Human ESC-derived Neural Rosettes and Neural Stem Cell Progression

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Neural stem cells (NSCs) are defined by their ability to self-renew while retaining differentiation potential toward the three main central nervous system (CNS) lineages: neurons, astrocytes, and oligodendrocytes. A less appreciated fact about isolated NSCs is their narrow repertoire for generating specific neuron types, which are generally limited to a few region-specific subtypes such as GABAergic and glutamatergic neurons. Recent studies in human embryonic stem cells have identified a novel neural stem cell stage at which cells exhibit plasticity toward generating a broad range of neuron types in response to appropriate developmental signals. Such rosette-stage NSCs (R-NSCs) are also distinct from other NSC populations by their specific cytoarchitecture, gene expression, and extrinsic growth requirements. Here, we discuss the properties of R-NSCs within the context of NSC biology and define some of the key questions for future investigation. R-NSCs may represent the first example of a NSC population capable of recreating the full cellular diversity of the developing CNS, with implications for both basic stem cell biology and translational applications in regenerative medicine and drug discovery.

The isolation of NSCs from the developing and adult brain has transformed our understanding of CNS development and presented new avenues for regenerative medicine (McKay 1997; Gage 2000). Much effort in the field has been devoted to the identification of factors directing the self-renewal and differentiation of NSCs in vitro and in vivo (Alvarez-Buylla and Lim 2004; Shen et al. 2004; Conti et al. 2005). Such studies demonstrated that single factors act instructively to specify neuronal versus glial fate choice (Johe et al. 1996). However, the ability to direct NSCs toward specific neuronal fates has remained remarkably poor. It is well established that multipotent NSCs from many CNS regions can clonally give rise to neurons, oligodendrocytes, and astrocytes in vitro. However, the neuronal subtype potential of in-vitro-expanded NSCs is largely restricted to GABAergic and, to a lesser extent, glutamatergic fates (Caldwell et al. 2001; Jain et al. 2003). Recent in vivo studies suggest that adult subventricular zone (SVZ) stem cells also exhibit region-specific biases toward specific neuronal subtypes such as Pax6⁺/TH⁺ cells or calretinin⁺/GABA⁺ cells (Merkle et al. 2007). Remarkably, restrictions in neuron subtype potential in the adult SVZ are cell autonomous and retained following extensive in vitro culture (Merkle et al. 2007). These studies suggest that both endogenous NSC populations and NSCs cultured in vitro exhibit developmental and regional bias in neuron subtype differentiation.

In contrast to NSC studies, work with neural plate-stage explants or studies with embryonic stem cells (ESCs) suggest that early neural cells can be specified towards a wide range of neuronal fates. For example, combined exposure to sonic hedgehog (SHH) and retinoic acid (RA) or SHH and FGF8 induces populations enriched in spinal motoneuron (MN) or midbrain dopamine neurons, respectively. These protocols work well in neural-plate-stage explants (Roelink et al. 1995; Ye et al. 1998) and in neural progeny of ESCs (Lee et al. 2000; Wichterle et al. 2002; Barberi et

al. 2003) but not in cultured NSCs (Caldwell et al. 2001; Jain et al. 2003). The striking difference in neuron subtype fate potential of ESCs and NSCs suggests that neural patterning response becomes rapidly restricted during neural development. One of the major challenges in NSC biology is therefore the development of novel strategies to enhance the differentiation potential of NSCs. This could be achieved either by manipulating the genetic or epigenetic state of later-stage NSCs or by isolating and maintaining an earlier NSC stage with intact patterning potential.

We have recently reported the isolation of a novel NSC type from human ESCs (hESCs), termed rosette-stage NSCs (R-NSCs) (Elkabetz et al. 2008) based on their characteristic cytoarchitecture. R-NSCs are capable of extensive self-renewal and broad differentiation potential along CNS and peripheral nervous system (PNS) lineages and readily yield a wide range of neuron types inaccessible to later-stage NSCs (Lee et al. 2007a; Elkabetz et al. 2008). The differentiation potential of R-NSCs corresponds to that of neural-plate-stage cells, and R-NSC-like populations can indeed be isolated directly from neural-plate-stage embryos (Elkabetz et al. 2008). The broad differentiation potential of R-NSCs is reflected by the specific molecular signature and distinct extrinsic growth factor requirements. A major goal of future studies is the identification of the molecular mechanisms that enable broad patterning potential of R-NSCs. In this chapter, we summarize our current understanding of R-NSCs and discuss their properties in the context of ESC and NSC biology.

