Multipotent and Restricted Precursors in the Central Nervous System

MAHENDRA S. RAO*

Acquisition of cell type-specific properties in the nervous system is likely a process of sequential restriction in developmental potential. At least two classes of pluripotent stem cells, neuroepithelial (NEP) stem cells and EGF-dependent neurosphere stem cells, have been identified in distinct spatial and temporal domains. Pluripotent stem cells likely generate central nervous system (CNS) and peripheral nervous system (PNS) derivatives via the generation of intermediate lineage-restricted precursors that differ from each other and from multipotent stem cells.

Neuronal precursors termed neuronal-restricted precursors (NRPs), multiple classes of glial precursors termed glial-restricted precursors (GRPs), oligodendrocyte-type 2 astrocytes (O2As), astrocyte precursor cells (APCs), and PNS precursors termed neural crest stem cells (NCSCs) have been identified. Multipotent stem cells and restricted precursor cells can be isolated from embryonic stem (ES) cell cultures providing a non-fetal source of such cells.

Analysis in multiple species illustrates similarities between rat, mouse, and human cell differentiation raising the possibility that similar factors and markers may be used to isolate precursor cells from human tissue or ES cells.

KEY WORDS: stem cell; neuronal stem cell; NRP; GRP; oligodendrocytes; astrocytes; differentiation; mouse; rat

Development of the nervous system involves differentiation, migration to an appropriate location, projection to an appropriate target, synaptogenesis, and acquisition of an appropriate rostrocaudal and dorsoventral identity. Our knowledge of the sheer number of cells present in the nervous system and an increasing awareness of neural diversity has led to an appreciation of the magnitude of the problem a developing organism faces in trying to specify the appropriate neuronal and glial fates of each precursor cell.

Seminal work by a number of laboratories has led to rapid advances in our understanding of phenotypic specification. Of particular importance has been our growing appreciation of the similarities between neurogenesis and tissue genesis in the liver, skin, and bone marrow. In each of these systems, development proceeds by a progressive restriction in developmental potential. In the hematopoietic system, for example, differentiation into macrophages, granulocytes, erythrocytes, lymphocytes, and platelets occurs by a sequential differentiation of progressively more committed cells. Primitive stem cells (PSCs) generate lymphoid and myeloid (committed) progenitor cells (see Fig. 1). Committed progenitor cells undergo self-renewal and give rise to a large number of identical cells by a process termed clonal amplification. These more restricted cells then undergo differentiation in response to environmental and non cell autonomous cues to generate particular subclasses of differentiated cells (Fig. 1). The validity of this model of differentiation in hematopoiesis has been confirmed by in vivo transplant experiments and identification of cell surface markers that clearly distinguish between classes of restricted precursors and their differentiated progeny.

If differentiation in the nervous system is indeed similar to differentiation in other tissues, one should be able to identify pluripotent stem cells and restricted stem cells, differentiate between classes of stem cells using markers, establish a lineage relationship between classes of stem cells, and identify growth factor and differentiation signals. Considerable progress has been made on each of these fronts. This article will focus on results from our laboratory and cite relevant related literature that demonstrates differentiation does indeed follow a pattern of sequential fate determination. The types of cells described and their antigenic differences are summarized in Table 1.

MULTIPO TENT STEM CELLS ARE PRESENT IN THE NERVOUS SYSTEM

Using single cell clonal analysis, retroviral tracing techniques and transplant assays, several groups have unambiguously demonstrated the presence of multipotent stem cells (reviewed in

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Kalyani and Rao\textsuperscript{18} in the central nervous system (CNS). At least two major classes of stem cells are present in the CNS (Fig. 2). One class of cell has been termed a neurosphere and was first described by Reynolds et al.\textsuperscript{42} Neurosphere stem cells isolated from multiple regions of the brain have been shown to undergo extensive self-renewal and to differentiate into neurons, astrocytes, and oligodendrocytes. Data from multiple laboratories show that epidermal growth factor (EGF)-dependent neurosphere cells are multipotent, self-renewing stem cells that can generate all major cell types in the brain. Neurosphere stem cells are characterized by nestin immuno-reactivity, growth in suspension culture, and by the requirement of high doses of EGF (50 ng/ml) for isolation. These cells may subsequently be supported by fibroblast growth factor (FGF) but neurosphere stem cells cannot be isolated using any growth factor other than EGF and transforming growth factor-alpha (TGF-alpha).\textsuperscript{52}

Another class of stem cells identified in the nervous system is FGF-dependent stem cells. FGF-dependent stem cells, like neurospheres, are self-renewing and can generate neurons, astrocytes, and oligodendrocytes (Fig. 3). FGF-dependent stem cells appear to grow in both suspension and adherent cultures and have been shown to self-renew for months\textsuperscript{16} or years.\textsuperscript{41} EGF cannot substitute for FGF during the process of isolation or maintenance and both acidic and basic FGF seem equally capable of maintaining FGF-dependent stem cells.

