Origin and Diversification of Neural Stem Cells during Mammalian Brain Development

by

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Thesis submitted in conformity with the requirements for the Degree of Doctor of Philosophy, Graduate Department of Anatomy and Cell Biology in the University of Toronto

This dissertation is dedicated to the memory of
Alberto Messore
(1971-1990)

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Abstract

Neural stem cells are self-renewing precursor cells that have a fundamental role in generating cellular diversity in the developing mammalian brain. However, the establishment of this unique class of cells during the course of embryogenesis has previously not been characterized. In this thesis, I investigate mammalian neural stem cell ontogenesis using mouse as a model system. First, evidence is presented suggesting that neural stem cell formation is preceded by a primitive neural stem cell stage before the onset of neurogenesis. Primitive neural stem cells display distinct growth factor requirements for the production of progenitor cells and have a broad range of neural and non-neural lineage potential. The transition from primitive neural stem cell to definitive neural stem cell is correlated with an alteration in growth factor dependence and a restriction in multilineage potential. Acquisition of primitive neural stem cell identity is negatively regulated by TGFβ signaling proteins, which act to inhibit neural differentiation. Second, results from experiments performed after the onset of neurogenesis reveal that the entire germinal zone is initially composed of a small population of neural stem cells that are critically dependent upon FGF as a stimulus for generating progenitor cells. As neurogenesis proceeds, the FGF-responsive neural stem cell population expands and also gives rise to a distinct EGF-responsive neural stem cell population. A heterogeneous population of FGF- and EGF-responsive neural stem cells (both with self-renewal and multilineage potential) persists in germinal zone compartments throughout the brain. Finally, experiments show that during neurogenesis, neural stem cells and their early progenitors isolated from distinct brain compartments maintain a region-specific pattern of regulatory gene expression in the absence of their in vivo environment. However, this regional specification may not be irreversible and can be altered by local inductive cues. Overall, the results of this research provide the first description of mammalian neural stem cell ontogenesis. An ontogenetic model for the origin and diversification of mammalian neural stem cells is discussed in the context of brain development and adult neural homeostasis. The thesis concludes with an attempt to consolidate what is currently known about stem cell ontogenesis and behavior in various tissues and diverse organisms in order to elucidate the role of stem cells during metazoan evolution.

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List of Abbreviations

AMCA	7-amino-4-methylcoumarin-3-acetic acid
β-gal	β-galactosidase
BrdU	bromodeoxyuridine
CD1	mouse albino strain from Charles River, Quebec
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
DMEM	Dulbecco's modified eagle's medium
dNTP	2'-deoxynucleoside-5'-triphosphate
DNA	deoxyribonucleic acid
DTAF	dichlorotriazinyl amino fluorescein
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis[β-aminoethyl ether]-N, N, N', N'-tetraacetic acid
FBS	fetal bovine serum
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GFAP	glial fibrillary acidic protein
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
Hoechst	bisbenzamide trihydrochloride
IgG	immunoglobulin type G
IgM	immunoglobulin type M
MAP	microtubule associated protein
mg	milligram
ml	milliliter
mM	millimolar
μg	microgram
μl	microliter
μm	micrometer
μM	micromolar
MuMLV	murine Molooney leukemia virus
n	number of cases
ng	nanogram
NGS	normal goat serum
NP-40	nonidet P-40
O4	O-antigen sulfatide type 4
p	probability of error
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
ROSA	reverse orientation splice acceptor
RT-PCR	reverse transcription polymerase chain reaction
SEM	standard error of the mean
t	Student's t statistic
Taq	*Thermus aquaticus*
Tris	tris(hydroxymethyl) aminomethane hydrochloride
TRITC	tetramethyl rhodamine isothiocyanate
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter I

General Introduction

"...community in embryonic structure reveals community of descent; but dissimilarity in embryonic development does not prove discommunity of descent, for in one of two groups the developmental stages may have been suppressed, or may have been so greatly modified through adaptation to new habits of life, as to be no longer recognizable."

- Charles Darwin, *The Origin of Species By Means of Natural Selection*, 1859

In his *Origin of Species*, Darwin recognized that as a vehicle of evolution, natural selection could modify an existing pattern of embryogenesis by virtue of its adaptive significance to the adult body form. In principle, natural selection can influence any or all of the fundamental processes of metazoan development – cell division, differentiation and tissue morphogenesis. Perhaps one of the most important processes to have evolved in multicellular organisms is the ability to call upon these same developmental mechanisms in adulthood, long after the organism is fully formed.

Tissue regeneration, the ability of cells to be replaced as a result of natural attrition or injury, manifests itself in many ways and to varying degrees, but is believed to be a direct consequence of stem cell activity. Stem cells can divide asymmetrically to produce one identical daughter cell and one differentiated daughter (progenitor) cell. In this manner, stem cells can continually divide to produce a progenitor cell that is the same in each generation or one that is distinct from its older sibling. Thus, stem cells can self-renew and have multilineage potential. Other features have been ascribed to metazoan stem cells, but whether they are applicable to all organisms remain to be determined. For instance, stem cells in the nematode *Caenorhabditis elegans* have a relatively invariant mode of asymmetric cell division to produce all of the somatic lineages and the germline precursors during development (Sulston et al., 1983; Schnabel, 1997), but the original stem-like cells have very limited self-renewal ability. On the other hand, mammalian stem cells have the ability

to expand in number through symmetric division during development (the production of two identical stem cells) and subsequently persist through regular asymmetric divisions (e.g. crypt of intestinal epithelium) or in a relatively quiescent state in adulthood (Potten and Loeffler, 1990; Morrison et al., 1997). Although relatively inactive, these stem cells can be called upon to produce new tissue-specific cell types. A range of stem cell lineage potential among various metazoan species is also evident. For instance, the mammalian interfollicular epidermal stem cell appears to generate only one type of differentiated progenitor, a keratinocyte that undergoes several rounds of division to amplify the numbers of cells (Jones, 1997). In contrast, putative blood stem cells in botryllid ascidians can regenerate the entire organism (Rinkevich et al., 1995).

It is often speculated that stem cell activity is highly regulated and very conservative under homeostatic conditions. This is usually based on the fact that very small numbers of stem cells are maintained in tissues, especially in larger multicellular organisms such as mammals. Furthermore, remodeling in adult tissues by the production of new cells may largely be a function of stem cell-derived transient amplifying cells that undergo several rounds of cell division before terminally differentiating (Jones, 1997). Thus, the stem cell itself may not divide frequently at all, but remain in a relatively quiescent state. The cellular and molecular mechanisms that regulate the maintenance of the stem cell population as well as the unique stem cell properties remain somewhat elusive, but significant insight has come from various compelling, and often creative, perspectives. For instance, the asymmetric localization of intracellular determinants may play a primary role in stem cell self-renewal and the establishment of differentiated progeny (Jan and Jan, 1998). Cellular competition for limited resources (e.g. growth factors), in which the rare stem cell in a large population generally loses, may ultimately regulate the numbers of stem cell divisions in homeostasis (Conlon and Raff, 1999). Finally, an interesting but largely unsubstantiated theory invokes the nonequivalent segregation of DNA strands to protect dividing stem cells from the accumulation of genetic mutations (Potten et al., 1978). One can imagine how

these stem cell characteristics may have an adaptive advantage in allowing an organism to overcome limitations in achieving reproductive success, while minimizing the accumulation of too many genetic mutations (with few rounds of cell division) that would otherwise be detrimental to stem cells and their progeny.

The adult mammalian CNS has a rather low level of cellular turnover (primarily glial cells) and historically was believed to lack neuronal cell replacement altogether. Earlier observations made by Altman (1962) and Bayer and colleagues (1982), that discrete regions of the adult rodent forebrain produce new neurons have only recently come to fruition. There is currently considerable evidence that the adult CNS has regenerative potential and that neural stem cells orchestrate the production of new nerve cells (reviewed in Weiss et al., 1996; McKay, 1997). However, many features of neural stem cell biology remain to be determined. We have empirical evidence that neural stem cells can self-renew and generate neurons and glia, using both cell culture and in vivo experimental paradigms. Any cell in the embryonic or adult CNS that did not demonstrate these abilities as part of their behavioural repertoire would, by definition, not be classified as a stem cell. However, these properties alone do not reveal how neural stem cells arise during development, which has been the focus of my research interests.

In this dissertation, I have chosen to apply our knowledge of neural histogenesis and pattern formation to investigate the cellular mechanisms of neural stem cell ontogeny in the murine brain. As a conceptual foundation, I summarize in Chapter I of this thesis three relevant areas in the field of developmental neurobiology that have provided the rationale for my experimental approach.

Acquisition of Neural Identity in the Gastrulating Vertebrate Embryo

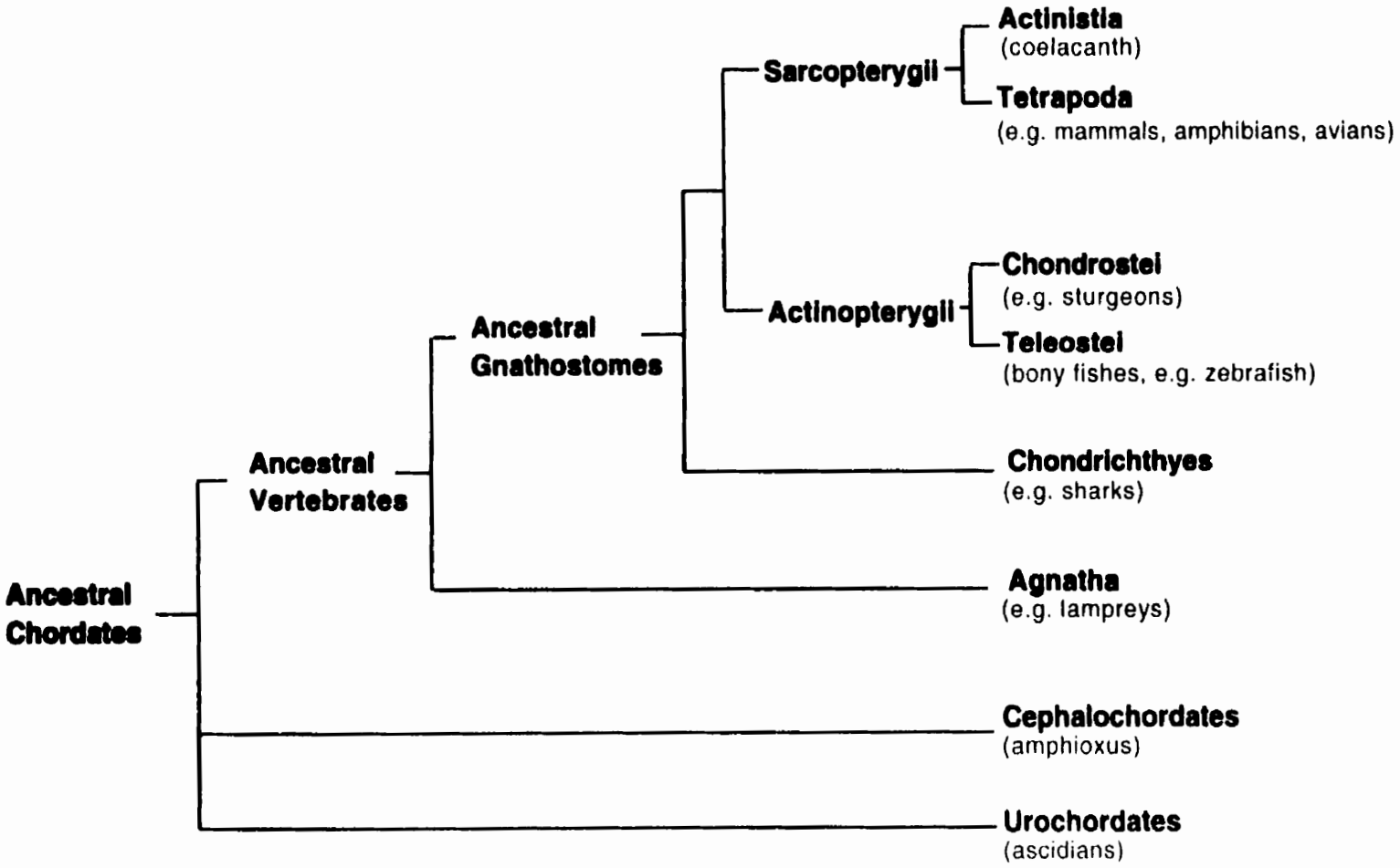
The formation of a relatively complex vertebrate embryo from a simple ball or sheet of cells in early developmental stages involves a substantial rearrangement of cell position. On the surface of the embryo, cells invaginate through a discrete opening (amphibian

blastopore) or groove (amniote primitive streak), or involute and converge from the edge of a cell sheet (dorsal blastoderm margin in teleosts) and eventually come to lie beneath other cells that do not undergo inward migration and remain on the surface (Stern, 1992). This elaborate morphogenesis has been extensively documented in representative vertebrate species of the actinopterygian (including teleosts) and sarcopterygian (including tetrapods) radiations (Figure 1.1) and defines the embryonic period of gastrulation. What emerges during this epoch of development is an embryonic bilaterian body plan with three separate primordia (germ layers): endoderm, mesoderm and ectoderm. These layers become organized into multiple distinct cell types.

In a broad sense, endodermal precursors gives rise to such tissues as the liver and the gastrointestinal tract, while mesoderm gives rise to such tissues as muscle, bone, kidney and blood. Cells derived from the embryonic ectoderm are organized into either neural or epidermal tissues. Given that extensive cell intermixing occurs during the onset of teleost and mammalian gastrulation (Warga and Kimmel, 1990; Gardner and Croft, 1998), the establishment of these separate lineages must rely on differences in the extent of cell mixing. Thus, cellular interactions ultimately effect the direction of clonal dispersion and demarcate regional boundaries with a unique composition in the embryo. During amphibian gastrulation, cell intermixing is less extensive and this contributes to a more constant (from one animal to the next) fate map at early stages (Wetts and Fraser, 1989). A recent dramatic demonstration of the maintenance of unique boundaries through cell interaction comes from experiments demonstrating that the chick organizer (node) is defined as a dynamic position in the embryo. The organizer is a small group of cells that has a crucial role in patterning the midline axis. Cells were found to immigrate and emigrate constantly through the node, rather than being defined as a static cohort (Joubin and Stern, 1999). Thus, the "dynamic node" may exemplify an underlying principle for establishing the various embryonic tissues during gastrulation, which involves specification of cohorts of cells through cell-to-cell

Figure 1.1. Vertebrate phylogeny. Schematic representation of the descent of extant groups of chordates from ancestral chordates. Only some of the groups are depicted in the schematic for clarity and branching lines are representative of lineal relationships and do not reflect the time of divergence acurately. Figure is adapted from Butler, A.B., Hodos, W. (1996). Comparative vertebrate neuroanatomy. Wiley-Liss, Inc. New York.

Vertebrate Radiation



interactions, rather than delineation from a strict ancestry. Nonetheless, fatemapping, sometimes at the resolution of a single cell, has proven extremely useful in determining how the tissues of the primary germ layers are formed and compartmentalized. By using interspecies tissue grafting methods (quail-chick chimeras) or intracellular labeling techniques (e.g. horse radish peroxidase, rhodamine dextran, carbocyanine dyes), a more precise fate map for the cellular descendents of precursors within the germ layers of avian (Selleck and Stern, 1991; Schoenwolf et al., 1992; Psychoyos and Stern, 1996), mammalian (Lawson et al., 1991; Parameswaran and Tam, 1995; Quinlan et al., 1995), amphibian (Wilson and Keller, 1991; Delarue et al., 1992) and teleost (Strehlow and Gilbert, 1993; Helde et al., 1994) embryos is emerging. From these studies the topography of the prospective tissues appears to be conserved between vertebrate species allowing for more meaningful comparisons. Particularly relevant for this thesis, mammalian anteroposterior deployment of neural subdivisions is fundamentally similar in zebrafish (Woo and Fraser, 1995), *Xenopus* (Eagleson and Harris, 1990) and chick (Couly and Le Douarin, 1988). However, the spatial allocation of the different neural subdivisions do differ, not only between these different vertebrates but also within one vertebrate class (e.g. between different teleost species, Woo and Fraser, 1995). Nonetheless, these lineage studies indicate that the acquisition of neural fate and fundamental neural organization in the embryo are conserved among vertebrate species.

Our understanding of the mechanism that regulates vertebrate neural specification in the uncommitted ectoderm has been fostered by the neural induction model, which was first conceptualized from investigations of amphibian embryology (Spemann and Mangold, 1924) and later supported by similar avian (Waddington and Schmidt, 1933), teleost (Oppenheimer, 1936) and mammalian (Beddington, 1994) experiments. Heterotopic transplants of the dorsal blastopore lip (the organizer) of amphibian embryos (the node in amniotes and shield in teleosts) to the undifferentiated ectoderm on the ventral side of a second embryo of the same developmental stage resulted in a secondary axis including a

patterned nervous system. It was postulated that the nascent embryonic ectoderm received a positive "vertical" signal from the dorsal organizer tissue during gastrulation, which caused the ectodermal cells to adopt a neural fate in a restricted manner. Furthermore, it was presumed that in the absence of this signal, the ectodermal cells differentiated into epidermis, independent of any cellular communication. In addition to vertical induction of neural tissue, experiments in *Xenopus* exogastrulae revealed that neural differentiation can be achieved by signals from mesoderm in the same plane as the ectoderm, without the need for vertical induction (Doniach, 1992; Keller et al., 1992). Remarkably, in these explant cultures much of the neurectoderm had appropriate anteroposterior patterning indicated by the expression of genes such as *En-2* (midbrain), *Krox-20* (hindbrain) and *XlHbox6* (spinal cord). Although these regional markers could be induced in ectopic sites along the ventral ectoderm, it was not clear from these studies whether organizer-derived signals were naturally capable of influencing ectoderm at distances far greater than the proximal influence of underlying mesoderm during vertical induction. Instead, these results offered an alternative possibility that the ectoderm may be predisposed towards a neural fate in the absence of organizer signals.

The identification of organizer-derived molecules, that alone were capable of inducing a second nervous system in analogous organizer transplant experiments performed in *Xenopus* (Smith et al., 1993; Sasai et al., 1995), revealed that the mechanism by which organizer signals promoted neural differentiation of ectodermal cells was not entirely consistent with a "positive induction" model. Neural inducers such as noggin and chordin, were shown to act by binding extracellularly to bone morphogenetic proteins (BMP), members of the TGFβ superfamily of molecules, previously shown to inhibit neural differentiation (Hemmati-Brivanlou and Melton, 1994). In this way, these antagonists could prevent the binding of BMP to their cognate receptors expressed on the surface of ectodermal cells (Piccolo et al., 1996; Zimmerman et al., 1996). In fact, BMP4 was shown to act as a positive signal for epidermal fate determination in the Xenopus

ectoderm (Wilson and Hemmati-Brivanlou, 1995). As a result of these findings, an alternate model for vertebrate neural fate determination was proposed, one in which neural fate determination was considered to be a "default" state (i.e. a state achieved autonomously from the lack of any cell-cell communication).

The first experimental evidence supporting the default neural fate hypothesis came from in vitro experiments of isolated ectodermal (animal cap) cells derived from amphibian gastrula. Prolonged dissociation of ectodermal cells, in the absence of organizer tissue, resulted in most of the cells expressing neural markers or forming neural structures upon reaggregation (Godsave and Slack, 1989; Grunz and Tacke, 1989; Sato and Sargent, 1989). Furthermore, ectodermal explants (undissociated cells) expressing a dominant-negative receptor for activin (member of the TGFβ superfamily of growth factors) were shown to become neural when cultured in vitro (Hemmati-Brivanlou and Melton, 1994). These findings from amphibian experiments were consistent with the notion that the establishment of neural identity from the uncommitted ectoderm occurs in the absence of neural inducing signals from the organizer. Thus, the powerful signals derived from the organizer are required to antagonize the effects of neural inhibitors. But does this antagonism have a role in the anteroposterior patterning of the nervous system?

It was realized early on that the earliest cells to involute during amphibian gastrulation (anterior endoderm and mesendoderm) were potent inducers of secondary cement glands and heads, respectively, while posterior organizer-derived tissue (chordamesoderm) was a potent trunk inducer (reviewed in Niehrs, 1999). The distinction between the head and the trunk inducing capacity of organizer tissues was hypothesized by Nieuwkoop (1952) to describe an initial anterior neural induction (activation) and subsequent posteriorization (transformation) of neural tissue. It was subsequently shown that during development in *Xenopus*, posterior neural regions are initially induced as phenotypically anterior and later respecified to give rise to a posterior identity (Sive et al., 1989). However, "activation" is not a necessary antecedent for posterior neural fate since

posterior neural tissue can be induced without initial anterior specification in animal cap experiments (Cho and De Robertis, 1990; Sive and Cheng, 1991; Kolm and Sive, 1995). With the recent elucidation of specific molecules that mediate head and trunk induction, the observations from classical experiments have been reincarnated as a "two-inhibitor" model (Niehrs, 1999). The model proposes that the nascent ectoderm expresses in addition to BMP, other neural inhibitors of the Wnt class of signaling molecules originally characterized in *Drosophila* (Harland and Gerhart, 1997). Both BMP and Wnt signals in the ectoderm prevent neuralization. Co-injection of mRNAs encoding BMP inhibitors (e.g. chordin, noggin and cerberus) and Wnt inhibitors (e.g. cerberus, dickkopf-1 and frzb) induces secondary heads (Glinka et al., 1997; Leyns et al., 1997; Wang et al., 1997; Glinka et al., 1998). These results suggested that the anterior endoderm and prechordal mesendoderm, where the inhibitors are naturally co-expressed, are poised to antagonize BMP and Wnt, thereby inducing anterior neurectoderm. Furthermore, the diminution of Wnt inhibitor expression posteriorly along the anteroposterior axis allows for progressively higher concentrations of Wnt, which in association with FGF or retinoic acid can pattern the posterior nervous system (Durston et al., 1989; Sive et al., 1990; Ruiz i Altaba and Jessell, 1991; McGrew et al., 1997; Popperl et al., 1997; Sasai and De Robertis, 1997; Chang and Hemmati-Brivanlou, 1998). Interestingly, cerberus alone is sufficient to induce a secondary head and anterior neural tissue in vivo (as well as in cultured animal caps) and is known to simultaneously antagonize BMP, Wnt and nodal proteins, all of which can inhibit head formation (Piccolo et al., 1999). Thus, the two-inhibitor model is sufficient to explain the establishment of broad anterior (forebrain and midbrain) and posterior (hindbrain and spinal cord) neural identity. Furthermore, other vertebrate species seem to employ similar strategies (with some significant differences) and utilize similar molecules in order to orchestrate the formation of patterned neural tissue. This molecular conservation provided me with a basis to test the nature of neural stem cell specification in mouse as a model mammalian system.

Prior to gastrulation in mouse, the inner cell mass (ICM) becomes segregated into two distinct lineages: the primitive endoderm and the primitive ectoderm (or epiblast). A component of the primitive endoderm, the anterior visceral endoderm (AVE) comes to lie directly beneath the anterior end of the epiblast in the presumptive head region (reviewed in Beddington and Robertson, 1999). In contrast to amphibian mesendoderm (organizer tissue), the AVE (a non-organizer derived tissue) in mouse is required for head and anterior neural formation.

The AVE is itself sufficiently patterned before the onset of gastrulation, expressing such genes as *Otx2* (Ang and Rossant, 1994; Acampora et al., 1995), *Lim-1* and *cerberus-like* (Belo et al., 1997) and *Hex* (Thomas et al., 1998) and several findings implicate the AVE in regulating neural specification. First, targeted null mutations in the *HNF3β* gene (a transcription factor expressed in cells of the node during gastrulation) in mouse lead to an absence of overt node (organizer) differentiation, yet patterned neural tissue is generated (Ang and Rossant, 1994) apparently independently of the node-derived factors. In a similar manner, a specialized population of cells at the anterior neural plate boundary is necessary and sufficient for forebrain patterning in the zebrafish embryo independent of shield (organizer) tissue (Houart et al., 1998). Second, the expression of nodal (a secreted molecule of the TGFβ superfamily) in the mouse AVE is required for anterior neural development (Waldrip et al., 1998). Given that nodal antagonism is sufficient for neural induction but is expressed in the AVE (Piccolo et al., 1999), these results suggest that nodal is necessary for the expression of neural antagonists in the AVE, such as cerberus that then antagonize neural inhibitory signals, in the ectoderm such as BMP (Waldrip et al., 1998). Consequently, the expression of anterior neural genes like *Otx2* and *Hesx1* (Varlet et al., 1997) is permitted. However, a recent study in zebrafish provides a slightly different scenario, which implicates direct nodal inhibition in the ectoderm itself as the mechanism for neural specification. It was demonstrated that the inhibition of nodal related or activin signaling (by the antagonist antivin) is required for the expression of anterior neural genes

in cells at the animal pole (Thisse et al., 2000). Clearly, the absence of nodal and other neural inhibitors in the tissue that will eventually become anterior neural tissue (anterior epiblast in mouse and animal pole ectoderm in zebrafish) is necessary, but the strategies from these two vertebrates differ. This raises the possibility that an alternative mechanism has evolved during anterior neural specification in mammals.