NEURAL STEM CELLS

The in vivo persistence of immature neural precursors in the adult rodent brain was first described more than four decades ago (Altman and Das 1965). However, these studies were largely ignored until studies in the 1980s confirmed a link between dividing precursors in the SVZ and

postmitotic neuronal progeny in the adult canary brain (Goldman and Nottebohm 1983). Many subsequent studies confirmed and extended these results for both the developing and adult CNS, providing a quantitative description of endogenous murine precursor populations *in vivo* (Frederiksen and McKay 1988). These early studies revealed strong evidence for the presence of endogenous NSCs *in vivo*. However, efforts to manipulate NSC fate in a systematic manner required the establishment of relevant *in vitro* culture systems. Early attempts at isolating and manipulating the fate of neural precursors (Cattaneo and McKay 1990) got a significant boost with the availability of the neurosphere culture technique (Reynolds and Weiss 1992; Kilpatrick and Bartlett 1993; Gritti et al. 1995). Under these conditions, neural precursors are grown as free-floating aggregates on nonadherent plates in the presence of fibroblast growth factor-2 (FGF-2), epidermal growth factor (EGF), or both. For human neurospheres, supplementation with leukemia inhibitory factor (LIF) in addition to EGF and FGF-2 has been proposed (Galli et al. 2000). The demonstration of clonal neurosphere formation, self-renewal, and multilineage potential provided a valuable assay to probe NSC properties *in vitro*. Neurosphere assays were used for the prospective identification of NSC-like populations from primary tissue based on expression of CD133 (Uchida et al. 2000), SSEA-1 (Capela and Temple 2002), or combinations of positive/negative marker sets (Rietze et al. 2001). However, neurospheres are composed of heterogeneous cell types including many differentiated cells. Furthermore, in the adult SVZ, proliferating neurospheres are obtained more readily from transient-amplifying cells (type C cells) than from the SVZ stem cell compartment (type B cells) (Doetsch et al. 2002). Therefore, neurosphere data need to be interpreted cautiously, and neither primary nor secondary neurosphere formation is a conclusive assay of NSC identity.

An alternative approach for isolating NSCs *in vitro* is based on monolayer cultures in the presence of FGF-2 (Davis and Temple 1994; Palmer et al. 1995; Johe et al. 1996). Monolayer techniques allow direct observation of lineage relationships and the establishment of complete lineage trees *in vitro* (Qian et al. 2000). A recent variation of this technique is the neurosphere (NS) culture system yielding fairly homogeneous populations of radial glial-like NSC progeny in the presence of FGF-2 and EGF (Conti et al. 2005). NS cultures exhibit improved maintenance of neurogenic fate potential following long-term culture and represent one of the few examples of a symmetrically dividing stem cell population (Conti et al. 2005). However, the predominant neuron types generated from NS cells are GABAergic and glutamatergic neurons, similar to the neurosphere and FGF-2-expanded monolayer cultures.

DEVELOPMENTAL POTENCY

The two key criteria that define NSCs are capacity for extensive self-renewal and multilineage potential toward neurons, astrocytes, and oligodendrocytes (McKay 1997; Gage 2000; Temple 2001). Maintenance of NSCs *in vitro* or *in vivo* requires that at least one of the two daughter cells retains NSC potential. In the developing CNS, cells

undergo various phases of expansion and differentiation. At the earliest stages of neural development, cells symmetrically expand to yield the precursor pool subsequently generating the broad range of neural cell types. At later stages, cells undergo both asymmetric and symmetric divisions to yield transit-amplifying cells, neurons, and glia. There is evidence that cells at the neural-plate stage contain precursors with plasticity capable of changing anteroposterior (AP) and dorsoventral (DV) identity in response to inducing tissues (Yamada et al. 1993; Hynes et al. 1995) or defined patterning signals (Roelink et al. 1994; Ye et al. 1998). However, it is unclear whether cells at the neural-plate stage represent true NSC populations that undergo many self-renewal divisions before losing patterning potential. Accordingly, the identification of an "authentic" NSC *in vivo* with the ability to recreate the whole cellular diversity of the CNS has remained controversial (Mukouyama et al. 2006). The situation in the CNS is contrasted by the hematopoietic system, where a single transplanted hematopoietic stem cell (HSC) can successfully reconstitute hematopoiesis throughout the life span of the graft recipient (Morrison et al. 1995).

