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Original Article

Development and Differentiation of Neural Rosettes Derived From Human Embryonic Stem Cells

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Abstract

Neurons and glia are important targets of human embryonic stem cell research, promising a renewable source of these differentiated cells for biomedical research and regenerative medicine. Neurons and glia are derived in vivo from the neuroepithelium of the neural tube. Concomitant to development along the anterior to posterior axis, gradients of morphogens across the dorsal and ventral axis of the neural tube establish positional codes that generate distinct progenitor domains and ultimately specify subtype identity. The neural rosette is the developmental signature of neuroprogenitors in cultures of differentiating embryonic stem cells; rosettes are radial arrangements of columnar cells that express many of the proteins expressed in neuroepithelial cells in the neural tube. In addition to similar morphology, neuroprogenitors within neural rosettes differentiate into the main classes of progeny of neuroepithelial cells in vivo: neurons, oligodendrocytes, and astrocytes. Despite these similarities, important differences exist and the extent to which neural rosettes can model neurogenesis in vivo is not yet clear. Here, the authors review the recent studies on the development and differentiation of neural rosettes from human embryonic stem cells. The authors focus on efforts to generate motor neurons and oligodendrocytes in vitro as representative of the challenges to obtaining the progeny of a single progenitor domain with in vitro methods. Opportunities for further progress are discussed.

Index Entries: Embryonic stem cells; neuroprogenitors; neural stem cells; gliogenesis; neurogenesis; neurons; glia.

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Introduction

Since the first report that human embryonic stem cells (hESCs) could be cultured in vitro *(1)*, research teams worldwide have directed efforts toward utilizing hESCs as a source of differentiated cells. ESCs are derived from the inner cell mass of the blastocyst embryo, a population of proliferating totipotent stem cells that generates all of the somatic as well as germline cells in adults. In contrast to somatic cells that undergo senescence, ESCs proliferate indefinitely in culture and can be maintained as diploid cells with a normal karyotype. Thus, hESCs promise a renewable source of differentiated cells and the proliferating precursors that produce them.

Neurons and glia in the central nervous system (CNS) are important targets of stem cell research. Neurons send and receive instructions in the form of electrochemical signals that underlie such normal activities as movement, memory, sensation, and learning. Each of these activities is threatened by neuronal injury; neurons are terminally differentiated cells that do not proliferate to replace those lost to damage or disease. Neuron function, viability, and response to damage rely heavily on glia, most of which arise during the development from the same pool of precursors as the do neurons. Oligodendrocytes form an insulating sheath that surrounds axons in the CNS and enables rapid transmission of nerve impulses.

Astrocytes are important to nutrition and exchange of metabolites as well as in regulation of neurotransmitters as integral parts of nerve synapses. Thus, both neurons and glia are involved in brain and spinal cord injuries and in neurodegenerative diseases such as multiple sclerosis, epilepsy, and chronic pain among others *(2,3)*. Initial efforts to derive neural lineages from hESCs in vitro relied on spontaneous differentiation *(4)*, subculture of neuroprogenitors in neurospheres *(5)*, or immunoselection of living precursor cells within embryoid bodies *(6)*, aggregates of differentiating hESCs *(7,8)*. Recent studies have produced new insights into derivation of neural lineages from hESCs, based in part, on parallel advances in developmental neurobiology. Here, the authors integrate neurogenesis in vivo with derivation of neural lineages from hESCs. The authors emphasize derivation of motor neurons and oligodendrocytes that arise sequentially from a single progenitor domain during embryogenesis. Models for neurogenesis in human embryogenesis draw on the wealth of consolidating information available from mouse, chick, zebra fish, and flies. Although neurogenesis is highly conserved across species, important differences exist, even between humans and rodent systems *(9,115,117)*.

Specification of Neuroectoderm and Formation of the Neural Tube

The nervous system and the epidermis emerge from the ectoderm germlayer of the gastrulated embryo. Signaling by members of the bone morphogenic protein (BMP) family of transforming growth factors specifies ectoderm to become epidermis *(10,11)*, but blocks specification of neuroectoderm. Specification of neuroectoderm is thought to be a default pathway in which the absence of BMP signaling is permissive rather than instructive *(12)*. Antagonism of BMP signaling is supported by the essential role of Ectodermin, a conserved Smad4 ubiquitin ligase, in ectoderm specification in *Xenopus (13)*. BMP signaling triggers phosphhorylation of a subset of Smad proteins, allowing them to bind to Smad4 and enter the nucleus in which they activate or repress genetic networks. Thus, destruction of Smad4 through Ectodermin actively denies an epidermal fate to ectoderm, allowing specification of neuroectoderm as the default. Smad4 also mediates signaling through the Activin/Nodal branches of the transforming growth factor-β family *(10)*, but it is not yet clear if the inactivation of the Activin/Nodal pathways is also necessary for specification of neuroectoderm. Alternative views of neuroectoderm specification cite evidence for positive instructive signals, based in part, on studies showing that loss or diminished signaling through basic fibroblast growth factor (bFGF; also known as FGF2) and Wnt (wingless) pathways prevents neurogenesis *(14,15)*. Because these molecules mediate signaling through other pathways as well, the effects of bFGF and Wnt on neurogenesis could be indirect measures of BMP activity *(16)*. Nonetheless, alternative pathways to neuroectoderm specification cannot be excluded.

In response to specification, the neuroectoderm forms the neural plate, cytologically detectable by its dorsal position on the embryo and the elongated columnar shape of constituent cells (Fig. 1). Development of the neural plate is polar, moving in the rostral (anterior/head) to caudal (posterior/tail) direction. Two neural folds form in the neuroectoderm, rising up to form a groove that deepens and takes on the hallmark key-hole shape of the neural tube. The neural folds fuse along the length of the groove to form a hollow open-ended tube. Concomitantly, cells proximal to the neural plate fuse to form an overlying sheet of epidermis. Fusion events are mediated at least in part by cell adhesion molecules (CAM) like NCAM in the neural plate and the subsequent development of cell–cell junctions. The open ends of the neural tube, known as the rostral and caudal neuropores, close later in their development. Failure of neuropores to close generate congenital defects like spina bifida and anencephaly *(17)*. The neuroepithelium of the forming

