

induction with about 80% of resulting neurons positive for the cortical markers Emx1 and VGlut [Eiraku et al., 2008]. This study also demonstrates a role for exogenous FGF8 in promoting rostral-most fates, as observed for ANR induction in the forebrain.

Midbrain dopaminergic and hindbrain serotonergic neurons are generated by evoking the signals found in the isthmic organizer. This includes FGF8 and SHH treatment following embryoid body-mediated differentiation and expansion of neural progenitors in the presence of FGF2 [Lee et al., 2000]. Addition of Wnt1 and Wnt3a to these cultures promotes the generation of cerebellar neurons [Salero and Hatten, 2007]. The stromal cell co-culture method of neural differentiation selectively induces high levels of tyrosine hydroxylase (TH)-positive dopaminergic neurons [Kawasaki et al., 2000] and, when supplemented with FGF8 and SHH, effectively generates almost 80% TH-positive neurons [Perrier et al., 2004].

Spinal motor neurons can be specified in vitro using both mouse and human ESCs, and recently using human iPSCs [Dimos et al., 2008]. These protocols depend on first caudalizing the neural progenitors using RA and then ventralizing the cultures with either SHH or a synthetic SHH pathway agonist [Lee et al., 2007]. Protocols have also been developed for the generation of spinal cord interneurons, which are distinct from cortical interneurons in that they are born in dorsal regions. Sequential treatment of progenitors with RA followed by BMP2, Wnt3A, and low concentrations of SHH yields Lim2 and GAD67-positive spinal interneurons [Murashov et al., 2004].

These studies demonstrate that reproducing in vivo inductive and patterning events in vitro effectively directs the differentiation of ESCs into selected neural derivatives.

IN VITRO ROSETTE FORMATION OF NSCs: A MODEL SYSTEM FOR IN VIVO NEUROGENESIS

ESC-derived NSCs, generated from both adherent and embryoid body-intermediate approaches, characteristically arrange themselves radially on a flat surface, eventually forming a central lumen. This radial, floral-like arrangement is known as a rosette. Rosettes have an uncanny structural and functional similarity to the embryonic neural tube (Fig. 2A). In addition to their morphological similarities, both display localized zones of proliferation and can be patterned by signaling molecules and growth factors, suggesting their formation and differentiation are governed by similar mechanisms [Wilson and Stice, 2006; Elkabetz et al., 2008].

NSC IDENTITY AND POTENTIAL

Radial glia are the NSCs of the embryonic neural tube, and give rise to the abundant variety of neurons and glia of the nervous system [Noctor et al., 2004]. Recent data have shown that rosette NSCs are radial glia-like and also have the ability to generate a variety of neural cell types from the forebrain, midbrain, hindbrain, and spinal cord. Mouse ESC-derived NSCs exhibit distinct bipolar cell shapes, a morphological characteristic of radial glia. These cells express proteins characteristic of radial glia—RC2 and brain lipid binding protein (BLBP) (Fig. 2A) [Bibel et al., 2004]. These cells give rise to neurons with a variety of spatial and functional phenotypes, characterized by the expression of neuronal subtype-specific transcription factors and neurotransmitters. Human ESC-derived rosette NSCs also demonstrate radial glia-like properties. Global