In the adult brain, there is ample evidence for continuous neurogenesis in specific regions of the CNS, such as in the SVZ along the lateral ventricle (Doetsch et al. 1999) and in the hippocampus (Palmer et al. 1997). However, adult NSC populations display an even more limited neuronal fate potential (Merkle et al. 2007). Although studies in the SVZ have reported broadened differentiation potential toward striatal neuron types upon noggin and BDNF (brain-derived neurotrophic factor) exposure (Chmielnicki et al. 2004), evidence for more general AP or DV respecification of adult NSCs is lacking.

One important question is whether restricted *in vivo* NSC potential can be overcome following *in vitro* culture. An expanded differentiation repertoire of isolated "glial-restricted" O2A progenitors toward neuronal fates has been reported after FGF-2 treatment (Kondo and Raff 2000). FGF-2 treatment has also been shown to alter expression of DV markers in neural precursors and to broaden differentiation from bipotent to tripotent fate characteristics *in vitro* (Gabay et al. 2003). Such studies raise the question of whether *in vitro* culture and FGF-2 exposure can dysregulate patterning and broaden fate potential. However, FGF-2 has complex roles during development and serves as a patterning factor in its own right (Crossley et al. 1996; Lumsden and Krumlauf 1996; Shimamura and Rubenstein 1997; Shimamura et al. 1997; Bertrand et al. 2000). Therefore, FGF signaling may have roles in both broadening and restricting potential depending on developmental context.

During normal development, there is evidence for regional specification even before neurulation (Quinlan et al. 1995), and early neural-plate cells appear to quickly adopt regional subtype identities (Chang and Hemmati-Brivanlou 1998) that impact their ability to respond to patterning morphogenetic cues. A number of studies isolated NSC-like populations from specific regions of the CNS including forebrain (He et al. 2001), midbrain (Ling et al. 1998; Studer et al. 1998), hypothalamus (Markakis et al. 2004), and spinal cord (Mayer-Proschel et al. 1997). These

studies generally confirm that in-vitro-expanded NSC populations exhibit temporal region-specific neuronal differentiation potential. The issue of whether such restrictions can be overcome in an “instructive” environment remains controversial. Numerous studies have reported remarkable plasticity of grafted neural precursor populations upon heterotopic transplantation in the developing CNS (Brüstle et al. 1995; Campbell et al. 1995; Fishell 1995; Suhonen et al. 1996; Shihabuddin et al. 2000). However, these studies are limited largely to the analysis of morphological criteria and generic neuron marker expression; they did not carefully probe for reexpression of region-specific transcription factors. There is evidence that grafted cells can undergo morphological adaptation in the host region without undergoing respecification such as NSCs grafted into the retina (Takahashi et al. 1998). Future studies will need to revisit these questions using genetic lineage-marking strategies before and after transplantation.

Temporal fate restrictions have been investigated in heterochronic transplantation assays. Such studies have shown that early-stage NSCs have the ability to populate orthotopic regions in older-stage hosts but not vice versa (Frantz and McConnell 1996). Other examples of temporal restrictions are the increased bias of late NSC and neural precursor populations to undergo gliogenic differentiation (Quinn et al. 1999) and, conversely, the unresponsiveness of early-stage cortical NSCs to undergo glial differentiation in response to instructive gliogenic cues based on activating JAK-STAT signaling (Molne et al. 2000). The latter phenomenon has been linked to methylation of critical components of JAK-STAT signaling in early-stage NSCs (Fan et al. 2005). One interpretation of these data is that at any given stage of development, no single NSC possesses the capacity for recreating the full cellular diversity of the CNS (Mukouyama et al. 2006) and that NSCs progress through sequential stages of fate potential from the time of neural induction to adulthood.

A practical example for the difficulties of using NSCs to derive a specialized neuron type is the generation of midbrain dopamine neurons for application in models of Parkinson's disease. Functional midbrain dopamine neurons can be derived from short-term expanded precursors isolated from the early rodent or human midbrain (Studer et al. 1998; Sánchez-Pernaute et al. 2001; Parish et al. 2008). However, more extensive in vitro expansion results in a dramatic decrease in the efficiency to generate dopamine neurons (Yan et al. 2001). Many strategies have been developed in an effort to overcome this limitation, including treatments with extrinsic factors and genetic modification of key developmental genes (for review, see Kriks and Studer 2009). However, the goal of obtaining long-term expandable populations of NSCs yielding authentic midbrain dopamine neurons has remained elusive.