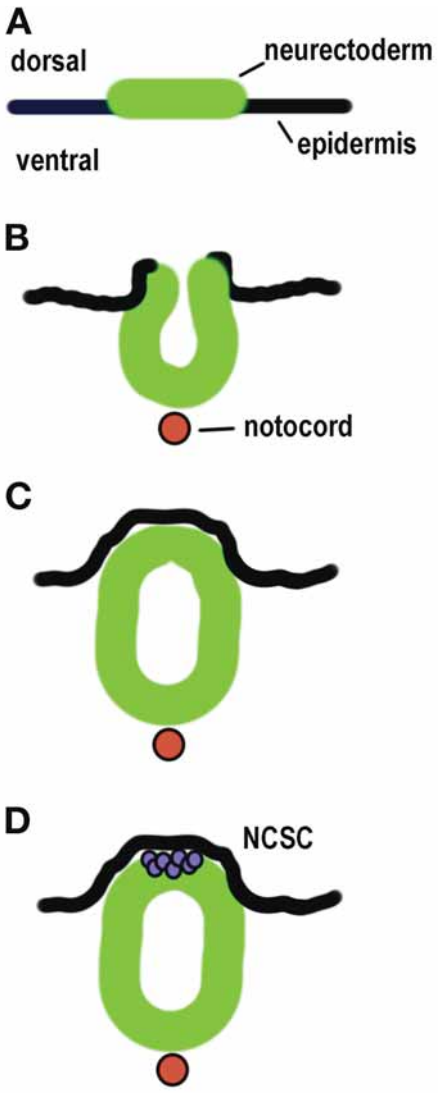

Fig. 1. Sagittal view of forming neural tube. **(A)** Following specification, neuroectoderm cells on the dorsal side of the embryo acquire a columnar morphology, represented here as thick green line. The neural plate is flanked by epidermis, represented by black line. For simplicity, the underlying mesoderm and endoderm is not shown. **(B)** Neural folds rise up and form a groove that overlies the forming notocord. **(C)** The neural folds and the epidermis fuse, forming the open ended neural tube underneath a sheet of epidermis. **(D)** Neuroepithelial cells delaminate from the neural tube and form a cluster (purple balls) between the epidermis and the dorsal neural tube before migrating throughout the embryo.

neural tube is a single cell layer thick and shows definitive characteristics of epithelia *(18,19)*. After fusion of the neural folds, the neuroepithelium thickens by mitotic proliferation. Neuroepithelial cells show characteristic interkinetic nuclear migration in which nuclei of mitotic cells lie proximal to the lumen, whereas the nuclei of interphase cells undergoing DNA synthesis lie distal to the lumen near the basement membrane at the outer surface *(20,21)*. When neuroepithelial cells exit the cell cycle and initiate differentiation, they move away from the lumen and into a mitotically quiescent region. The relative position of mitotically active and inactive neural cells in postnatal and adult CNS mirrors that of the developing neural tube, known as the ventricular and mantle zones, respectively. Here, the authors designate cells in the neuroepithelium of the forming neural tube as neuroepithelial cells and neuroprogenitors as the progeny of the neuroepithelial cells in vivo and neuroprecursor cells derived from ESCs in culture.

Historically, the neural tube was thought to consist of two different cell types, neuroepithelial cells, which produced neurons and radial glia that produced glia *(18,19,22,23)*. Radial glia extend long processes to both surfaces of the neural tube and show interkinetic nuclear migration. The processes of radial glia appear to guide newly born neurons moving away from the luminal region of the neural tube. Radial glia transform into astrocytes near the end of the neurogenic period of development and, with the exception of small populations of closely related Bergmann's glia and Muller glia, radial glia are not present in adults. Results from several recent studies show that radial glia are both neurogenic and gliogenic *(19)*, including live imaging of radial glia undergoing asymmetric cell division to generate another radial glial cell and a neuron. Functional studies show that radial glia are essential for normal CNS development *(24)*, but it has been difficult to establish the developmental potential of these cells, in part because few markers are available to distinguish radial glia from neuroprogenitors and astrocytes and in part because the expression profile of radial glia is dynamic *(19)*. Like neuroprogenitors, radial glia express intermediate filament proteins nestin as well as the transcription factor PAX6 that is expressed in some neuroprogenitors in the ventral half of the neural tube. Radial glia also express proteins characteristic of astrocytes, including the widely used glial fibrillar acidic protein (GFAP), among others *(19)*. Cytological markers that might be unique to radial glia include modified forms of nestin identified by the RC1 and RC2 antibodies that recognize the murine antigens. Although it is not yet clear whether some, or all neurons in the CNS have a radial glial origin, radial glia have become an active area of stem cell research as radial glia in culture might prove to be most like the neuroepithelial cells of the neural tube. Radial glia have already been derived from mouse ESCs and differentiated into neurons and glia in vitro *(25)*.

Neurons and glia in the peripheral nervous system are derived from a cluster of neural crest stem cells (NCSCs) that delaminate from the neural tube and accumulate between the epidermis and the neural tube (Fig. 1). NCSCs migrate throughout the embryo and differentiate into a diverse array of cell types, including the neurons and glia in the peripheral *(26,27)* and enteric nervous systems *(28)*, smooth muscle, bone and melanocytes *(29)*. Founded on studies in mouse and primate ESCs *(30,31)*, recent work indicates that NCSC can be obtained from hESCs *(32)*. Isolation of NCSCs in vitro is an important advance in biomedical research and it is important that recovery of NCSCs is considered when differentiating hESCs toward neural lineages. However, NCSCs and their derivatives will not be considered further here.

Morphogen Gradients and Specification of Progenitor Domains

The diversity generated among the progeny of the neuroepithelial cells is initiated by morphogens that elicit a concentration-dependent response in target cells. The rostrocaudal and dorsoventral position of a neuroprogenitor within the developing neural tube determines its exposure to morphogen gradients that will restrict its developmental potential and define its fate *(33–35)*. Retinoic acid (RA) is a caudalizing signaling molecule that mediates sequential specification of the neural tube into four main subdivisions along its rostrocaudal axis: the forebrain, midbrain, hindbrain, and spinal cord. Following specification and in response to additional signaling molecules *(12,15)*, the neuroectoderm initially shows a rostral character and is secondarily induced to form more caudal structures. Within the spinal cord, the dorsoventral axis correlates with neuronal subtype. Motor neurons emerge from ventral regions of the neural tube and communicate with muscles, glands and other effecter tissues. Spinal motor neurons are organized in columns along the rostrocaudal axis in which the axons of neurons within a column are directed to a common target *(36,37)*. The cell bodies of sensory neurons of the peripheral nervous system are organized in clusters called ganglions that lie out side, but next to the spinal cord. The axons of many sensory neurons enter the spinal cord in which they terminate near interneurons in both the dorsal and ventral regions of the spinal cord. Interneurons are important to processing sensory information and coordinating motor activity and represent the vast majority of neuronal subtypes *(34,38)*.