In fish, as in amphibians, organizer derived factors are sufficient for both anterior neural (head) and posterior neural (trunk) induction through the graded expression of antagonists to the TGFβ (BMP, nodal) and Wnt family of neural inhibitors. On the other hand, in mammals, separate head (AVE) and trunk (node) organizers may exist. This notion, in a strict sense, has been recently challenged by the finding that null mutations in both *chordin* and *noggin* genes in mouse, which are normally expressed in the node and its derivatives and not the AVE, display severe forebrain and head deficits (Bachiller et al., 2000). In these double knockout mice, the onset of expression of AVE genes (Hesx1, Lim-1 and cerberus-like) is normal, as is the expression of BMP4 in the extraembryonic ectoderm. Thus, the AVE in these animals is formed normally but is not sufficient for anterior neural development. However, it remains formally possible that noggin and chordin are indirectly responsible for maintaining the capacity of the AVE to regulate anterior neural patterning because the expression of cerberus-like in the AVE is not maintained in the noggin/chordin double knock-out mice (Bachiller et al., 2000). Perhaps even more revealing is that the midbrain, hindbrain and spinal cord are patterned normally even though the primary antagonists of BMP signaling are absent. Thus, the AVE may control the formation of anterior neural tissue, but BMP antagonists and other organizer-derived antagonists (e.g. antivin) may be needed to maintain AVE function in order to properly pattern all regions of the nervous system. It is interesting to note that BMP inhibition alone in chick embryos is not sufficient for neural induction. Misexpression of chordin or noggin in embryonic non-neural ectoderm does not generate ectopic neural tissue (Streit et al., 1998; Streit and Stern, 1999). However, the existence of combinations

of a multitude of neural antagonists like noggin, chordin, follistatin, frzb, dikkopf-1, antivin and cerberus in any one species clearly reveals a redundancy in this conserved mechanism underlying vertebrate neural fate specification and broad anteroposterior patterning.

Overall, the evidence from in vivo ectopic expression and targeted null mutation experiments in mouse and zebrafish substantiates the notion, originally conceived from amphibian studies, that vertebrate neural fate is achieved autonomously in the absence of a positive inducing signal. The variety of antagonists described above is a clear testament to this hypothesis. However, the complex cellular interactions that occur in vivo preclude a direct assessment of the autonomy of neural specification (i.e., default mechanism). In Chapter II, I test whether primitive cells in mouse can adopt a neural stem cell fate directly in an analagous low cell-density tissue culture paradigm that was used in the amphibian studies. Using immunocytochemistry, RT-PCR and in vivo mouse chimeric analyses, I demonstrate a novel stage in the development of the neural stem cell lineage, one that can be established directly from the most primitive embryonic mouse cells through a default mechanism.

Role of Neural Stem Cells in Generating Cellular Diversity

Cells in the vertebrate nervous system are richly diversified with respect to morphological, biochemical and hodological characteristics. How the generation of such an array of cell type diversity from relatively few cytologically indistinct precursor cells occurs continues to be a major question in developmental neurobiology.

Ever since the observations made by Cajal and Golgi in the late nineteenth century, it has been recognized that the emergence of the anatomical structure of the nervous system at the cellular level is sequential. This sequence can be described simply as the proliferation of precursor cells, migration to their final destination, differentiation into a specific and often predetermined phenotype and finally their maturation and functional integration with

one another. In mammals, the entire nervous system is initially organized as a flat columnar epithelium (neural plate) that is flanked by non-neural ectoderm (epidermal primordium) generated during gastrulation. Toward the end of gastrulation, the neural plate is drawn up into folds and a floorplate hinge is formed at the ventral midline. The neural tube is formed by the fusion of the dorsal tips of the neural folds generating roof plate cells at its dorsal midline (Schoenwolf and Alvarez, 1989) and emigrating neural crest cells, which populate a diverse array of tissues including the peripheral nervous system, bone and cartilage of the face and epidermal melanocytes (Le Douarin, 1980; Bronner-Fraser, 1993).

The neural tube is lined by germinal zone cells that proliferate and subsequently migrate to their final destination in a radial or tangential manner. The pattern of neural cell production and deployment may differ depending on the anatomical structure. For instance, in the multilayered mammalian neocortex (a dorsal forebrain structure), the first neurons to be produced will occupy deep layers while the subsequent neurons to be produced will occupy progressively superficial layers (Angevine and Sidman, 1961; Rakic, 1974). On the other hand, in the mammalian striatum (a ventral forebrain structure) early born neurons form aggregates or "patches," while later born neurons surround the patches in a more diffuse matrix (van der Kooy and Fishell, 1987; Fishell and van der Kooy, 1987; Krushel and van der Kooy, 1993). Thus, the acquisition of regionally specialized structures in the brain depend on various types of cells that emerge at different times and from different points of origin, reflecting a non-uniform distribution of a variety of cell lineages.

Studies in the rodent embryonic forebrain have generally supported the view that the pool of precursor cells in the germinal zone of the developing brain is heterogeneous. In vitro clonal analyses of isolated germinal zone tissue demonstrated the presence of unique precursor cell types with varying potentials to generate the main cell types found in the central nervous system: neurons, astrocytes and oligodendrocytes. For example, neuron-only precursors derived from the embryonic striatum (Cattaneo and McKay, 1990), septal precursors that could generate either neurons-only or neurons and astrocytes (Temple, 1989)

and cortical precursors that give rise to neurons and oligodendrocytes (Williams et al., 1991). Furthermore, precursors in the optic nerve (O-2A precursors) that can differentiate into oligodendrocytes or type-2 astrocytes (Raff et al., 1983; Hughes et al., 1988; Noble et al., 1988) and retinal precursors that can generate retinal neurons and glia (Anchan et al., 1991; Lillien and Cepko, 1992) have also been identified. Although capable of generating several progeny, these unique progenitor cells have a limited proliferative capacity.

The presence of such a rich composition of precursor cells has also been observed using clonal analyses in vivo, albeit more controversial. Limiting dilutions of reporter gene constructs, such as retroviruses housing the bacterial β-galactosidase gene *LacZ*, have been widely used as an indelible marker of cell lineage (Sanes, 1989; Cepko et al., 1990). These studies have generally shown mixed results. In some cases relatively small retrovirally labeled clones were primarily composed of only neurons or only glia (Luskin et al., 1988; Goldman and Vaysse, 1991; Parnavelas et al., 1991; Grove et al., 1993; Luskin et al., 1993; Mione et al., 1994). However, other studies demonstrated that retrovirally labeled clones may be composed of a greater number of different neuronal and glial cell types spanning several functional regions of the forebrain (Walsh and Cepko, 1992; Levison and Goldman, 1993; Reid et al., 1995). In considering these disparate conclusions from in vivo studies, Weiss and colleagues (1996) proposed that the small clone size and close proximity of clone members (obligate criteria for defining a clonally derived cohort of cells) limited the interpretation of some in vivo studies. Hence, the migratory nature of the progeny and the rare labeling of large clones (which could indicate the labeling of a multipotent precursor) were considered unreliable and presumably a result of multiple retroviral infections (Weiss et al., 1996). Indeed, when combined with cell cycle analyses, some retroviral experiments revealed a heterogeneity of precursor cells both in clone composition and cell cycle kinetics in the rodent forebrain germinal zone (Acklin and van der Kooy, 1993; Reznikov et al., 1997). Taken together, the contribution of in vitro and in vivo studies suggested the presence of a variety of progenitor cell phenotypes in the germinal zone of the developing

mammalian nervous system. However, whether these progenitors reflected more committed descendents from a multipotential stem cell was not clear and the designation of these cells as "stem cells" in some cases may have been overstated.

Among several properties that have been ascribed to stem cells, three have been used as definitive characteristics: multilineage potential, self-renewal capacity and longevity (Potten and Loeffler, 1990; Morrison et al., 1997). Early evidence for neural stem cells in the rodent forebrain came from in vitro clonal analyses of cells that could divide to produce neurons (of a variety of neurotransmitter subtypes), astrocytes and oligodendrocytes from both the striatal germinal zone (Reynolds et al., 1992) and the cortical germinal zone (Davis and Temple, 1994). Striatal stem cells had extensive proliferative behavior and generated large colonies of cells that expressed the intermediate filament protein nestin, a marker for neural precursor cells (Lendahl et al., 1990), prior to their differentiation. When primary stem cell colonies were dissociated and subcloned, a small proportion of individual progeny retained the ability to generate new colonies of cells with similar multilineage potential, and hence could self-renew (Reynolds and Weiss, 1996). In fact, stem cells with such properties could be similarly isolated from the adult forebrain subependyma (Reynolds and Weiss, 1992; Morshead et al., 1994; Reynolds and Weiss, 1996; Tropepe et al., 1997; Chiasson et al., 1999), the remnant of the germinal zone lining the lateral ventricles (Privat and Leblond, 1972; Takahashi et al., 1996). Hence, fulfilling the third criterion of longevity. The forebrain subependyma is a source of new olfactory bulb interneurons that are constantly being generated in the adult rodent brain, albeit at a low rate (Altman, 1969; Lois and Alvarez-Buylla, 1994). The presence of a relatively quiescent stem cell population in this region (Morshead et al., 1994) makes teleonomic sense. One can speculate that the constant turnover of olfactory interneurons conferred some adaptive advantage for olfactory sensory processing in rodents and thus the presence of a continued source of new neurons (a similar source used during development) could be positively selected. The same can be said for the constant replacement of neurons in the high vocal center of adult songbirds (Nottebohm,

1985; Alvarez-Buylla et al., 1990). The loss of specific neurons that were experimentally removed from this region was correlated with song deterioration, but was reported to be reversible with the subsequent recruitment of new neurons (Scharff et al., 2000). In these avian species, song production is intimately linked to mating behaviours.

Our knowledge of neural stem cell biology, especially in the adult stage, has been almost exclusively derived from studies in mammals. There are, however, some studies that reveal putative neural stem cell activity in non-mammalian and even invertebrate species. For instance, persistent neurogenesis in the brain of adult arthropods, like crustacea (Schmidt and Harzsch, 1999) and insects (Cayre et al., 1996), has been observed as well as post-embryonic neurogenesis in the optic tectum of goldfish (Raymond and Easter, 1983) and the high vocal center in song birds cited above. In these studies, a neural stem cell was implicated in facilitating the production of new neurons although the extent to which these stem cells were thought to express multilineage potential was not discussed. Furthermore, it is not yet clear whether tissue specific stem cells (e.g. neural stem cells) exist in other metazoan phylla.

Those of us that have investigated neural stem cells have been greatly influenced by stem cell biology in other mammalian organ systems, such as liver (Thorgeirsson, 1996), gut (Potten and Loeffler, 1990) bone (Aubin, 1998) or blood (Ogawa, 1993). In many of these systems, however, an understanding of the developmental profile of the tissue-specific stem cell lineage has facilitated and indeed helped to define our concept of stem cells. An ontological framework for neural stem cells has hitherto been conspicuously absent. In Chapter III, I continue to investigate the development of neural stem cells. Specifically, I test whether there are quantitative and qualitative differences in the temporal-dependent (embryonic age) and spatial-dependent (dorsal or ventral forebrain) growth factor responsiveness, self-renewal and multilineage potential of neural stem cells. I demonstrate for the first time that two separate, but lineage-related neural stem cell populations co-exist in the embryonic mouse forebrain.

Regional Diversification in the Developing Vertebrate Nervous System

In both the classical organizer transplantation experiments and the more recent ectopic expression experiments using organizer-derived (or extraembryonic) molecules, a rudimentary anteroposterior pattern in the neural ectoderm could be reliably observed. As previously mentioned these observations lead to a model in which the anterior and the posterior segments of the neural axis are differentially patterned by proximal non-neural tissues. Interestingly, hypotheses concerning the anatomical organization of the developing vertebrate nervous system predate the early observations from transplant studies and for over one hundred years have been largely investigated and debated in parallel to the body of work on neural induction. This has changed recently, and the two fields of inquiry are beginning to enjoy a fruitful convergence.

There are two main models for how the developing nervous system becomes regionalized with respect to morphological, molecular and functional domains.

His-Herrick Model. The first model, often called the His-Herrick model after two of the original proponents, emphasizes the longitudinally arrayed cellular columns along the neuraxis, which represent functional domains (e.g. sensory dorsal columns and motor ventral columns) divided by sulci (deep furrows), the most prominent one being the sulcus limitans that divides the dorsal and ventral columns [His' work (1888-1893) reviewed in Northcutt, 1995; Herrick, 1899, 1922]. This view of the fundamental organization of the nervous system continues to be recognized (Heijdra and Nieuwenhuys, 1994), especially in the hindbrain and spinal cord. Although the model is rooted in classical neuroembryology, there is currently a wealth of experimental evidence demonstrating the existence of polarized sources of inductive signals in the region adjacent to the hindbrain and spinal cord that could provide a mechanistic basis for elaborating a pattern of longitudinal columns.

In mammals, molecularly distinct dorsoventral subdivisions of the neural progenitor cells are evident in the ventricular zone surrounding the lumen of the central spinal cord canal (derivative of the posterior neural tube). The homeodomain containing transcription factors *Pax3* and *Pax7* (Liem et al., 1995) are preferentially localized to the dorsal aspect of the spinal neural tube, as are certain bHLH transcription factors such as Math1 (Helms and Johnson, 1998) and Neurogenin (Ma et al., 1996). Other bHLH transcription factors such as *Mash1* (Lo et al., 1991) are expressed in an intermediate dorsoventral position of the spinal neural tube along the anteroposterior axis. On the other hand, *Pax6* expression exists in the ventral neural tube in a gradient from strongest (dorsal aspect) to weakest (ventral aspect) (Walther and Gruss, 1991; Ericson et al., 1997). Therefore, at this early stage of neural differentiation, domains of molecularly distinct precursors are evident even though the cells are morphologically very similar.

Subclasses of neurons can be generated at distinct dorsoventral positions in the ventral spinal cord and hindbrain. The identity and position of these cells can be controlled by a gradient of Sonic Hedgehog (Shh) protein, which is first expressed in the underlying axial mesoderm and later induced in the non-neural floor plate cells at the ventral midline (Roelink et al., 1995; Tanabe and Jessell, 1996). As a result, the ventral-most subset of somatic or visceral motor neurons sensing high concentrations of Shh protein along the neuraxis (e.g., hypoglossal and vagal motor neurons of the hindbrain or lateral and medial thoracolumbar motor neurons) are specified. These neurons express a unique combination of molecular markers, such as *Isl1* and *Gsh4* (hindbrain) or *Isl1* and *Isl2* (spinal cord) (Ericson et al., 1992; Pfaff et al., 1996; Ericson et al., 1997). Lower concentrations of Shh expression in dorsal aspects of the ventral hindbrain and spinal neural tube specify interneuron fates with different repertoires of molecular markers (Ericson et al., 1997). Thus, through a gradient of Shh emerging from the floor plate and an opposing gradient of Pax6 in the ventricular zone of the ventral neural tube, longitudinal domains are generated in distinct dorsoventral positions.

In the dorsal half of the spinal cord and posterior hindbrain, cells situated lateral to the roof plate cells in the dorsal midline differentiate into several classes of sensory interneurons. Many genes are expressed in restricted dorsal domains of the neural tube and are thus poised to function as regulators of dorsal fate specification. The Wnt1 and Wnt3a glycoproteins (Cadigan and Nusse, 1997) are expressed in the dorsal neural tube (Parr et al., 1993; Hollyday et al., 1995), but targeted single mutations of these genes in mouse do not alter dorsoventral patterning of the spinal cord, even though these genes are essential for midbrain development (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). However, there is evidence for Wnt1 causing an expansion of the dorsal neural precursor population (Dickinson et al., 1994) and Wnt7b can induce the expression of the early neural crest markers *Slug* and *Twist* in *Xenopus* ectodermal explants (Chang and Hemmati-Brivanlou, 1998). The role of Wnt proteins in expanding dorsal progenitor populations is supported further by the finding that in mice targeted mutations of both the *Wnt1* and *Wnt3a* genes causes a severe reduction in the numbers of neural crest derived melanocytes and skeletal structures as well as cranial and spinal sensory neurons (Ikeya et al., 1997).

Epidermal ectoderm is implicated in patterning dorsal neural cell types, which is primarily mediated by TGFβ proteins. Prior to neural tube closure, BMP signaling promotes the maintenance of *Pax3* and *Pax7* expression in the lateral aspects of the neural plate (future dorsal neural tube) and induces the expression of *Slug*, a zinc finger transcription factor, in premigratory neural crest cells and roof plate cells after neural tube closure (Dickinson et al., 1995; Liem et al., 1995). Once the neural tube has fused, the epidermal ectoderm is no longer in close proximity to the cells of the neural tube ventricular zone. However, roof plate cells have been shown to subsequently pattern the specification of dorsal interneurons. Several members of the TGFβ family of proteins, including BMP4, activin and GDF7 are expressed in roof plate cells and have been implicated in activating discrete populations of ventricular zone precursors to differentiate into specific classes of interneurons (Liem et al., 1997; Lee et al., 1998). For instance, two recent studies further

demonstrate the requirement for the roof plate in patterning the dorsal neural tube. First, the selective elimination of roof plate cells, using the gene encoding the diphtheria toxin A subunit inserted into the *Gdf7* locus, resulted in the non-cell autonomous loss of Math1$^+$ and Neurogenin$^+$ progenitor cells in the dorsal ventricular zone (Lee et al., 2000). Consistent with previous results, the loss of both progenitor subtypes prevented the differentiation of D1A and D1B interneurons (Lee et al., 2000). Second, mutations in the LIM homeodomain gene *Lmx1a* in mouse, which is normally expressed in the roof plate, are responsible for the spontaneous neurological *dreher* mutant phenotype, which causes a diminution of D1 interneurons in the dorsal spinal cord (Millonig et al., 2000). Taken together, these results demonstrate that TGFβ signaling in the roof plate is involved in specifying unique dorsoventral identity. Thus, such a phenomenon occurring throughout the entire extent of the hindbrain and spinal cord would suggest that a pattern of longitudinal "columns" of sensory interneurons could be reasonably accounted for. However, at each anteroposterior level, sensory interneurons do not share an identical phenotype or pattern of axonal projection.

Dorsal and ventral cell fate specification in the vertebrate hindbrain, midbrain and posterior forebrain also appear to utilize Shh induction (ventrally) and BMP induction (dorsally). The expression of Shh in the anterior portion of the notochord and prechordal plate enables ventral neural tube cells of the hindbrain, midbrain and forebrain to express markers of ventral identity, such as *Pax6*, *Nkx2.2* and the related gene *Nkx2.1* (Walther and Gruss, 1991; Ericson et al., 1995; Shimamura et al., 1995). The function of Shh in regulating the ventral identity of neurons in the anterior neuraxis is similarly conserved (Barth and Wison, 1995; Ekker et al., 1995; MacDonald et al., 1995; Ericson et al., 1997). The expression of *BMP2*, *BMP4* and *BMP7* extends into the anterior roof plate and can promote dorsal cell fates in the hindbrain (Lyons et al., 1995; Arkell and Beddington, 1997; Dudley and Robertson, 1997). Furthermore, the transcription factor *Math1* is similarly expressed in the dorsal ventricular zone precursors in a specialized region of the

anterior hindbrain called the rhombic lip that gives rise to the external granule layer cells of the cerebellum (Alder et al., 1996; Ben-Arie et al., 1997), and the expression of Math1 in these precursors may be induced by BMP4 (Lee and Jessell, 1999). It is interesting, however, that the *Math1*-expressing progenitor cells in the rhombic lip are the predecessors of cerebellar granule cells, while *Math1*-expressing cells of the spinal cord are the predecessors of D1A spinal sensory interneurons.

Thus, these studies seem to suggest that the entire neural tube may utilize a common mechanism for establishing patterned longitudinal arrays of neurons along the anteroposterior axis. However, there are several caveats to the supposition, set forth at the beginning of this section, that this longitudinal organization represents a continuous columnar array of cells. First, a longitudinal array of cell columns continued rostrally from the spinal cord is difficult to reconcile anatomically in regions of the forebrain (discussed below). Second, although Shh can specify ventral neurons along the entire neuraxis, the same Shh signal specifies several different classes of neurons (based on both biochemical and hodological criteria) depending on the anteroposterior position. For instance, in the cervical spinal cord, Shh can specify a motor neuron identity to neurons of the phrenic nerve projecting to visceral organs, while in the ventral midbrain Shh can specify the identity of dopaminergic neurons that innervate the forebrain (Hynes et al., 1995; Ye et al., 1998). Similarly in the dorsal neural tube, *Math1* expression coincides with the spinal sensory interneuron progenitor cells in the spinal cord, and also granule neurons of the presumptive cerebellum, both of which are induced by members of the TGFβ family of molecules expressed in the roof plate. Finally, FGF8 which is expressed in the isthmus and in the anterior neural ridge in mouse can induce the expression of *BF1*, a critical transcription factor for forebrain development, when applied to forebrain tissue, whereas it induces *En1* expression when applied to midbrain tissue (Shimamura and Rubenstein, 1997). It appears, therefore, that although Shh and TGFβ molecules are used throughout the neuraxis to broadly specify dorsal and ventral fates, the differential competence of the

precursor cells responding to these signals in the anteroposterior dimension is critical in establishing different neuronal identities and structures. This is also true for the regulation (by FGF8) of relatively discrete domains such as the isthmus and the anterior neural ridge. Therefore, the His-Herrick model, in its original conception of longitudinal columns of cells bordered by sulcal landmarks that may or may not cross neuronal boundaries, is alone insufficient to explain the specification of distinct neuronal populations that are derived from different levels along the entire length of the neural tube.

Neuromeric Model. Early investigators noted that the longitudinal organization of cell "columns", that was quite apparent in the hindbrain and spinal cord, was anatomically rather obscure and controversial in the forebrain. The complex morphology of the tetrapod forebrain required a re-evaluation of the longitudinal model that had been widely applied to the relatively simplified neuroanatomy of the chondrichthyans (e.g. sharks) and the actinopterygian fish. In fact, Herrick (1922) recognized certain general morphological exceptions to the longitudinal organization: the cerebellum on the dorsal surface of the hindbrain (developing outside of the neural tube proper) and the primary olfactory sensory area in the forebrain, which encompassed both dorsal and ventral regions. Furthermore, the importance of sulci in clearly defining longitudinal boundaries in the hindbrain and spinal cord is less convincing in the forebrain. It has been proposed (Herrick, 1899) that additional horizontal sulci are required to divide several regions within forebrain subdivisions, for instance, even though these sulci do not seem to coincide with cortical or nuclear boundaries (Northcutt and Bradford, 1980).

In early stages of vertebrate neural development the inner ventricular surface of the neural tube consists of a series of ridges that are separated by shallow depressions along the entire extent of the anteroposterior axis. From the outer surface, the neural tube appears as a series of iterative bulges separated by constrictions and these have been termed neuromeres (rhombomeres in the hindbrain or prosomeres in the forebrain) (von Baer, 1928 reviewed in Northcutt, 1995; Kuhlenbeck, 1973). This neuromeric morphology is

transient and is not evident at later stages of development. However, there is now compelling evidence that the neuromeric model is currently most adequate for describing the fundamental organization of the developing vertebrate nervous system. The segmented pattern of neuronal development in teleost (Hanneman et al., 1988; Trevarrow et al., 1990; Woo and Fraser, 1995); amphibian (Jacobson, 1959; Eagleson and Harris, 1990) and avian (Vaage, 1969; Couly and Le Douarin, 1987; Lumsden and Keynes, 1989) embryos was revealed by analyzing cell lineages. For example, the descendents of single precursor cells were restricted to their segment of origin in the hindbrain neural tube (Lumsden and Keynes, 1989) or diencephalic (early posterior forebrain) neural tube (Fidgor and Stern, 1993). More recently, the results of cell lineage analyses in the forebrain neural plate in mouse are consistent with the notion of transverse segments in the anterior-most CNS (Inoue et al., 2000). Several genes are regionally expressed in restricted patterns from the hindbrain to the forebrain and these patterns seem to agree topologically with the proposed boundaries of neuronal lineages. For example, the genes *Emx1* and *Emx2*, vertebrate homologues of the *Drosophila empty spiracles* segmentation gene, are expressed in the dorsal forebrain in mouse, whereas the genes *Dlx1*, *Dlx2* and *Dlx5*, vertebrate homologues of the *Drosophila distal-less* segmentation gene, are expressed in the ventral forebrain (Simeone et al., 1992; Boncinelli et al., 1993; Liu et al., 1997; Eisenstat et al., 1999). Interestingly, although these genes demonstrate a dorsoventral restriction pattern, their expression does not extend further posterior and terminates with fairly sharp boundaries. A similar restriction in the extent of gene expression can be observed in other domains within the anterior portions of the CNS. For example, *En1* expression in the isthmic region bordering the posterior midbrain and anterior hindbrain (Joyner, 1996) or *Krox20* expression in only rhombomeres three and five (Wilkinson et al., 1989). The expression of the *Hox* genes also form a nested and ordered pattern along the anteroposterior axis in the hindbrain and spinal cord in such a way that unique combinations of different *Hox* genes define a specific segment of the neuraxis (Lumsden and Krumlauf, 1996). Taken together,

the lineage tracing analyses, gene expression patterns and a recent re-evaluation of the morphological boundaries, especially in the anterior portion of the neural tube (Puelles, 1995), these experimental findings strongly support the neuromeric model for the organization of vertebrate neural development.