While clearly both EGF- and FGF-dependent stem cells can self-renew in culture and can generate multiple phenotypes of cells under appropriate signals, self-renewal has not yet been demonstrated in vivo. Transplants of stem cells in vivo have clearly demonstrated that these cells can differentiate into neurons, astrocytes, and oligodendrocytes but self-renewal has been harder to demonstrate. In the hematopoietic system, transplanted stem cells can be harvested, and stem cells reisolated and then retransplanted into fresh recipients, and this entire process was repeated to show that transplanted stem cells undergo self-renewal in vivo and that the daughter stem cells generated could repopulate the bone marrow with all hematopoietic phenotypes. Experiments of this sort, a technical tour de force, clearly demonstrate self-renewal in vivo. Such experiments, to my knowledge, have not been undertaken in the central nervous system or the peripheral nervous system. Labeling cells, harvesting a small population and reisolating and transplanting cells are still major technical challenges that need to be solved before one can demonstrate self-renewal in the CNS. Nevertheless, the overall consensus is that EGF- and FGF-dependent progenitor cells are self-renewing stem cells that likely self-renew in vivo. Demonstrating this unambiguously is likely to be simply a matter of time.

Many factors that can promote neuron growth, differentiation, and survival have been described. Because this article’s focus is on stem cell lineages dependent on EGF or FGF, the reader is directed to reference 18 for a more complete discussion of other effector molecules and additional references. Other classes of neuronal precursor cells have only been recently characterized and the stage-specific role of effector molecules remains to be fully defined. FGF and Wnt-1, for example, seem to play a key role in maintaining neuroepithelial cell proliferation (e.g., see reference 41). FGF’s appear to be the only cytokines that promote mitosis of NEP cells. EGF, PDGF, NT-3, and a variety of other cytokines tested do not appear to stimulate NEP cell division (e.g., see references 16, 17, 52). Though FGF promotes NEP cell proliferation, an additional, undefined factor is required to completely prevent their differentiation.\textsuperscript{16} Another group of negative regulators, the Id family of genes.
is expressed in the spinal cord and may also play an important role in inhibiting stem cell differentiation. Positive differentiation factors have also been described, such as the BMPs. Neuroepithelial stem cell differentiation into neural crest stem cells (NCSCs) is promoted by BMPs 2 and 4; since differentiation does not require cell division, BMPs likely play an instructive role in the differentiation process. Determinants have yet to be found that bias NEP cell differentiation toward neuroblasts and glioblasts. Homologs of notch and numb may impinge on the decision by a stem cell to generate either a daughter stem cell or a differentiated progenitor. However, no single molecule that "instructs" stem cells to become neuroblasts or glioblasts has yet been found. Factors regulating postmitotic neuron survival have been identified. NT-3, LIF, and CNTF each may have tropic

### TABLE 1. Neuronal precursors and neurons express distinct cell-specific antigenic markers

<table>
<thead>
<tr>
<th>Name</th>
<th>Cell Type</th>
<th>Antigens Present</th>
<th>Antigens Absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEP cell</td>
<td>FGF-dependent, multipotent stem cell</td>
<td>FGFRs, Brain fatty acid protein, nestin, Bm-1, Musashi 1</td>
<td>All markers expressed by differentiated cells, EGFR, PDGFR</td>
</tr>
<tr>
<td>Neurosphere</td>
<td>EGF-dependent multipotent stem cell</td>
<td>EGFR, FGFR1 and 2, Brain fatty acid protein, nestin, Bm-1, Musashi 1</td>
<td>All markers expressed by differentiated cells</td>
</tr>
<tr>
<td>NCSC</td>
<td>Neural crest stem cell</td>
<td>Nestin, FGFRs, p75</td>
<td>All markers expressed by differentiated cells</td>
</tr>
<tr>
<td>NRP</td>
<td>Neuron-restricted precursor</td>
<td>nestin, E-NCAM, β-III tubulin, MAP2</td>
<td>Astrocyte, oligodendrocyte and glioblast cell markers. No late neuronal markers</td>
</tr>
<tr>
<td>Neuron</td>
<td>Differentiated postmitotic neuronal cell</td>
<td>Late appearing markers such as Neurofilament-H, synaptic proteins, neurotransmitter synthesizing enzymes</td>
<td>Astrocyte, oligodendrocyte and glioblast cell markers. No late neuronal markers</td>
</tr>
<tr>
<td>GRP</td>
<td>Glial-restricted precursor cell</td>
<td>A2B5, nestin, FGFR 1, 2, and 3, PLP, DM-20</td>
<td>No early or late neuronal markers. GalC, myelination antigens, S-100β, GFAP, Ran-2</td>
</tr>
<tr>
<td>O2A</td>
<td>Oligodendrocyte type-2 astrocyte precursor cell</td>
<td>A2B5, nestin, FGFR 1, 2, 3, PLP, DM-20, PDGF-R-alpha</td>
<td>No early or late neuronal markers. GalC, myelination antigens, S-100β, GFAP, Ran-2</td>
</tr>
<tr>
<td>Oligodendrocyte</td>
<td>Differentiated postmitotic glial cell</td>
<td>O4, O1, myelination antigens, PLP, DM-20</td>
<td>No early or late neuronal markers. No astrocytic markers. Nestin, PCNA</td>
</tr>
<tr>
<td>Astrocyte Precursor Cell (APC)</td>
<td>Restricted precursor that generates only one kind of astrocyte</td>
<td>A2B5, nestin, S-100β</td>
<td>No early or late neuronal markers. GalC, myelination antigens, S-100β, GFAP, Ran-2</td>
</tr>
<tr>
<td>Type 1 Astrocyte</td>
<td>A differentiated glial cell</td>
<td>Ran 2, GFAP, S-100β, nestin, FGFR3</td>
<td>A2B5. No early or late neuronal markers. No GalC, or myelination antigens</td>
</tr>
<tr>
<td>Type 2 Astrocyte</td>
<td>A differentiated glial cell that has not been identified in vivo</td>
<td>A2B5, GFAP, S-100β, nestin, Musashi 1</td>
<td>Ran2, FGFR3. No early or late neuronal markers. No GalC, or myelination antigens</td>
</tr>
</tbody>
</table>