Sonic hedgehog (Shh) and BMP family members play pivotal roles in specifying progenitor domains in the ventral *(33,35,39)* and dorsal *(34,40–42)* regions of the neural tube, respectively. Shh is first secreted from the notocord (Fig. 1), a specialized region of mesoderm that lies between the forming neural tube and the underlying endoderm of the gastrula stage embryo. Cells in the neural tube immediately adjacent to the notocord, known as floorplate, respond to Shh signally and then express Shh as well. As a result, a concentration gradient is created in which notocord-proximal cells in the neural tube are exposed to a higher concentration of Shh than more distal cells. Shh mediates its concentration-dependent effects through Gli1, Gli2, and Gli3, zinc finger containing transcription factors *(43)* that act combinatorially to activate and/or repress expression of Shh responsive targets *(44,45)*. The dorsal epidermis that overlies the neural tube secretes several BMP proteins that induce proximal cells, collectively known as the roof plate, to express BMPs as well. As a result, a gradient of BMP signaling is generated in the dorsal neural tube that mirrors that of Shh in the ventral neural tube. Additional signaling molecules also contribute to dorsoventral patterning of the neural tube *(46)*; notably, retinoic acid secreted by the paraxial mesoderm is necessary for Shh dependent patterning *(47)*. The end result of dorsoventral patterning is 11 distinct domains of neuroprogenitors; 10 of these domains will ultimately

produce interneurons, whereas only one domain will first produce motor neurons and then oligodendrocytes *(41)*.

Sequential Emergence of Neurons and Glia

The neuroepithelium of the neural tube gives rise to all of the neurons as well as glia in the CNS; however, neurogenesis precedes gliogenesis. Neuroepithelial cells must first proliferate; otherwise, differentiation will produce too few neurons and glia to sustain normal development. The Sox family of transcription factors are involved in a broad range of developmental pathways, including neurogenesis and gliogenesis. Among more than 20 members of the Sox family, members of the SoxB1 group, Sox1-3, are expressed in broadly overlapping regions of the developing CNS *(48)* and in adult neural stem cells *(49)*. Although family members are structurally very similar and can partially compensate for loss-of-function of other family members, SoxB1 proteins are not functionally identical. Mice lacking Sox2 fail to form primitive ectoderm, whereas mice deficient in either Sox1 or Sox3 develop without significant defects in development. Forced expression of Sox2 maintains neuroprogenitor identity in vivo and loss of its expression in proliferating neuroprogenitors correlates with the onset of differentiation *(50,51)*. Recent work indicates that Sox2 plays a role in self-renewal of hESCs *(52)* as well as self-renewal of neuroprogenitors in adults *(50,53)*. Although Sox genes are among the earliest expressed, it is important to note that Sox genes are not the sole regulators of neuroprogenitor proliferation in vivo or in vitro *(54–58)*.

The molecular mechanism underlying the switch from the neurogenic to gliogenic phases of early development is not well established; however, the switch from self-renewal to differentiation of neuroprogenitors is thought to involve antagonism of SoxB1 function, in part by the activity of the SoxB2 group of genes. Sox proteins belong to the high-mobility group (HMG) of DNA-binding proteins that require a binding partner to activate gene expression *(48)*. For example, Sox2 co-occupies promoters with Oct4 in epiblast cells of the inner cell mass *(59)*, with Brn2 in CNS stem cells *(60)* and with PAX6 during the lens development *(61)*. SoxB1 function is antagonized by proneural transcription factors that drive neuroprogenitors to exit a proliferating cell cycle and initiate postmitotic differentiation *(62)*. Proneural genes increase expression of SoxB2 genes, *Sox21* and *Sox 14 (48)*. SoxB2 proteins are thought to bind to the same sites as SoxB1 proteins, but repress rather than activate gene expression and thereby neutralizing SoxB1 activity. Precisely how the levels of SoxB1 and SoxB2 proteins are regulated is not yet clear. Before SoxB1 expression is lost in vivo, members of the SoxE group, Sox8–10, begin to show expression, marking the onset of gliogenesis. Expression of SoxB1 and SoxE genes is associated with a proliferative state, but some Sox genes continue to be expressed in select subsets of differentiated cells *(49)*. Sequential expression of *SoxB1* and *SoxE* genes correlates well with the general theme in developmental neurobiology in which neurogenesis precedes gliogenesis.

Specification and Differentiation of Progenitors in the pMN Domain

A collection of studies provide insight into the molecular basis of specification of neuroprogenitor fate and differentiation of the diverse array of neuronal subtypes that includes the CNS. The spinal cord region of the developing neural tube is one of the most studied *(33,63,64)*. In the ventral half of the neural tube, homeodomain, and basic helix-loop-helix transcription factors translate the Shh/Gli signaling gradient into progenitor fate. Graded levels of Shh signaling repress expression of class I genes and induce expression of class II genes (Fig. 2). The vast majority of both classes are repressors, suggesting that specification of neuroprogenitors identity is mediated by derepression. Complementary pairs of classes I and II genes within abutting domains are cross repressive, sharpening the boundaries between domains and consolidating progenitor identity within domains. For example, the combined activities of class I/class II genes Pax6.1/Nkx2.2 ventrally and Irx3/Olig2 dorsally delineate the boundaries of the pMN domain, the progenitor domain that will ultimately produce motor neurons and oligodendrocytes. Expression of Nkx6 proteins induces expression of Olig2 that in turn maintains pMN identity by repressing expression of Irx3, a potent repressor of pMN specification and development of motor neurons and oligodendrocytes.