The neuromeric paradigm in its most modern version can be considered a composite between the contribution of non-neural derived patterning signals along the anteroposterior axis (e.g. notochord and floor plate ventrally; epidermal ectoderm and roof plate dorsally), and the transversely arranged precursor cells representing the primordia of the divisions of the vertebrate neural tube (Rubinstein et al., 1998). One can immediately appreciate that such a Cartesian arrangement can easily generate unique positional information at various levels of the neural tube. It remains to be determined, however, how the neuroepithelial precursor cells acquire the differential competence to respond appropriately to generic proximal signals, which seems inherent to the cells at the time of neural tube morphogenesis. Are the cells predisposed or "fated" to be a specific anterior neural phenotype and subsequently specified to a posterior identity by signals generated from the floor plate and roof plate at different levels of the neuraxis, as Nieuwkoop originally proposed? Furthermore, is regionalization regulated by each cell lineage autonomously (in isolation of other lineages), or does the regionalized maintenance of cell identity critically depend upon the cooperative interactions between groups of cells?

In Chapter IV I test the extent to which the neural stem cell lineage along the dorsoventral and anteroposterior domains of the CNS is specified to a particular regional identity. By assaying for region-specific gene expression with RT-PCR, I show that isolated neural stem cell colonies are intrinsically specified to their region of origin. Furthermore, using a transgenic mouse with a reporter gene construct under the control of ventral-specific forebrain gene expression, I demonstrate that local regional cues can substantially alter the molecular identity of dorsal stem cell colonies when placed in an ectopic ventral environment.

Chapter II

Neural stem cell fate specification

This chapter has been submitted for publication:

Tropepe, V., Hitoshi, S., Sirard, C., Mak, T., Rossant, J., van der Kooy, D. (2000). Direct neural fate specification from embryonic stem cells: a primitive neural stem cell stage acquired through a default mechanism.

Summary

Mouse embryonic stem (ES) cells cultured in low cell density, completely defined media adopt a neural identity. Using a clonal colony-forming assay, we identify a novel primitive neural stem cell stage as a component of neural lineage specification, which is negatively regulated by TGFβ-related signaling. These results are consistent with a default mechanism for neural fate specification. Primitive neural stem cells are formed directly from single ES cells in a LIF- and FGF-dependent manner, express multiple neural precursor markers and give rise to neurons and glia. Moreover, in vivo mouse chimera experiments reveal that these primitive ES-derived neural stem cells have a broad range of neural and non-neural lineage potential. These results support a model whereby definitive neural stem cell formation is preceded by a primitive neural stem cell stage during neural lineage commitment.

Introduction

Neural stem cells have a fundamental role in generating cellular diversity in the developing mammalian nervous system. However, there is very little known about how neural stem cells are initially formed in embryogenesis. Evidence from studies primarily in *Xenopus* suggest that the acquisition of a neural fate in ectoderm cells is actively repressed and that escaping the repressive signal is the predominant mechanism by which cells reveal their default neural identity (Hemmati-Brivanlou and Melton, 1997). However, it is uncertain whether default neural specification occurs in mammalian development, and if so whether the process of default neural fate specification is homologous among vertebrate species.

During mouse gastrulation, cells derived from the embryonic ectoderm are organized into either neural or epidermal primordia. The concept of vertebrate neural

induction, borne out of studies in amphibian embryology, was proposed to account for the segregation of these two vertebrate ectodermal lineages (Spemann and Mangold, 1924; Waddington and Schnidt, 1933; Oppenheimer, 1936; Beddington, 1994). It was postulated that the nascent embryonic ectoderm received a positive inducing signal from the dorsal organizer tissue during gastrulation, which caused the ectodermal cells to adopt a neural fate in a restricted manner. In the absence of this signal, ectodermal cells were presumed to differentiate into epidermis, independent of any cellular communication.

Results from in vitro experiments of isolated ectodermal (animal cap) cells derived from amphibian gastrula supported a different model for neural fate specification. Prolonged low-density dissociation of ectodermal cells, in the absence of organizer tissue, resulted in most of the cells expressing neural markers or forming neural structures upon reaggregation (Godsave and Slack, 1989; Grunz and Tacke, 1989; Sato and Sargent, 1989). Furthermore, ectodermal explants (undissociated cells) expressing a dominant-negative receptor for activin (a member of the TGFβ superfamily of growth factors) were shown to become neural when cultured in vitro (Hemmati-Brivanlou and Melton, 1994). In studies aimed at identifying the nature of the organizer signals, molecules isolated from mesendodermal tissue, such as noggin and chordin, were found to be sufficient for inducing a second neural axis in analogous ectopic experiments performed in *Xenopus* (Smith et al., 1993; Sasai et al., 1995). However, the biochemical mechanism by which organizer signals promoted neural differentiation of ectodermal cells was not entirely consistent with a positive induction model for neural fate determination. Noggin and chordin were shown to act by binding extracellularly to bone morphogenetic proteins (BMPs), members of the TGFβ superfamily of molecules that strongly inhibit neural differentiation (Hemmati-Brivanlou and Melton, 1994). Thus, in a restricted manner, noggin and chordin prevent the binding of BMPs to their cognate receptors expressed on the surface of ectodermal cells (Piccolo et al., 1996; Zimmerman et al., 1996). In fact, BMP4 was shown to act as a positive signal for epidermal fate determination in the

Xenopus ectoderm (Wilson and Hemmati-Brivanlou, 1995). These findings from amphibian experiments were consistent with the notion that the establishment of neural identity from the uncommitted ectoderm occurs by default (i.e. a state achieved autonomously after the removal of the inhibitory signals) in the absence of neural-inducing factors emanating from the organizer.

We sought to determine whether a default-like mechanism underlies neural specification in uncommitted mammalian embryonic stem (ES) cells – the precursors to all embryonic lineages. ES cells are derived from the inner cell mass (ICM) of the pre-implantation mouse embryo (Evans and Kaufman, 1981; Martin, 1981) and can be sustained in an undifferentiated state in vitro while maintaining ICM/epiblast characteristics. We show that in low-density cell culture assays, in the absence of serum-derived or feeder cell-derived factors and in the absence of embryoid body (EB) formation, ES cells directly differentiate into neural cells. The transition from ES cell to neural cell can be enhanced by the inhibition of TGFβ-related signaling, in a manner that is consistent with a default model of neural fate specification, but one which is distinct from *Xenopus* default neuralization. Furthermore, we report the identification of a novel primitive neural stem cell stage in the neural lineage, which defines the transition between ES cell and neural stem cell.

Experimental Procedures

Propagation and maintenance of ES cells

The ES cell line R1 was grown on mitotically inactive fibroblast feeder layers maintained in DMEM + 10% FCS culture medium containing LIF (1000 U/ml) at low passage number (6-11) as previously described (Nagy and Rossant, 1993). For passaging ES cells, cultures were disaggregated with 0.05% trypsin dissolved in Tris-saline/EDTA for 5-10 minutes, mechanically dissociated, centrifuged and resuspended in culture medium.

Culturing ES cells

Passaged ES cells were washed (2 times), centrifuged and resuspended in chemically defined serum-free media (Reynolds et al., 1992) composed of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM; GIBCO) and F-12 nutrient (GIBCO) including 0.6% glucose (Sigma), 2 mM glutamine (GIBCO), 3 mM sodium bicarbonate (Sigma), and 5 mM HEPES buffer (Sigma). A defined hormone and salt mixture (Sigma) that included insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (20 nM), putrescine (60 μM), and selenium chloride (30 nM) was used instead of serum. ES cells were plated at various cell densities in 24-well culture plates (Nunclon) in the presence of either LIF (1000 U/ml), LIF + FGF2 (10 ng/ml; Upstate Biotech or Sigma) and 2 μg/ml heparin (Sigma), or in the absence of any exogenous growth factors. For short term (4-24 hours) neural differentiation, ES cells were plated in identical culture conditions in 24-well culture plates (Nunclon) that were precoated with poly-L-ornithine (15 μg/ml, Gibco).

Limiting dilution analysis was performed as previously described (Bellows and Aubin, 1989; Tropepe et al., 1999). ES cells were plated in 24-well plates containing LIF (1000U/ml). Cell numbers were adjusted to give a starting concentration of 5000 cells/ml from which serial dilutions were made. Final cell dilutions ranged from 1000 cells per well to 1 cell per well in 0.5 ml aliquots. Cultures were left undisturbed for 7 days after which time the fraction of wells not containing sphere colonies for each cell plating density was calculated and those points were plotted against the number of cells plated per well. The number of cells required to form one sphere colony, which reflected the proportion of neural stem cells in the entire population, was then determined from the point at which the regression line crossed the 0.37 level (37%). That is $F_0 = e^{-x}$, where F_0 is the fraction of wells without sphere colonies and x is the mean number of cells per well. Based on a Poisson distribution of cells, $F_0 = 0.37$ corresponds to the dilution at which there is one neural stem cell per well. The linear relationship observed between the cell density and the

number of sphere colonies generated (regression coefficient $R^2 = 0.99$) can be accounted for by the clonal proliferation of a single rare population of cells.

To assess colony formation at clonal densities, ES cells were plated in serum-free media containing LIF (as above) at 5 x 10^4 cells per 94 mm Greiner hybridoma tissue culture dish (Fedoroff et al., 1997). which is subdivided into approximately 700 microwells, 0.04 cm^2 each (Greiner Labortechnik, Bellco Glass, Inc., Vineland, NJ). Using this procedure, microwells contained ~15 viable cells per well (randomly assorted). Cultures were maintained for a 7-day period.

Self-renewal of primary colony-forming ES cells was assessed as previously described (Tropepe et al., 1999). Single sphere colonies were isolated, mechanically dissociated into a single cell suspension in 0.2 ml of serum-free media containing various combinations of LIF (1000 U/ml), FGF2 (10 ng/ml), heparin (2 μg/ml), EGF (20 ng/ml; Upstate Biotech) or B27 supplement (1X) and cultured in 96-well (Nunclon) plates. Secondary sphere colonies were quantified after 7-10 days. A similar procedure was used for repeated passaging experiments. Cell viability after a 7-day culture period (sphere colony assay) or after 4-24 hours (short-term ES differentiation assay) was determined using trypan blue exclusion (1:2 dilution of 0.4% trypan blue; Gibco).

To determine the effect of a targeted null mutation in the *Smad4* gene on neural colony formation, we used clones C8-13 (-/-), C8-24 (-/-), F9-2 (-/-), F9-5 (-/-) and the wildtype E14K (+/+) ES cell lines (Sirard et al., 1998). There were no differences in colony formation between the various (-/-) ES cell clones and thus the analysis included the pooled results from all of the clones. Human recombinant BMP4 protein (stock 0.812 mg/ml) was provided by Genetics Institute Inc. and human recombinant Noggin protein (stock 1.05 mg/ml) was provided by Regeneron Pharmaceuticals Inc.

Embryonic and adult forebrain dissections

Pregnant CD1 mice (Charles River, Quebec) of gestational age 9.5 (E9.5) or E14.5 (see below) were killed via cervical dislocation and embryos were removed as previously described (Tropepe et al., 1999). Dissected germinal zone from the E9.5 telencephalon was transferred to serum-free media and mechanically dissociated into a cell suspension with a fire-polished Pasteur pipette. Cell viability was assessed using trypan blue. Cells were plated at 10 cells/μl in 24-well (0.5 ml/well) uncoated plates (Nunclon) in serum-free media containing either FGF2 (10 ng/ml) + heparin (2 μg/ml) or FGF2 + heparin + LIF (1000U/ml). Self-renewal of neural stem cells that generated primary sphere colonies (selecting mainly floating colonies after 7 days in vitro) were routinely subcloned by mechanically dissociating a single colony in 0.2 ml of serum-free media, in identical growth factor conditions as the primary culture, and plated in uncoated 96-well (0.2 ml/well) plates (Nunclon). The number of new secondary colonies was quantified after a further 6-7 days in vitro. Neural stem cell colonies from the adult forebrain subependyma were isolated as previously described (Chiasson et al., 1999) and cultured as above.

Immunocytochemistry

Single sphere colonies were transferred to a well coated with MATRIGEL basement membrane matrix (15.1 mg/ml stock solution diluted 1:25 in serum-free media; Becton-Dickinson) in individual wells of a 24-well culture plate (Nunclon) (0.5 ml/well). For nestin immunolabeling, sphere colonies were allowed to adhere for 24 hours in serum-free media prior to fixation. Adherent colonies were fixed with 4% paraformaldehyde in PBS (pH 7.2) for 20 min at room temperature and then washed (3x) with PBS (5 min each). Colonies were then permeabilized with 0.3% Triton X-100 for 5 min, washed (2x) with PBS (5 min each) and then incubated for 1 hour in 10% normal goat serum (NGS) at room temperature in order to presaturate non-specific protein binding sites. A rabbit polyclonal antiserum (a gift from Dr. R. McKay) (Tohyama et al., 1992) was diluted to 1:1000 (in

PBS + 10% NGS) and colonies were incubated overnight at 4°C. The next day, sphere colonies were washed (3x) in PBS (5 min each) and subsequently incubated with a secondary goat anti-rabbit FITC-conjugated antibody (1:200; Sigma) for 30 min at 37°C. After rinsing three times (5 minutes each), all cultures were incubated in Hoechst 33258 nuclear stain (0.015 mg/ml stock solution diluted to 0.001 mg/ml; Boehringer Mannheim) for 5 minutes at room temperature in order to facilitate cell quantification. After washing (3x) (5 min each), colonies were coated with Fluor-mount mounting medium. Fluorescence was detected on a Nikon inverted fluorescence microscope. A similar procedure was used for longer-term differentiation of sphere colony cells. After 7 days in culture, colonies that were spread out on the substrate were fixed in 4% paraformaldehyde (in PBS, pH 7.2) for 20 minutes at room temperature followed by 3 (5 minutes each) washes in PBS (pH 7.2). Cells were then permeabilized for 5 minutes in PBS containing 0.3% Triton-X, rinsed for 5 min (2x) in PBS and blocked for 1 hour in PBS containing 10% normal goat serum (NGS). After blocking, cultures were incubated in anti-MAP-2 mouse monoclonal (IgG) (1:1000; Boehringer Mannheim) and anti-GFAP rabbit polyclonal (IgG) (1:400; Chemicon) antibodies diluted in PBS containing 10% NGS over night at 4°C. Cultures were then rinsed in PBS three times (5 minutes each) and subsequently incubated in FITC goat anti-rabbit (1:200; Jackson ImmunoResearch) and TRITC goat anti-mouse (1:200; Jackson ImmunoResearch) secondary antibodies at 37°C for 30 min. Cultures were rinsed three times (5 minutes each) in PBS. Separate cultures (from similar conditions) were used for oligodendrocyte immunolabelling. Cultures were incubated in anti-O4 mouse monoclonal (IgM) antibody (1:40; Boehringer Mannheim) in PBS containing 10% NGS at 4°C overnight. The next day, cultures were rinsed three times (5 minutes each) and subsequently incubated in DTAF goat anti-mouse-IgM (1:200; Jackson ImmunoResearch) secondary antibody in PBS containing 10% NGS at 37°C for 30 minutes. After rinsing three times (5 minutes each), all cultures were incubated in Hoechst 33258 nuclear stain (0.015 mg/ml stock solution diluted to 0.001 mg/ml; Boehringer Mannheim) for 5 minutes

at room temperature in order to facilitate cell quantification. Colonies were washed (3x) in PBS (5 min each) and then coated with Fluormount and fluorescence was visualized using a Nikon inverted-fluorescence microscope. Secondary antibody-only control cultures were processed simultaneously using the identical protocol except dilution solutions were devoid of primary antibodies. All secondary controls were negative for immunolabeling.

For short-term (24 hours) differentiation experiments, ES cells were adhered to a poly-L-ornithine substrate (15 μg/ml; Sigma), fixed in 4% paraformaldehyde (as above) and immunolabeled using primary mouse monoclonal anti-βIII-tubulin antibody (1:1000; Sigma), anti-nestin antibody (as above) and rabbit anti-mouse Oct-4 antibody (1:400; a gift from Dr. J. Cross). Cultures were counter-labeled with Hoechst (as above) and quantifed by counting 3-4 random standardized areas (using an ocular grid) at 20X objective magnification per culture.

To cyrosection ES derived or forebrain derived sphere colonies, colonies were rinsed (2x) by transferring to PBS (pH 7.2) for a few seconds with a Pasteur pipette. Colonies were then transferred to 4% paraformaldehyde containing 0.4% picric acid in 0.16 M phosphate-buffer (pH 6.9) and fixed for 1 hour at room temperature. Sphere colonies were then rinsed (3x) in 10 mM PBS for 5 min each prior to being resuspended in 10% sucrose (in 10 mM PBS) overnight at 4°C. The following day, sphere colonies were placed in tissue freezing media (Tissue Tek) in order to quick freeze to -50°C. Using a cryostat, 14 μm sections were taken and collected on gelatin coated slides. Slides were stored at -70°C and subsequently processed for nestin or nuclear Oct4 immunolabeling (as above).

RT-PCR analysis

Total RNA was isolated using the RNeasy extraction kit (Qiagen) and 1 μg of total RNA was used to synthesize cDNA with oligo-d$(T)_{12-18}$ primers and MuMLV reverse transcriptase (Superscript II; Boehringer-Mannheim) at 42°C for 1 hour. The PCR reaction

mixture (20 µl) consisted of 1 µl cDNA, 16 pmol 5' primer, 16 pmol 3' primer, 0.2 mM dNTP, 2 µl PCR reaction buffer and 0.8 U of Taq polymerase (Promega). cDNA was amplified in a thermal cycler (Perkin-Elmer). For all primer pairs denaturation for 30 sec at 94°C, annealing for 30sec and extension at 72°C was used. The sense and antisense primers, Mg^{2+} concentration, annealing temperature, extension time and number of PCR cycles were used for the following genes. *Emx2*: sense 5'-GTCCCAGCTTTTAAGGCTAGA-3', antisense 5'-CTTTTGCCTTTTGAATTTCGTTC-3', 1.65 mM Mg^{2+}, 56°C, 40 sec, 40 cycles. *HoxB1*: sense 5'-CCGGACCTTCGACTGGATG-3', antisense 5'-GGTCAGAGGCATCTCCAGC-3', 1.35 mM Mg^{2+}, 58°C, 40 sec, 40 cycles. *Otx1*: sense 5'-TCACAGCTGGACGTGCTCGA-3', antisense 5'-GCGGCGGTTCTTGAACCAAA-3', 1.65 mM Mg^{2+}, 58°C, 40 sec, 40 cycles. *Six3*: sense 5'-CGCGACCTGTACCACATCCT-3', antisense 5'-GCCTTGGCTATCATACGTCA-3', 1.35 mM Mg^{2+}, 56°C, 40 sec, 40 cycles. *Brachyury*: sense 5'-AGTATGAACCTCGGATTCAC-3', antisense 5'-CCGGTTGTTACAAGTCTCAG-3', 1.65 mM Mg^{2+}, 56°C, 1 min, 35 cycles. *GATA4*: sense 5'-AGCCTACATGGCCGACGTGG-3', antisense 5'-TCAGCCAGGACCAGGCTGTT-3', 1.35 mM Mg^{2+}, 58°C, 1 min, 35 cycles. *HNF-4*: sense 5'-CCATGGTGTTAAAGGACGTGC-3', antisense 5'-TAGGATTCAGATCCCGAGCC-3', 1.35 mM Mg^{2+}, 56°C, 1 min, 35 cycles. As a control, cDNA amplification of the *GAPDH* gene (glyceraldehyde-3-phosphate dehydrogenase) was simultaneously run in each PCR experiment. Primers for *GAPDH*: sense 5'-ACCACAGTCCATGCCATCAC-3', antisense 5'-TCCACCACCCTGTTGCTGTA-3' and PCR reaction conditions were similar to conditions used for Emx1 amplification (see above). Amplified products were electrophoresed in 2% agarose gel containing ethidium bromide (25 µg/ml) and bands were visualized with UV light (DualLite Transilluminator, Fisher Biotech).

Expression of mouse *Cerberus-like* in Neuro2a cells

Neuro2a (a murine neuroblastoma cell line) cells were seeded at 1 x 10^6 cells per 100 mm petri dish and transiently transfected with 10 μg of plasmid DNA by means of LipofectAMINE (Gibco) according to the manufacturer's instructions. After 6 hours, the culture media was changed to 10 ml of DMEM+10% FBS (Gibco). Twenty-four hours after transfection, culture media was changed to 10 ml of serum-free media. Seventy-two hours after transfection, cell supernatant was collected and centrifuged to remove cellular debris. Supernatant media was aliquoted and stored at -70°C. Addition of 4% (v/v) of supernatant (3 separate experiments) resulted in a similar increase in colony formation compared to the addition of 20% (v/v) supernatant (2 separate experiments), but this effect was considerably variable from one experiment to the next, whereas addition of 20% (v/v) supernatant resulted in a very consistent increase between experiments. Thus data from the 20% (v/v) experiments were used for the analysis. The plasmids pCS-V2 (gift from Dr. R. Moon) (Hoppler et al., 1996) and pCS-*cer-l* (a gift from Dr. E. De Robertis) (Belo et al., 1997) were used.

Generation of chimeras

ES sphere colonies were generated using ES cells harboring a yellow-fluorescent protein (YFP) transgene or cyan-fluorescent protein (CFP) transgene (gifts from Drs K. Hadjatonakis and A. Nagy). Embryonic or adult telencephalon-derived sphere colonies were generated from either green fluorescent protein (GFP) transgenic mice (a gift from Drs. K. Hadjantonakis and A. Nagy) or ROSA mice ubiquitously expressing the LacZ gene product β-galactosidase (β-gal) (Jackson Laboratory) (Friedrich and Soriano, 1991). ES-derived, E9.5, E14.5 or adult telencephalon-derived sphere colonies were aggregated with diploid CD1 morula-stage embryos for 24 hours in vitro as previously described (Nagy and Rossant, 1993). Once integrated, the colony-embryo aggregates were then transferred into pseudo-pregnant CD1 females, harvested at embryonic day E8.5-E9.5 and

either stained for β-gal activity (for ROSA-CD1 chimeras) or visualized for fluorescence (GFP-CD1 chimeras). β-gal activity was detected by rinsing embryos in a 100 mM sodium phosphate buffer (pH 7.3), fixing in 0.2% gluteraldehyde, 2 mM $MgCl_2$, 5 mM EGTA and 100 mM sodium phosphate (pH 7.3) at room temperature for ~15 min. Embryos were then rinsed (3x) in a wash buffer containing 0.02% NP-40, 0.01% deoxycholate, 2 mM $MgCl_2$, and 100 mM sodium phosphate (pH 7.3) for ~10 min each. Embryos were stained in 1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 0.02% NP-40, 0.01% deoxycholate, 2 mM $MgCl_2$, and 100 mM sodium phosphate buffer (pH 7.3) at 37°C overnight.