A list of the cell types discussed and their salient characteristics. The markers listed are by no means exhaustive but are the selection of published markers that appear to distinguish between different cell types. Note that different groups have reported the expression of E-NCAM on NEP-like cells, GRP cells and astrocytes at specific stages of development. Note likewise A2B5 expression has been reported on subsets of neurons. The reader is referred in the text to specialized reviews for a detailed discussion on the expression profile of these markers.

Abbreviations: FGFR, fibroblast growth factor receptor; EGFR, epidermal growth factor receptor; PDGFR, platelet growth factor receptor; Bm-1, POU domain protein; nestin, intermediate filament protein; Musashi 1, RNA binding protein; MAP2, microtubule associated protein 2; E-NCAM, polysialated neural cell adhesion molecule; β-III tubulin, a neuron-specific tubulin; PCNA, proliferation specific nuclear antigen; A2B5, antibody that recognizes a specific non protein epitope; Ran2, an epitope specific to type 2 astrocytes; GFAP, glial fibrillary acid protein, an astrocyte specific marker; S-100β, an epitope expressed predominantly by astrocytes; PLP, proteolipid protein; DM-20, an alternate transcription product of the PLP gene.
In addition, motoneurons—the earliest developing neurons—have a unique set of factors in addition to FGF that affect their survival. The key takeaway message is that the effect of a particular cytokine(s) is stage specific, and when used in combination, they may have distinct effects. For example, sonic hedgehog (Shh) ventralizes neural tube cells, promoting both motoneuron and oligodendrocyte differentiation, but may act on other cell types to promote mitosis.

**WHEN AND WHERE ARE EGF-DEPENDENT NEUROSPHERE STEM CELLS AND FGF-DEPENDENT STEM CELLS PRESENT?**

If stem cells play a role in normal development, these cells should be present prior to the onset of neurogenesis and gliogenesis. In the spinal cord of rats, neurogenesis begins at or around embryonic day (E) 12.5–13.0 and proceeds rostrally (headwards) and caudally (tailwards), with most neurogenesis being completed by E17. Select regions of the brain show neurogenesis at later stages. Cerebellar granule cell are born perinatally and neurogenesis is completed during the first two weeks of postnatal life. Olfactory bulb and hippocampal neurogenesis appear to continue throughout life with a peak in the first postnatal week. The presence of precursor cells has been correlated with these developmental stages and the ability of stem cells to contribute to ongoing neurogenesis has been determined. FGF-dependent stem cells have been shown to be present prior to the onset of neurogenesis in rat spinal cord as well as in the cortex, multipotent stem cells are present as early as embryonic day E10.5 (our unpublished results). We and others have shown that FGF-dependent stem cells are present at equivalent stages in embryonic mouse neural tubes. Indeed, we have noted that at early stages the neural tube has a homogenous population of FGF-dependent, pluripotent stem cells and that these FGF-dependent stem cells do not respond to EGF and do not express detectable levels of EGF receptor. Both groups report that no EGF-dependent neurosphere stem cells could be detected at this stage, making it likely that only FGF-dependent stem cells contribute to neurogenesis at this early developmental stage.

The absence of EGF-dependent stem cells at these early stages of development is also supported by other independent experiments. Weiss et al. have noted that EGF-dependent neurosphere cells cannot be isolated earlier than E14.5, a time period following the onset of neurogenesis. At earlier developmental stages, dividing cells [as identified by bromodeoxyuridine (BrdU) incorporation] are present in the ventricular zone and neurons are being born. Early-born neurons are therefore likely to arise from non EGF-dependent stem cells. These observations are consistent with results seen in transgenic animals, where EGF-R expression was abolished. In these animals, the ventricular zone develops normally and no proliferative or stem cell abnormalities were reported. Further, early neurogenesis is unaltered though neuronal heterotopias were observed, suggesting that while stem cell proliferation may not be affected, EGF may be important in regulating neuronal migration. Similar conclusions can be drawn from the EGF receptor overexpression studies, where increasing the EGF response biases differentiation towards the astrocytic fate. Overall, the data suggest that stem cells requiring EGF for proliferation are not present at early developmental stages.