Olig2 plays a central role in coordinating expression of pan-neural genes expressed in all neurons with expression of subtype identity genes that will produce motor neurons (Fig. 3). Olig2 promotes expression of Ngn2 that accumulates heterogeneously in different cells *(65)*. A subset of cells accumulate sufficient Ngn2 to out-compete Olig2 for shared DNA-binding sites *(65)*, downregulating Olig2 in that cell. Ngn2 promotes expression of the proneural genes *NeuroD* and *NeuroM*; these genes trigger exit from a mitotic cell cycle and expression of pan-neural genes like β-*III tubulin* and *MAP2*. Concomitantly, Olig2 promotes expression of subtype identity transcription factors, including Isl1, Isl2, and Lhx3 *(66–69)* and choline acetyltransferase (ChAT), an essential enzyme in production of the neurotransmitter acetylcholine. Isl proteins and Lhx3 have Lim domains that mediate interaction with Lim domain binding protein (Ldb/NLI/Clim) and form a ternary complex. Synergism with pronuclear gene products coordinates expression of pan-neural genes with expression of genes like the homeodomain transcription factor HB9 that is expressed primarily in motor neurons. Olig2 and Ngn2 expression is lost in cells undergoing motor neuron differentiation, but HB9 expression becomes Shh independent and continues in postmitotic neurons throughout adult development. Consistent with the roles ascribed, ectopic expression of Olig2 alone *(70–72)* or with Ngn2 *(73)* generates ectopic motor neurons in the neural tube as does ectopic expression of Nkx6.1 and Isl1 together with Lhx3 *(74,75)*. One implication of these findings is that forced expression of one or more of these same genes in hESC-derived neuroprogenitors might also increase recovery of motor neurons in vitro.

The mechanism underlying sequential differentiation of motor neurons and oligodendrocytes in the pMN domain is not fully established. Two Olig closely related genes, *Olig1* and *Olig2*, are expressed in the pMN domain. *Olig2* expression is essential for production of oligodendrocytes as well as motor neurons; in the absence of *Olig2* function, pMN progenitors differentiate into astrocytes and interneurons *(70,72)*. Near the onset of gliogenesis, the previously sharp boundaries between Olig2 and Nkx2.2 expressing cells is blurred by the appearance of Olig2/Nkx2.2 expressing cells *(71,76)*. Concomitantly, Ngn2

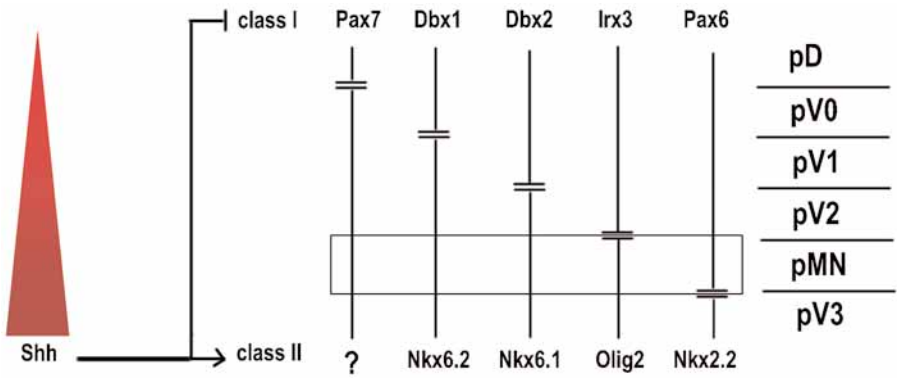

Fig. 2. Specification of progenitor domains in the ventral neural tube. A gradient of Shh (red triangle) is expressed by the ventrally located notocord and floorplate (not shown). Shh blocks expression of class I genes and induces expression of class II genes in a concentration dependent manner. For example, less Shh is needed to block expression of Pax7 than is needed to induce expression of Nkx6.2. Complimentary pairs of class I and class II genes establish the boundaries between domains. For example, Irx3 blocks expression of Olig2, establishing the dorsal boundary of the pMN domain. The predicted class II repressor (?) of Pax7 has not been identified. Progenitor domains are indicated by horizontal bars and the pMN domain is highlighted in box for emphasis. The six progenitor domains in dorsal neural tube are collectively designated pD. (Modified from refs. *33,35,39.*)

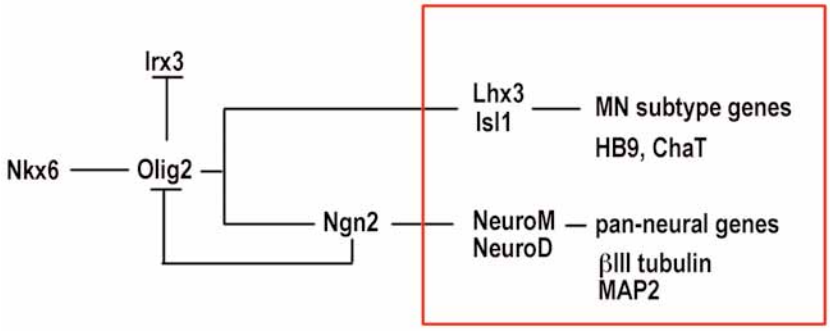

Fig. 3. Specification and differentiation of motor neurons. Expression of Nkx6 proteins promotes expression of Olig2, which represses expression of Irx3, an inhibitor of motor neuron specification. Olig2 promotes expression of Isl1, Lhx3, and Ngn2, transcription factors that induce expression of motor neuron (MN) subtype genes. Ngn2 accumulates heterogeneously in cells and induces pan-neural genes. Ngn2 eventually outcompetes Olig2 for common binding sites and represses Olig2 expression.

expression is lost *(71,76)*, allowing continued Olig2 expression. Consistent with these observations, ectopic expression of Olig2 and Nkx2.2 together is sufficient to induce production of oligodendrocytes *(71,76,77)*. The function of Olig1 has been puzzling as it is not required to generate either oligodendrocytes or motor neurons during embryogenesis. Recent work reveal that in contrast to Olig2, Olig1 relocates from the nucleus to the cytoplasm early in the development, but returns to the nucleus of cells in response to demyelinated lesions *(78)*. Transient nucleotransfection of Olig1 in mouse ESCs increases the number of oligodendrocytes in differentiating populations *(79)*, suggesting that nuclear transport of Olig1 might be regulated in response to demyelinated lesions in vivo. It is not yet known whether forced expression and nuclear localization of Olig1 in human neuroprogenitors can elicit motor neurons as does Olig2.

Development of Neural Rosettes

The signature of neuroprogenitors in culture is the neural rosette, a radial arrangement of elongated columnar cells (Fig. 4) that resembles a sagittal view of the neural tube (Fig. 1). Here, the authors discuss recent advances that utilized neural cell rosettes as the basis for enriching neuroprogenitor populations. Although differing in detail, development of neural rosettes proceeds under alternative conditions in which pluripotent hESCs proliferate with or without serum or serum replacers, on feeder cells or in feeder-conditioned media, as adherent or suspension cultures (Table 1).