Results

Single ES cells differentiate into colony forming neural stem cells in the absence of serum, feeder layers or the formation of EB

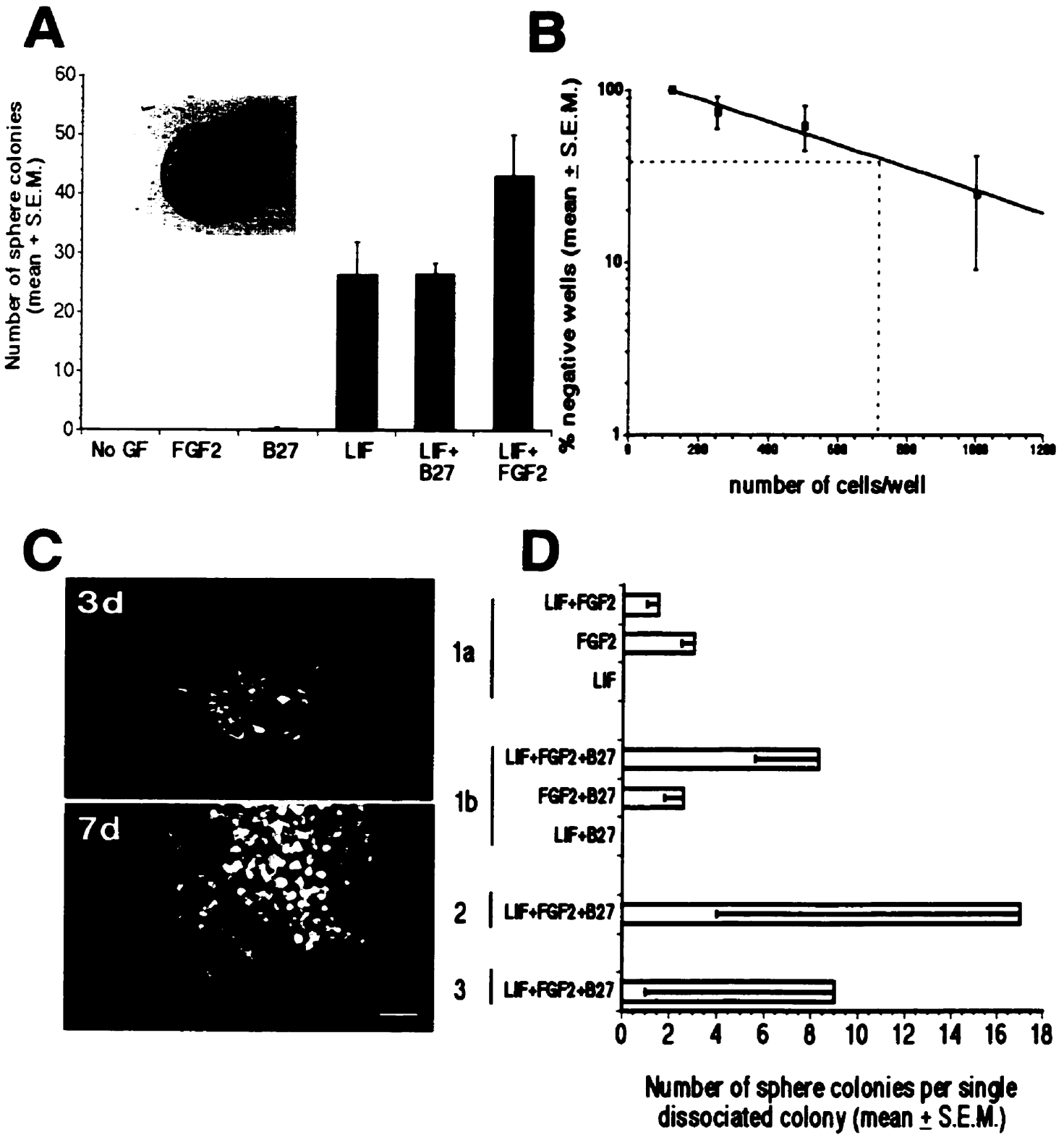
To determine directly the capacity for ES cells to adopt a neural fate in the absence of serum-derived or feeder layer-derived factors and in the absence of cell-cell contact, we cultured ES cells at relatively low cell densities in a chemically-defined, serum-free media. Under similar conditions, single neural stem cells isolated from the embryonic germinal zone of the neural tube can proliferate in response to exogenous EGF or FGF2 to give rise to clonal colonies of undifferentiated neural precursor cells that form floating spheres (Reynolds et al., 1992; Tropepe et al., 1999). The colony-forming neural stem cells have the classical stem cell properties of self-renewal and multipotentiality (Potten and Loeffler, 1990; Morrison et al., 1997). That is, a small percentage of cells isolated from single dissociated colonies can generate new clonal colonies (self-renewal), while the majority of cells within the colonies will differentiate into either neurons, astrocytes or oligodendrocytes (Reynolds and Weiss, 1996).

When ES cells were cultured at relatively low cell densities in the presence of either EGF or FGF2 or in the absence of exogenous growth factors, no cell colonies were

generated (Figure 2.1A). In contrast, in the presence of exogenous leukemia inhibitory factor (LIF), which is normally used to maintain ES cells in an undifferentiated state (Smith et al., 1988; Williams et al., 1988), floating sphere-like colonies were generated after 7 days in vitro. There was no significant difference in the numbers of neural stem cell colonies generated when either EGF or FGF2 were combined with LIF compared to LIF alone, although the presence of FGF2 produced a non-significant trend toward facilitating LIF-dependent colony formation (Figure 2.1A). Thus, exogenous EGF and FGF2 were neither necessary nor sufficient for colony formation in primary cell cultures. Furthermore, CNTF, another member of the cytokine family of signaling molecules to which LIF belongs (Kishimoto et al., 1994), was unable to substitute as a colony-promoting factor (data not shown), suggesting that the effects of LIF are specific.

To determine the frequency of cell colony formation, we cultured ES cells at various cell densities (from 1 cell/well to 20 cells/μl) in 24-well culture dishes in a limiting dilution assay (Bellows and Aubin, 1989; Tropepe et al., 1999). The estimated frequency of sphere colony forming cells in the presence of LIF was ~0.2% (Figure 2.1B). No sphere colonies were observed at cell densities of less than 500 cells per well (0.5 ml of media), suggesting that a threshold number of cells may be required in order to facilitate the clonal proliferation of a single ES cell. To further test this possibility, ES cells were cultured at ~15 cells per microwell randomly distributed in Greiner hybridoma culture dishes subdivided into 700 microwells (0.04 cm^2 each). Even though the majority of microwells contained cells, an average of 35 colonies were generated (2 separate cultures) over the entire dish. Hence, a similar frequency of sphere colony formation was observed over the entire culture dish (i.e. 15x700 = 10,500 cells; an average of 35 colonies ÷ 10,500 = 0.3%). Furthermore, in one additional experiment, single ES cells were cultured in 96-well plates (0.2 ml) and 1 sphere colony was generated in 600-700 wells scored. Thus, the

Figure 2.1. LIF-dependent neural cell colonies are clonally derived from single ES cells. (A) ES cells cultured at 20 cells/μl in chemically defined serum-free media proliferate to form sphere colonies in the presence of LIF (1000 U/ml). Photo inset shows a ES derived sphere colony after 7 days in culture (scale bar 100 μm). The addition of FGF2 (10 ng/ml) and heparin (2 μg/ml) causes a slight, but non-significant increase in the numbers of primary sphere colonies compared to LIF alone ($t = 1.1$, $p>0.05$) or LIF+B27 ($t = 1.2$, $p>0.05$). The presence of FGF2+heparin alone or B27 supplement alone (diluted to one-tenth the stock concentration: 1X) is not sufficient for colony formation. Data represent 6-12 cultures per group from 4-11 separate experiments. (B) Cells plated at limiting dilution in the presence of LIF revealed that the frequency in which at least one neural stem cell will proliferate to form a sphere colony (37% mark on the ordinal scale) was ~0.2% (dashed line). Each data point represents the average of 6 cultures from 2 separate experiments. (C) Sphere colonies are composed of cells with neural precursor identity. After 3 days in vitro (relatively small) or 7 days in vitro (relatively large) individual sphere colonies (n=6 from each of 2 separate experiments) were transferred to a poly-ornithine substrate and allowed to adhere for 24 hours. The expression of the neural precursor marker nestin was determined using immunocytochemistry. Scale bar 100 μm. (D) Neural colony forming ES cells displayed neural stem cell self-renewal characteristics. Single primary colonies generated in the presence of LIF alone (1a) were subcloned in LIF+FGF2, FGF2 or LIF to generate secondary colonies. Single primary colonies generated in the presence of LIF+FGF2+B27 (1b) were subcloned in LIF+FGF2+B27, FGF2+B27 or LIF+B27 to generate secondary colonies. Single secondary colonies generated in LIF+FGF2+B27 (2) were subcloned in LIF+FGF2+B27 to generate tertiary colonies. Single tertiary colonies generated in LIF+FGF2+B27 (3) were subcloned in LIF+FGF2+B27 to generate quaternary colonies. Single sphere colonies from primary culture (n=6-24 isolated colonies per condition from at least 2 separate experiments) were dissociated into a single cell suspension after 7 days in vitro and re-cultured. Secondary colonies derived from single primary colonies were quantified after 7-10 days in vitro and a similar procedure was used to subclone secondary and tertiary sphere colonies. LIF (1000 U/ml), FGF2 (10 ng/ml), heparin (2 μg/ml), B27 supplement (1X).


A
Number of sphere colonies (mean + S.E.M.)
60
50
40
30
20
10
0
No GF
FGF2
B27
LIF
LIF+ B27
LIF+ FGF2
B
% negative wells (mean ± S.E.M.)
100
10
1
number of cells/well
C
3d
7d
D
LIF+FGF2
FGF2
LIF
1a
LIF+FGF2+B27
FGF2+B27
LIF+B27
1b
2
LIF+FGF2+B27
3
LIF+FGF2+B27
0 2 4 6 8 10 12 14 16 18
Number of sphere colonies per single dissociated colony (mean ± S.E.M.)

results demonstrate that a very small percentage of single ES cells generate sphere colonies under these conditions as predicted by the limiting dilution analysis.

Colony-forming ES cells show neural stem cell characteristics

Sphere colonies generated in the presence of LIF grew to a size of approximately 300-500 μm in diameter after 7 days and were composed of cells that all expressed the intermediate filament protein nestin, which is expressed in neural precursor cells in embryonic and adult CNS tissues and transiently in muscle progenitors (Lendahl et al., 1990) (Figure 2.1C). An analysis of smaller sized colonies identifiable at 3 days in culture (composed of 20-30 cells) demonstrated that all of the cells within these colonies (determined by counting Hoechst stained nuclei) appeared to express nestin. Thus, nestin expression is correlated with the initial formation of the sphere colony, coinciding with nestin expression in single ES cells at the onset of the cell culture period prior to sphere colony formation, and no cells within the colonies retained nuclear expression of the ES cell marker Oct-4 (see below). These data suggest that individual ES cells acquire a neural precursor cell identity before they proliferate to generate neural colonies.

Individual colonies were dissociated and subcloned as previously reported (Reynolds et al., 1992; Tropepe et al., 1999) in the presence of exogenous LIF, FGF2 or EGF alone, or in combinations. Regardless of the primary culture conditions the formation of secondary neural stem cell colonies was dependent upon the presence of exogenous FGF2. LIF alone was not sufficient for secondary colony formation (Figure 2.1D). The colony-forming ability in tertiary and quaternary subcloned cell cultures could be sustained with combined FGF2 and LIF. However, substituting for LIF with a B27 media supplement (thought to prevent excessive cell death by inhibiting free radical-induced cellular damage) in the FGF2 cultures was sufficient for repeatedly generating new sphere colonies (Figure 2.1D). Furthermore, the ability to generate sphere colonies in the presence of exogenous EGF alone was not observed. The relatively small expansion of ES sphere

colonies (2-16 new clonal colonies arise from the dissociation of a single ES derived neural colony; Figure 2.1D) is similar to the primary subcloning of FGF-responsive neural stem cells isolated from the E8.5 anterior neural plate (Tropepe et al., 1999). Under our conditions, however, new ES derived colonies maintain their FGF2 and LIF (or B27) dependence upon repeated subcloning, whereas the E8.5 derived neural stem cell colonies require only FGF2. Furthermore, a separate EGF-responsive population of colony forming cells, which occurs during the development of the neural stem cell lineage between E10.5 and E14.5 in vivo (Tropepe et al., 1999; Martens et al., 2000) was not established from the ES-derived colonies.

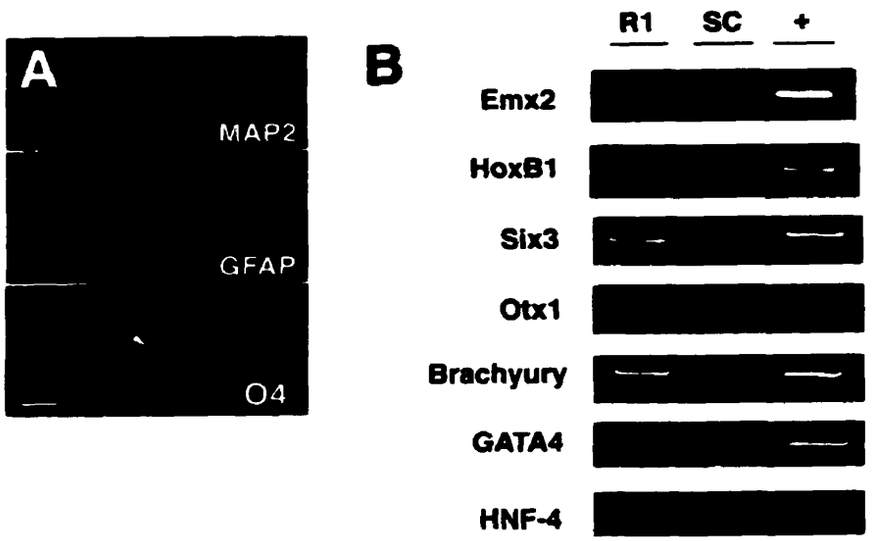
To determine if the individual cells giving rise to the neural colonies had neural multilineage potential, individual colonies were encouraged to fully differentiate (placed on a MATRIGEL substrate and in the presence of 1% FBS) for a period of 7 days. Under these conditions, each of the differentiated colonies contained neurons ($MAP2^+$ or β-III $tubulin^+$), astrocytes ($GFAP^+$) and oligodendrocytes ($O4^+$) using these conventional cellular markers of differentiation (Figure 2.2A). The neural cells identified in these differentiated cultures (including undifferentiated, $nestin^+$ cells) accounted for all of the cell types present in the colonies. At least one non-neural marker, the muscle determination gene product MyoD, was not detectable by immunocytochemistry in these colonies, even though $MyoD^+$ cells were identified in control explant cultures of E9.5 somitic mesoderm (data not shown).

To further examine the lineage commitment of the ES-derived sphere colonies, we analyzed the expression of genes restricted to neural and non-neural lineages using RT-PCR analysis (Figure 2.2B). Sphere colonies did not express the early mesoderm-specific transcription factor brachyury (Beddington et al., 1992), which is abundant in EB (Elefanty et al., 1997). Sphere colonies expressed the early endodermal marker GATA4, a zinc finger transcription factor that binds to a core GATA motif in the *cis* regulatory elements of many genes (Arceci et al., 1993). However, the gene HNF-4, which is a later endodermal

marker (Taraviras et al., 1994; Li et al., 2000), was not expressed in ES-derived neural colonies, suggesting only partial endodermal potential within the colonies, unlike full endodermal potential documented for EB differentiation. Consistent with this observation, the absence of Otx1, expressed during the formation of the anterior visceral endoderm and later in the forebrain (Acampora et al., 1998), suggest that sphere colonies do not engage in full visceral endoderm differentiation.

Specific neural mRNAs were expressed in isolated ES-derived sphere colonies. The dorsal telencephalon-specific homeodomain transcription factor Emx2 (Simeone et al., 1992), and the hindbrain and spinal cord specific transcription factor HoxB1 (Wilkinson et al., 1989) were expressed in the ES-derived neural sphere colonies (Figure 2.2B). However, the anterior neural gene Six3 (Oliver et al., 1995), like Otx1, was not expressed. As a control, neural colonies derived from E14.5 forebrain germinal zone were assayed for the expression of lineage-specific genes. Although neural specific gene expression was confirmed in these samples (Figure 2.2B) expression of the non-neural genes brachyury, GATA4 and HNF-4 was not observed (data not shown). In addition, ES cells freshly trypsinized from their feeder-layers were also used as controls. With the exception of GATA4, Otx1 and HNF4, the unmanipulated ES cells express all of the genes tested, and indeed are known to non-specifically express a variety of genes (Elefanty et al., 1997). Interestingly, neural-specific gene expression persisted in the sphere colonies, whereas the mesodermal marker Brachury was downregulated in the transition from ES cells to neural colonies. Thus, sphere colonies generated through the proliferation of a single neural cell are specified to primarily a neural identity and are composed of both neuronal and glial lineages. The fact that some non-neural genes (e.g. GATA4) are expressed in sphere colonies may suggest that these specified neural stem cell derived colonies are not completely committed to a neural fate, but may retain pluripotent or more primitive characteristics (see below) than the neural stem cells isolated from the embryonic and adult CNS. The absence of Otx1, which is expressed in the anterior neural tube and anterior

Figure 2.2. Cells from ES-derived sphere colonies express neural-specific genes and can differentiate into neurons and glia. (A) ES sphere colonies were cultured under differentiation conditions and the presence of neurons ($MAP2^+$), astrocytes ($GFAP^+$) and oligodendrocytes ($O4^+$, arrowhead) was determined using immunocytochemistry. Data are representative of 18 cultures from 2-3 separate experiments. (B) Gene expression analysis was also determined using RT-PCR. RNA was isolated from sphere colonies after 7 days in vitro and analyzed for the expression of markers for neural differentiation [Emx2 (151 bp), HoxB1 (325 bp), Six3 (571 bp), and Otx1 (128 bp)], endoderm differentiation [GATA4 (809 bp), HNF4 (629 bp)] and mesoderm differentiation [Brachyury (857 bp)]. To normalize for the amount of cDNA present in the sample, the cDNA for GADPH (452 bp) was amplified. R1 refers to primary ES cells; SC refers to ES-derived sphere colony; + refers to positive tissue control (forebrain, hindbrain, somitic mesoderm, liver). Data are representative of at least 3 separate experiments. Scale bar: 20 μm.


A
MAP2
GFAP
O4
B
R1
SC
+
Emx2
HoxB1
Six3
Otx1
Brachyury
GATA4
HNF-4

visceral endoderm, further may indicate that early anterior-posterior polarity is not intrinsic to sphere colonies.

LIF functions as a permissive factor for neural stem cell differentiation of ES cells

The ability of LIF to specifically promote neural colony formation in serum-free media (in the absence of exogenous growth factors) may indicate that LIF induces uncommitted ES cells to a neural fate in primary cultures. However, there are numerous examples in the literature where the presence of LIF was necessary to maintain ES cells in an undifferentiated state (reviewed in O'Shea, 1999), while LIF withdrawal was coincident with differentiation (e.g. Doetschman et al., 1985). Two observations in the present study suggest that LIF may act in a permissive manner to enable ES cells to adopt a neural stem cell fate.

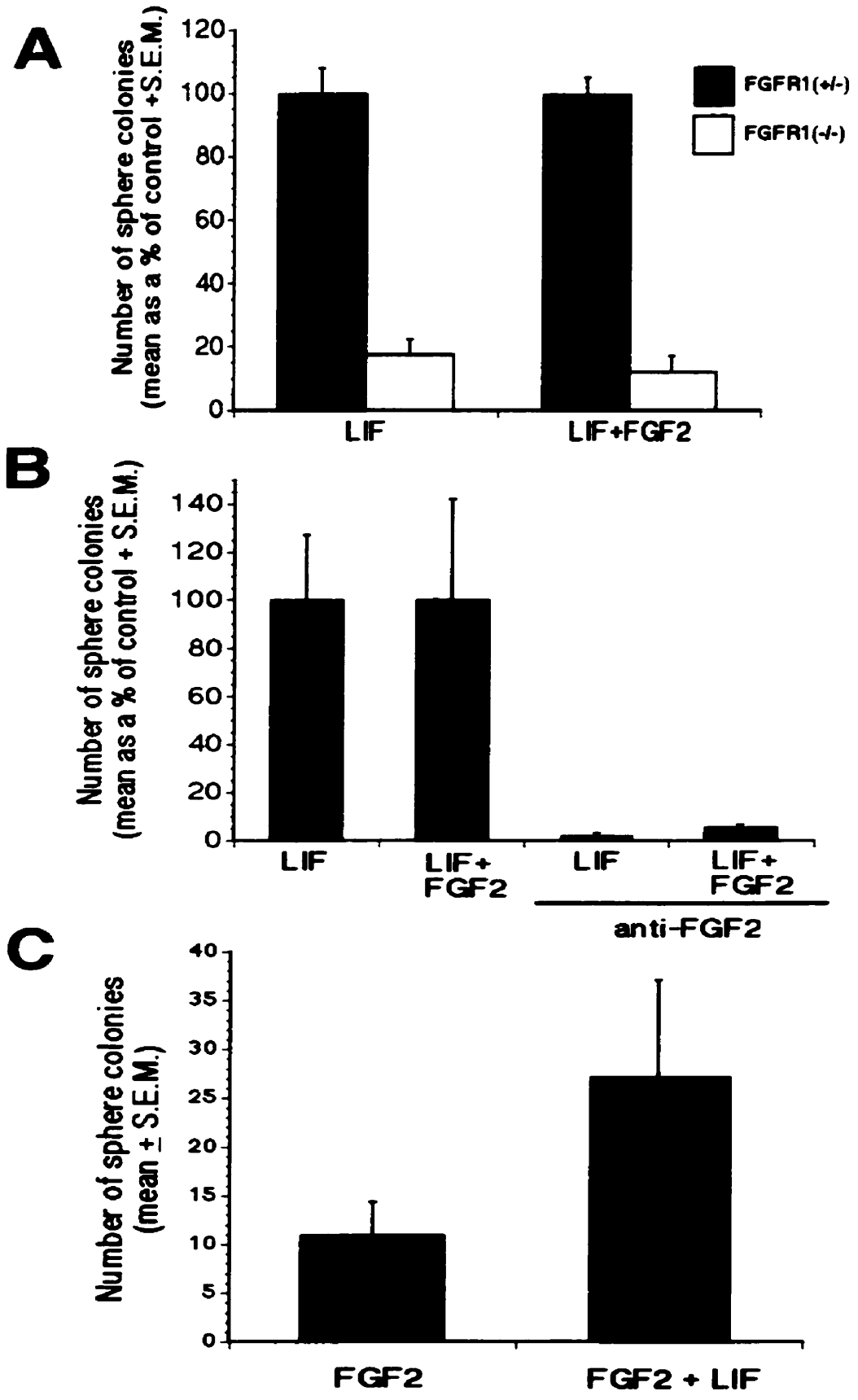
First, since neural stem cells isolated from the E8.5 neural plate are dependent upon FGF (Tropepe et al., 1999), we tested whether endogenous FGF signaling mediates neural colony formation in primary ES cell cultures in the presence of LIF. We utilized a FGF-receptor-1-deficient (*FGFR1*$^{(-/-)}$) ES cell line (compared to a *FGFR1*$^{(+/-)}$ control cell line; Ciruna et al., 1997) and assayed for neural colony formation. In the absence of functional FGFR1 signaling, the ability of ES cells to adopt a neural stem cell fate and generate colonies after 7 days in vitro was diminished by 82% in the presence of LIF (Figure 2.3A), suggesting that ES cells may be responding to endogenous FGF that is released by the ES cells. Consistent with this notion, the addition of an anti-FGF2 antibody to a primary ES cell culture in the presence of LIF caused a >95% decrease in the number of neural colonies observed after 7 days (Figure 2.3B). These results demonstrate that although the addition of exogenous FGF2 is not necessary for neural colony formation in the presence of LIF, endogenous FGF signaling is required.

Second, exogenous LIF can enhance the numbers of FGF-responsive neural stem cells from the E9.5 forebrain that proliferate to form sphere colonies in the presence of FGF2, compared to cultures with FGF2 alone (Figure 2.3C), but LIF alone is not sufficient for E9.5 neural stem cell proliferation. Furthermore, LIF (as well as B27) can promote the repeated subcloning of ES-sphere colonies that are FGF-dependent. These results indicate that although LIF is critical for the early transition of ES cells into colony-forming neural stem cells, it may act primarily as a permissive factor to maintain cell survival in these minimal conditions. In contrast, FGF signaling is critical at all stages of neural stem cell colony formation, but it is unclear if it is involved in the induction of the neural differentiation of ES cells or simply in promoting proliferation in our colony-forming assay.