Isolation of stem cells from E14.5 onwards suggests that both EGF- and FGF-dependent stem cells are present at appropriate locations and may contribute to neurogenesis at later stages of development. Transplanting both stem cell types has provided evidence that stem cells can contribute to ongoing neurogenesis. The question of whether endogenous, neurosphere stem cells or FGF-dependent stem cells...
contribute to ongoing neurogenesis is still under investigation. An interesting set of experiments were performed to address this question. Endogenous stem cell proliferation was enhanced by infusion of either FGF or EGF and differentiation of newly born cells was assessed by BrdU pulse labeling and staining for the appearance of neuron specific markers. The results suggest that FGF-dependent stem cells may contribute significantly more to neurogenesis. Both EGF and FGF infusion causes expansion of the ventricular zone and an increase in the number of BrdU-incorporating (dividing) cells. EGF stimulates cell division to a larger extent but the percentage of neuronal gen. 

The question of why two types of multipotent stem cells coexist in the CNS remains unresolved. More recently, Johansson and colleagues have suggested that stem cells are indeed present in the adult brain and that they are localized to the ependymal layer lining the ventricular system. In a series of elegant experiments, the authors showed that ependymal cells are a slowly dividing stem cell population that generate a more rapidly dividing "transit cell" that is localized to the subventricular zone. The rapidly dividing cell can subsequently generate neurons and astrocytes. It is unclear from their experiments whether the EGF-dependent neurosphere or the FGF-dependent stem cell represented the transit cell population or the ependymal stem cell population. These results are consistent with earlier work and are exciting, since identifying and localizing stem cells to a specific location in vivo provides an unprecedented opportunity to examine the response of stem cells to in vivo manipulations.

**ARE EGF-DEPENDENT NEUROSPHERE STEM CELLS AND FGF-DEPENDENT STEM CELLS LINEARELY RELATED MULTIPOTENT CELLS?**

The possible lineage relationships between EGF- and FGF-dependent stem cells are summarized in Figure 2. Important characteristics of FGF-depen-
dent neuroepithelial (NEP) stem cells appear to be their ability to grow in adherent cultures in vitro and their absolute requirement of FGF for survival. Neither EGF nor any other cytokine tested can substitute for FGF, whether cells are grown in adherent or in suspension (neurosphere-like) cultures. Examination of epidermal growth factor receptor (EGF-R) expression in NEP cells provides an explanation for their failure to respond to EGF. EGF-R expression is not seen on FGF-dependent NEP cells by either PCR or by immunocytochemistry, both in vitro or in vivo. In addition to the difference in growth factor dependence, another important difference appears to be the frequency of neuron generation. NEP stem-cells, as well as other FGF-dependent stem cells, appear to generate neurons at high frequency, whereas EGF-dependent neurospheres have a very low frequency of neuron generation and appear to lose the ability to generate neurons after multiple passages. Based on these differences, we have argued that EGF-dependent neurospheres and FGF-dependent stem cells represent two distinct classes of stem cells. Apart from their phenotypic and other differences, perhaps the most compelling supportive arguments are the reports of Santa-Ollala and Covarrubias and Tropepe et al. Both groups showed that both FGF- and EGF-dependent stem cells co-exist in similar brain regions at later stages of development. Using population and statistical analyses, both groups argued that these represent two distinct populations of cells. The coexistence of these two populations suggests that the FGF-dependent stem cell does not necessarily transform into an EGF-dependent stem cell; rather, both populations coexist.

In a technically difficult set of experiments, Tropepe and colleagues constructed chimeras from fibroblast growth factor receptor 1-null (FGFR1-) and normal animals and showed that EGF-dependent stem cells must go through a FGF-dependent stage. These data argue that FGF-dependent cells are the precursors of the EGF-dependent neurosphere stem cells. These results are compelling but do not address the stage at which the two lineages diverge. Some groups have suggested that FGF-dependent neurospheres will generate EGF-dependent neurospheres arguing that FGF-dependent cells are indeed the precursor of the EGF-dependent stem cell. Why these two populations co-exist and what regulates the transition from one multipotent stem cell to another, however, remains unknown.

**MULTIPLE CLASSES OF GLIAL PRECURSORS EXIST**

Oligodendrocyte-type 2 astrocyte (O2A) cells, which represent one of the best defined glial precursors of the CNS, were initially isolated from the postnatal rat optic nerve, and subsequently from the postnatal cortex and spinal cord. O2A cells have a default pathway of differentiation into oligodendrocytes and this differentiation can be modulated by growth factors. In culture, O2A cells can also differentiate into type 2 astrocytes. Type 2 astrocytes differ from the more common type 1 astrocyte in their expression of A2B5 immunoreactivity and the absence of Ran 2 immunoreactivity (see Table 1). O2A cells will not differentiate into neurons under any culture condition and upon transplantation will differentiate into myelinating oligodendrocytes. O2A cells thus represent glial-restricted precursor cells that can generate a subset of the glial population present in the adult brain.