A collection of studies highlights the importance of initiating derivation efforts with early passages of karyotypically normal hESCs *(80–83)*. A recent study reports a fully feeder free method of culturing hESCs *(84)*, but derivation of neural rosettes hESCs cultured with this system has not yet been reported.

Similar structures form in hESC-derived teratomas in mice, cited as evidence for pluripotentcy *(1,4,85)*. Like neuroepithelial cells in the neural tube, cells within rosettes express multiple neural cell markers, including among others nestin *(86,87)*, NCAM, and SOXB1 proteins as described earlier as well as Musashi-1 *(88)*, a RNA-binding protein that is expressed in proliferating neural stems cells. It is worth noting that none of the markers used to identify hESC-derived neuroprogenitors in culture are definitive for this cell type. However, expression of multiple early neural markers together with differentiation of rosette derivatives into neurons and glia suggest that neural rosettes contain mutipotential neuroprogenitors. Clonal

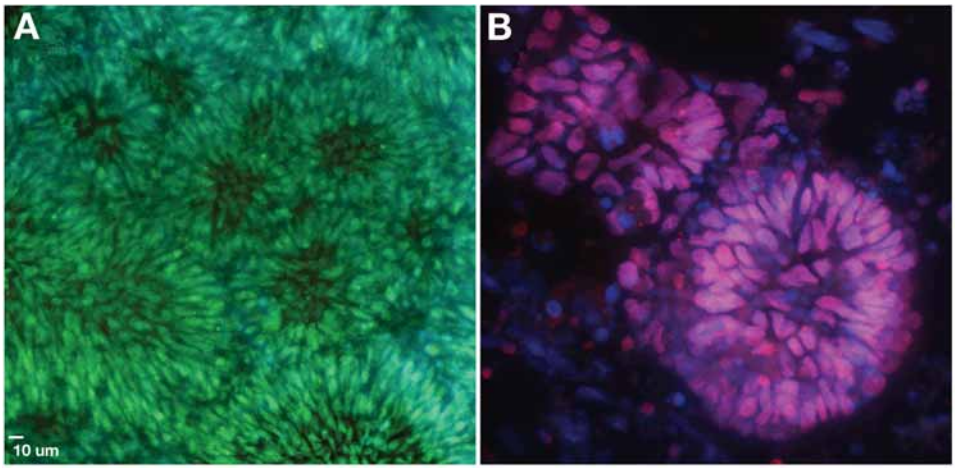

Fig. 4. Neural rosettes in differentiating cultures of hESCs. Neural rosettes in cultures of H9 hESCs induced with RA and Shh. Rosette cultures immunostained with antibodies directed against human **(A)** PAX6 (green) or **(B)** human Sox2 (red) and stained with a fluorescent chromatin dye (blue). Images collected with Olympus DSU imaging system (www.olympusamerica.com). Scale 10 μ; image in B ×2 magnification of image in A.

Table 1
Derivation of Neural Rosettes and Neuroprogenitors From hESCs

	1. hESCs	2. induce differentiation	3. neural rosettes	4. propagate NPs	
hESC[a]					*Refs.*
WA01(H1) WA09(H9)	MEFS[b] SR[c] heparin bFGF	SR heparin	bFGF supplement cocktail[d]	bFGF	*91* *103*
BG01 BG02	MEFS FBS/SR LIF[e]	bFGF LIF	N2 bFGF LIF	NB B27 bFGF LIF	*104* *105*
ES01(HES1) ES02(HES2)	MEFS FBS LIF	B27 bFGF noggin	B27 bFGF noggin	B27 bFGF noggin	*98*
WA01(H1) WA07(H7) WA09(H9)	CM[f] bFGF	DMEM/ F12/NB N2/ B27 noggin	DMEM/ F12/NB N2/ B27 noggin	DMEM/ F12/NB N2/ B27 noggin	*97*

[a]NIH registered name; common name in parenthesis.
[b]Mouse embryonic fibroblasts. Unless stated otherwise, media base was DMEM or a combination of DMEM and F12 (Invitrogen). All formulations included supplements of glutamine and antibiotics. N2 and B27 are commercial cocktails of media supplements and NB is Neural Basal media. Media for hESC culture include nonessential amino acids and β-mercaptoethanol.
[c]Commercial serum replacer.
[d]Cocktail includes putrescine, insulin, sodium selenite, transferin, progesterone, and serum albumin.
[e]Leukemia inhibitory factor.
[f]hESCs propagated in CM, media conditioned by MEFs, on Matrigel or polyornithine/laminin-treated dishes.

analysis has not yet been reported and it is not yet clear whether neuroprogenitors within neural rosettes are comparable to neuroepithelial cells of the neural tube that are capable of generating all neural and glial subtypes found in vivo.

The similarity between the neural rosette and the radial arrangement of neuroprogenitors in the neural tube raise the question of whether these structures are produced by similar mechanisms. The neuroepithelium of the neural tube shows

apicobasal polarity, in part through formation of specialized adherens belts and tight junctions that exist only transiently. The presence or absence of similar junctions in neural rosettes has not yet been reported. Mitosis within the neural tube occurs primarily near the luminal surface and although a number of reports note mitotic cells near the center of neural rosettes, quantitative analysis has not been offered. Asymmetric division of many stem cell types allows one daughter to remain a stem cell, the property of self-renewal, and one daughter to become more fate restricted. The mitotic spindle is frequently positioned in stem cells such that the new stem cell daughter retains the position of the preceding stem cell and the more fate-restricted daughter is displaced distally *(89,90)*. However, the functional relationship between cells within the neural rosettes has not yet been examined. Furthermore, it is not clear whether formation of neural rosettes is a necessary prelude to derivation of multipotential human neuroprogenitors or the result of neuroprogenitor specification.