Inhibition of TGFβ-related signaling enhances neural stem cell differentiation of ES cells

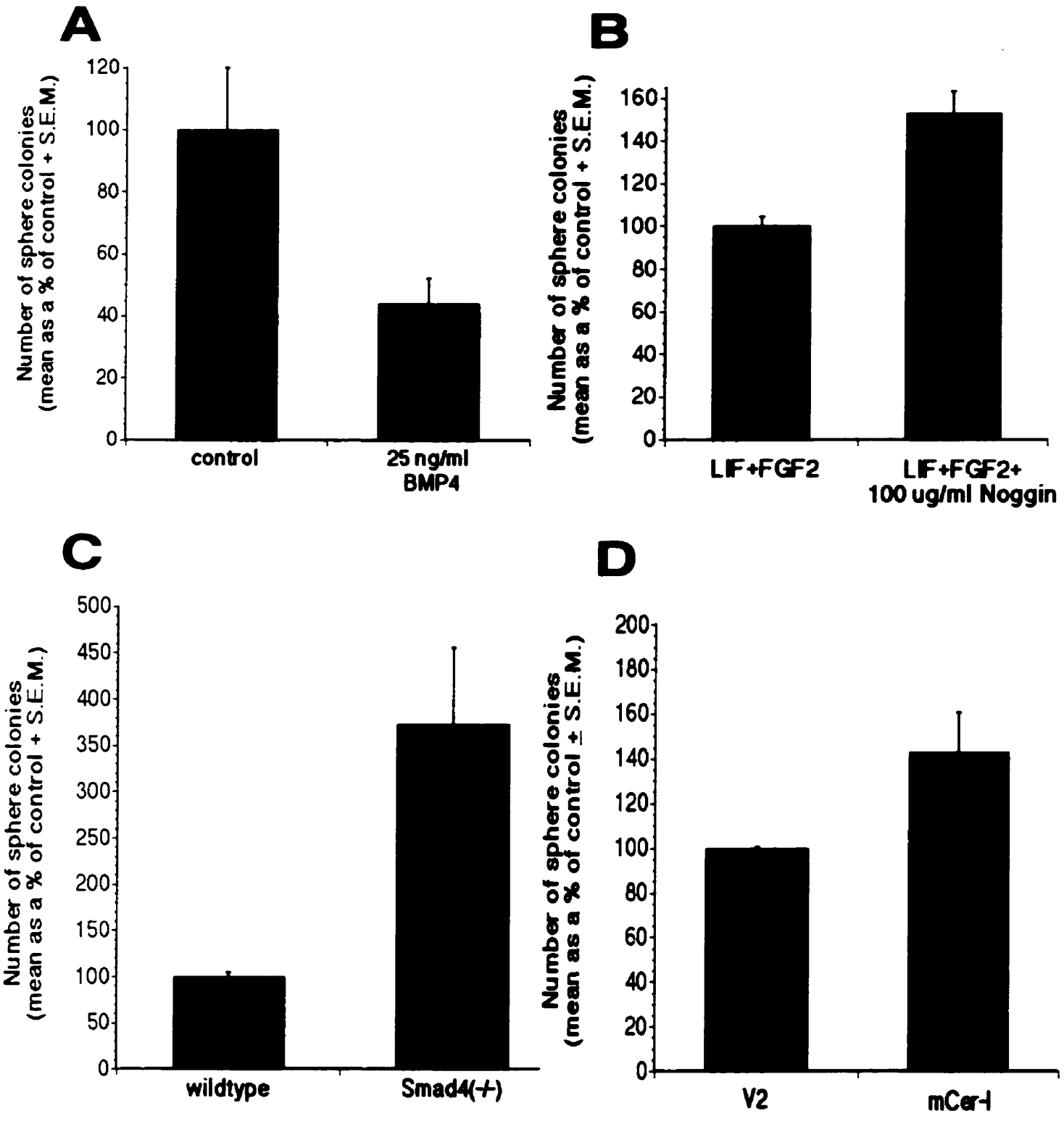
Given that very few of the cultured ES cells generated sphere colonies (~0.2%), we sought to determine if the release of endogenous BMP from the ES cells inhibited neural sphere colony formation, as would be predicted from the neural default model. To test whether BMP could inhibit ES sphere colony formation, we added BMP4 (5 ng/ml) to ES cell cultures containing LIF and FGF2. We observed a greater than 50% decrease in the number of sphere colonies generated and this effect appeared to be maximal since a 5-fold increase in BMP4 concentration did not further significantly attenuate the number of sphere colonies generated (Figure 2.4A). The addition of the BMP protein antagonist Noggin (100 μg/ml) to the primary ES cell cultures caused a 50% increase in the number of sphere colonies generated (Figure 2.4B). This increase appeared to be maximal since an increase in Noggin concentration from 10 μg/ml to 100 μg/ml resulted in no additional increase in the numbers of sphere colonies generated.

Figure 2.3. Endogenous FGF-signaling mediates LIF-dependent primary neural colony formation. (A) Signaling through FGF-receptor-1 is required for neural colony formation. ES cells with a homozygous null mutation in the gene encoding FGF-receptor-1 (*fgfr1*$_{\Delta}^{tmk}$/*fgfr1*$_{\Delta}^{tmk}$) or control heterozygous ES cells (*fgfr1*$_{\Delta}^{tmk}$/+) were cultured at 20 cells/μl in LIF (1000 U/ml) alone or LIF+FGF2 (10 ng/ml)+heparin (2 μg/ml) and sphere colonies (n=12 cultures per group) were quantified after 7 days in vitro ($t = 8.5$, $p<0.05$ comparing mutant and wildtype cells in LIF alone; $t = 8.9$, $p<0.05$ comparing mutant and wildtype cells in LIF+FGF2). (B) Anti-FGF2 antibodies block neural colony formation. ES cells were cultured at 20 cells/μl in the presence of LIF (1000 U/ml) or LIF+FGF2 (10 ng/ml)+heparin (2 μg/ml) alone or in the presence of 1.25 μg/ml mouse monoclonal IgG anti-FGF2 antibodies. Data represent the average of 6 cultures per group from 2-3 separate experiments ($t = 3.66$, $p<0.05$ comparing LIF alone in presence or absence of antibody; $t = 2.21$, $p<0.05$ comparing LIF+FGF2 in presence or absence of antibody). (C) LIF facilitates colony formation in FGF2 from neural stem cells isolated from the E9.5 forebrain vesicles compared to FGF2 alone ($t = 2.9$, $p<0.05$). Germinal zone tissue was cultured at 10 cells/μl in either FGF2 (10 ng/ml)+heparin (2 μg/ml) or in the presence of FGF2+heparin and LIF (1000 U/ml) and colonies were quantified after 7 days in vitro. Data represent the average of 6-9 embryos per group.


A
Number of sphere colonies
(mean as a % of control +S.E.M.)
120
100
80
60
40
20
0
FGFR1(+/-)
FGFR1(-/-)
LIF
LIF+FGF2
B
Number of sphere colonies
(mean as a % of control + S.E.M.)
140
120
100
80
60
40
20
0
LIF
LIF+
FGF2
LIF
LIF+
FGF2
anti-FGF2
C
Number of sphere colonies
(mean ± S.E.M.)
40
35
30
25
20
15
10
5
0
FGF2
FGF2 + LIF

It is evident that although Noggin can enhance the numbers of ES cells that differentiate into neural colony-forming stem cells, the effect is moderate. It is possible that Noggin may eventually lose its activity and degrade with our extended culture periods. Alternatively, Noggin is known to be less effective than Chordin in neural induction assays in *Xenopus* (Lamb et al., 1993) and targeted null mutations in both Noggin and Chordin are required to demonstrate anterior neural development deficits in mice in vivo (Bachiller et al., 2000). Thus, the moderate increase in the numbers of ES cells that will differentiate into neural sphere colony-forming stem cells in the presence of exogenous Noggin may underestimate the role for BMP-mediated inhibition of neural stem cell colony formation. Certainly, BMP4 and BMP-receptor-1 are expressed by undifferentiated ES cells (Elefanty et al., 1997). To determine more directly the effect of blocking BMP signaling, we utilized an ES cell line with a targeted null mutation in the *Smad4* gene (Sirard et al., 1998), an intracellular transducer of TGFβ-related signaling (Wrana, 2000). Since Smad4 is a critical common component for multiple TGFβ-related signaling pathways, we reasoned that a null mutation in the *Smad4* gene would abrogate most of the BMP signaling that could potentially inhibit neural sphere colony formation. *Smad4*$^{(-/-)}$ ES cells cultured in the presence of LIF generated a 4-5 fold increase in the numbers of neural sphere colonies, compared to the wildtype E14K cell line used to generate the targeted mutation (Figure 2.4C). The baseline numbers of sphere colonies generated by wildtype R1 ES cells (26.3 ± 5.4) and wildtype E14K ES cells (25.7 ± 6.7) cultured at 20 cells/μl were not significantly different ($t = 0.08$, $p>0.05$). Interestingly, the rate of proliferation between wildtype and *Smad4*$^{(-/-)}$ cells in high or low serum concentration is similar, indicating that the increase in the number of colonies from mutant ES cells is likely not a result of a general increase in proliferation. The increase in neural colonies in *Smad4*$^{(-/-)}$ was greater than the augmented numbers of sphere colonies observed in the presence of exogenous Noggin, possibly

Figure 2.4. TGFβ signaling can modulate neural stem cell differentiation from ES cells. (A) BMP4 inhibits neural colony formation compared to controls ($t = 4.45$, $p<0.05$). ES cells were cultured at 20 cells/μl in the presence of LIF (1000 U/ml)+FGF2 (10 ng/ml)+heparin (2μg/ml) alone or in the presence of BMP4. Sphere colonies were quantified after 7 days in vitro. Data represent the average of 6 cultures per group from 2 separate experiments. (B) Noggin enhances neural colony formation compared to controls ($t = 4.78$, $p<0.05$). ES cells were cultured at 20 cells/μl in the presence of LIF (1000 U/ml)+FGF2 (10 ng/ml)+heparin (2 μg/ml) alone or in the presence of Noggin (100 μg/ml). Sphere colonies were quantified after 7 days in vitro. Data represent the average of 6 cultures per group from 2 separate experiments. (C) A null mutation in the *Smad4* gene enhances neural colony formation compared to wildtype controls ($t = 2.67$, $p<0.05$). $Smad4^{(-/-)}$ and wildtype E14K ES cells were cultured at 20 cells/μl in the presence of LIF (1000 U/ml) and sphere colonies were quantified after 7 days in vitro. Data represent 6-12 cultures per group from 3-5 separate experiments. (D) Mouse Cerberus-like (mCer-l), enhances neural colony formation compared to controls ($t = 2.4$, $p<0.05$). ES cells were cultured at 20 cells/μl in the presence of LIF (1000 U/ml) and B27 (1X) alone or in the presence of 20% (v/v) in 0.5 ml culture wells of media supernatant from Neuro2a cell lines transiently expressing a mCer-l transgene. Sphere colonies were quantified after 7 days in vitro. Data represent an average of 6 cultures per group from 2 separate experiments.


A
Number of sphere colonies (mean as a % of control + S.E.M.)
120
100
80
60
40
20
0
control
25 ng/ml BMP4
B
Number of sphere colonies (mean as a % of control + S.E.M.)
160
140
120
100
80
60
40
20
0
LIF+FGF2
LIF+FGF2+ 100 ug/ml Noggin
C
Number of sphere colonies (mean as a % of control + S.E.M.)
500
450
400
350
300
250
200
150
100
50
0
wildtype
Smad4(-/-)
D
Number of sphere colonies (mean as a % of control ± S.E.M.)
200
180
160
140
120
100
80
60
40
20
0
V2
mCer-I

because *Smad4* inactivation is more effective in inhibiting BMP signaling. Taken together, these results indicate that BMP4 signaling has a specific effect in limiting the numbers of single ES cells that differentiate into colony forming neural stem cells and that inhibition of this pathway is sufficient to enhance neural stem cell colony formation. Importantly, the *Smad4*$^{(-/-)}$ primary neural stem cell derived colonies did not passage at a greater rate compared to control primary neural stem cell colonies (data not shown), suggesting that the effect of the mutation is on the transition from ES cell to neural stem cell and not on the later symmetrical division of the neural stem cells.

The secreted factor Cerberus is a potent neural inducer in *Xenopus* (Bouwmeester et al., 1996), [as is the mouse homologue Cerberus-like (Belo et al., 1997)] and acts by antagonizing BMP signaling (Pearce et al., 1999; Piccolo et al., 1999). To determine whether Cerberus can interfere with neural stem cell commitment in mammalian cells, we cultured primary ES cells in the presence of LIF in media containing supernatant collected from transiently transfected Neuro2a cell lines producing mouse Cerberus-like (mCer-l) protein. The presence of 20% (v/v) of mCer-l supernatant in 0.5 ml serum-free media + LIF resulted in close to a 50% increase in the numbers of primary neural stem cell colonies generated, compared to control ES cell cultures containing equivalent proportions of supernatant from cell lines similarly transfected with the backbone vector without the mCer-l gene (Figure 2.4D). A similar increase in sphere colony formation was also observed when using supernatant collected from a transiently transfected COS7 cell line (data not shown). Again consistent with the default model, mCer-l-mediated inhibition of BMP signaling can enhance the frequency with which single ES cells differentiate into colony forming neural stem cells. Wnt proteins are known to inhibit neural differentiation (Harland and Gerhart, 1997) and Cerberus can antagonize Wnt signaling (Piccolo et al., 1999). However, the effect of exogenous mCer-l on neural colony formation was not greater than exogenous Noggin, and substantially less than the effect of a *Smad4* mutation, suggesting

that under these conditions additional Wnt antagonism may not be required for ES-derived neural colony formation.

Neural cell fate is rapidly established from ES cells in the absence of exogenous factors

In addition to TGFβ-related inhibition, the low frequency of ES cells differentiating into neural cells may be a result of extensive cell death in long-term culture assays. Thus, an analysis of neural cell differentiation from ES cells at an earlier time period in culture would facilitate a more accurate estimate of the number of ES cells that differentiate into neural cells.

We predicted that if ES cells were acquiring a neural identity by default, they would express neural markers at very early stages during the culture period. To test this, ES cells (seeded at 10 cells/μl) were allowed to adhere to a poly-ornithine substrate and the proportion of ES cells that differentiated into neural cells after 24 hours in the absence of serum and LIF was determined. After 24 hours in culture, 69.9 ± 4.6% of ES cells were non-viable in the absence of growth factors (estimated using trypan blue exclusion, n=4 separate culture wells). However, of the remaining 30% of viable cells, 82% were immunoreactive for the neuroepithelial marker nestin in the absence of growth factors (Figure 2.5A). The majority of the nestin$^+$ cells had a relatively large, flattened and irregular morphology with prominent filamentous immunolabeling within the cytoplasm. A smaller subpopulation (51%) of the nestin$^+$ cells were also immunolabeled for the immature neuronal marker βIII-tubulin, many of which had a relatively small soma with very little perinucler cytoplasm, and evidence for thin cytoplasmic processes resembling leading and trailing processes of a bipolar neuronal morphology (Figure 2.5B). The addition of LIF and FGF2 to these culture conditions did not significantly alter the percentage of ES cells that differentiated into neural cells. These data indicate that within 24 hours, ES cells may

be competent to directly differentiate into neural cells at low cell densities and serum-free conditions in the absence of exogenous growth factors.

A second prediction that can be made from the default model of neural fate specification is that an increase in cell density will facilitate inhibitory intercellular communication (cells in close proximity) and attenuate the numbers of ES cells differentiating into neural cells. To test this, we cultured ES cells in identical conditions for 24 hours, but increased the cell density by 5-fold (to 50 cells/μl). At this relatively higher cell density, the proportion of nestin$^+$ cells was reduced from 82% to 40% ($t = 2.98$, $p<0.05$) in absence of growth factors and from 70% to 51% ($t = 2.79$, $p<0.05$) in LIF+FGF2. The proportion of βIII-tubulin$^+$ cells was reduced from 51% to 13% ($t = 4.07$, $p<0.05$) in absence of growth factors and from 53% to 7% ($t = 5.63$, $p<0.05$) in LIF+FGF2 (Figure 2.5A, B).

To exclude the possibility that a subpopulation of ES cells at the start of the 24-hour culture period were already committed to a neural fate, we tested whether ES cells just prior to culturing expressed the ICM/ES cell nuclear marker Oct-4, a POU transcription factor (Nichols et al., 1998). After ES cells were trypsinized from their feeder layers and washed in serum-free media, the cell suspension was fixed in 4% paraformaldehyde and allowed to adhere to a poly-ornithine substrate before immunolabeling with an anti-Oct-4 anbtibody. Using this method, all of the ES cells retained their rounded morphology and were immunoreactive for Oct-4 (localized to the nucleus), but none expressed nestin. Next, we tested whether the remaining non-nestin immunoreactive population after 24 hours in our low-density cultures retained their ES cell identity. All of the non-nestin immunoreactive cells (17%) expressed nuclear Oct-4 at cell densities of 10 cells/μl (Figure 5C). The Oct-4$^+$ cells had a rounded morphology with a thin rim of perinuclear cytoplasm that was distinct from the morphology of nestin$^+$ cells. Furthermore, we observed a trend toward an increase in nuclear Oct-4-immunoreactivity (up to 26%) when ES cells were cultured at a 5-fold higher cell density (Figure 2.5C), which was inversely proportional to the relative decrease

Figure 2.5. Neural cell fate inhibition is attenuated in relatively low cell density cultures. ES cells were cultured on a poly-ornithine substrate for 24 hours at 50 cells/μl or 10 cells/μl in the absence of exogenous growth factors or, where indicated, in the presence of LIF (1000 U/ml)+FGF2 (10 ng/ml)+heparin (2 μg/ml). Subsequently, the expression of nestin (A), βIII-tubulin (B) and Oct-4 (C) were assessed using immunocytochemistry. Cultures were counter-labeled with Hoechst nuclear dye to facilitate cell quantitation. In a similar manner, βIII-tubulin expression was assessed in *Smad4*$^{(-/-)}$ ES cells, compared to the E14K wildtype controls (D). Data represent the average proportion of phenotype-specific cells (positively immunolabeled) per total numbers of cells (Hoechst-labeled) obtained from 4-6 random standardized areas (using an ocular grid) at 20X objective magnification from 3-6 separate cultures. Arrows point to neuronal processes. Scale bar: 20 μm.

in nestin and βIII-tubulin expression at the same high cell densities. Thus, increased cell density inhibits neural cell differentiation and may facilitate the maintenance of ES cells in an undifferentiated state.

Neuronal differentiation is enhanced in *Smad4*$^{(-/-)}$ ES cells

To determine whether TGFβ signaling influences the extent to which ES cells adopt a neuronal phenotype in the short term differentiation assay, as it did the acquisition of the neural stem cell phenotype (see above), we cultured *Smad4*$^{(-/-)}$ ES cells at relatively high cell densities (50 cells/μl) for 24 hours and double-immunolabeled for nestin and βIII-tubulin. Under these conditions, neuronal differentiation from wildtype ES cells is relatively low. The number of nestin^{+} cells that differentiated from *Smad4*$^{(-/-)}$ ES cells after a 24 hour culture period increased slightly, but not significantly to 71% compared to 58% in the E14K wildtype control ES cells (Figure 2.5D). However, a more substantial increase in β III-tubulin^{+} neurons (26%) was observed from the *Smad4*$^{(-/-)}$ ES cells, compared to the E14K control ES cells (10%; $t = 2.62$, $p<0.05$), and a greater number of the *Smad4*$^{(-/-)}$ ES cells demonstrated a more elaborate neuritic morphology. Thus, at a relatively high cell density, inhibition of the BMP signaling pathway resembles increased cell dilution in its effectiveness in facilitating neural cell differentiation from ES cells.

ES-derived neural stem cell colonies contribute extensively to all embryonic tissues in chimeric mice

Neural stem cells derived from the embryonic and adult central nervous system demonstrate neural multilineage potential (Weiss et al., 1996). Similarly, neural stem cells derived from ES cells generate progeny that are specified to a neural fate and differentiate into neurons and glia. To determine if neural stem cell colonies have a broader potential to generate non-neural lineages, we performed mouse chimeric analyses. ES cells introduced into a blastocyst or aggregated with a morula predominantly contribute to the epiblast of the

developing embryo, whereas extraembryonic tissues are primarily of host origin (Beddington and Robertson, 1989). As mentioned previously, cells within ES-derived neural colonies (adhered for 24 hours) express the undifferentiated neural marker nestin throughout all stages of colony formation. We further determined that within 14 μm cryosections of whole ES sphere colonies after 7 days in culture all of the cells appeared to express nestin, which is similar to nestin expression in sectioned forebrain derived neural stem cell colonies. However, we did not observe any nuclear Oct4-expressing cells in ES sphere colony sections or forebrain colony sections (data not shown), suggesting that no cells within ES derived neural colonies maintained an undifferentiated ES cell phenotype.

We used blastocyst-stage or morula-stage embryos as hosts and neural stem cell colonies derived either from: (a) embryonic or adult forebrain tissue from mice harboring a ubiquitously expressed *LacZ* transgene (ROSA) (Friedrich and Soriano, 1991) or a ubiquitously expressed green fluorescent protein transgene (GFP) (Hadjantonakis et al., 1998); or (b) ES cells harboring a yellow or cyan fluorescent protein transgene (YFP, CFP) (gifts from Drs. Hadjantonakis and Nagy). Approximately 92% (22/24) of the single YFP or CFP ES-derived colonies aggregated with morulas after 24 hours in vitro contributing to the ICM in normally developed blastocysts (Figure 2.6A, inset) and had substantial contribution to all embryonic tissues in embryos recovered from pseudopregnant females at E9.5 (Figure 2.6A). However, blastocyst injections of cells derived from E14.5 or adult ROSA neural colonies did not integrate into the ICM of the host embryos after 24 hours and in many cases tended to adhere to the host mural trophectoderm. Embryos recovered between E7.5 and E8.5 from these chimeras did not contain any *LacZ*$^+$ cells (0/19). Furthermore, E14.5 ROSA or E9.5 GFP neural stem cell colonies were unable to aggregate with morulas over a 24-hour period. Neural stem cell colonies were apparently unable to adhere to the host embryonic cells in the morula aggregates. Consequently, host morulae developed normally over the 24-hour culture period into healthy blastocysts while the sphere colonies remained outside of the embryo

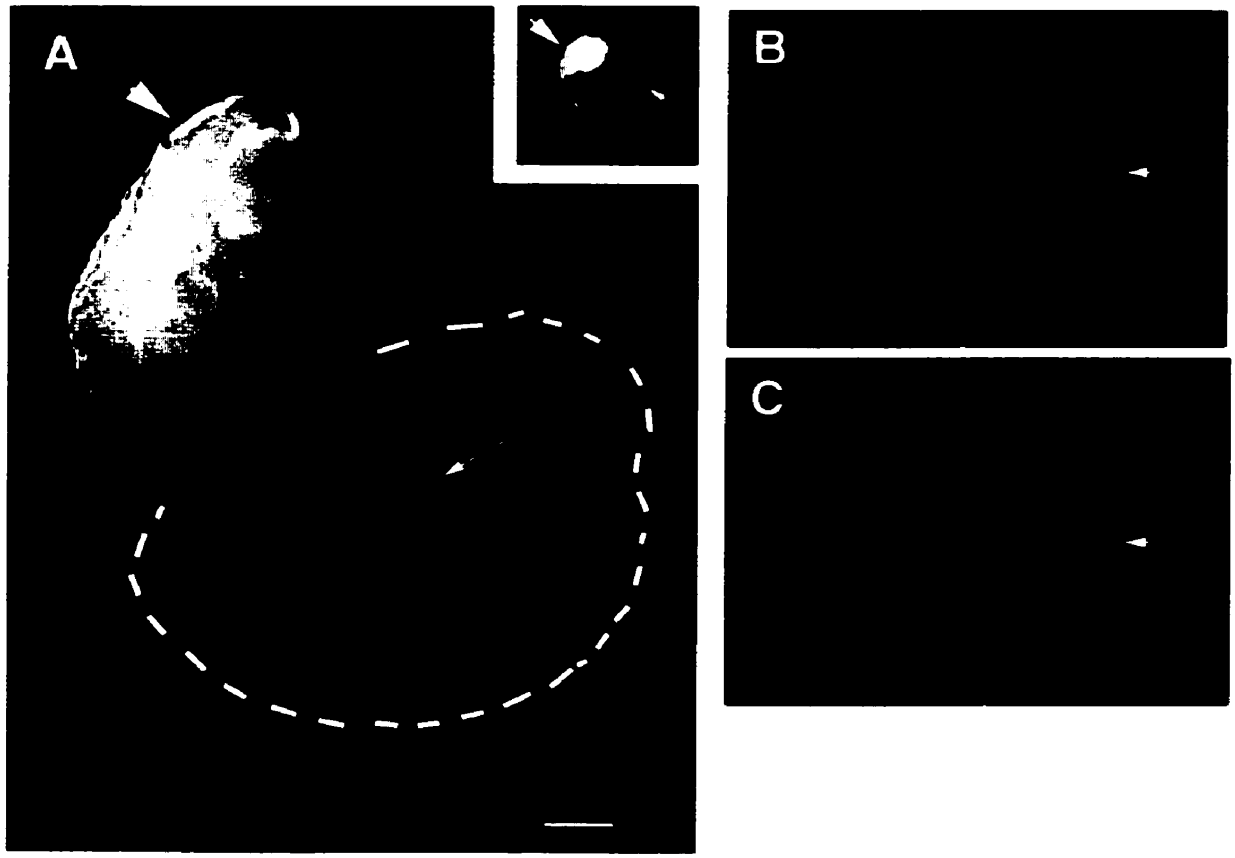
(Figure 2.6B, C). To test whether ES-derived sphere colonies (that readily adhere to morula cells) could facilitate the integration of colonies derived from the E9.5 forebrain, we cultured CFP ES colonies with GFP E9.5 colonies together with the host morula. In all cases, no E9.5 GFP colonies were observed to integrate (0/18), even though in many cases the CFP ES colonies did. These data suggest that ES derived neural stem cell colonies are competent to colonize many different tissues when exposed to an appropriate environment. However, this ability is only transient since neural stem cell colonies isolated from embryos in the earliest stages of neural development do not appear to have the same capacity (e.g. adhere to morula cells or integrate into ICM) to contribute to chimeric mice. Thus, the pluripotency of neural stem cells may only be evident in the earliest stages of the ES to neural transition, before the neural cells become more restricted.