**ASTROCYTE-RESTRICTED PRECURSORS**

Another class of precursor cells restricted to glial differentiation are astrocyte restricted precursors (APCs). Seidman et al. have described astrocyte restricted precursor cells isolated from the E16 mouse cerebellum that do not express glial fibrillary acid protein (GFAP), an astrocyte marker, and are EGF dependent. Upon differentiation, the cells begin to express high levels of GFAP but do not differentiate into oligodendrocytes. APCs are not A2B5 immunoreactive and the astrocytes that differentiate appear to be type 1 astrocytes. Mi and Barres have independently provided unambiguous evidence for an astrocyte precursor cell or APC. The authors show that this cell expressed A2B5 immunoreactivity and expresses some, but not all, astrocytic markers. This cell can differentiate into astrocytes under appropriate culture conditions. The authors argue that since these APC cells do not default to an oligodendrocyte pathway of differentiation and but rather differentiate into type 1 and not type 2 astrocytes, they are clearly distinct from O2A cells. The authors did not, however, show that this cell could not make oligodendrocytes under conditions in which the O2A precursor cells do, raising the possibility that this might be a distinct type of oligodendrocyte-astrocyte precursor cell, rather than solely an astrocyte-restricted precursor cell.

Examining oligodendrocyte differentiation from spinal cord, Richardson and colleagues noted that while oligodendrocyte differentiation occurred in the ventral cord and required platelet-derived growth factor receptor (PDGFR)-alpha expression, astrocytes can be generated from dorsal spinal cord from cells that do not express the PDGFR-alpha. Their results suggested the existence of an astrocyte precursor that is present in the dorsal spinal cord. The authors also noted that at E13.5 A2B5 immunoreactive/PDGFR-alpha- cells were present in the dorsal spinal cord, raising the possibility that the spinal cord astrocyte precursor may be A2B5 immunoreactive, as compared to APCs from the brain.

**GRP CELLS ARE GLIAL-RESTRICTED PRECURSOR CELLS THAT CAN DIFFERENTIATE INTO OLIGODENDROCYTES AND TYPE 1 ASTROCYTES**

While glial restricted precursors (GRPs) clearly exist, almost all data suggest that these are late-glial precursors. The questions that remained unanswered were: did an embryonic glial-restricted precursor exists prior to the first generation of oligodendrocytes and astrocytes and did this precursor arise from a multipotent stem cell. In a series of in vitro experiments, we identified and characterized a glial-
restricted precursor (GRP) that is present in the developing spinal cord. GRPs can be identified as early as E12 by their A2B5 and nestin immunoreactivity. Dividing glial precursors with similar properties can be identified as late as postnatal day 2. GRP cells lack PDGFR-alpha immunoreactivity (at least initially) and synthesize detectable levels of PLP/DM-2050 (see Table 1). GRPs arise ventrally from a restricted region of the proliferating neuroepithelium, although the potential to generate GRPs appears much more widespread.5,12,16 GRP cells differ from a previously characterized, later appearing O2A precursor.40 An important difference is the absence of PDGF-R alpha expression on GRP cells. Perhaps the most significant difference, however, is the ability of GRP cells to generate two kinds of astrocytes, while O2A cells generate only one kind of astrocyte. This observation is important as it provides a possible lineage relationship between oligodendrocytes and type 1 astrocytes (Fig. 4).

GRPs can generate type-1 astrocytes, type-2 astrocytes, and oligodendrocytes, while O2A cells and APCs generate subsets of these populations. Further, all three classes of glial precursors are present in the developing spinal cord at different stages of development. It is therefore tempting to suggest a lineage relationship between these precursor cell types. A hypothetical relationship is schematized in Figure 3. While such a relationship is intellectually appealing, no direct evidence exists suggesting that this is indeed the case. However, clear elucidation of the similarities and differences between the cell types and the identification of cell surface markers suggests that determining whether a lineage relationship exists is simply a matter of time. Indeed, preliminary results from our laboratory suggest that GRP cells generate astrocyte-restricted precursor cells that differentiate solely into type 1 astrocytes.

**Neuron-restricted precursors are present in multiple regions of the brain**

In addition to neural crest stem cells (NCSCs) and GRPs, precursors limited in their differentiation potential to neurons have also been reported to exist in the developing brain. Mayer-Proschel et al.27 were able to isolate a neuronal restricted precursor from the developing spinal cord. The authors showed that this neuronal restricted precursor (NRP) expresses E-NCAM (high polysialic-acid NCAM) and is morphologically distinct from neuroepithelial (NEP) cells,16 NCSCs,32 and spinal GRPs.40 The authors showed that E-NCAM-immunoreactive neuronal precursors can be maintained as undifferentiated precursors for over 3 months and can differentiate into multiple neuronal phenotypes but cannot differentiate into oligodendrocytes or astrocytes. The authors used clonal analysis to show that individual neuroblasts could generate excitatory, inhibitory, and cholinergic neurons that synthesized multiple neurotransmitter receptors and that they establish synapses in culture. These results were complemented by in vivo retroviral labeling experiments which confirm that neuron-restricted clones are present in the chick spinal cord and that these clones may contain multiple kinds of neurons.