The first study to use human neural rosettes to enrich for neuroprogenitors allowed multilineage differentiation of hESCs in embryoid bodies *(91)*. When embryoid bodies formed in suspension were plated on adherent substrates (Table 1), cells migrated out of the plated embryoid bodies and columnar cells appeared that subsequently formed rosette structures. Rosettes expanded in chemically defined media *(92,93)* containing bFGF generated multilayered structures. Rosette structures could then be released from surrounding cells by mild dispase treatment and propagated in suspension culture *(91)*. Enrichment for neuroprogenitors within the released rosettes was significant; almost all of the cells expressed nestin and many expressed Musashi-1 and PSA-NCAM, a modified form of NCAM. The basis for the differential effects of dispase on rosette structures is not yet clear, but might reflect a preference of neural cells to adhere to each other, possibly through cell adhesion molecules like NCAM and/or tight junctions and adherens belts. Similar interactions might also contribute to formation of neurospheres, proliferating aggregates of neuroprogenitors derived from ESCs or from fetal or adult CNS tissue. Whatever be the mechanism, selective recovery of neural rosettes generates enriched populations of neuroprogenitors.

BMP Signaling Inhibits Development of Neural Rosettes

BMP signaling inhibits specification of neuroectoderm during in vivo development *(42)*, raising the question of BMP effects on formation of neural rosettes. BMPs expressed in cultures of pluripotent hESCs *(8,94,95)* and present in serum replacers *(96)* promote loss of pluripotency and differentiation of hESCs toward nonneural lineages *(96)*. Although basal levels of BMP signaling does not block neural induction in vitro under standard culture conditions, BMP antagonists increase recovery of neural rosettes from differentiating cultures of hESCs *(94,97,98)* whether differentiation of hESCs proceeded (Table 1) on adherent substrates *(97)* or in suspension cultures *(98)*. Noggin was more effective than folistatin as a BMP antagonist *(97)*, attributed to the higher binding affinity of noggin for BMPs. Transcript analysis by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) indicated that expression of neural markers increased during noggin treatment whereas those of nonneural lineage markers decreased. The transcription profile stabilized after a few weeks in culture such that noggin could be withdrawn without reducing the proportion of cells expressing neural markers *(97,98)*. Increased recovery of neural rosettes in the presence of noggin is consistent with the view that development of rosettes in vitro is sensitive to BMP signaling, as in the case of development of the neural tube in vivo.

The molecular underpinnings of the effects of noggin on neural induction in vitro are not yet clear. Competition experiments indicated specificity of noggin on BMP signaling; added BMP increased phosphorylation of Smad-1, Smad-5, and Smad-8 in aggregates of hESCs differentiating in suspension *(98)*, showing that differentiating hESCs respond to BMPs as expected. Phosphorylation of these Smads was largely prevented by concomitant addition of noggin *(98)*, suggesting that noggin blocked induction of BMP signaling cascades. Consistent with this view, noggin decreased expression of endoderm makers, GATA6 and α-feto protein in adherent cultures *(97)*. BMP2 is thought to be responsible for induction of hESCs toward extraembryonic endoderm *(94)*; however, BMP2 transcripts actually increased with noggin treatment and BMP4 levels were unchanged *(97)*. Id proteins mediate BMP signaling *(42)* and noggin decreases expression of Id proteins in cultures of hESCs *(96,99)*, but the impact of noggin on the level of Id transcripts was modest in differentiating cultures *(97)*. Although the precise molecular basis is not yet clear, these results *(97,98)* together with other studies *(94,96,100)* show that noggin increases recovery of neural rosettes from hESC cultures.

Adherent Self-Renewing Neuroprogenitors Derived From Rosettes

Neuroepithelial cells in the forming neural tube undergo continual development, quickly acquiring regional and subtype identities as the neural tube extends from the rostral to caudal direction. Thus, an outstanding question is whether neuroepithelial-like cells be captured from rosettes before their developmental potential is irreversibly restricted. Although this question requires clonal analysis for a definitive answer, if it can be answered at all, recent advances show that multipotential neuroprecursors can be derived from neural rosettes. When hESCs are allowed to expand without feeder renewal and then deprived of serum or serum replacer (Table 1), a portion of the population penetrates the feeder layer and adheres to the tissue culture dish *(101)*. Following manual removal of the overlying feeder layer, neural rosettes appear among other adherent cells within a few days. Although rosette structures can also form on overlying feeder layers, targeting adherent cells provides an easy and convenient method to isolate neural rosettes.

Neural rosettes can be manually isolated and then subcultured on polyornithine/laminin treated substrates in proliferating monolayer cultures of neuroprogenitors *(101)*. The purity of adherent neuroprogenitor cultures approached 90% as assayed by expression of nestin and Musashi-1, but not markers of other lineages. The advances of this and other adherent monolayer culture systems *(97,102)* include more uniform application of extrinsic factors and accessible cytology to follow differentiation of neuroprogenitors into neurons and glia.

Although routinely used in culture media, the effects of bFGF on rosette development and differentiation of neuroprogenitors are not completely understood. Early studies

suggested an essential role for bGFG in deriving neuroprogenitors from hESCs (91). Subsequent studies tested the effects of bFGF on derivation of neural rosettes in the presence and absence of bFGF (97,98). Quantitative analysis revealed that bFGF expanded the number of neuroprogenitors obtained, but was not required for derivation of neural rosettes *per se*, with or without the BMP inhibitor noggin (97,98).

Although one method to induce differentiation of neuroprogenitors is simply to withdraw bFGF (97,98), it is important to note that inclusion of bFGF might not block the differentiation of neuroprogenitors. Transcript analysis of neuroprogenitor cultures showed expression of genes like *Otx2* that are expressed in anterior region of neural tube and subsequently in midbrain structures (98). Furthermore, transcript analysis of proliferating neuroprogenitor cultures at regular intervals for more than several months showed expression of MAP2, a microtubule associated protein that is expressed in immature neurons (97). Together with a morphology suggestive of early neurons (97), these observations raise the question of whether some neuroprogenitors escape the mitotic cell cycle and initiate differentiation even in the presence of bFGF. If so, continued proliferation of cultures might select for one or more subsets of neuroprogenitors that resist differentiation. Further research to address this possibility could advance the field.

Differentiation of Rosette-Derived Neuroprogenitors

In contrast to the morphogen gradients in vivo that establish subtype identity, neuroprogenitors within neural rosettes in culture are exposed to largely uniform environmental conditions. Thus, the position code established by sequential steps of specification in a spatial and temporal pattern in vivo must be attained differently in vitro. Here, the authors discuss efforts to differentiate rosette-derived neuroprogenitors into motor neurons and oligodendrocytes as representative of the challenges to obtaining the progeny of a single progenitor domain in the developing neural tube.