EMBRYONIC STEM CELLS AND THE DERIVATION OF NEURAL FATES

The challenges for obtaining patternable NSC populations have been addressed using two main strategies: the development of improved protocols for expansion and differentiation of NSC-like populations with defined regional

and temporal identity and the search for a novel NSC source with intact neural-plate-stage patterning potential. Mouse ESCs and the more recent isolation of human ESCs (Thomson et al. 1998) offer a powerful paradigm for generating unlimited numbers of early-stage neural progeny. Many protocols have been developed for inducing neural differentiation in ESCs. These include embryoid body (EB) differentiation in combination with retinoic acid treatment, conditioned medium, or neural survival factors (Bain et al. 1995; Okabe et al. 1996; Lee et al. 2000; Zhang et al. 2001; Rathjen et al. 2002); the use of neuron-inducing stromal feeders (Kawasaki et al. 2000, 2002; Barberi et al. 2003; Perrier et al. 2004); and the development of adherent feeder-free approaches (Reubinoff et al. 2001; Cibelli et al. 2002; Ying et al. 2003).

All three basic strategies yield neural progeny at stages suitable for neural patterning studies. Comparative analysis of developmental progression in vitro and in vivo showed a remarkable correlation from the stage of gastrulation to neurulation, patterning, neurogenesis, and gliogenesis (Barberi et al. 2003). Clonogenic experiments have demonstrated that ESC-derived neural progeny can self-renew extensively and clonally give rise to neurons, astrocytes, and oligodendrocytes (Barberi et al. 2003). These results in mouse ESCs provided access to early stages of neural development and yielded ESC-derived multipotent NSC populations in vitro. NSC-like populations have been also obtained from hESCs and demonstrated to engraft in the adult rodent SVZ in vivo and contribute human cells to the host rostral migratory stream and olfactory bulb (Tabar et al. 2005). However, the isolation of ESC-derived NSCs illustrated again the importance of developmental stage and neuronal fate potential. ESC-derived neural progeny readily respond to appropriate developmental cues that direct fate toward midbrain dopamine neurons (Lee et al. 2000; Barberi et al. 2003; Perrier et al. 2004), somatic motoneurons (Wichterle et al. 2002; Yan et al. 2005; Lee et al. 2007b), or forebrain neuron types (Barberi et al. 2003; Watanabe et al. 2005, 2007). However, patterning potential is restricted to narrow developmental windows and is lost progressively after in vitro expansion and isolation of FGF-2/EGF-expanded NSC populations (Tabar et al. 2005; Elkabetz et al. 2008). The broad differentiation potential of ESC-derived early-stage neural progeny raises the question of whether these cells reflect a novel, distinct NSC state that can be isolated in vitro and propagated long term under appropriate conditions.

NEURAL ROSETTES

Original Description of Neural Rosettes

A striking feature during neural differentiation of hESCs is the formation of neural rosettes. Neural rosettes are an early intermediate composed of radially organized columnar epithelial cells resembling the stage of neurulation. Neural rosette formation has been previously described under pathological conditions such as during tumor formation in teratomas or primitive neuroepithelial tumors (PNETs). Initial studies in hESCs suggested that neural rosettes represent abortive neural tube structures

(Zhang et al. 2001) expressing a broad range of neural precursor markers including Nestin, NCAM, Pax6, and Sox1. One critical feature of neural rosettes is the close temporal link between rosette formation and neural patterning potential (Perrier et al. 2004; Yan et al. 2005). Neural rosettes also exhibit a high proliferative capacity and a broad differentiation potential along both neuronal and glial lineages (Reubinoff et al. 2001; Zhang et al. 2001). There has been some controversy whether neural patterning is restricted to the earliest stages of neural rosette formation or whether patterning potential is maintained in later-stage rosettes. It has been suggested that expression of Sox1 marks later-stage rosettes with reduced patterning potential (Yan et al. 2005). However, other studies did not observe an obvious delay in the onset of Pax6 versus Sox1 expression and reported intact patterning potential toward midbrain dopamine neuron (Perrier et al. 2004) and spinal motoneuron fates (Lee et al. 2007b) in later-stage rosettes.