Discussion

Neural cell fate specification during mammalian development

In the present study, we demonstrate that mouse ES cells adopt either a primitive neural stem cell or neuronal fate in the absence of exogenous serum- or feeder layer-derived signals and in the absence of cell-to-cell contact in a low cell density, chemically-defined culture environment. Furthermore, blocking TGFβ-related signaling can augment the proportion of either neural stem cell colony formation or neuronal differentiation, consistent with similar neuronal differentiation evidence in a variety of vertebrate species (Sasai et al., 1995; Wilson et al., 1997; Fainsod et al., 1997; Hoodless and Hemmati-Brivanlou, 1997; Grinblat et al., 1998). Thus, our observations reveal a striking similarity between mammals and other vertebrates in the propensity of uncommitted precursor cells to directly adopt a neural fate and support the default model of neural specification (Wilson and Hemmati-Brivanlou, 1997). Furthermore, we demonstrate that even at relatively low cell densities, ES cells secrete TGFβ-related neural inhibitors (e.g. BMP4) to limit the proportion of cells

Figure 2.6. ES-derived neural sphere colonies contribute extensively in mouse chimeras. (A) Chimeric E9.5 embryo (illuminated with UV light) generated with a YFP ES sphere colony and a CD1 host morula. YFP-expressing cells are evident in all embryonic tissues (large arrow) and yolk sac (small arrow), but are absent from the placenta (outlined with dashed lines). Inset shows a normally developed blastocyst after 24 hours in vitro from the aggregation of a YFP ES sphere colony and a CD1 host morula. YFP cells (illuminated with UV light) integrate extensively into the ICM (large arrow), whereas the trophectoderm (faintly illuminated with a low intensity white light) is normally devoid of YFP cells (small arrow). (B) Twenty-four hours after the attempted aggregation of a GFP sphere colony derived from the E9.5 telencephalon and a CD1 host morula, the morula develops normally into a blastocyst (arrow), while the sphere colony remains unintegrated outside of the embryo (both visualized with low intensity white light). (C) GFP-expressing cells were not observed within the embryo. Scale bar: 1 mm (A), 750 μm (A, inset), 250 μm (B, C).


A
B
C

adopting a neural phenotype, a process that is similarly thought to occur in the epiblast during gastrulation in vivo (Beddington and Robertson, 1999). However, our findings also suggest that the default neuralization in mammalian cells may not be homologous with default neuralization in amphibian cells.

In *Xenopus*, ectodermal cells differentiate into epidermis as their alternate fate when neural differentiation is inhibited. Given that we assessed neural fate specification in totipotent ES cells, three possibilities exist for the acquisition of non-neural cell fates in this model. First, an alternate fate for ES cells under our defined culture conditions may be epidermis, which would indicate that mouse default neuralization is homologous with *Xenopus* default neuralization. Second, any non-neural cell type (including epidermis) may be established in a stochastic manner. Finally, an ES cell phenotype may be maintained in the absence of neural differentiation. These latter two possibilities would indicate that mouse default neuralization may be analogous to, but not homologous with, *Xenopus* default neuralization. Our results (Figure 2.5) are consistent with the third possibility; the alternative to neural default for mouse ES cells may be to maintain the undifferentiated ES cell fate. When cell density is increased in our 24 hour differentiation paradigm, the proportion of nuclear Oct-4 expressing ES cells increased, compared to the decrease in β III-tubulin expressing neurons. Thus, although the establishment of a neural phenotype may be under inhibitory control (a default mechanism), additional signals may be required to drive ES cell differentiation into various non-neural lineages – signals that are likely to be absent of below threshold in our culture conditions.

Both LIF and FGF are required for the initial transition of ES cells into neural colony forming stem cells. This raises the question of whether the functions of LIF and FGF are to induce neural stem cell differentiation of ES cells, which would not support the notion of neural fate being achieved autonomously. Although LIF and FGF are required for neural colony formation, the majority of ES cells can take on a neural identity within 24 hours in culture in the absence of any exogenous growth factors. Furthermore, the

influence of *Smad4* inactivation on neuronal differentiation (at relatively high densities) indicates that simply attenuating TGFβ-related signaling can promote neural specification under relatively inhibitory (high-density) conditions. Finally, preliminary experiments reveal that blocking extracellular FGF signaling using anti-FGF2 antibodies in relatively low cell density cultures does not appreciably decrease the percentage of nestin-expressing cells after 24 hours of differentiation in the absence of LIF. The hypothesis that LIF and FGF (specifically FGF2) are acting permissively to specify a neural fate is supported by the results of targeted null mutations. For instance, the formation of neural tissue and subsequent early neural morphogenesis is relatively normal in mice lacking the LIF receptor (LIFR) (Li et al., 1995). Moreover, in the *FGFR1* null mice (the primary receptor for FGF2), early gastrulation and neural tube formation was relatively normal (Yamaguchi et al., 1994). However, evidence for neural stem cell proliferation deficits in $FGFR1^{(-/-)}$ mice (Tropepe et al., 1999) as well as motor neuron differentiation deficits in $LIFR^{(-/-)}$ mice (Li et al., 1995) indicate that these factors are important for neural development at slightly later stages. Thus, we speculate that the primary roles for FGF and LIF are permissive ones and that ES cells autonomously adopt a neural cell fate. We propose that LIF may initially maintain ES cell survival in these minimal culture conditions, whereas FGF may act primarily as a mitogen for neural stem and progenitor cell proliferation.

In contrast to the ICM and subsequent epiblast cells, ES cells can express the neural precursor marker nestin and the early neuronal marker βIII-tubulin within 24 hours when dispersed in culture in the absence of exogenous factors. The onset of nestin expression in vivo occurs at approximately E7.5 within the neuroepithelium of the presumptive neural plate (Lendahl et al., 1990) and neuronal differentiation begins thereafter. One possibility that emerges from our findings is that the potential for cells within the ICM or epiblast to behave like primitive neural stem cells in vivo is actively suppressed. For example, epiblast cells in vivo may be competent to differentiate into neurons, but the absence of neurons prior to neurulation (even after a neural fate has been specified) suggests these cells may be

inhibited from precocious neuronal differentiation. One intriguing possibility is that the Notch signaling pathway may partially prevent neuronal differentiation by maintaining newly generated neural stem cells in an undifferentiated state. For example, functional inactivation of the mouse *Su(H)/RBP-Jk* gene, a downstream intracellular target of multiple Notch receptors, results in premature neuronal differentiation within the neural plate (de la Pompa et al., 1997). Similarly, functional inactivation of the mouse bHLH transcription factor *HES1*, which negatively regulates neuronal differentiation via Notch activation, resulted in diminished forebrain neural stem cell self-renewal and a concomitant increase in neuronal differentiation (Nakamura et al., 2000).

Studies aimed at testing the role of BMP inhibition in neural fate specification using avian epiblast cells have come to different conclusions. BMP inhibition (by noggin or chordin) was not sufficient for ectopic neural cell differentiation in extraembryonic tissue (Streit et al., 1998; Streit and Stern, 1999), and dissociated epiblast cells preferentially adopted a muscle cell phenotype in culture (George-Weinstein et al., 1996). Combinations of multiple BMP inhibitors, or BMP/Wnt inhibitors may be required for avian neural differentiation to occur. Both BMP and Wnt inhibition causes a more complete secondary axis to form in *Xenopus* transplantation experiments (Glinka et al., 1997). In addition, the generation of mice harboring targeted null mutations in both *Noggin* and *Chordin*, indicate that these two BMP inhibitors may function in concert to exert their effects on neural inhibition during mouse development (Bachiller et al., 2000). In the present study, mCer-l (known to antagonize BMP, nodal and Wnt signaling) (Piccolo et al., 1999) was similarly effective at augmenting neural stem cell colony formation when compared to Noggin, suggesting that Wnt signaling alone may not actively suppress the ES to neural transition.

Cell density and culture media conditions employed in some chick studies (George-Weinstein et al., 1996) indicate that the results may in fact be consistent with the neural default model. Although the epiblast cells were cultured at relatively low cell densities (~15 cells/μl), they were pre-treated at high cell densities (~400 cells/μl) for up to 5 hours in the

presence of serum and chick embryo extract (George-Weinstein et al., 1996) a condition likely to suppress neural cell differentiation. Interestingly, these authors reported that neurofilament-expressing chick neurons, when present, were found in relatively cell-dispersed regions of the cultures, whereas muscle cells were typically aggregated. Thus, these data provide clear examples of how neural differentiation can be inhibited in epiblast cells upon aggregation.

An ES cell paradigm for neural stem cell fate specification

ES cell differentiation assays typically involve the formation of embryoid bodies (EB) (Martin et al., 1977; Coucouvanis and Martin, 1995) that are generated from the aggregation of numerous ES cells in the presence of serum and in the absence of LIF, a factor which normally prevents differentiation. EB resemble early embryos: in the interior, EB contain ectodermal and mesodermal tissue surrounding a cystic cavity, while externally, EB are encapsulated by primitive endoderm (Coucouvanis and Martin, 1995; 1999). Given that EB formation in very high-density cultures (25-75 times greater than the densities used in the present study) contains many different cell types (derived from all three germ layers) and are generated in the presence of 10-20% serum (which contains undefined factors), EB formation precludes a more direct analysis of the mechanisms regulating the differentiation of a specific cell lineage. Indeed, consideration of cell density and culture media parameters in studies using dissociated *Xenopus* ectodermal cells (discussed above) initiated a significant change in our understanding of vertebrate neural patterning.

The derivation of neural cells (among other cell types) from EB derived cells in vitro has been previously documented (Doetschman et al., 1985). Several studies have shown that the differentiation of neurons and glial precursors from EB derived cells can be enriched in the presence of retinoic acid (Bain et al., 1995; Fraichad et al., 1995; Strubing et al., 1995), FGF2 (Okabe et al., 1996), or PDGF (Brustle et al., 1999). Also, BMP4 has been shown to suppress neuronal differentiation of EB derived cells (Finley et al., 1999).

Although these observations clearly demonstrate the potency of such factors to promote or attenuate neuronal differentiation of ES cells, each experiment was preceded by EB formation in the presence of serum. Here we present an alternative and specific paradigm for neural cell fate specification directly from ES cells. Neural colonies can develop from ES cells in serum-free conditions in the absence of EB formation, and many single ES cells can adopt a neural (nestin$^+$) or neuronal (βIII-tubulin$^+$) phenotype in the absence of exogenous growth factors.

This paradigm may be useful in analyzing the role of single genes in the regulation of neural fate specification. For instance, the utilization of an expression-based gene trap library of ES cell lines (Stanford et al., 1998) offers a unique opportunity to employ a strategy for isolating genes that positively and negatively regulate the transition from an ES cell to a neural cell (Seaberg et al., 1999). Thus, our present findings underscore the potential for using ES cell models of mammalian neural development.

A primitive stage in the neural stem cell lineage

With the exception of the hematopoietic stem cell (Weissman, 2000), our knowledge of the ontogeny of stem cells in other mammalian organ systems is comparatively limited. Clonal neural colonies generated from ES cells share similar features to clonal neural stem cell colonies described from the embryonic forebrain germinal zone. At a very low frequency (~0.2%), single ES cells proliferate in a LIF- and FGF-dependent manner to form neural colonies that express multiple neural precursor markers (e.g. nestin, *Emx2, Hoxb1*), even though the vast majority of ES cells upregulate nestin expression and down-regulate nuclear Oct-4 expression within 24 hours. We previously demonstrated that the proportion of FGF-dependent neural stem cells from the E8.5 anterior neural plate was similar (~0.3%) (Tropepe et al., 1999), and forebrain neural stem cell colonies express similar region-specific patterning genes (present study). Thus, the mechanism for segregating a subpopulation of colony-forming neural stem cells among a larger population

of neural cells may be recapitulated during neural fate specification from ES cells. This raises the question of whether the first neural cell to arise in the nervous system is a neural stem cell or whether the first neural derivative is a general neural precursor cell that precedes (or is generated simultaneously with) the emergence of the neural stem cell lineage (van der Kooy and Weiss, 2000).

The ES cell-derived colonies are typically spheroid in morphology and many float in suspension as has previously been described for neural stem cell-derived colonies at all ages (Reynolds and Weiss, 1996). ES cell-derived colonies do not retain ES cell characteristics (e.g. do not express *Brachyury* or Oct-4 protein), but are specified to a neural identity, retaining the expression of neural genes such as *Emx2* and *HoxB1*. Furthermore, cells derived from the neural colonies can differentiate into neurons and glia, suggesting that the initial colony-forming cell had neural multilineage potential. However, neural stem cells derived from ES cells display other features than those derived from the embryonic forebrain, which may indicate an earlier primitive stage in the neural lineage.

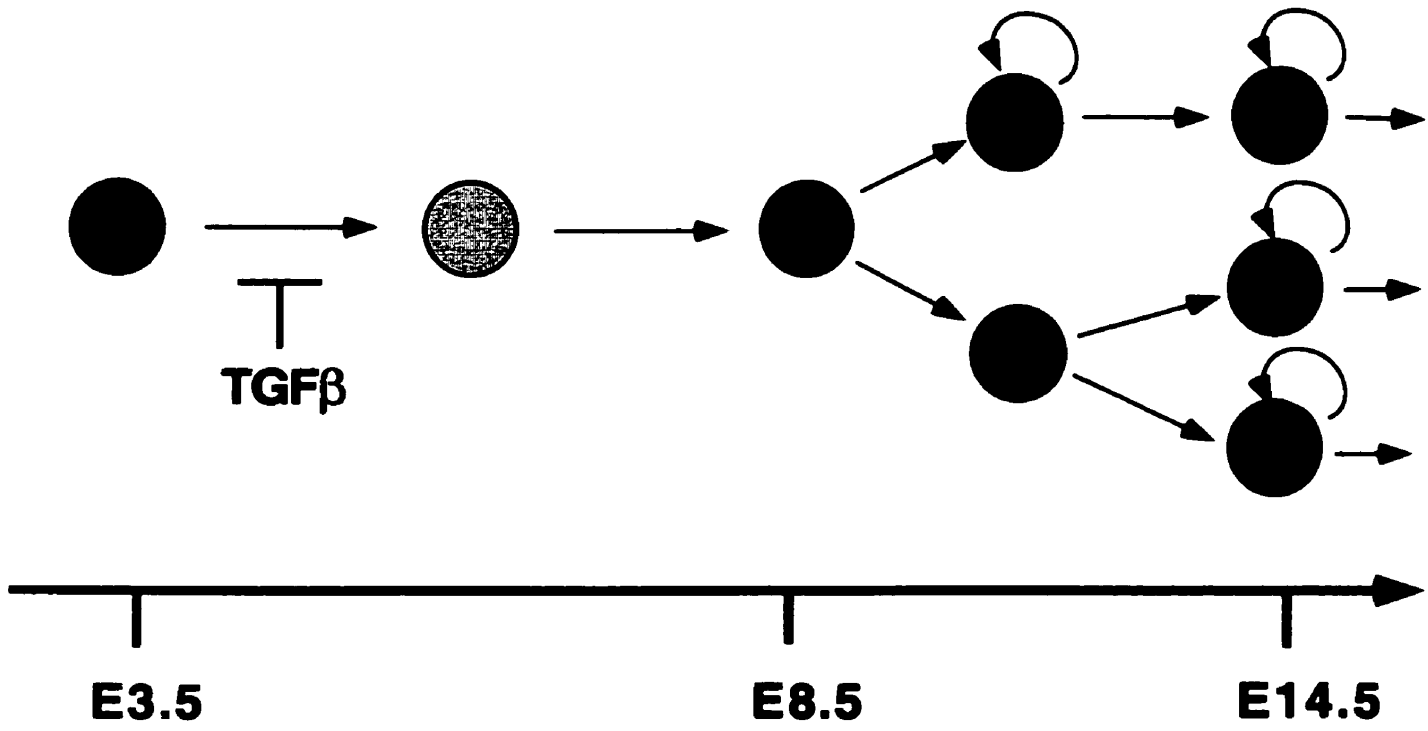
First, LIF and FGF are critical for ES-derived neural stem cell colony formation and subsequent subcloning (stem cell self-renewal). This is in contrast to neural stem cells isolated from embryonic or adult tissues, where either exogenous FGF or EGF is sufficient for colony formation and subcloning (Reynolds et al., 1992; Reynolds and Weiss, 1992). The nature of the LIF effect on the ES to neural transition is not completely understood. Although CNTF can substitute for LIF in maintaining ES cells in an undifferentiated state (Conover et al., 1993; Nichols et al., 1994), it does not substitute for LIF in promoting neural colony formation from ES cells, and LIF alone can not elicit neural colony formation from embryonic derived tissue. Thus, LIF does not appear to maintain neural colonies in an undifferentiated ES state or act as a mitogen. Instead, LIF may act as a survival factor (reviewed in Mehler and Kessler, 1997) that is initially required for ES cell viability. Subsequently, LIF can facilitate colony formation from early embryonic neural stem cells (presumably by keeping more stem cells alive longer), but it is not absolutely required.

Hence, growth factor requirements may be sequentially modified from a primitive neural stem cell stage (LIF- and FGF-dependent) to an early embryonic neural stem cell stage (only FGF-dependent), and finally to a relatively mature neural stem cell stage where both FGF- and EGF-dependent subpopulations co-exist from late embryogenesis into adulthood (Figure 2.7). A similar role has been attributed to LIF with respect to the survival of primordial germ cells in cultures. Congruent with our results, CNTF was not able to substitute for LIF in keeping primordial germ cells alive, even though embryonic germ cells (an ES-like cell derived from primordial germ cells) could be propagated with several members of the LIF family of ligands, including CNTF (Koshimizu et al., 1996). However, further studies will be required to determine more precisely the factors that mediate the transition from a LIF- and FGF-dependent primitive neural stem cell to a definitive FGF-dependent neural stem cell that can give rise to EGF-dependent stem cells at later embryonic ages.

Second, the expression of neural genes and at least one non-neural gene (GATA4) indicates that the neural stem cell giving rise to neural colonies may retain a certain degree of pluripotency or primitive characteristics. Most important, this retained pluripotency can be observed in the ability of sphere colony derived cells to extensively colonize various embryonic tissues under appropriate influences in the chimeric embryos in vivo (no such pluripotency is shown by neural tube derived neural stem cells under similar conditions). Under the culture conditions employed in the present study the colony-forming neural stem cells derived from ES cells are specified, but not committed, to a neural fate.

Therefore, we have identified a novel cell type in the neural lineage based on the degree of neural commitment and growth factor responsiveness in vitro and the potential to give rise to neural and non-neural progeny in vivo. This cell type may be suitably described as a primitive neural stem cell or a pre-neural stem cell, a term that has been used by others (Morrison et al., 1997) to describe a stem cell that is primarily tissue-specific, but that retains a certain degree of pluripotency during a restricted early period of development.

Figure 2.7. A model depicting the establishment of the early neural lineage from ES cells. Totipotent ES cells derived from the E3.5 ICM directly differentiate (limited by the inhibitory control of TGFβ molecules) to give rise to LIF- and FGF-dependent pluripotent primitive neural stem cells that undergo relatively few symmetric (expansionary) divisions. These primitive neural stem cells can generate neurons and glia, but under appropriate environmental conditions (chimeric embryos) have the potential to generate cells with the capacity to differentiate into various cell types. As development proceeds, primitive neural stem cells give rise to FGF-dependent (and not LIF-dependent) neural stem cells that are present at the neural plate stage at E8.5 (Tropepe et al., 1999). The FGF-responsive neural stem cells initially undergo mostly asymmetric divisions, but at later stages divide symmetrically to expand their population. By E14.5, FGF-responsive neural stem cells also give rise to a relatively separate EGF-responsive neural stem cell population, both of which have the potential to generate neurons and glia (Tropepe et al., 1999; Martens et al, 2000).



Totipotent ES cell

LIF- and FGF-dependent pluripotent primitive neural stem cell

FGF-dependent multipotent neural stem cell

EGF-dependent multipotent neural stem cell

Lineage restriction in developing neural stem cells may be reversible

To what extent can the microenvironment dictate the identity of neural stem cells and their ability to produce different progeny? We demonstrate that ES-derived primitive neural stem cells can produce progeny that colonize neural and non-neural tissues in chimeric mice in vivo. In contrast, we were unable to generate chimeras using neural stem cell colonies derived from either the early embryonic or adult forebrain. This difference would suggest that primitive neural stem cells transiently retain their pluripotency, but through development neural stem cells become restricted in their ability to generate non-neural cell types. This restriction, however, may be reversible.

Clarke et al. (2000) recently demonstrated that a very low percentage (6 chimeras out of 600 viable embryos, or 1%) of adult neural stem cell colony cells could contribute to neural and non-neural tissues in a mouse chimera paradigm similar to the one we utilized in the present study. An increase in the frequency of chimeras was observed when undissociated stem cell colonies were injected into the mouse blastocoel or chick amniotic cavity (Clarke et al., 2000). Consistent with our findings, the degree to which definitive neural stem cell-derived progeny (after isolation from embryonic or adult brain) can contribute to non-neural tissue in mouse is very restricted, compared to the proportion of ES cell derived primitive neural stem cell progeny that contribute to neural and non-neural tissues in the present study (22 chimeras out of 24 viable embryos, or 92%). However, it is clear from their analysis of the inductive influence of EB on adult neural stem cell colonies in vitro (to form muscle cells), that appropriate inductive signals can reveal some potential of neural stem cells to give rise to non-neural cells independent of the in vivo environment (Clarke et al., 2000). One intriguing possibility is that these inductive cues could enable some of the definitive neural stem cells to revert to a pluripotent primitive neural stem cell stage and subsequently produce progeny indicative of all three germ layers.

Thus, the delineation of a pluripotent primitive neural stem cell stage during neural stem cell ontogeny may provide a basis for further understanding the mechanisms governing this remarkable cellular plasticity.

Chapter III

Neural stem cell heterogeneity

This chapter has been previously published:

Tropepe, V., Sibilia, M., Ciruna, B., Rossant, J., Wagner, E., van der Kooy, D. (1999). Distinct neural stem cells proliferate in response to FGF and EGF in the developing mouse telencephalon. Dev. Biol. 208, 166-188.

Summary

Multipotent, self-renewing neural stem cells reside in the embryonic mouse telencephalic germinal zone. Using an in vitro neurosphere assay for neural stem cell proliferation, we demonstrate that FGF-responsive neural stem cells are present as early as E8.5 in the anterior neural plate, but EGF-responsive neural stem cells emerge later in development in a temporally- and spatially specific manner. By separately blocking EGF and FGF2 signaling, we also show that EGF alone and FGF2 alone can independently elicit neural stem cell proliferation, and at relatively high cell densities separate cell nonautonomous effects can substantially enhance the mitogen-induced proliferation. At lower cell densities, neural stem cell proliferation is additive in the presence of EGF and FGF2 combined, revealing two different stem cell populations. However, both FGF-responsive and EGF-responsive neural stem cells retain their self-renewal and multilineage potential, regardless of growth factor conditions. These results support a model in which separate, lineage-related EGF- and FGF-responsive neural stem cells are present in the embryonic telencephalic germinal zone.

Introduction

The vertebrate central nervous system emerges from a layer of cytologically indistinct neuroepithelial cells along the dorsal midline of the embryo, called the neural plate. The neural plate subsequently undergoes a series of morphogenetic movements to form a neural tube consisting of prominent vesicles anteriorly which represent the anlage of the forebrain, midbrain and hindbrain, and a slender portion posteriorly which develops into the spinal cord. Clonal analysis has shown that single cells isolated from the neural tube along the spinal segment are competent to give rise to clones containing both dorsal

(e.g sensory ganglion neurons, and presumptive pigment cells) and ventral (e.g. motor neurons and floor plate cells) derivatives, indicating that neural precursor cells at an early stage are not restricted in their potential to generate different cell types along the dorsoventral axis (Artinger et al., 1995). Indeed, the generation of diverse cellular phenotypes has been shown to depend upon signals emanating from surrounding tissues that can regulate phenotypic and positional specification of neural precursor lineages along the rostrocaudal and dorsoventral axes (reviewed in Lumsden and Krumlauf, 1996; Tanabe and Jessell, 1996). Although the fate of the progeny of multipotential precursors can be specified by extrinsic mechanisms at early stages of neural development, the regulation of the multipotential precursor population itself at these early stages is poorly understood. It is not known if, for example, all precursors terminally differentiate along with their progeny throughout the course of neurogenesis. The recent identification of stem cells in the embryonic and adult mammalian central nervous system (reviewed in Weiss et al., 1996) indicates that not all lineage precursors differentiate. Therefore, the regulation of neural stem cell proliferation may be required for the initial events leading to the elaboration of neural tissue and the maintenance of discrete regions of neurogenesis in the adult brain.