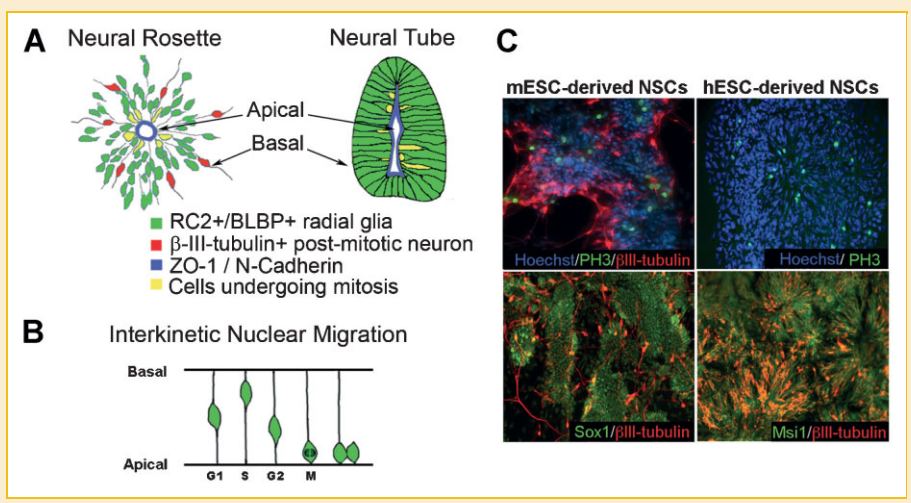

Fig. 2. ESC-derived neural rosettes in vitro bear striking resemblance to the neural tube. A: Apicobasal polarity is similar between rosettes and the neural tube, with tight junctions (indicated by ZO-1 expression, blue) at the apical surface forming a lumen. Radially arranged progenitors in the rosette, as well as the neural tube, express RC2 and BLBP (green). B: Cell division by interkinetic nuclear migration occurs in both the neural tube and in rosettes. C: mESC and hESC derived neural rosettes undergo cell division (marked by phospho-histone H3 labeling in green, upper panels) at the apical surface and neural differentiation (marked by βIII-tubulin labeling in red) at the basal surface. Pools of neural stem cells remain at the interior of rosettes (labeled by Sox1 and Msi1 in green, lower panels). Nuclei are labeled by Hoechst in blue. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

gene expression and immunocytochemistry analysis performed using human ESC-derived rosette NSCs illustrate the capacity of these cells to give rise to multiple neuronal subtypes of both the central and peripheral nervous systems [Elkabetz et al., 2008]. Glial subtypes, such as astrocytes [Elkabetz and Studer, 2008] and oligodendrocytes [Hatch et al., 2009], have also been produced from rosette NSCs.

ESC-derived neural cell types arise in vitro in a temporal manner reminiscent of in vivo development—the earliest differentiation events give rise to neurons while later-staged cultures of NSCs exhibit reduced differentiation plasticity, preferentially producing cells of the glial lineage [Okada et al., 2008].

APICAL–BASAL POLARITY

The neural tube consists of a radially arranged neurepithelium surrounding a central lumen. The side of the epithelial cell layer facing the lumen (ventricles) is considered apical, while the side facing the outer pial surface is considered basal. Apicobasal polarity is quickly established following the closure of the neural tube, in part by the asymmetric distribution of proteins involved in adherens and tight junction formation [Miyata, 2007]. Tight junction protein zona occludens 1 (ZO-1) is expressed exclusively at the apical surface of cells surrounding the lumen of the neural tube. Its localization coincides with expression of the neurepithelial adhesion marker N-cadherin (N-Cad) (Fig. 2A) [Marthiens and Ffrench-Constant, 2009].

This asymmetric expression pattern is also observed in ESC-derived neural rosettes, where ZO-1 protein is observed initially at tight junctions between cultured ESCs. However, at the onset of neural differentiation, this protein is rapidly redistributed and restricted to the center of emerging rosettes [Elkabetz et al., 2008]. Additionally, similar to in vivo immunohistochemical analysis of the neural tube, ZO-1 and N-Cad also show significant overlap in domains of expression in vitro at the luminal side of the neural rosette (Fig. 2A).

GEOMETRY OF PROLIFERATION

While the early neural tube appears to be organized into a stratified epithelium, it is actually comprised of a single layer of radial glia. These cells make contact with both the apical and pial surfaces of the neural tube, but have nuclei arranged at different heights, producing the appearance of a multi-layered epithelium [reviewed in Kriegstein and Alvarez-Buylla, 2009]. Proliferation of radial glia within the neural tube is largely restricted to the ventricular and subventricular zones, adjacent to the lumen (Fig. 2A). Recent data suggest that a third neurogenic zone exists within the human embryonic cortex, known as the outer ventricular zone (OSVZ), which may provide a mechanism for generating the larger cortical area of the human versus rodent brain [Hansen et al., 2010].