NRP-like cells have also been identified from other cortical regions. Several laboratories have shown that neuronal precursors exist in the hippocampus, subventricular zone and developing cortex.25,41 Other laboratories have used retroviral labeling techniques to show that restricted neuronal precursors are present in the developing spinal cord. Levison21 has shown by retroviral injections that neuronal-only precursors are present in the subventricular zone. Luskin and her colleagues25 have combined retroviral labeling and immunocytochemistry to show that migrating olfactory neuroblasts are E-NCAM-immunoreactive, dividing neuroblast cells. The external

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**Figure 4.** Hypothetical relationship between glial restricted precursor cells. A model of how NEP cells, GRPs, O2A cells, and astrocyte precursor cells are related is shown. NEP cells may generate GRPs that subsequently differentiate into an even more restricted glial precursor the O2A cell and APC cells. These cells can be distinguished from each other on the basis of their antigenic properties. Note: Direct lineage relationships between GRPs, O2As, and APCs still need to be established. See Table 1 for definitions of terms.
granule cell layer of the cerebellum may also contain neuron-restricted precursor cells. Thus, NRPs that generate more differentiated progeny are likely a general feature of CNS differentiation.

Whether these neuronal precursor cells identified from different brain regions are related to each other, however, remains to be determined. It would be tempting to assume that these cells represent a common neuronal precursor, harvested from multiple sources. Indeed, in vitro assays suggest that spinal cord neuroblasts, for example, can generate neuronal phenotypes that are normally not present in the spinal cord. Other data, however, indicate that while neuroblasts may be morphologically similar, a bias in the differentiation potential may already have occurred. Progenitors from the hippocampus, but not from the cerebellum or midbrain, produced hippocampal pyramidal neurons. A recent report showed that under appropriate conditions as many as 50% of the neurofilament immunoreactive neurons that differentiated from midbrain precursors appeared dopaminergic. This frequency was much higher than that obtained from any other neuron precursor cell population. Transplant experiments that directly assess the differentiation potential of regionally distinct populations of E-NCAM cells in side-by-side comparisons may reveal similarities and differences.

**DIRECT LINEAGE RELATIONSHIP BETWEEN THE MULTIPOTENT STEM CELL AND RESTRICTED PRECURSORS**

Retroviral lineage tracing has suggested that at early developmental stages, multipotent stem cells are present while, at later stages, colonies are phenotypically more restricted. Using cultures of acutely dissociated cells from different embryonic ages we have shown that most of the rat neuroepithelium at E10.5 comprises multipotent stem cells, while a short time later, more restricted precursor cells are present. These data suggest that differentiated cells must be derived from an initially pluripotent stem cell population.

How this process of differentiation occurs is only now being clarified. We have been able to demonstrate a direct lineage relationship between FGF-dependent stem cells and spinal cord neuron and glial-restricted precursor cells. We showed that NEP stem cells can be induced to differentiate into E-NCAM<sup>+</sup> and A2B5<sup>-</sup> cells by replating and reducing FGF concentration. In this condition, approximately 50% of the cells will differentiate into A2B5-immunoreactive cells and a smaller percentage will differentiate into E-NCAM-immunoreactive cells. These two distinct populations of cells are morphologically and antigenically similar to NRPs and GRPs directly isolated from more mature neural tubes and, like E13.5 derived A2B5<sup>-</sup> or E-NCAM<sup>-</sup> cells, are glial-restricted or neuron-restricted precursors. Equally importantly, we were able to show by complement-mediated lysis experiments that NEP cell differentiation into postmitotic neurons and oligodendrocytes likely requires an obligate transition through a restricted-precursor cell stage. We were thus able to show a direct lineage relationship between multipotent NEP stem cells and more restricted neuronal- and glial-precursor cells present in vivo at E13.5.

This finding demonstrates a transition from an NEP cell to an NRP cell was the first evidence that restricted neuronal precursors are an intermediate stage between pluripotent stem cells and fully differentiated postmitotic neurons (summarized in Fig. 5). Similar lineage relationships between other classes of potential restricted precursors (neuron-astrocyte or neuro-oligodendrocyte) have not been described and it is not clear whether EGF-dependent neurosphere stem cells generate neurons, astrocytes, and oligodendrocytes via a similar mechanism of progressive cell fate restriction.