Evidence from in vivo lineage analyses and in vitro cell culture experiments revealed that the rodent telencephalic germinal zone (GZ) at embryonic day (E) 14 is composed of a heterogeneous population of multipotential and committed precursor cells (Gage et al., 1995; Weiss et al., 1996; McKay, 1997). Neural stem cells, exhibiting the fundamental stem cell properties of multipotentiality and self-renewal (Potten and Loeffler, 1990), have been shown to make up a relatively small percentage of this heterogeneous E14 GZ population (Temple, 1989; Reynolds et al., 1992; Vescovi et al., 1993). In the adult forebrain, neural stem cells are present as a relatively quiescent subpopulation in the subependyma, a remnant of the embryonic GZ, (Reynolds and Weiss, 1992; Morshead et al., 1994; Craig et al., 1996; Gritti et al., 1996) and this population persists into senescence (Tropepe et al., 1997).

Epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF2) have been shown to mediate cell proliferation in the embryonic retina (Anchan et al., 1991; Lillien and Cepko, 1992), telencephalon (Gensburger et al., 1987; Drago et al., 1991; Kilpatrick and Bartlett, 1993), hippocampus (Ray et al., 1993), mesencephalon (Murphy et al., 1990; Santa-Olalla and Covarrubias, 1995), and spinal cord (Ray and Gage, 1994). Furthermore, EGF and FGF2 are critical for the proliferation of neural stem cells isolated from the embryonic and adult forebrain GZ (Reynolds et al., 1992; Reynolds and Weiss, 1992; Vescovi et al., 1993; Gritti et al., 1996), as well as the embryonic and adult spinal cord (Weiss et al., 1996; Kalyani et al., 1997). FGF2 is believed to act primarily through FGF-receptor-1 (FGFR1) (Johnson and Williams, 1993), and both receptor binding affinity and growth factor-mediated mitogenicity in many cell types, including neuroepithelial cells, are dependent on heparin (Ornitz and Leder, 1992; Roghani et al., 1994; Brickman et al., 1995). In the rat telencephalon, FGFR1 is expressed as early as E8.5-E9.5 and this expression is relatively confined to the ventricular zone during later stages of development (Orr-Urtreger et al., 1991; Wanaka et al., 1991), while peak expression of FGF2 occurs at mid-neurogenesis in predominantly postmitotic cells (Weise et al., 1993). Targeted null mutations of FGFR1 cause defects in cell proliferation and mesoderm patterning, and embryos die between E7.5 and E9.5 (Deng et al., 1994; Yamaguchi et al., 1994; Ciruna et al., 1997). Thus, FGF-dependent cellular proliferation has a prominent and ubiquitous role during embryogenesis. The in vitro evidence for FGF-dependent proliferation of neural precursor cells and the early expression of FGFR1 in the telencephalon indicates that neural precursor cell proliferation is predominantly regulated by FGFs. Low levels of transforming growth factor-α (TGFα) and the EGF-receptor (EGFR) are similarly present at relatively early stages of telencephalic development in mostly ventral regions, with an increase in intensity and dorsal distribution at later embryonic stages (Eagleson et al., 1996; Kornblum et al., 1997). Both EGF and TGFα bind preferentially to the EGFR (Massague, 1983; Marquardt et al., 1984), but TGFα is thought to be the predominant endogenous

ligand in the rodent brain (Kornblum et al., 1997). Although there is in vitro evidence for EGF-dependent proliferation of neural precursor cells, targeted disruption of the EGFR has no apparent neural phenotype at early stages of development, but does cause forebrain cortical dysgenesis at late embryonic and postnatal ages with evidence of an attenuated forebrain, cortical cell death and hippocampal ectopias (Threadgill et al., 1995; Sibilia and Wagner, 1995; Sibilia et al., 1998). Depending on the background genetic strain, the $EGFR^{(-/-)}$ mutation can cause implantation defects, while some mice can survive for several weeks postnatally. Both EGF and FGF2 can elicit the in vitro proliferation of neural stem cells isolated from the embryonic GZ, but evidence from the null receptor mutations suggests that there may be differential in vivo influences of these mitogens on telencephalic neural stem cell proliferation at different stages of embryonic development.

To determine if EGF and FGF2 have differential roles in neural stem cell proliferation (and indeed act on different cells) we analyzed the EGF- and FGF-responsiveness of neural stem cells from early stages of neural development, and tested whether there were quantitative and qualitative differences between the two mitogenic signals. In serum-free conditions neural stem cells proliferate in the presence of FGF2 as early as E8.5, but respond to EGF only at later stages of embryonic development. Neural stem cells independently proliferate in response to EGF and FGF2, and at low cell densities the EGF- and FGF2-induced proliferation of stem cells is additive. FGF2 signaling via FGFR1 is critical for proliferation of FGF2-responsive neural stem cells isolated from the E8.5 anterior neural plate. EGF signaling through the EGFR is critical for the proliferation of EGF-responsive neural stem cells, but not FGF2-responsive neural stem cells, isolated from the E14.5 telencephalic GZ. Furthermore, in the absence of a functional FGFR1, the expansion of the FGF2-responsive neural stem cell population, as well as the emergence of EGF-responsive neural stem cells, is severely diminished at E14.5. The distinct proliferative responses to EGF and FGF2 reveal a heterogeneity among the neural stem cell population itself. Furthermore, our results suggest that the EGF-responsive stem cells that

are present in the E14.5 GZ are the lineage descendents of FGF-responsive stem cells that are present as early as E8.5. In addition, the greater than proportional increase in the number of embryonic neural stem cells proliferating in high density cultures demonstrates that the proliferation of neural stem cells can be actively regulated by additional cellular interactions that were shown to be separate of both EGF and FGF2 signaling.

Materials and Methods

Isolation of neuroepithelial cells from embryonic day 8.5 embryos

Pregnant CD1 mice (Charles River) at the specified gestational age of 8.5 days (E8.5) were killed via cervical dislocation and the uteri were aseptically removed and transferred to petri dishes containing sterile Dulbecco's phosphate-buffered saline (PBS; GIBCO) with 30% glucose (Sigma) and 2% penicillin/streptomycin (5000 IU; GIBCO). Each decidua from the uterine sac was dissected out and transferred to a new sterile petri dish containing PBS in order to rinse away excess blood. Deciduae were then transferred to a chemically defined serum-free media (Reynolds et al., 1992) composed of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM; GIBCO) and F-12 nutrient (GIBCO) including 0.6% glucose (Sigma), 2 mM glutamine (GIBCO), 3 mM sodium bicarbonate (Sigma), and 5 mM HEPES buffer (Sigma). A defined hormone and salt mixture (Sigma) that included insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (20 nM), putrescine (60 μM), and selenium chloride (30 nM) was used instead of serum. All subsequent dissection procedures were adapted from Drago et al. (1991). Under a dissecting microscope (Zeiss), sterile, fine forceps were used to make a single superficial incision through the narrow end of the decidual wall. The amniotic sac was gently removed and transferred to a new petri dish containing fresh media. Embryos were removed from the amniotic sac and the head primordia were dissected using fine forceps and microprobe by excising rostral to the first branchial arch. Using a sterile, fire-polished Pasteur pipette head

primordia were transferred to media containing 0.1% (w/v) trypsin (Sigma) and 0.001% (v/v) deoxyribonuclease I (10 mg/ml stock DNase I; Boehringer Mannheim) and incubated at 4°C for approximately 15 minutes. After enzymatic dissociation, head primordia were washed in PBS and subsequently transferred to media containing 0.7 mg/ml trypsin inhibitor (Boehringer Mannheim). Anterior neural plate tissue was gently teased away from surrounding head mesenchyme and overlying epidermal ectoderm. The neuroepithelium was washed in Ca^{2+}-Mg^{2+}-free Hank's buffer (GIBCO) to remove loosely adherent cells. Each anterior neuroepithelium was transferred to serum-free culture media containing either 10 ng/ml FGF-2 (human recombinant; Upstate Biotech) and 2 μg/ml heparin (Upstate Biotech), or 20 ng/ml EGF (mouse submaxillary; Upstate Biotech), mechanically dissociated into single cells and plated in uncoated 24-well plates (0.5 ml/well; Nunclon). Each dissection yielded an average of 5000 viable cells/well assessed using trypan blue exclusion (0.4%; GIBCO). Due to the limited amount of viable cells recovered from these small tissue samples, one E8.5 anterior neural plate dissection per well was plated. The average cell plating density was estimated at 10 cells/μl (from n=6 embryos).

Isolation of ventricular zone cells from embryonic day 13.5-15.5 embryos and postnatal day 3 mice

Pregnant CD1 mice of gestational age 13.5, 14.5, or 15.5 days (E13.5, E14.5, E15.5) were killed via cervical dislocation and embryos were removed as described above. Postnatal day 3 mice were anesthetized at 4°C and decapitated. In PBS (as above) the brains of each embryo postnatal mouse was removed and overlying meninges and blood vessels were removed. Dissected cortical or striatal GZ tissue was transferred to serum-free media and mechanically dissociated into a cell suspension with a fire-polished Pasteur pipette. Cell viability was assessed using trypan blue (as above). Cells were plated in 96-well (0.2 ml/well), 24-well (0.5 ml/well) or 6-well (2 ml/well) uncoated plates (Nunclon) depending on the experimental conditions in serum-free media containing growth factors

(as above). In order to assess self-renewal, E8.5, E13.5-15.5 and P3 primary stem cell derived sphere colonies (neurospheres) were passaged (selecting mainly floating neurospheres after 6-7 days in vitro) by mechanically dissociating a single colony in 0.2 ml of serum-free media, in identical growth factor conditions as the primary culture, and plated in uncoated 96-well (0.2 ml/well) plates (Nunclon). Passagibility was assessed by identifying new sphere colonies after a further 6-7 days in vitro. For E14.5 and P3 dose-response experiments, the molarity values for each of the concentration points chosen are: (1) for FGF2, 0.05 ng/ml = $3.5x10^{-12}$ M; 0.2 ng/ml = $1.4x10^{-11}$ M; 0.6 ng/ml = $3.8x10^{-11}$ M; 2 ng/ml = $1.2x10^{-10}$ M; 10 ng/ml = $5.7x10^{-10}$ M; 20 ng/ml = $1.14x10^{-9}$ M; 40 ng/ml = $2.28x10^{-9}$ M; 80 ng/ml = $4.57x10^{-9}$ M; and (2) for EGF, 0.05 ng/ml = $1.0x10^{-11}$ M; 0.2 ng/ml = $4.0x10^{-11}$ M; 0.6 ng/ml = $1.1x10^{-10}$ M; 2 ng/ml = $3.3x10^{-10}$ M; 10 ng/ml = $1.64x10^{-9}$ M; 20 ng/ml = $3.28x10^{-9}$ M; 40 ng/ml = $6.56x10^{-9}$ M; 80 ng/ml = $1.31x10^{-8}$ M.

For the growth factor dose-dependent clonal analyses, E14.5 GZ tissue was isolated as above and cells were plated at 4 x 10^5 cells per 94 mm Greiner hybridoma tissue culture dish (Fedoroff et al., 1997), which is subdivided into approximately 700 microwells, 0.04 cm^2 each (Greiner Labortechnik, Bellco Glass, Inc., Vineland, NJ). Using this procedure, microwells containing 0 cells, 1-3 cells, or greater than 3 cells per well were scored after approximately 24 hours (randomly assorted cells), and only the wells containing 1-3 cells were followed for the 7 day culture period for the presence of sphere colonies in 3 separate growth factor concentration conditions (0.6, 20, 80 ng/ml) for both EGF and FGF2. Single colonies generated in all conditions were either passaged to assay for the generation of secondary and tertiary colonies (as above) or plated to assess their differentiation potential (see below).

Generation of FGFR1$^{(-/-)}$ tetraploid and diploid chimeras

The generation of FGFR1 tetraploid and diploid chimeric mice was exactly as previously described by Ciruna et al. (1997). Briefly, ES cell lines that were homozygous

for the *fgfr1*$_{\Delta}^{tmk}$ allele, previously identified as a functional null mutation (Yamaguchi et al., 1994), and marked with a ubiquitously expressed *lacZ* marker (Friedrich and Soriano, 1991) were used for generating chimeras (Ciruna et al., 1997). These ES cells (as well as *fgfr1*$_{\Delta}^{tmk}$/+ control ES cells) were aggregated with two tetraploid CD1 embryos, produced by electrofusion of embryos at the two cell stage, and aggregates were transferred to the uteri of CD1 foster mothers. Embryos were subsequently isolated at 8.5 days of gestation and anterior neural plate tissue was dissected, dissociated and plated in serum-free media (as above) in order to assay for neurosphere formation. Diploid chimeric embryos were generated by aggregating 8-10 cell morulae of *fgfr1*$_{\Delta}^{tmk}$/*fgfr1*$_{\Delta}^{tmk}$ ES cells (or *fgfr1*$_{\Delta}^{tmk}$/+ control ES cells) with CD1 8-cell embryos. Aggregates were transferred into the uteri of CD1 foster mothers, and chimeric embryos were isolated at E14.5. Striatal GZ tissue was dissected from one hemisphere (the other was used for X-gal staining - see below), dissociated and plated in serum-free media (as above), and the number of sphere colonies generated in the presence of either EGF or FGF2 was assayed after 7 days. Tetraploid and diploid chimeric animals will be refered to as ROSA26-FGFR1$^{(-/-)}$ or ROSA26-FGFR1$^{(+/-)}$, mutant and control respectively, throughout.

Whole mount β-Gal staining for E14.5 chimeric embryonic brains and sphere colonies

E14.5 embryonic brain hemispheres (or colonies) generated from the E14.5 diploid chimeras were rinsed in 100 mM sodium phosphate buffer (pH 7.4), and then fixed in 0.2% gluteraldehyde, 2 mM $MgCl_2$, 5 mM EGTA and 100 mM sodium phosphate (pH 7.3) at room temperature for 5 min. (colonies) or 15 min. (brains). Single brain hemispheres (or colonies) were then washed (3x) in wash buffer containing 0.02% NP-40, 0.01% deoxycholate, 2 mM $MgCl_2$ and 100 mM sodium phosphate (pH 7.3) for 5-15 min. each. Brain hemispheres (or colonies) were stained in 1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 0.02% NP-40, 0.01% deoxycholate, 2 mM $MgCl_2$ and 100 mM sodium

phosphate buffer (pH 7.3) at 37°C overnight. Single brain hemispheres (or colonies) were then rinsed with wash buffer and stored at 4°C. For forebrain sections, brain hemispheres were postfixed overnight in 4% paraformaldehyde at 4°C, subsequently cryoprotected with 20% sucrose, cryosectioned at 14 μm, and then counterstained with 0.5% safranin (stains the entire cell light red). In order to estimate the percent chimerism in the striatal germinal zone region, 4-6 sections (obtained from every 10th serial section through the forebrain) were used to determine the proportion of blue (X-gal-positive) cells. Cell counts (a total of approximately 500 cells counted in any one section) were obtained using a phase-contrast Nikon microscope (20x objective).

Genotyping mouse embryos from EGFR heterozygote matings

Targeted disruption of the mouse EGFR was carried out as previously described (Sibilia and Wagner, 1995). A total of 12 pregnant female mice, from EGFR heterozygote matings (129/B6xB6) were analyzed in order to obtain an adequate number of mutant embryos. DNA was prepared from yolk sacs or tails and genotyped by PCR and primers used for amplification of wildtype and mutant alleles was as previously described (Sibilia and Wagner, 1995).

Dose-response analysis for EGFR-inhibitor and anti-FGF2 antibody effects on sphere colony formation

The generation of primary sphere colonies isolated from the E14.5 GZ ($1x10^5$ cells in 2 ml) in the presence of EGF (20 ng/ml) and various concentrations of the EGFR inhibitor (referred to as PD) (Park Davis, PD 153035; Fry et al., 1994) was determined. At plating densities of 50 cells/μl and n=6 embryos for each group, the number of neurospheres (mean percent of control ± S.E.M.) generated were as follows: Dimethyl sulphoxide (DMSO) control = 100 ± 9.0; 0.0036 μM PD = 51.5 ± 8.5; 0.032 μM PD = 3.24 ± 0.9; 0.16 μM PD = 2.37 ± 0.8; 0.8 μM PD = 1.45 ± 0.4. Since there was some

evidence for reduced viability at the 0.8 μM concentration, we used 0.16 μM for subsequent analyses, which resulted in similar viability to the DMSO control. The same analysis was performed for the anti-FGF2 antibody (IgG, Upstate Biotech) in the presence of FGF2 (10 ng/ml) and heparin (2 μg/ml) at plating densities of 50 cells/μl and n=6 embryos for each group. A non-specific control antibody (conAb) of similar species (mouse) and isotype (anti-MAP2 monoclonal IgG) was also used to compare to the PBS control. The percentages of spheres colonies (labeled as neurospheres in the figures) (mean percent of PBS control ± S.E.M.) generated were as follows: PBS control = 100 ± 5.0; 0.125 μg/ml conAb = 100.5 ± 5.2; 1.25 μg/ml conAb = 96.6 ± 5.5; 0.125 μg/ml anti-FGF2 = 12.4 ± 2.14; 1.25 μg/ml antiFGF2 = 1.14 ± 0.6; 12.5 μg/ml anti-FGF2 = 0.21 ± 0.1. There were no significant differences between the PBS control and the conAb conditions. There was some evidence for compromised viability at the 12.5 μg/ml concentration of the anti-FGF2. Therefore, we used 1.25 μg/ml anti-FGF2 for subsequent analyses, which resulted in similar viability of cells when compared to the PBS control and the 1.25 μg/ml conAb.

Cryosectioning sphere colonies

Using a Pasteur pipette, colonies cultured for 6 or 7 days in vitro were transferred and washed (2x) in 10 mM phosphate buffered saline (PBS pH 7.2) for a few seconds for each wash. Colonies were then transferred to 4% paraformaldehyde containing 0.4% picric acid in 0.16 M phosphate buffer (pH 6.9) (Zamboni and de Martino, 1967) and fixed for 1 hour at room temperature. Colonies were washed (3x) in 10 mM PBS for 5 minutes each prior to being resuspended in 10% sucrose (in 10 mM PBS) overnight at 4°C. The following day, colonies were placed in tissue freezing media (Tissue Tek) in order to quick freeze to -50°C. Using a cryostat, serial 14 μm sections were taken and collected on gelatin-coated slides. Slides were stored at -70°C.

Immunocytochemistry on sectioned whole sphere colonies

Gelatin-coated slides containing sphere colony sections were allowed to rehydrate with PBS for 10 minutes. For nestin immunocytochemistry, a rabbit polyclonal antiserum (a gift from Dr. R. McKay) (Tohyama et al., 1992) was diluted to 1:1000 (in PBS + Triton-X) and sections were incubated overnight at 4°C. Sections were then washed (3x) in 10 mM PBS (5 minutes each) and subsequently incubated with secondary anti-rabbit FITC-conjugated antibody (1:20; Sigma) for 1 hour at room temperature. Sections were washed (3x) (5 minutes each) and then cover-slipped with Fluor-mount mounting medium. Fluorescence was detected on a Nikon microscope. For EGFR (Sugiyama et al., 1989) and FGFR1 (Werner et al., 1993) immunocytochemistry, colony sections were rehydrated with PBS for 10 minutes and incubated overnight at 4°C in sheep anti-mouse EGF-receptor (1:100; Biodesign International) in PBS containing 0.3% Triton-X and 10% donkey serum. Sections were rinsed with PBS for 10 minutes and then incubated in Cy3 donkey anti-sheep secondary antibody (1:300; Jackson ImmunoResearch) in PBS containing 0.3% Triton-X and 10% donkey serum. After rinsing in PBS for 10 minutes, sections were incubated in rabbit anti-mouse FGF-receptor-1 (1:250; a gift from Dr. L. Williams) in PBS containing 0.3% Triton-X and 10% donkey serum. Sections were again rinsed with PBS for 10 minutes and subsequently incubated in biotinylated donkey anti-rabbit (1:200; Jackson ImmunoResearch) for 1 hour at 37°C, followed by rinsing in PBS for 10 minutes, and finally incubated in streptavidin Cy2 (1:1000; Jackson ImmunoResearch) in PBS only for 1 hour at 37°C. Sections were rinsed (2x) for 10 minutes each and cover-slipped in Fluor-mount. Fluorescence was detected on a Nikon Microphot microscope.

Immunocytochemistry on differentiated sphere colonies

Six days after primary culture, single sphere colonies were transferred using a Pasteur pipette onto glass coverslips coated with MATRIGEL basement membrane matrix (15.1 mg/ml stock solution diluted 1:50 in serum-free media; Becton-Dickinson) in

individual wells of a 24-well culture plate (Nunclon) (0.5 ml/well) in serum-free media containing various growth factor concentrations, or in media containing 10% FBS and no extra growth factor. Media was not changed for the rest of the culture period. Coverslips were processed 6-7 days later using immunocytochemistry. Coverslips were fixed in 4% paraformaldehyde (in PBS, pH 7.2) for 30 minutes at room temperature followed by 3 (5 minutes each) washes in PBS (pH 7.2). Cells were then permeabilized for 5 minutes in PBS containing 0.3% Triton-X, rinsed for 5 min (2x) in PBS and blocked for 1 hour in PBS containing 10% normal goat serum (NGS). After blocking, coverslips were incubated in anti-MAP-2 mouse monoclonal (IgG) (1:1000; Boehringer Mannheim) and anti-GFAP rabbit polyclonal (IgG) (1:400; Chemicon) antibodies diluted in PBS containing 10% NGS for 2 hours at 37°C. Coverslips were then rinsed in PBS three times (5 minutes each) and subsequently incubated in FITC goat anti-rabbit (1:200; Jackson ImmunoResearch) and TRITC goat anti-mouse (1:200; Jackson ImmunoResearch) secondary antibodies at 37°C for 30 min. Coverslips were rinsed three times (5 minutes each) in PBS. Separate coverslips (from similar culture conditions) were used for oligodendrocye staining. Coverslips were incubated in anti-O4 mouse monoclonal (IgM) antibody (1:40; Boehringer Mannheim) in PBS containing 10% NGS at 4°C overnight. The next day, coverslips were rinsed three times (5 minutes each) and subsequently incubated in DTAF goat anti-mouse-IgM (1:200; Jackson ImmunoResearch) secondary antibody in PBS containing 10% NGS at 37°C for 30 minutes. After rinsing three times (5 minutes each), all coverslips were incubated in Hoechst 33258 nuclear stain (0.015 mg/ml stock solution diluted to 0.001 mg/ml; Boehringer Mannheim) for 20 minutes at room temperature.

For secondary sphere colonies generated from primary clonal microwell cultures in various concentrations of EGF or FGF2 (see above), triple labelling was performed. The procedure was identical to the above described double-labelling procedure except that the secondary antibody used to detect GFAP-positive cells was an AMCA goat anti-rabbit antibody (1:200 dilution; Jackson ImmunoResearch) and counterstaining with Hoechst was

not performed. All coverslips were rinsed for 5 minutes in PBS (3x) and mounted in Fluor-mount. Fluorescence was visualized using a Nikon Microphot microscope. Secondary antibody-only control coverslips were processed simultaneously using the identical protocol except dilution solutions were devoid of primary antibodies. All secondary controls were negative for staining.

Limiting dilution analysis

Limiting dilution analysis was performed according to Bellows and Aubin (1989). Cells isolated from the E14.5 striatal germinal zone were plated in 96-well microwell plates containing either EGF (20 ng/ml), FGF2 (10 ng/ml) + heparin, or EGF + FGF2 + heparin. Cell numbers were adjusted to give a starting concentration of 4000 cells/ml from which serial dilutions were made. Final cell dilutions ranged from 500 cells per well to 1 cell per well in 0.2 ml aliquots. Cultures were left undisturbed for approximately 7 days after which time the fraction of wells not containing sphere colonies for each cell plating density was calculated and those points were plotted against the number of cells plated per well. The number of cells required to form one colony, which reflected the proportion of neural stem cells in the entire population, was then determined from the point at which the line crossed the 0.37 level. That is, $F_0 = e^{-x}$, where F_0 is the fraction of wells without colonies and x is the mean number of cells per well. Based upon a Poisson distribution of cells, $F_0 = 0.37$ corresponds to the dilution at which there is one neural stem cell per well.