During cell division, nuclei of actively dividing radial glia migrate towards the apical surface, where they undergo mitosis and cytokinesis. This migration is known as interkinetic nuclear migration (Fig. 2B) [Sauer and Walker, 1959]. Early divisions within the neural tube are symmetric, with each subsequent round of division giving rise to two daughter radial glia cells. This initial division pattern creates a large pool of NSCs at the apical surface. However, following the onset of neurogenesis, asymmetric division begins at the ventricular zone, with each round of division giving rise to both a daughter NSC, as well as a neuronal progenitor cell [Noctor et al., 2004]. This asymmetrical division is due, in part, to the apically restricted distribution of Numb, a repressor of the Notch signaling pathway [Zhong et al., 1997]. The resulting daughter NSC remains in contact with both the ventricular and pial surfaces, while the neuronal progenitor breaks contact with the ventricular zone just hours after division, and migrates along the radial glia cells, towards the basal surface [Miyata et al., 2001; Noctor et al., 2001]. This creates a layer of post-mitotic neurons at the basal region of the neural tube. The brief retention of contact with the ventricular zone following division may provide the neuronal progenitor with fate determining information, based upon asymmetric distribution of adherens and tight junction proteins, as well as cell-fate determining factors, like Numb, at the luminal face [Gotz and Huttner, 2005; Miyata, 2007].

Proliferation in the ESC-derived rosette is also spatially restricted. Labeling of rosettes with phospho-histone H3 (PH3) shows that mitosis occurs near the apical surface (Fig. 2C) [Elkabetz et al., 2008]. The establishment of a "pool" of NSCs at the apical surface, and neuronal markers at the basal surface, is also observed within the rosette structure. Immunocytochemistry analysis of NSC markers, such as the RNA-binding protein Musashi1 (Msi1), demonstrates localization of these proteins to the luminal surface. Additionally, markers indicative of terminal neuronal differentiation, such as βIII-tubulin, are present at the outermost region of the rosette (Fig. 2C) [Grabel, unpublished work]. To date, the nature of NSC divisions within the rosette have not been characterized as extensively as divisions within the neural tube. For example, further investigation is necessary to determine whether Numb is involved in determining symmetry of division within rosette NSCs.

DIFFERENTIATION AND PATTERNING

The neural tube is divided into discrete regional domains during embryonic CNS development (discussed earlier). NSC rosettes also have regional identities. For example, rosettes expressing the forebrain specific antigen Forse-1, emerge amidst Forse-1-negative but engrailed1-positive rosettes [Grabel, unpublished work]. In addition, individual rosettes may display concentric rings with distinct region identities. How do these distinctly patterned rosettes emerge in a single dish in which the cells are theoretically exposed to the same soluble components? There are two apparent explanations: first, local signaling centers are established in vitro. For example, we have shown that a subset of differentiating cells in monolayer make and secrete SHH [Cai et al., 2008]. Second, cells can respond differently to the same signals based upon their history and fate.

RECAPITULATION OF IN VIVO CORTICOGENESIS BY ESC-DERIVED NSCs

Emerging data suggest that ESC-derived NSCs recapitulate in vivo corticogenesis in vitro [Eiraku et al., 2008] [Gaspard et al., 2008]. The process of corticogenesis gives rise to the cerebral cortex, a complex and stratified brain structure coordinately patterned by sequential

gene expression and migration of cortical neurons from the ventricular zone to their respective cortical layers. This brain region plays a significant role in cognitive functions, as well as vision, memory, and language. The molecular mechanisms that govern corticogenesis are largely unknown. Therefore, the ability of ESC-derived NSCs to mimic corticogenesis in vitro provides an ideal model system for dissecting and discovering key regulators of cortical development.