**FGF-DEPENDENT STEM CELLS REPRESENT A COMMON CNS-PNS PRECURSOR**

The neural crest contains a multipotent cell termed a neural crest stem cell (NCSC), which can generate craniofacial mesoderm, melanocytes, and the neurons and glia of the PNS. Several laboratories have analyzed the properties of NCSCs and have shown that these cells undergo self-renewal, can be maintained in cultures, and that individual cells can generate neurons, Schwann cells, and other PNS derivatives. NCSCs do not appear to generate CNS derivatives and transplanting NCSCs into the CNS results in Schwann cell differentiation (unpublished results, Bronner-Fraser, personal communication). Thus NCSCs likely represent a restricted stem cell.
A variety of evidence from chick embryo experiments at early developmental stages further suggest that NCSCs and differentiated CNS cells share a common progenitor. Perhaps the most direct evidence is from neural fold ablations, which demonstrate that cells of the remaining neural tube have the regulative capacity to compensate for the ablated neural crest cells (reviewed in Kalyani and Rao19). These results suggest that precursor cells normally destined to form the CNS possess the ability to regulate their prospective fates to form PNS derivatives.

Recent work from two different laboratories has now shown that FGF-dependent, pluripotent CNS stem cells likely represent such a common CNS-PNS precursor. Mujtaba et al.31 have shown that NEP cells can generate p75-immunoreactive cells. These results are independently confirmed, NEP-derived, p75-immunoreactive cells differentiate into peripheral neurons, smooth muscle, and Schwann cells in both mass and clonal culture. More importantly, the authors31 showed by clonal analysis that individual NEP cells can generate both CNS and PNS derivatives providing evidence for the first time of a direct lineage relationship between these two distinct cell types.

More recently, McMay and colleagues have shown that cortical stem cells that were isolated at a stage well after neural crest migration has taken place still retain the ability to differentiate into PNS derivatives.14 These results demonstrate that FGF-dependent stem cells are less restricted in their developmental potential than was previously believed; they have the capacity to differentiate into cells of both the CNS and PNS, and that PNS differentiation involves a transition from an NEP stem to another more limited, p75-immunoreactive, neural crest stem cell. No data on the ability of the EGF-dependent neurosphere cell to generate crest or PNS derivatives are available. Our initial attempts to generate smooth muscle and Schwann cells from neurospheres were unsuccessful (unpublished results). If these results are independently confirmed, then this may represent an additional distinction between EGF- and FGF-dependent cells and may provide further evidence that FGF-dependent cells represent a distinct, more pluripotent population of stem cells.

**HUMAN NEURAL DEVELOPMENT MIRRORS THAT IN MOUSE AND RAT**

CNS differentiation in humans is similar to that described for the rat and mouse. Analysis of human fetal development shows that initially the neural tube is a homogenous population of dividing, nestin-immunoreactive cells. Proliferation is restricted to the ventricular zone and neurogenesis occurs first, followed by differentiation of oligodendrocytes and astrocytes. Neurons and glial cells express antigens similar to those identified in mouse and rat and differentiate in similar spatiotemporal locations. More recently, direct evidence has been provided for the existence of multiple classes of stem cells from human tissue.

Culture of human fetal tissue has shown that multipotent stem cells exist in the human CNS. These cells are nestin-immunoreactive, self-renewing cells that can generate neurons, astrocytes, and oligodendrocytes. Pluripotent stem cells with the characteristics of neurospheres as well as FGF-dependent stem cells have been described4 (Melissa Carpenter, Stem Cell Inc., Ronghao Li., Signal Pharmaceuticals, personal communication). While some differences exist, the overall growth properties and cytokine responses of human and rat stem cells appear similar. Xenotransplant experiments in which human stem cells were transplanted into rats showed that these cells can integrate and differentiate into multiple types of cells in vivo.310

In addition to multipotent stem cells, E-NCAM-immunoreactive neuroblasts have been identified. We have shown that human fetal spinal cord cultures contain dividing neuron-restricted precursor cells. More recently, Li and colleagues21 have reported the generation of an immortalized, human neuron-restricted precursor cell line that is limited in its differentiation potential to multiple kinds of neurons. This cell line, termed HSP-1 (human spinal precursor cell) cannot differentiate into astrocytes or oligodendrocytes under conditions where other cells readily differentiate into astrocytes. Morphologically and phenotypically the HSP-1 cell line appears similar to the rat and mouse NRP cell, suggesting that they have immortalized a human NRP cell.

Glia precursors have also been identified in human tissue. Rivkin et al.43 identified A2B5+ cells that have a typical bipolar morphology characteristic of GRPs and O2A cells. The authors showed that in longer-term cultures, these cells could generate oligodendrocytes and that cells with similar characteristics exist in the human fetal brain. Similarly we have shown (unpublished results) that A2B5+ cells present in fetal spinal cord cultures express glial but not neuronal markers. Likewise Murray et al.33 have shown that oligodendrocyte precursor cells expressing the markers DM-20 and O4 were present in the human CNS (see Table 1). While additional experiments are clearly required to characterize glial precursors more fully and to determine whether this cell is a O2A precursor or a GRP cell, the available data do lead to the conclusion that glial-restricted precursors exist in the developing human brain and that these cells likely express A2B5 and DM-20. Thus, the overall evidence suggests that human neural development involves multipotent stem cells generating more differentiated progeny via the generation of an intermediate, more-restricted, precursor cell. While direct lineage relationships between multipotent and lineage restricted human stem cells have not been established, it is likely that these exist and are similar to the lineage relationships established in rodent stem cell differentiation.