Recent work from our lab has characterized the patterning potential of neural rosette-stage cells in more detail and confirmed extensive AP and DV respecification in rosettes (Elkabetz et al. 2008), including the potential to yield neural crest stem cells (NCSCs) and precursor populations (Lazzari et al. 2006; Lee et al. 2007a). Interestingly, neural rosettes do not express distinct markers that define specific DV domains during neural tube formation, as best characterized for the developing spinal cord *in vivo* (Jessell 2000). The lack of defined DV domains within rosettes suggests that neural rosettes correspond to the neural-plate/neural-fold stage before DV specification, rather than representing abortive neural tube structures.

Stem Cell Nature, Cytoarchitecture, and Progression in Neural Rosettes

The broad differentiation potential and proliferative capacity of neural rosettes suggest that they may contain patternable NSC-like populations. The demonstration of a self-renewing rosette-stage NSC with intact differentiation potential has been challenging. Propagation of rosette-stage cells in FGF-2 and EGF, the growth factors most commonly used in NSC culture, results in the rapid loss of radial organization and the concomitant loss of patterning potential. These findings suggest that the extrinsic milieu (e.g., FGF-2/EGF exposure) suppresses rosette potential, or that proliferation in the presence of FGF-2 and EGF causes progression toward a later nonpatternable state.

The classic definition of NSCs is based on self-renewal potential and trilineage differentiation. In the case of rosette-stage stem cells, this definition may need to be adjusted to include maintenance of rosette cytoarchitecture and—most importantly—patterning potential. Therefore, NCSs at the rosette stage are expected to exhibit self-renewal and differentiation capacities broader than those observed in classic NSCs.

Neural rosettes and early-stage neuroepithelial cells *in vivo* share many morphological and functional characteristics including a pronounced apical-basal polarity. The apical side of neuroepithelial cells *in vivo* faces the amniotic fluid at the neural-plate stage and the neural tube lumen fol-

lowing neural tube closure. The basal side is linked to the basal lamina. Neural rosettes maintained *in vitro* lack equivalents of basal lamina or amniotic cavity. Therefore, apical-basal polarity must be defined based on marker expression and functional characteristics. Throughout the cell cycle, neuroepithelial cells are anchored to one another apically through junctional complexes (Hinds and Ruffett 1971; Aaku-Saraste et al. 1996) characterized by ZO1 expression. Toward mitosis, neuroepithelial cells round up at the apical side, concomitant with interkinetic migration of their nuclei along the apical-basal axis. *In vitro*, the expression of ZO1 and the location of mitotic nuclei marked by phosphohistone 3 (PH3) define the rosette lumen as the apical side of the rosette structure (Lazzari et al. 2006; Elkabetz et al. 2008). Early-stage neuroepithelial cells, similar to early rosettes *in vitro*, form a pseudostratified epithelium with all cells touching the lumen (Sauer 1935). As neural rosettes continue to grow *in vitro*, they mimic the processes occurring during neurulation and neural tube growth. Rosette cells gradually give rise to differentiated cells such as neurons migrating radially away from the rosette structure and a set of more restricted progenitors. The more complex organization of late-stage rosettes is reflected in their multilayered structure. Future studies must address whether the rosette lumen at these late stages continues to exert a niche function and retain a population with intact patterning and proliferation potential or whether all cells in late rosettes undergo progressive fate restrictions.

Early neural rosettes represent a symmetrically dividing undifferentiated neuroepithelial cell population with very small numbers of spontaneously differentiating neurons. This early expansion phase is followed by a neurogenic stage where rosette cells start to efficiently produce neurons. Concomitant to onset of neurogenesis, rosettes give rise to radial glial-like cells expressing classic markers such as brain lipid-binding protein (BLBP) and GLAST. These radial glial-like populations retain features of neuroepithelial cells such as interkinetic nuclear migration and apical endfeet. At later stages, there is a loss of epithelial polarity and increased EGF responsiveness associated with a loss of neurogenic bias and increased production of glial fates.

Forse-1: Anterior Bias and Neural Patterning in Rosettes

The broad differentiation potential of hESC-derived neural rosettes raises the question of whether cells with true NSC potential can be isolated at that stage. Such rosette-stage NSCs are predicted to combine self-renewal and multilineage differentiation potential with the ability to respond to patterning cues. Forse1 was previously characterized as a surface marker distinguishing anterior and posterior CNS precursor fates (Tole et al. 1995). The Forse1 antibody allows the separation of rosette-stage cells enriched for anterior markers such as BF-1 (FoxG1B). Forse1-negative rosette cells are enriched for precursors expressing posterior CNS and neural crest markers (Fig. 1). In addition, Forse1⁺ cells also include populations of more differentiated neurons and glia as well as nonneural progeny.