The cerebral cortex consists of varied cortical neuronal subtypes, with the cell bodies of specified subtypes arranged into six layers (Fig. 3). The horizontal layering of densely packed cell bodies within these layers gives the cortex its shading and subsequent distinction as gray matter. These layers are formed by an inside-out patterning mechanism; the earliest cortical progenitors form the innermost layers of the cortex, while neurons born at later stages of corticogenesis migrate away from the ventricular zone to form the outermost layers [Rakic, 1988]. Layer I, consisting largely of Cajal-Retzius neurons is an exception to this pattern however, as these cortical cells are born first, around embryonic days 10–11.5.

Cortical neurons, generated from the radial glia at the ventricular surface of the developing cortex, are classified broadly into two categories, pyramidal and stellate neurons, based upon the distinct morphological characteristics of their dendritic arborization. These classes of cortical neurons can further be divided into smaller subclasses, based on distinct cortex-specific gene and protein expression. Layer I is the most superficial layer of the cortex, and is also referred to as the molecular layer. This layer, composed of the Cajal-Retzius neurons and pyramidal cortical cells, is characterized by stereotyped expression of the cortical migratory neuronal marker, reelin and the transcription factor T-box brain 1 (TBR1), respectively [Gaspard et al., 2008]. The deepest layers are established throughout mouse E11.5–E14.5, producing cortical neurons expressing the transcription factors OTX1 and TBR1, as well as corticoneuronal zinc finger protein CTIP2. The upper layers of the cortex, layers II and III, develop last, appearing at mouse E13.5–E16.5, and are populated by both pyramidal and stellate neurons expressing SATB2 and CUX1, DNA-binding proteins involved in cortical cell fate determination [Gaspard et al., 2008].