Restricted Differentiation Potential of Neural Rosettes

Specification of the pMN domain in vivo requires RA and Shh signaling. A recent study suggests that neuroprogenitors in developing rosettes have a restricted window in which application of RA and Shh can efficiently induce specification of motor neurons (103). During the development of neural rosettes on adherent substrates (Table 1), Pax6 expression preceded detectable expression of Sox1, which did not appear until rosettes became well formed and easily identifiable. This is in contrast to development in vivo in which Sox1 expression is detected before Pax6 expression. Rosettes expressing Pax6 and Sox1 showed a rostral character, expressing transcription factors like Otx2, but did not show ventrally expressed transcription factors like Olig2 and Nkx2.2 that are expressed in the pMN domain. Differentiation of neural rosettes confirmed ongoing developmental changes; when rosettes expressing Sox1 and Pax6 were simultaneously exposed to RA and Shh, approximately half of the neurons produced expressed Isl1 and Lhx3. Because very few cells expressed HB9, the Isl1/Lhx3 expressing cells were ascribed to specification of interneurons rather than motor neurons. However, if differentiation was initiated with RA before Sox1 expression was detected and before application of Shh, approximately half of the Isl1 positive cells were also positive for HB9. As a result, about 20% of the cells expressed HB9 and a portion of mature neurons showed the electrophysiological activity of motor neurons (103). These results suggested that the developmental potential of neuroprogenitors in rosettes was restricted by the time that Sox1 expression was detected in well formed neural rosettes.

Transcript analysis with semiquantitative RT-PCR was undertaken to determine why neuroprogenitors in well-formed rosettes could not be efficiently induced to produce motor neurons (103). The results suggested that RA was required to caudalize early neural rosettes, as assayed by decreased expression of the rostrally expressed Otx2 and increased expression of caudally expressed Hox genes that play a role in specifying spinal-motor-neuron column identity. Interestingly, RA induced expression of endogenous Shh gene expression in early rosettes as well as expression of Shh dependent *Nkx6.1* and *Olig2* genes, but RA did not induce expression of these genes in well-formed late rosettes. Olig2 was detected cytologically in a few cells in early rosettes treated with RA alone, but many more were detected when RA-induced cultures were later treated with exogenous Shh as well. These results suggested that efficient induction of motor neuron identity requires the ventralizing effects of Shh. One implication of these findings is that the developmental potential of neuroprogenitors in forming rosettes is dynamic such that most Pax6/Sox1 expressing cells are refractory to specification of a pMN domain fate. A second implication is that efficient induction of a motor neuron fate requires RA and Shh to caudalize and ventralize neuroprogenitors in rosettes, respectively.

The results of this study raise the broader question of how the development of neuroprogenitors in rosettes differs from the development of neuroprogenitors in the neural tube. Given that SoxB1 genes show extensive functional redundancy early in the development (48) and neural development in mice lacking Sox1 or Sox3 is normal, Sox1 expression likely provides a convenient marker of rosette development, but might not be functionally significant. Sox2 expression was not examined in this study; however, Sox2 is expressed in pluripotent hESCs and required for proliferation of neuroprogenitors. Thus, an outstanding question is whether Sox2 provides SoxB1 function in early rosettes. In any case, the apparent inability to efficiently induce well-formed Sox1 expressing rosettes to produce motor neurons is unexpected, particularly in light of the absence of Irx3 expression in these rosettes that normally represses specification of pMN fate in vivo (Fig. 2). Further studies might show that Sox1 expressing rosettes could be efficiently induced to produce motor neurons with alternative methods.

Motor Neurons Derived From Cultured Self-Renewing Neuroprogenitors

Many would agree that the optimal source of motor neuron precursors would be self-renewing neuroprogenitors in monolayer cultures that could be induced at will to produce motor neurons. Recent studies suggest that this goal is achievable. Proliferating neuroprogenitors derived from neural rosettes express Patch (104), the cell surface receptor for Shh that is expressed in target tissues responsive to Shh signaling

(41,105). One inference of Patch expression was that these self-renewing neuroprogenitors could respond to Shh signaling. Consistent with this possibility, transcript analysis by RT-PCR of neuroprogenitors exposed simultaneously to bFGF, RA, and Shh showed expression of both Olig2 and HB9 when these factors were withdrawn during differentiation. Moreover, neurons expressing Isl1 as well as ChaT were estimated to include 20% of the differentiated progeny. Because these neuroprogenitor populations were derived from well formed rosettes and express Sox1, Sox2, and Sox3 transcripts (101), these findings show that motor neurons can be recovered from populations of self-renewing neuroprogenitors expressing SoxB1 genes. However, it is not known whether Olig2 and HB9 coexpressed in a subset of cells that were Sox1 negative.

Transcript analysis of differentiating neuroprogenitors in this study raised the question of whether bFGF might have a role in specifying motor neuron identity. Neuroprogenitors were cultured in media with bFGF before induction and the effects of extrinsic factors were tested by maintaining cultures in media with or without bFGF, RA, and/or Shh for induction. All extrinsic factors were then withdrawn for differentiation and transcript analysis by RT-PCR to examine Olig2 and HB9 expression was performed on days 1 and 14, respectively, after withdrawal of all extrinsic factors. The results suggested that bFGF might play a role in motor neuron specification, as both Olig2 and HB9 showed higher levels of expression when cultures were induced in the presence bFGF (104). These results are consistent with previous work showing that bFGF treatment primes fetal neural stem cells to generate ChaT expressing neurons (106). In addition, another study showed that RA together with bFGF can specify motor neuron fate in chick explants that is independent of Shh signaling (47), suggesting that bFGF might be a part of a Shh-independent pathway (43) that operates in vitro as well.

Genetic Methods to Recover Enriched Populations of Motor Neurons

Pure populations of motor neurons precursors would benefit stem cell-based therapies, not only targeting a specific neuronal subtype, but reducing the risks associated with transplantation of undifferentiated and potentially neoplastic cells. Nearly pure populations of motor neurons precursors have been recovered from differentiating hESCs by retrieving cells expressing GFP under the control of an enhancer element associated with the HB9 promoter (107). Cultures of hESCs were allowed to become confluent by prolonged culture without feeder replacement (107), a method previously shown to generate neural lineages from murine ESCs (108). Differentiating cultures were transiently transfected with a selection cassette based on the promoter of HB9 (109) that directed expression in somatic motor neurons derived from transgenic mice and dissociated human fetal spinal cord cells (108). Embryoid bodies derived from transfected cultures were formed in the presence of RA alone or with Shh and allowed to differentiate. The quantitative analysis of unsorted differentiated populations showed that addition of Shh did not increase the number of neurons generated, but increased the proportions of motor neurons as assayed by immunostaining of HB9 and ChaT, consistent with a ventralizing effect of Shh on differentiation of neuroprogenitors. Further, the proportion of motor neurons among differentiated cells was roughly similar to the proportion reported by other methods (103,104). Florescence-activated cell sorting of GFP expressing cells from dissociated embryoid bodies generated populations in which nearly 90% of cells were immunopositive for HB9, Islet1, and ChaT and showed electrophysiological activity expected of motor neurons. Because neither HB9 (110) nor Islet1 (34,111) are unique to motor neurons, coexpression of these markers with ChaT indicates that the procedure produced motor neurons rather than Iset1 and/or HB9 expressing interneurons.