Figure 1. NSC progression model. During neural induction, hESCs form early neuroepithelial structures developing into neural rosette structures. At the rosette stage, cultures can be separated based on Forse1 expression into cells with anterior CNS character and into cells with more posterior markers including neural crest markers. Both anterior and posterior R-NSC-stage cells can be patterned toward a broad range of fates along the AP and DV axes. Depending on culture conditions, R-NSCs can be propagated and give rise to NSC^{FGF/EGF} or yield self-renewing populations of R-NSCs. NSC^{FGF/EGF} also exhibit self-renewal potential and multilineage differentiation toward astrocytes, oligodendrocytes, and neurons but lose patterning potential.

Forse1⁺ cells express classic NSC markers including nestin, BLBP, and vimentin while retaining expression of neuroepithelial markers such as Pax6, Sox1, and N-cadherin. Importantly, at the rosette stage, both Forse1⁺ and Forse1⁻ cells contain population of cells that can be respecified in response to appropriate AP and DV patterning cues (Elkabatz et al. 2008). Clonal studies have revealed that single Forse1⁺ cells are capable of self-renewal, multilineage differentiation, and neural pattern-

ing, the key features that define NSCs at the rosette stage. These data indicate that rosettes contain regionally biased NSC populations that can be prospectively identified and that retain broad differentiation potential at the clonal level, termed R-NSCs. Figure 2 presents a summary of markers that distinguish R-NSCs from both undifferentiated hESCs- and hESC-derived NSCs^{FGF/EGF}.

Identification of an R-NSC-specific molecular profile was based on the hypothesis that independent of their

Figure 2. Marker and functional panel distinguishing ESCs, R-NSCs, and NSCs^{FGF/EGF}. Representative markers show rosette cytoarchitecture ("morphology"), cell polarity, NSC identity, R-NSC state, and the ability of the cells to differentiate in region-specific neurons such as somatic motoneurons (patterning).

regional bias (Forse1⁺ or Forse1⁻), R-NSCs share a molecular profile distinct from undifferentiated hESCs and NSCs^{FGF-2/EGF}. R-NSC markers identified by global transcriptome analysis were highly enriched in nuclear proteins including transcription factors and transcriptional regulators (Elkabetz et al. 2008). Most R-NSC genes have not been previously associated with neural development and NSC biology, although most of them are expressed during early neural development in the mouse (www.informatics.jax.org). Null mutations for many of the R-NSC-specific markers do not result in major CNS defects, suggesting functional redundancy. However, these studies did not directly study CNS impact of these genes and may have missed more subtle neural phenotypes. On the basis of our studies, we predict—given the narrow developmental windows of expression—that more dramatic effects may be observed in gain-of-function rather than in loss-of-function studies. Gene ontology analysis revealed that gene expression in R-NSCs is enriched for components of the Notch, SHH, and Wnt signaling pathways and is distinct from NSC^{FGF/EGF}.

Growth Requirements and Intrinsic Growth Potential

A strong temporal correlation exists between patterning potential and rosette cytoarchitecture, although there is no evidence that rosette organization is directly required for mediating patterning response. When R-NSCs are first derived from hESCs, they rapidly expand in number and spontaneously form secondary rosettes, suggesting that a default intrinsic program drives R-NSC self-renewal. However, as R-NSCs progress in culture, this program is down-regulated as reflected by reduced proliferation potential, and the loss of rosette cytoarchitecture and patterning potential and becomes dependent on extrinsic factors. Therefore, a key question in R-NSC biology is the identification of extrinsic factors that promote R-NSC self-renewal.