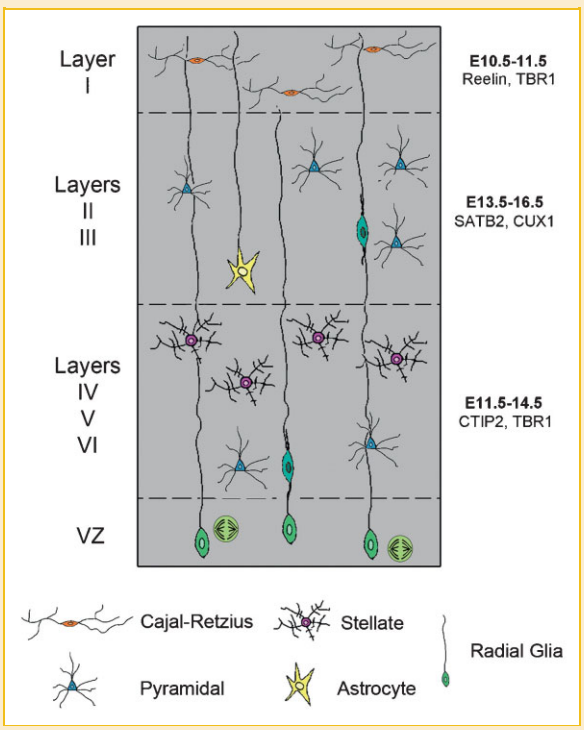

Fig. 3. Corticogenesis. The cortex is comprised of six distinct layers, each containing a variety of cells born at different times during development. Characteristic markers of each layer and approximate birthdates of cells expressing these markers are indicated. New neurons are generated from radial glia in the ventricular zone that then migrate along glial processes towards the pial surface to form the cortical layers. The earliest born cells reside in the innermost layers while later born cells reside in outer layers, except the Cajal-Retzius cells which are born first but migrate to form layer I. Following neurogenesis, radial glia differentiate into astrocytes, lose attachment from the ventricular zone, and populate higher layers of the cortex. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Recent data illustrate the ability of ESCs—both mouse and human—to not only generate cortical progenitors, but to do so in a spatial and temporal pattern reminiscent of corticogenesis [Eiraku et al., 2008; Gaspard et al., 2008]. Combined birthdating and immunocytochemical analysis performed on mouse ESC-derived NSCs, through the use of adherent neural differentiation, revealed the establishment of progenitors representative of each cortical layer [Gaspard et al., 2008]. The earliest mouse ESC-derived cortical cells are visible 6 days following the onset of neural differentiation and are reelin-positive, representative of the Cajal-Retzius neurons that populate the molecular layer of the cortex. TBR1-positive neurons, reminiscent of the pyramidal cells present in layer I of the cortex, emerge shortly after the reelin-positive cortical progenitors, at day 7 of differentiation. Additional OTX1, CTIP2, SATB2, and CUX1-positive cortical neurons differentiate and appear sequentially at days 8, 9, and 12 of culture, respectively [Gaspard et al., 2008]. Following formation of the cortical layers, the radial glia, present in the ventricular zone, terminally differentiate, becoming astrocytes, and migrate upwards towards the pial surface (Fig. 3) [Okano and Temple, 2009].

This recapitulation of corticogenesis can also be accomplished by three-dimensional culture of either mouse or human ESCs, utilizing embryoid body intermediates cultured under serum-free culture conditions [Watanabe et al., 2005; Eiraku et al., 2008]. Mouse ESCs expressing GFP under the control of the cortical transcription factor BF1 were isolated from early stage embryoid bodies using fluorescence-activated cell sorting (FACS), and reaggregated to allow continued neural differentiation. Controlled exit of the cell cycle, by the addition of the gamma-secretase inhibitor DAPT, an inhibitor of the pro-proliferation signal Notch, reveals the sequential differentiation of layer-specific cortical neurons [Eiraku et al., 2008]. Similar to data obtained from adherent neural differentiation, DAPT treatment of a culture during early neural differentiation, at day 9, reveals a bias towards development of reelin-positive cortical progenitors [Eiraku et al., 2008]. However, DAPT treatment at later stages, at day 12, produces cultures largely expressing markers of deep cortical layers, like CTIP2. Human ESC-derived NSCs, cultured in the presence of Dkk1, the SMAD inhibitor SB431542, and the BMP antagonist BMPR-IA-Fc, preferentially

acquire forebrain identities and produce cortical progenitors in the same temporal manner as the isolated BF1::GFP mouse ESCs [Eiraku et al., 2008].

In addition to the expression of layer specific cortical markers, ESC-derived NSCs recapitulate the in vivo cytoarchitecture of the cortex [Eiraku et al., 2008]. Immunocytochemistry analysis of cryosectioned embryoid bodies, revealed distribution of cortical progenitors into horizontal layers much like the in vivo cortex structure. Reelin-positive cells were largely restricted to the periphery of rosettes, mimicking the cortical molecular layer, while Tbr1 expressing cells were confined to sublayers immediately adjacent to the reelin-positive region, as well as a small population of cells at the luminal surface, corresponding to layers I and VI of the cortex.

While ESC-derived layer-specific cortical progenitors are not produced in vitro in ratios similar to endogenous cortical neurons, the ESC-derived cortical progenitors respond to regional specific signals and adopt appropriate cell fates in culture. For example, 100% of ESC-derived cortical neurons differentiated in the presence of FGF8 become rostralized and are thus Tbr1 and reelin-positive. Subsequently, attenuation of FGF signaling by addition of FGFR3-Fc caudalizes the culture, as observed by increased expression of the caudal transcription factor COUP-TF1, and inhibits the expression of more rostral markers [Gaspard et al., 2008].

ESC-derived cortical progenitors also demonstrate the ability to extend appropriate cell-type specific projections when transplanted in vivo and functionally incorporate into host circuitry [Eiraku et al., 2008; Gaspard et al., 2008]. Endogenous cortical neurons extend layer-specific projections to neurons within a variety of regions of the cortex, such as the areas involved in language and vision, as well as extending projections to several subcortical regions, such as the thalamus and striatum. ESC-derived cortical progenitors, when transplanted in vivo, make preferential projections towards the pial surface, like their endogenous counterparts, as well as extending layer subtype-specific projections to cortical and sub-cortical brain regions [Eiraku et al., 2008].