Given the role of glia in supporting neuron viability (3), an outstanding question is whether pure populations of motor neurons can be sustained in culture. GFP expressing neurons showed good viability in unsorted populations, surviving at least 2 wk in culture (107). However, GFP-positive neurons in sorted populations showed low viability and failed to survive beyond a few days, even when the cultures were maintained in media supplemented with a cocktail of neurotropic factors (107) like brain-derived neurotropic factor that are widely used to improve viability of neurons (112). However, viability could be rescued by plating sorted GFP-positive cells on freshly isolated skeletal muscle derived from neonatal rats (107). GFP-positive cells became fully differentiated motor neurons, formed functional neuromuscular complexes and showed the expected electrophysiological properties. Although it is not clear whether muscle explants included glia, one inference of this study is that formation of neuromuscular junctions can maintain viability of motor neurons. This study provides a significant advance in the field and shows that selected subtypes of cells can be retrieved from neural rosettes with genetic methods, bypassing the limitations of variable induction and low yields. Furthermore, the use of GFP reporters for cells induced to become motor neurons like the recently identifies *GDE2* gene (113) or intermediates in this and other pathways would provide insights into the development and differentiation of neural rosettes in vitro.

Derivation of Oligodendrocytes and the Dynamic Differentiation Potential of Neuroprogenitors in Culture

Following the initial period of neurogenesis, progenitors in the pMN domain generate oligodendrocytes that will myelinate newly formed neurons. At first blush, induced differentiation of neural rosettes or their derivatives with RA and Shh is expected to generate oligodendrocytes as well as motor neurons. Although oligodendrocytes have been recovered, the frequency is invariably very low and quantification has not been offered. Highly enriched cultures of oligodendrocytes have been derived from hESCs with alternative methods (114), generating neurosphere-like structures from hESC cultured on mesoderm-derived stroma feeder layers that have neural inductive characteristics. This approach employed EGF as well as bFGF and utilized RA, but not Shh, during differentiation. Quantitative analysis showed expression of several markers for glial progenitors in the majority of proliferating cells, including Olig1, Sox10, and PDGFRa, a receptor for platelet-derived growth factor. Given the respective roles ascribed to Olig1 and Olig2, it is not clear whether the antibodies used in this study detected Olig2 as well as Olig1. Differentiation of these

populations showed nearly all of the cells differentiated into oligodendrocytes, as assayed by immunostaining of the oligodendrocyte markers GalC and O4 among others. Less than 10% of differentiated cells were immunopositive for GFAP or β-III tubulin. These results show that oligodendrocytes can be derived from hESCs and raise the question of why recovery of oligodendrocytes from neural rosettes or proliferating neuroprogenitors is so inefficient.

Further studies might show that oligodendrocytes could be derived with altered protocols such as inclusion of additional growth factors. However, if the developmental potential of neuroprogenitors change over time in culture, the developmental potential of the starting population might be important to the cell type recovered. A recent study suggests that the differentiation profile of neuroprogenitors in culture shift from a neuronal to glial fate *(98)*. Proliferating neuroprogenitors were differentiated by withdrawal of bFGF and the differentiation profile of neuroprogenitors was quantified by immunostaining of β-III tubulin and GFAP at weekly intervals for several months. The results showed that early cultures tested subsequent to derivation generated β-III tubulin expressing neurons, but did not generate GFAP expressing glia. Over sequential testing periods, the proportion of β-III tubulin positive neurons fell and GFAP expressing glia increased, such that GFAP expressing glia predominated at the end of the testing period. The frequency of undifferentiated cells was not reported and it is not clear whether the proportion of cells that could be differentiated changed over time or whether a subset of glial progenitors continued to proliferate. However, the shift in differentiation profile reported in this study is reminiscent of changes in the neural tube during development; neuroprogenitors in the pMN domain express either Olig2 or Nkx2.2 during neurogenesis (Fig. 2), but a subset cells near the boundary of pMN domain later expresses both Olig2 and Nkx2.2 before differentiating into oligodendrocytes *(71,76)*. Testing for the presence or emergence of Olig2 and Nkx2.2 coexpressing cells in culture might be important in understanding whether neural rosettes and their derivatives could serve as a model for the switch from neurogenesis and gliogenesis in vivo.

Summary and Perspectives

The neural rosette serves as a model for neurogenesis in vivo in several respects. Apparently multipotential neuroprogenitors in rosettes express many of the same genes as neuroepithial cells in the neural tube. The rosette arrangement itself is noteworthy and the molecular mechanism of its formation could reveal if and how formation of rosettes reflects specification of neuroectoderm in vivo. Formation of neural rosettes is sensitive to BMP signaling, as is formation of the neural tube. Neural rosettes produce all of the major classes of progeny of the neural tube, including motor neurons and at least some oligodendrocytes. However, it is not clear whether all of the expected subtypes can be produced or whether these same subtypes can be produced through alternative pathways in vitro that are not normally taken in vivo. There are important differences as well, including the apparent restriction of developmental fate of neuroprogenitors in rosettes as assayed by differentiation of motor neurons. Differentiation of motor neurons derived from embryoid bodies and from proliferating neuroprogenitors raises the question of whether it is the rosette structure itself that restricts developmental fate. Another possible difference is that radial glia have not yet been identified in hESC-derived neural rosettes or neuroprogenitors. Whether this reflects a biological difference or a technical oversight is not clear. In summary, the neural rosette is a three dimensional structure derived from hESCs in culture. Understanding how the neural rosettes are formed and how the constituent neuroprogenitors become specified in response to extrinsic factors might contribute to better understanding of how these events occur within the developmental niche of neuroprogenitors in vivo.

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