Two well-known factors for propagating NSCs are FGF-2 and EGF. Both factors have been shown to promote in vitro proliferation of NSCs and neural precursors from many regions of the developing and adult CNS (Murphy et al. 1990; Reynolds and Weiss 1992; Kilpatrick and Bartlett 1993; Ray et al. 1993; Gritti et al. 1995; Johe et al. 1996; Kalyani et al. 1997; Studer et al. 1998). FGF-2-dependent proliferation in vitro is compatible with the early expression of FGF-R1 (FGF-receptor-1) and FGF-2 in the CNS (Orr-Urtreger et al. 1991; Wanaka et al. 1991), defects in cell proliferation associated with targeted deletion of these receptors (Yamaguchi et al. 1994; Ciruna et al. 1997; Raballo et al. 2000), and the proliferative effects following FGF-2 administration in vivo (Kuhn et al. 1997). EGF exposure also has well-known effects in the developing and adult SVZ (Craig et al. 1996; Doetsch et al. 2002) and is required for normal forebrain development at late embryonic and postnatal stages (Sibilia and Wagner 1995; Threadgill et al. 1995). Despite the important role of FGF-2 and EGF for NSCs in vitro and in vivo, at the rosette stage, FGF-2 and EGF treatments cause a rapid loss of rosette cytoarchitecture and patterning potential (Shin et

al. 2006; Elkabetz et al. 2008). Another problem regarding FGF-2 treatment at the R-NSC stage is the induction of non-CNS cells with neural crest and mesenchymal precursor features (Lee et al. 2007a; Elkabetz et al. 2008).

There are multiple lines of evidence for the importance of Notch signaling at the R-NSC stage. First, components of Notch signaling were selectively enriched in R-NSCs (Elkabetz et al. 2008). Second, high cell density promotes growth of R-NSCs and maintenance of rosette cytoarchitecture, and both of these responses can be blocked by Notch inhibition via the γ -secretase inhibitor DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-*L*-alanyl]-*S*-phenylglycine *t*-butylester). Third, addition of recombinant proteins activating notch signaling including Dll4 and Jagged-1 promotes R-NSC maintenance (Elkabetz et al. 2008). However, Notch downstream effectors such as Hes1 and Hes5 are enriched both in R-NSCs and NSCs^{FGF/EGF}, and a role for Notch signaling is not restricted to the R-NSC state given its well-known effects in many NSC paradigms (Gaiano et al. 2000; Ohtsuka et al. 2001; Androutsellis-Theotokis et al. 2006; Mizutani et al. 2007). Therefore, it remains to be determined whether certain aspects of Notch signaling selectively impact the R-NSC state or whether Notch exerts a more generic self-renewal effect in R-NSCs, similar to its role in other NSC populations.

SHH has many fundamental roles in early neural development as both a morphogen and a proliferation factor. Ablation of SHH during mouse development causes many abnormalities in CNS patterning and beyond (Chiang et al. 1996). Conditional ablation studies in the CNS have revealed a role for SHH signaling in the maintenance of neural stem and precursor cells during development and adulthood (Ishibashi and McMahon 2002; Lai et al. 2003; Machold et al. 2003; Ahn and Joyner 2005; Palma et al. 2005). We observed that SHH is a potent mitogen for R-NSCs, inducing rapid proliferation over multiple passages without impacting rosette cytoarchitecture or gene expression profiles (see Fig. 3) (Elkabetz et al. 2008). However, it remains to be determined whether SHH acts by selectively promoting R-NSC proliferation, promoting R-NSC identity via inducing R-NSC-specific genes, repressing progression to NSC^{FGF/EGF} state, or, in part, preventing differentiation toward dorsal fates including neural crest precursors.

Molecular Dissection of R-NSC Development

A striking illustration of the R-NSC state is the unique molecular signature as defined by global gene expression analysis comparing undifferentiated hESCs to R-NSC and NSC^{FGF/EGF} progeny (Fig. 4). The identification of large sets of R-NSC-specific genes opens up new opportunities for defining the R-NSC state and the associated neural patterning potential at the functional levels. However, temporal analysis of gene expression also provides a better understanding for the molecular events involved in R-NSC induction and progression to the NSC^{FGF/EGF} state.

At the level of cell adhesion, an early switch from E-cadherin expression in undifferentiated hESCs to N-cadherin expression is retained in R-NSCs and NSCs^{FGF/EGF}.

Figure 3. R-NSC maintenance and proliferation. Labeling of cells using Ki67 reveals that SHH/DLL4/Jag1 and FGF-2 treatments induce proliferation compared with untreated control cultures. However, only SHH/DLL4/Jag1 treatment efficiently maintains R-NSC cytoarchitecture, whereas FGF-2 treatment causes a progressive loss of cell polarity.