CONCLUSIONS

Despite dramatic differences between the in vitro and the in vivo neurogenic environments, ESC neurogenesis has remarkable parallels to neural specification and neural differentiation during embryogenesis. Rosette NSCs mimic the neural tube's apicobasal polarity axis, establishment of proliferation at the ventricular zone, as well as the morphogenetic specification of neuronal subtypes. Three-dimensional differentiation of ESCs into NSCs via embryoid body intermediates closely recapitulates embryonic development of the cortical layers. These similarities suggest that morphogenesis and patterning of both the neural tube and ESC-derived NSCs are shaped by analogous mechanisms. These data suggest that a number of aspects of neural specification, for example, cell migration during corticogenesis, can be studied under in vitro conditions that can readily be manipulated via pharmacological or genetic intervention. These studies will define optimized conditions for production of specific neural subtypes, and therefore aid in the design of future therapeutic approaches.

REFERENCES

Anderson RM, Lawrence AR, Stottmann RW, Bachiller D, Klingensmith J. 2002. Chordin and noggin promote organizing centers of forebrain development in the mouse. Development 129:4975–4987.

Ang SL, Conlon RA, Jin O, Rossant J. 1994. Positive and negative signals from mesoderm regulate the expression of mouse Otx2 in ectoderm explants. Development 120:2979–2989.

Barberi T, Klivenyi P, Calingasan NY, Lee H, Kawamata H, Loonam K, Perrier AL, Bruses J, Rubio ME, Topf N, Tabar V, Harrison NL, Beal MF, Moore MA, Studer L. 2003. Neural subtype specification of fertilization and nuclear transfer embryonic stem cells and application in parkinsonian mice. Nat Biotechnol 21:1200–1207.

Bibel M, Richter J, Schrenk K, Tucker KL, Staiger V, Korte M, Goetz M, Barde YA. 2004. Differentiation of mouse embryonic stem cells into a defined neuronal lineage. Nat Neurosci 7:1003–1009.

Cai C, Grabel L. 2007. Directing the differentiation of embryonic stem cells to neural stem cells. Dev Dyn 236:3255–3266.

Cai C, Thorne J, Grabel L. 2008. Hedgehog serves as a mitogen and survival factor during embryonic stem cell neurogenesis. Stem Cells 26:1097–1108.

Chambers SM, Fasano CA, Papapetrou EP, Tomishima M, Sadelain M, Studer L. 2009. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nat Biotechnol 27:275–280.

Dimos JT, Rodolfa KT, Niakan KK, Weisenthal LM, Mitsumoto H, Chung W, Croft GF, Saphier G, Leibel R, Goland R, Wichterle H, Henderson CE, Eggan K. 2008. Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. Science 321:1218–1221.

Eiraku M, Watanabe K, Matsuo-Takasaki M, Kawada M, Yonemura S, Matsumura M, Wataya T, Nishiyama A, Muguruma K, Sasai Y. 2008. Self-organized formation of polarized cortical tissues from ESCs and its active manipulation by extrinsic signals. Cell Stem Cell 3:519–532.

Elkabetz Y, Studer L. 2008. Human ESC-derived neural rosettes and neural stem cell progression. Cold Spring Harb Symp Quant Biol 73:377–387.

Elkabetz Y, Panagiotakos G, Al Shamy G, Socci ND, Tabar V, Studer L. 2008. Human ES cell-derived neural rosettes reveal a functionally distinct early neural stem cell stage. Genes Dev 22:152–165.

Gaspard N, Bouschet T, Hourez R, Dimidschstein J, Naeije G, van den Ameele J, Espuny-Camacho I, Herpoel A, Passante L, Schiffmann SN, Gaillard A, Vanderhaeghen P. 2008. An intrinsic mechanism of corticogenesis from embryonic stem cells. Nature 455:351–357.