**ES CELLS MAY SERVE AS A SOURCE OF TISSUE-SPECIFIC CELLS**

Embryonic stem (ES) cells represent the earliest totipotent cells and are present at least until the late blastocyst stage. ES cells in vivo likely generate ectodermal, endodermal, and mesodermal precursor cells which generate progressively more tissue specific derivatives33,48 as fetal development proceeds (Fig. 6). ES cells, in principle, can generate every cell type in the embryo. Indeed, in chimeraic mouse experiments, ES cells can generate an
entire animal (including germ line cells). ES cell lines that are spontaneously immortal were isolated from mice in the early 1980s and have more recently been isolated from several species.

ES cell lines have been shown to recapitulate normal nervous system differentiation in vitro and to generate postmitotic neurons and glia that appear phenotypically normal and integrate and function normally after transplantation.9,35,48 Equally important, the sequential development of totipotent ES cells into specialized CNS derivatives appears to involve many of the same cytokines and transcription factors identified as being important in normal development. These observations raise the possibility of isolating late neural progenitors such as NRPs and GRPs from ES cell cultures.32 These results (summarized in Fig. 6) suggest that ES cells can be used as a source of early pluripotent and late, more-restricted precursor cells.

The importance of these results is twofold. First, it may obviate the need for fetal tissue. ES cells appear to be spontaneously immortal and have been passaged as undifferentiated cells for many years. Primate and human ES cell lines have been recently isolated35,37 and these cell lines can be used instead of harvesting cells from the fetus. Second, multiple ES cell lines can be generated that can be immunologically matched to the recipient, obviating problems arising from mismatch of ES-derived differentiated cell populations. While moral and ethical issues with regard to the acquisition and use of human ES cells need to be resolved, it now appears that a source of multiple kinds of precursor cells is assured.

**THE THERAPEUTIC IMPLICATIONS**

Stem cell therapy is already well established for hematopoietic disorders, cancer treatment, and in immune disorders. No such therapy is routinely available for CNS disorders. However, recent advances in identifying sources of stem cells, isolating subclasses of stem cells, maintaining cells in culture, and the more recent demonstration that ES cells can be used as a source of stem cells have all provided a major impetus to the use of stem cells for therapy in the CNS.

Several clinical trials and tests in animal models have begun to provide important insights into optimum cells for transplants, methods of injection, and criteria for assessment. What has become clear is that no single cell type will be universally used. The choice of cell type, be it stem cell, neuron- or glial-restricted precursor, will be dictated by the functional replacement that is desired. Neuron-restricted precursors will likely be favored in diseases such as Parkinson’s, in which specific subsets of neurons are lost, (GRP) cells, because of their ability to migrate over large distances in intact brain tissue, may be the optimum cells for delivering drugs or genes, in ganglioside disorders such as Tay-Sachs and Sandhoff disease for example, where replacement of a single gene (hexosaminidase A or sialidase, respectively) is sufficient to ameliorate symptoms. ES cells and stem cells may be

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**Figure 6.** ES cells are a source of early and late neural progenitors. ES cells can generate neurons, astrocytes, and oligodendrocytes. Recent evidence summarized in this figure suggests that differentiation involves the generation of neural specific and lineage specific stem cells. ?? indicate that these cell types have not been shown to derived from ES cell cultures. See Table 1 for definitions of terms.
used as sources of more differentiated cells or may be used in transplants in situations where multiple cell types have been lost, such as in strokes or traumatic injury to the brain. An important recent result that will likely broaden the number of disease targets is the demonstration that environment-specific differentiation, neurite outgrowth, and appropriate connectivity may be possible in the adult damaged brain (reviewed in Kalyani and Rao19).

As our ability to further define different neural lineages is enhanced, so will be our ability to identify and understand the different tumors derived from these cells. Determining the relationship between NEP or NRP cells and primitive neuroectodermal tumors such as medulloblastomas or between GRP cells or other glial progenitor cells and different gliomas will begin to establish a tumor classification that may in time emulate the sophisticated tumor classification system that exists for cancers of the hematopoietic system. As the cells of origin for various CNS tumors are identified, markers characteristic of each tumor will become available for diagnostics and specific gene targets will be identified for therapeutic intervention.

**SUMMARY**

Stem cell therapy has long held out the promise of totally replacing damaged and defective tissue. This prospect, initially farfetched, now appears much closer to fruition because of several new findings: 1) recent advances in the isolation and culture of multiple classes of stem cells, 2) the demonstration that human nervous system development follows the same principles of progressive fate restriction previously described in animal models, and 3) the finding that ES cells can generate early and late neural precursors. These exciting findings offer the hope that stem cell therapy will soon be feasible for a variety of human diseases of the nervous system.

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**LITERATURE CITED**