R-NSCs and NSCs^{FGF/EGF} also share expression of classic neuroepithelial and radial glial markers such as Sox1, Sox2, Hes5, and Pax6. The increasing radial glial nature of R-NSCs and NSCs^{FGF/EGF} is further illustrated by the temporal expression of FABP7 and SLC1A3 (GLAST) (Fig. 4). One striking feature of R-NSCs is their pronounced polarity, and several key polarity genes includ-

ing CDC42 and Par6 show peak expression in R-NSCs. The greatest temporal specificity is observed for bona fide R-NSC markers (Elkabatz et al. 2008) such as Plagl1, Dach1, and Zbtb16 (PLZF).

The patterning potential of R-NSCs likely reflects an epigenetic state permissive for plasticity. Recent studies have demonstrated reprogramming of somatic cells back

Figure 4. Expression of molecular markers: Time course analysis. Selected markers are shown grouped into specific functional categories (for details, see text).

to a pluripotent state using defined sets of transcription factors or RNA-binding proteins (Takahashi and Yamanaka 2006; Takahashi et al. 2007; Yu et al. 2007). We observed that expression of these reprogramming factors is rapidly abolished in the case of Oct4 (POU5F1) and Nanog. However, R-NSCs selectively retain high levels of LIN28, and levels of Sox2 and Klf4 were similar to those of NSCs^{FGF/EGF}. Although most anterior CNS markers such as FoxG1 are expressed highly in both R-NSCs and NSCs^{FGF/EGF}, Emx2 shows decreased expression at the NSCs^{FGF/EGF} state. However, other anterior markers including Dlx1 and Dlx2 show increased expression in NSCs^{FGF/EGF} (data not shown). Finally, our data indicate that neural crest potential (P1 R-NSCs) and glial differentiation bias (NSCs^{FGF/EGF}) can be predicted based on global gene expression profiles.

In summary, molecular analysis offers insights into the sequential events controlling NSC progression. These studies also identified many potential markers for the prospective isolation of R-NSCs and NSCs^{FGF/EGF} using genetic reporter lines. Importantly, molecular analysis also provides a high priority list of targets for functional interventions aimed at enhancing R-NSC self-renewal and preventing or reversing progression to the NSCs^{FGF/EGF} state.

CONCLUSIONS AND QUESTIONS FOR THE FUTURE

The isolation of hESC-derived R-NSCs raises many key questions for future exploration. One obvious task is defining the role of R-NSC-specific genes in self-renewal and patterning potential and testing whether ectopic expression of key R-NSC genes in NSCs^{FGF/EGF} is sufficient to reverse the R-NSC state. R-NSC markers are also critical for defining cell heterogeneity and lineage relationships within R-NSCs. Another issue relevant for future translation is the development of improved protocols harnessing the patterning potential of R-NSCs while reducing the risk for neural overgrowth, as commonly observed in hESC-derived neural progeny-grafting paradigms (Sánchez-Pernaute et al. 2005; Ferrari et al. 2006; Roy et al. 2006; Aubry et al. 2008; Elkabetz et al. 2008).

Beyond the role of R-NSCs in hESC biology, it will be important to identify potential endogenous R-NSC populations. Initial studies suggest that R-NSC-like populations can be isolated from mouse embryos at the neural-plate stage (E8.25) (Elkabetz et al. 2008). The availability of genetic markers should allow prospective isolation of R-NSCs from primary tissue and in vivo lineage marking. Although current data suggest that R-NSCs represent a highly transient cell population, it will be critical to confirm whether R-NSC-like cells can persist into postnatal and adult stages.

The striking cytoarchitecture of rosettes raises the question of whether cell organization and cell-to-cell contact is critical for aspects of R-NSC biology such as self-renewal and symmetric division capacity or patterning potential. To this end, tools need to be developed that selectively disrupt polarity at the R-NSC stage. Given the emergence of rosette structures in many neural tumor types such as PNETs or medulloblastomas, understanding these struc-

ture/function relationships may provide insights beyond the neural stem cell field.

One fundamental idea of rosette biology is that similar to ESCs at the inner cell mass (ICM) stage, R-NSCs may capture a defined developmental state such as the neural-plate stage and perpetuate early cell potential in vitro. Such an “ES cell equivalent of the nervous system” could become a powerful tool for translational medicine and for applications in high-throughput screening assays requiring large numbers of homogeneous cell populations. Such a paradigm may for the first time provide access to a universal NSC stage capable of recreating the complete cellular diversity of the central and peripheral nervous systems.

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