Gerrard L, Rodgers L, Cui W. 2005. Differentiation of human embryonic stem cells to neural lineages in adherent culture by blocking bone morphogenetic protein signaling. Stem Cells 23:1234–1241.

Gilbert SF. 2010. Developmental biology. Sunderland, MA: Sinaur Associates, Inc.

Gotz M, Huttner WB. 2005. The cell biology of neurogenesis. Nat Rev Mol Cell Biol 6:777–788.

Hansen DV, Lui JH, Parker PR, Kriegstein AR. 2010. Neurogenic radial glia in the outer subventricular zone of human neocortex. Nature 464:554–561.

Hatch MN, Nistor G, Keirstead HS. 2009. Derivation of high-purity oligodendroglial progenitors. Methods Mol Biol 549:59–75.

Helms AW, Johnson JE. 2003. Specification of dorsal spinal cord interneurons. Curr Opin Neurobiol 13:42–49.

Hoch RV, Rubenstein JL, Pleasure S. 2009. Genes and signaling events that establish regional patterning of the mammalian forebrain. Semin Cell Dev Biol 20:378–386.

Kawasaki H, Mizuseki K, Nishikawa S, Kaneko S, Kuwana Y, Nakanishi S, Nishikawa SI, Sasai Y. 2000. Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity. Neuron 28:31–40.

Kriegstein A, Alvarez-Buylla A. 2009. The glial nature of embryonic and adult neural stem cells. Annu Rev Neurosci 32:149–184.

Lamb TM, Harland RM. 1995. Fibroblast growth factor is a direct neural inducer, which combined with noggin generates anterior–posterior neural pattern. Development 121:3627–3636.

Lee SH, Lumelsky N, Studer L, Auerbach JM, McKay RD. 2000. Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. Nat Biotechnol 18:675–679.

Lee H, Shamy GA, Elkabetz Y, Schofield CM, Harrison NL, Panagiotakos G, Socci ND, Tabar V, Studer L. 2007. Directed differentiation and transplantation of human embryonic stem cell-derived motoneurons. Stem Cells 25: 1931–1939.

Levine AJ, Brivanlou AH. 2007. Proposal of a model of mammalian neural induction. Dev Biol 308:247–256.

Li XJ, Zhang X, Johnson MA, Wang ZB, Lavaute T, Zhang SC. 2009. Coordination of sonic hedgehog and Wnt signaling determines ventral and dorsal telencephalic neuron types from human embryonic stem cells. Development 136:4055–4063.

Liu A, Joyner AL. 2001. Early anterior/posterior patterning of the midbrain and cerebellum. Annu Rev Neurosci 24:869–896.

Liu JP, Laufer E, Jessell TM. 2001. Assigning the positional identity of spinal motor neurons: Rostrocaudal patterning of Hox-c expression by FGFs, Gdf11, and retinoids. Neuron 32:997–1012.

Maden M. 2002. Retinoid signalling in the development of the central nervous system. Nat Rev Neurosci 3:843–853.

Marthiens V, Ffrench-Constant C. 2009. Adherens junction domains are split by asymmetric division of embryonic neural stem cells. EMBO Rep 10:515–520.

Martinez S, Crossley PH, Cobos I, Rubenstein JL, Martin GR. 1999. FGF8 induces formation of an ectopic isthmic organizer and isthmocerebellar development via a repressive effect on Otx2 expression. Development 126:1189–1200.

Millet S, Campbell K, Epstein DJ, Losos K, Harris E, Joyner AL. 1999. A role for Gbx2 in repression of Otx2 and positioning the mid/hindbrain organizer. Nature 401:161–164.

Miyata T. 2007. Asymmetric cell division during brain morphogenesis. Prog Mol Subcell Biol 45:121–142.

Miyata T, Kawaguchi A, Okano H, Ogawa M. 2001. Asymmetric inheritance of radial glial fibers by cortical neurons. Neuron 31:727–741.

Murashov AK, Pak ES, Hendricks WA, Owensby JP, Sierpinski PL, Tatko LM, Fletcher PL. 2004. Directed differentiation of embyronic stem cells into dorsal interneurons. FASEB J 2:252–254.

Noctor SC, Flint AC, Weissman TA, Dammerman RS, Kriegstein AR. 2001. Neurons derived from radial glial cells establish radial units in neocortex. Nature 409:714–720.

Noctor SC, Martinez-Cerdeno V, Ivic L, Kriegstein AR. 2004. Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. Nat Neurosci 7:136–144.

Okabe S, Forsberg-Nilsson K, Spiro AC, Segal M, McKay RD. 1996. Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro. Mech Dev 59:89–102.

Okada Y, Matsumoto A, Shimazaki T, Enoki R, Koizumi A, Ishii S, Itoyama Y, Sobue G, Okano H. 2008. Spatiotemporal recapitulation of central nervous system development by murine embryonic stem cell-derived neural stem/progenitor cells. Stem Cells 26:3086–3098.

Okano H, Temple S. 2009. Cell types to order: Temporal specification of CNS stem cells. Curr Opin Neurobiol 19:112–119.

Perrier A, Tabar V, Barberi T, Rubio ME, Bruses J, Topf N, Harrison NL, Studer L. 2004. Derivation of midbrain dopamine neurons from human embryonic stem cells. Proc Natl Acad Sci 101:12546–12548.

Rakic P. 1988. Specification of cerebral cortical areas. Science 241:170–176.

Salero E, Hatten ME. 2007. Differentiation of ES cells into cerebellar neurons. Proc Natl Acad Sci USA 104:2997–3002.

Sauer ME, Walker BE. 1959. Radioautographic study of interkinetic nuclear migration in the neural tube. Proc Soc Exp Biol Med 101:557–560.

Shimamura K, Martinez S, Puelles L, Rubenstein JL. 1997. Patterns of gene expression in the neural plate and neural tube subdivide the embryonic forebrain into transverse and longitudinal domains. Dev Neurosci 19:88–96.

Smith JL, Schoenwolf GC. 1997. Neurulation: Coming to closure. Trends Neurosci 20:510–517.

Stern CD. 2005. Neural induction: Old problem, new findings, yet more questions. Development 132:2007–2021.

Tropepe V, Hitoshi S, Sirard C, Mak TW, Rossant J, van der Kooy D. 2001. Direct neural fate specification from embryonic stem cells: A primitive mammalian neural stem cell stage acquired through a default mechanism. Neuron 30:65–78.

Watanabe K, Kamiya D, Nishiyama A, Katayama T, Nozaki S, Kawasaki H, Watanabe Y, Mizuseki K, Sasai Y. 2005. Directed differentiation of telencephalic precursors from embryonic stem cells. Nat Neurosci 8:288–296.

Westmoreland JJ, Hancock CR, Condie BG. 2001. Neuronal development of embryonic stem cells: A model of GABAergic neuron differentiation. Biochem Biophys Res Commun 284:674–680.

Wilson PG, Stice SS. 2006. Development and differentiation of neural rosettes derived from human embryonic stem cells. Stem Cell Rev 2: 67–77.

Wurst W, Bally-Cuif L. 2001. Neural plate patterning: Upstream and downstream of the isthmic organizer. Nat Rev Neurosci 2:99–108.

Xu Q, Guo L, Moore H, Waclaw RR, Campbell K, Anderson SA. 2010. Sonic hedgehog signaling confers ventral telencephalic progenitors with distinct cortical interneuron fates. Neuron 65:328–340.

Ying QL, Stavridis M, Griffiths D, Li M, Smith A. 2003. Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. Nat Biotechnol 21:183–186.

Zhong W, Jiang MM, Weinmaster G, Jan LY, Jan YN. 1997. Differential expression of mammalian Numb, Numblike and Notch1 suggests distinct roles during mouse cortical neurogenesis. Development 124:1887–1897.