Results

Neural stem cells differentially proliferate in response to EGF and FGF depending on embryonic age and forebrain region

Given that neural stem cells are the lineage precursors to neuronal and glial progenitors, they are likely to be present in the GZ prior to E14 since postmitotic neurons

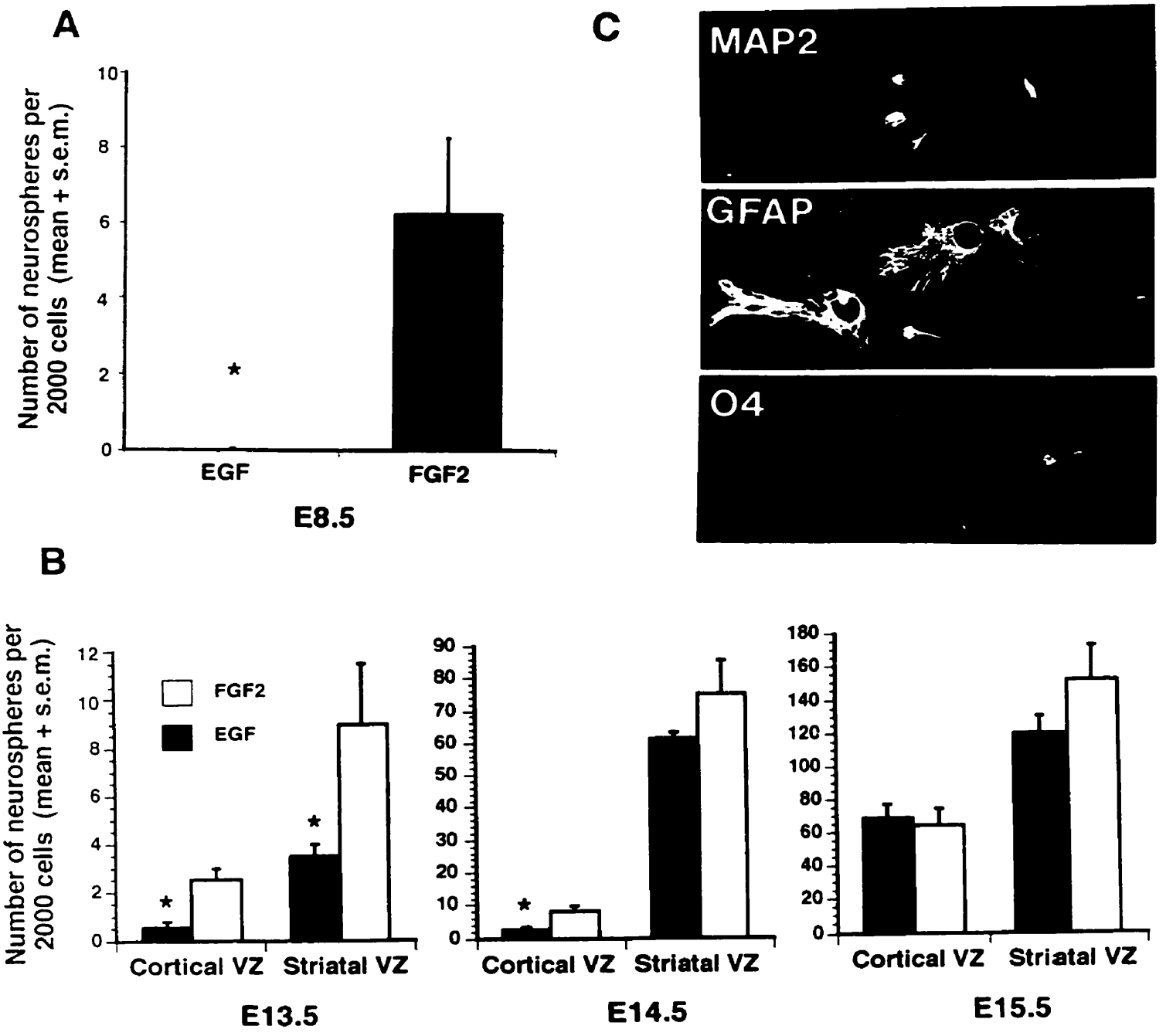
are present in the mouse forebrain as early as E11 (Smart and Smart, 1982). Neural stem cells isolated from the E14 striatal GZ have been shown to proliferate in response to EGF and FGF2 in serum-free culture conditions to give rise to clonal sphere-like colonies of undifferentiated neural precursors (refered to as neurospheres in the figures) (Reynolds et al., 1992; Vescovi et al., 1993). E14 neural stem cells have the classical properties of self-renewal and multipotentiality (Reynolds and Weiss, 1996). To determine if neural stem cells are present at very early stages of development, prior to neural tube formation, anterior neural plate tissue was isolated from E8.5 embryos and plated in the presence of EGF or FGF2. Neurospheres were generated from E8.5 anterior neural plate tissue in the presence of FGF2 alone, but not in EGF alone (Figure 3.1A). Increasing the concentration of EGF from 20 ng/ml to 80 ng/ml did not cause colonies to form; whereas, increasing the concentration of FGF2 to 80 ng/ml did not significantly increase the number of colonies generated compared to 10 ng/ml of FGF2 (data not shown).

Single FGF2-generated primary colonies from E8.5 anterior neural plate were capable of generating secondary colonies when passaged in either EGF (3.86 ± 1.0 secondary colonies generated from a single primary colony, n=7) or FGF2 (5.42 ± 1.3 secondary colonies generated from a single primary colony, n=7). This suggests that the cells isolated at E8.5 are not only capable of self-renewal in FGF2, but also that single FGF-responsive cells can give rise to stem cells capable of generating sphere colonies in EGF. Based on our estimate of the numbers of viable cells isolated and plated from a single embryonic anterior neural plate dissection (~5000; see Materials and Methods), the estimated frequency of neural stem cells that proliferate in the presence of FGF2 at this early developmental age was approximately 0.3% in the anterior neural plate. Thus, self-renewing neural stem cells are present in the developing nervous system as early as E8.5 and are competent to proliferate in response to FGF2, but not EGF. Furthermore, FGF-responsive stem cells at E8.5 appear to be the lineage precursors to EGF-responsive stem cells.

Since neural stem cells are not responsive to EGF at E8.5, but do proliferate in response to EGF at E14.5, we asked if the growth factor responsiveness of neural stem cells was dependent on the developmental stage and spatial localization within the forebrain GZ (i.e. the cortical and striatal germinal zones, the major E14.5 forebrain derivatives of the anterior neural plate germinal zone). Neural tissue was isolated from the striatal GZ and cortical GZ at E13.5, E14.5 and E15.5. At E13.5 the numbers of colonies generated per numbers of cells plated from both the cortical GZ and striatal GZ in the presence of EGF was significantly less than the numbers of colonies generated in the presence of FGF2 (Figure 3.1B). By E14.5, more colonies generated per numbers of cells plated in FGF2 compared to EGF from the striatal GZ; however, significantly fewer EGF-generated colonies than FGF2-generated colonies were isolated from E14.5 cortical GZ. At E15.5, the numbers of colonies generated from both the cortical GZ and striatal GZ were not significantly different in the presence of either EGF or FGF2 (Figure 3.1B). Therefore, FGF2-responsive neural stem cells are present before EGF-responsive neural stem cells in both the striatal and cortical GZs. By E15.5, however, the same frequencies of EGF- and FGF2-responsive neural stem cells are found within the striatal GZ or within the cortical GZ.

To determine if the single cells giving rise to the colonies were in fact multipotential, individual colonies were plated down on coverslips and cultured in the presence of 10% fetal bovine serum (FBS), or in similar concentrations of EGF or FGF2 as the primary or secondary colony cultures without FBS (see below) to allow for differentiation. After 6 days in vitro, the cultures were processed for immunocytochemistry to detect neuronal and glial cell types. Under these conditions, single colonies generated from each of the E8.5 anterior neural plate and the E14.5 striatal GZ and cortical GZ each contained neurons ($MAP2^+$), astrocytes ($GFAP^+$) and oligodendrocytes ($O4^+$) (Figure

Figure 3.1. Neural stem cells are differentially responsive to EGF and FGF2 at different embryonic ages of telencephalic development. (A) Neural stem cells isolated from the E8.5 anterior neural plate proliferate to form neurospheres after 7 days in vitro in the presence of FGF2 (10 ng/ml; n=22 embryos) and heparin, but not in the presence of EGF (20 ng/ml; n=20 embryos). Cells plated at an average density of 10 cells/μl (0.5 ml/well) from at least three separate experiments. (B) Neurospheres generated in EGF (20 ng/ml; n=6 embryos) or FGF2 (10 ng/ml; n=6 embryos) and heparin from striatal and cortical GZ cells isolated at E13.5, E14.5 and E15.5 and plated in duplicate at a density of 10 cells/μl (0.2 ml/well). Note that the Y-axes change with age. * indicates $p<0.05$ compared to immediately adjacent bar. (C) Representative triple-immunocytochemical labeling for neurons (anti-MAP2$^+$), astrocytes (anti-GFAP$^+$) and oligodendrocytes (anti-O4$^+$) derived from a single E14.5 neurosphere cultured for 6-7 days in serum-free media and then allowed to differentiate for a further 6-7 days in media containing 10% FBS. All three cell types are present in neurospheres generated from either the E8.5 anterior neural plate or the E13.5-E15.5 telencephalic GZ. Scale bar=25μm.


A
Number of neurospheres per 2000 cells (mean + s.e.m.)
10
8
6
4
2
0
EGF
FGF2
E8.5
C
MAP2
GFAP
O4
B
Number of neurospheres per 2000 cells (mean + s.e.m.)
FGF2
EGF
12
10
8
6
4
2
0
Cortical VZ
Striatal VZ
E13.5
90
80
70
60
50
40
30
20
10
0
Cortical VZ
Striatal VZ
E14.5
180
160
140
120
100
80
60
40
20
0
Cortical VZ
Striatal VZ
E15.5

3.1C). These findings indicate that multipotential neural stem cells are present at the earliest stages of development, and that FGF-dependent proliferation of neural stem cells occurs earlier in embryogenesis than the EGF-dependent proliferation of these cells.

Overlapping expression of EGF-receptor, FGF-receptors and nestin in the cells of neural stem cell-derived sphere colonies

Since both the EGF-receptor (EGFR) and the FGF-receptor-1 (FGFR1) are expressed in the E14 forebrain germinal zone it was of interest to determine if each of the EGF-derived and FGF-derived colonies expressed a single receptor or both receptors. There is indirect evidence that the in vivo distributions of these two receptors in the E14.5 germinal zone are not completely overlapping. Eagleson et al. (1996) reported that the greatest intensity of EGFR immunostaining was observed in the subventricular zone, while others report intense FGFR1 mRNA levels in the ventricular zone (Wanaka et al., 1991), suggesting that subpopulations of neural precursors may express only one type of receptor in slightly different regions of the forebrain germinal zone. In order to determine if neural stem cells isolated in EGF or FGF2 expressed EGFR, FGFR1 or both, colonies were generated in the presence of either EGF or FGF2. After 6 days in culture, 14 μm cryosections from single colonies were obtained and processed for EGFR, FGFR1 and nestin immunocytochemistry.

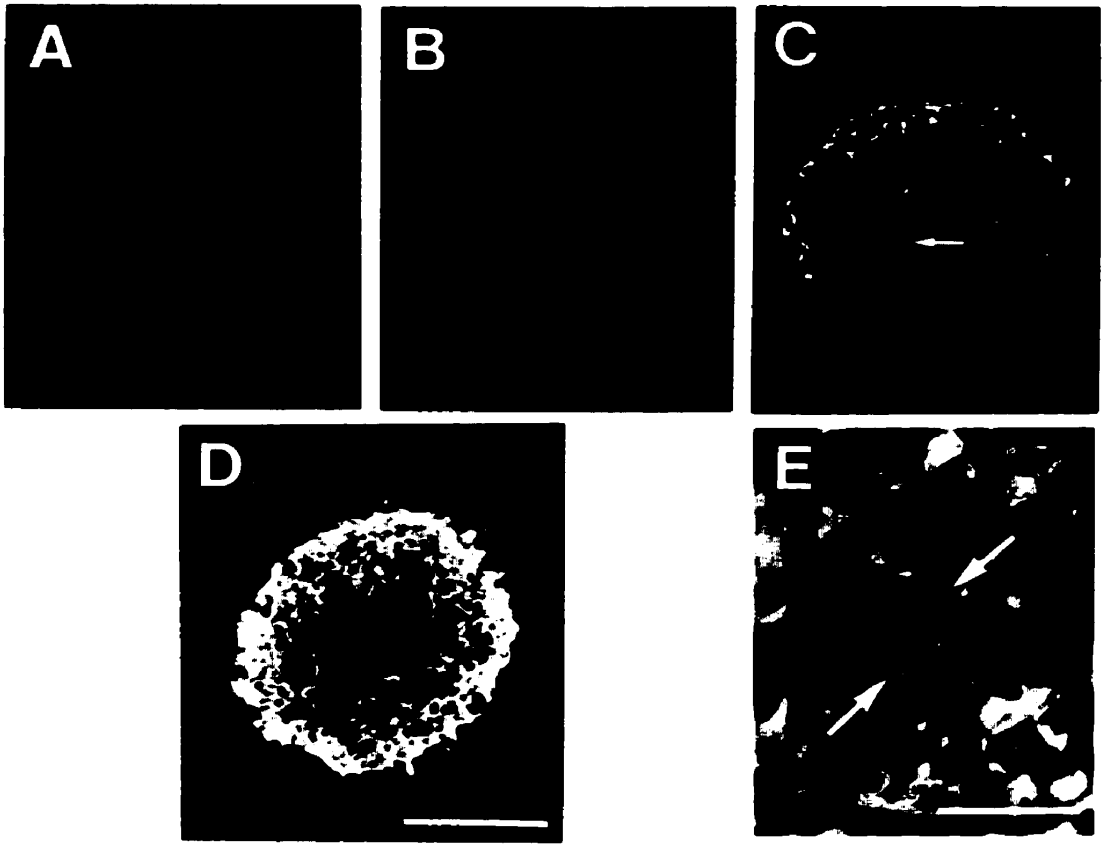
Single sphere colonies from a primary culture can be dissociated into single cells whereupon a small percentage of these cells generate new colonies (Reynolds and Weiss, 1996). Thus, only a small percentage of all of the cells within a colony are neural stem cells, thought to be derived from the symmetrical division of the initial stem cell, and the remaining cells are neural progenitor cells. Since the intermediate filament protein nestin is expressed in all neural precursor cells (Lendahl et al., 1990), we reasoned that a qualitative analysis of the expression pattern of the two growth factor receptors and nestin within a single colony generated in either EGF or FGF2 would be useful in identifying any

precursor cells, some of which are stem cells, within a colony that express only one or both growth factor receptors. The analysis revealed that virtually all of the cells within a single colony express nestin regardless of the growth factor conditions used to generate the colonies (Figure 3.2D). These results are consistent with previous reports that virtually all cells within sphere colonies generated from the adult subependyma express nestin (Gritti et al., 1996). The expressions of the EGFR (Figure 3.2A) and of FGFRs (Figure 3.2B) also were ubiquitous throughout the cells in the colonies, similar to nestin. Double labeling revealed that virtually all cells derived from a neural stem cell clone (isolated in either EGF or FGF2) express both EGFR and FGFR (Figure 3.2C). However, there were varying levels of expression of these receptors in the individual cells, and there were a few single cells within each colony which express mostly EGFR or mostly FGFR (Figure 3.2E). The presence of these few cells dominated by the expression of one receptor suggests the possibility that there may be a small number of differentially sensitive neural stem cells that remain undifferentiated and capable of self-renewal. Given that cells in the colonies generated in either EGF or FGF2 express both growth factor receptors, and that colonies can be passaged in either EGF or FGF2 regardless of initial growth factor conditions (data not shown), then we conclude that the stem cell initially responsive to either EGF or FGF2 can subsequently generate more stem cells that are responsive to both mitogens.

The proliferative response of neural stem cells to different concentrations of EGF and FGF2 is dependent upon cell density

Although a single neural stem cell can proliferate to form a sphere colony in vitro, the factors affecting the proliferative behavior of neural stem cells amongst a population of precursors, a situation more akin to the in vivo GZ environment is poorly understood. We examined whether the proliferative response of neural stem cells in the presence of varying concentrations (see Materials and Methods for conversions from molarity to ng/ml) of EGF and FGF2 was cell density-dependent by assaying the number of colonies generated from a primary E14.5 GZ dissection. A dose-response analysis revealed that at high cell densities

Figure 3.2. Expression of nestin, EGFR and FGFR1 in neural stem cell-derived neurospheres. Neurospheres generated from E14.5 VZ tissue cultured for 6 days in vitro in the presence of either EGF (20 ng/ml) or FGF2 (10 ng/ml) and heparin (2 μg/ml) were fixed and cryosectioned at 14 μm and immunocytochemically labeled for EGFR (red) (A), FGFR1 (green) (B) and EGFR+FGFR1 (yellow) (C) nestin (D). The vast majority of cells are nestin$^+$ and double-label for EGFR and FGFR1 regardless of the primary growth factor conditions. (E) Magnified view of arrowed region in (D). Arrows point to cells expressing mostly EGFR (red) or mostly FGFR1 (green). Scale bars A, B, C, D=200μm; E=50 μm.


A
B
C
D
E

(50 cells/μl) and low mitogen concentrations (0.05-2 ng/ml), EGF was an order of magnitude more effective than FGF2 in generating colonies, but at higher concentrations (10-80 ng/ml) both EGF and FGF2 elicited the proliferation of the same maximal number of neural stem cells to form colonies (Figure 3.3A). However, at relatively lower densities (10 cells/μl) and low mitogen concentrations, FGF2 was more effective than EGF in generating colonies and FGF2 generated a higher maximal number of colonies at higher concentrations (Figure 3.3B). Furthermore, the maximal numbers of colonies were more than proportionally higher at the high compared to the low densities. The five fold increase in cell density (from 10 cells/μl to 50 cells/μl) produced a greater than ten fold increase in the number of colonies generated, thus demonstrating cell nonautonomous effects that facilitate neural stem cell proliferation.

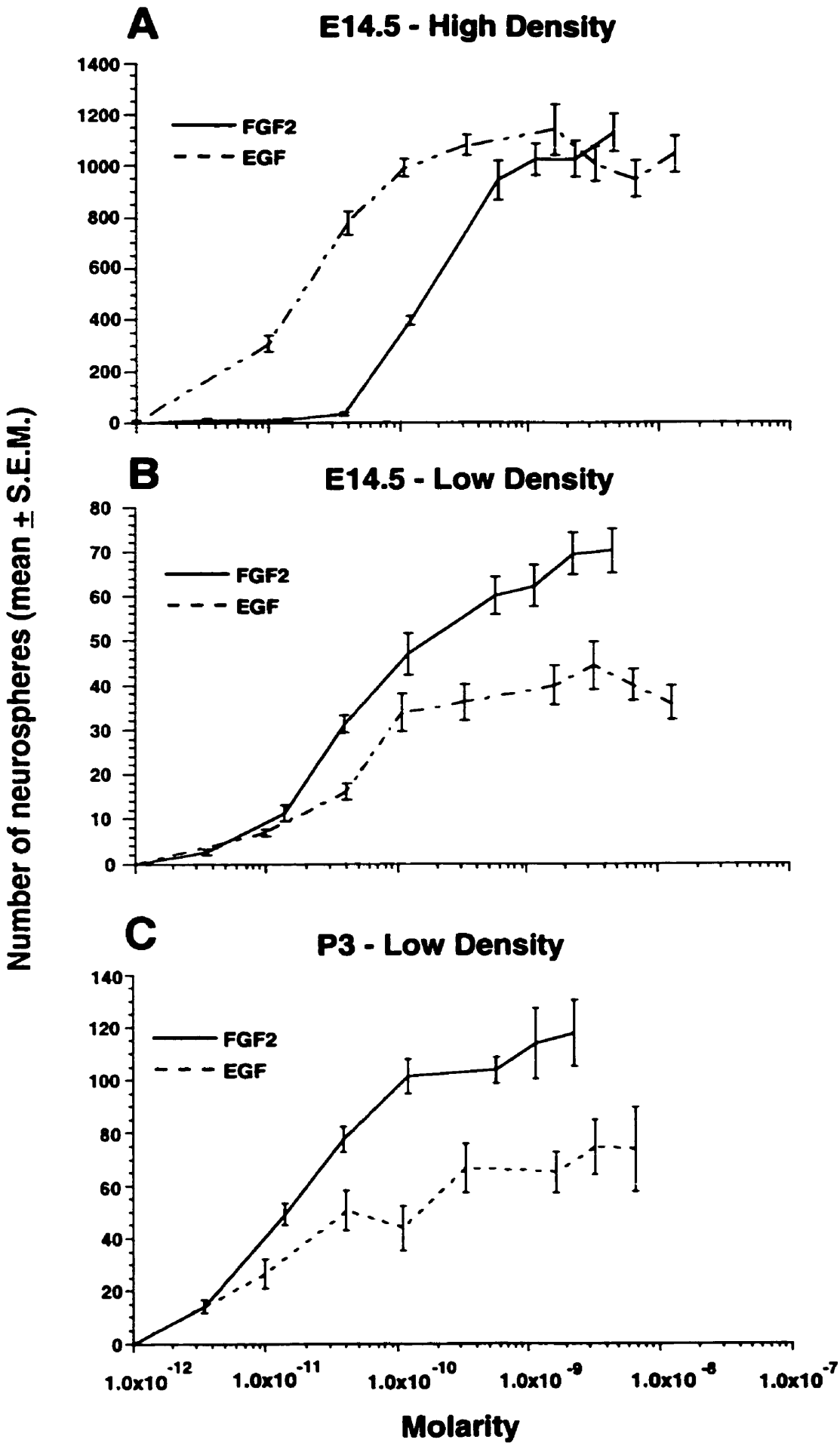
The self-renewing capacity of the stem cells that formed the colonies was assayed for at least one passage by dissociating single sphere colonies (Reynolds and Weiss, 1996) isolated from low (0.6 ng/ml) and high (20 ng/ml) growth factor concentration conditions from both low and high density primary cultures, and counting the number of secondary colonies generated. When single colonies generated from high density cultures (50 cells/μl) in 0.6 ng/ml of EGF or FGF2 were dissociated in the same growth factor conditions, new colonies were generated after ~7 days in either EGF (9.3 ± 1.7, n=8) or FGF2 (6.4 ± 1.8, n=8), respectively. Single sphere colonnies generated in high-density cultures in 20 ng/ml of EGF or FGF2 were dissociated, secondary colonies were generated in either EGF (19.6 ± 3.9, n=8) or FGF2 (20.4 ± 2.4, n=8), respectively. Similarly, in experiments where single colonies generated from low density cultures (10 cells/μl) in 0.6 ng/ml of EGF or FGF2 were dissociated in the same growth factor conditions, new colonies were generated in either EGF (13.2 ± 2.0, n=12) or FGF2 (5.0 ± 1.3, n=12), respectively. Single colonies generated in low-density cultures in 20 ng/ml of EGF or FGF2 were dissociated, secondary colonies were generated in either EGF (16.5 ± 2.6, n=12) or FGF2 (12.8 ± 1.8, n=12), respectively. Thus, single sphere colony passaging data demonstrate that individual

neural stem cells, regardless of cell density and growth factor concentration, can self-renew and expand the stem cell population through symmetrical divisions.

The proportionally greater maximal numbers of colonies generated at high density (EGF or FGF2) reflects a cell nonautonomous potentiation of neural stem cell proliferation at higher densities. Furthermore, the greater maximal numbers of colonies generated in FGF2 at low densities, compared to the same maximal number of EGF- and FGF2-generated colonies at high densities (Figure 3.3B versus 3.3A), indicates that these cell nonautonomous effects may have a greater influence on EGF-responsive stem cell proliferation. The finding that low density cultures appeared to produce a leftward shift from high density cultures in the dose-responsiveness of FGF2 on E14.5 colony formation, compared to the relatively stable EGF dose-responsiveness regardless of cell density, may suggest that FGF2 has a greater affinity for its receptor at lower densities. This may be due to the decreased availability of the FGF2 ligand (or heparin) at higher cell densities, or perhaps due to the down regulation of FGF receptors on stem cells at high cell densities.

The relative number of stem cells present in higher cell density cultures is greater than the number of stem cells in low density cultures. Since a relatively greater number of neural stem cells also can be isolated from older than younger embryonic GZs (see above), it is possible that the interactions between the older stem and progenitor cells may facilitate the observed cell nonautonomous effects in vivo, and that neural stem cells isolated from older GZ tissue and cultured at low densities will behave as neural stem cells isolated from younger GZ tissue and cultured at higher cell densities. In order to determine if the cell density-dependent facilitation observed reflects an age-dependent maturation phenomenon or is a true reflection of stem and progenitor cell interactions dependent only on cell density and not on age, we examined the dose-dependent proliferation of neural stem cells isolated from the postnatal day (P) 3 mouse forebrain at low densities. The results demonstrate that the dose-dependent proliferation of neural stem cells isolated from the P3 GZ and cultured

Figure 3.3 Dose-dependent proliferation of neural stem cells isolated from the E14.5 and P3 forebrain ventricular zone in EGF and FGF2 is sensitive to cell density. Neurospheres generated from neural stem cells isolated from the E14.5 GZ (n = 6 embryos/concentration) and plated at either a density of 50 cells/μl (A) or 10 cells/μl (B), and from neural stem cells from the P3 VZ (n = 6 embryos/concentration) and plated at 10 cells/μl (C) in varying concentrations (molarity) of EGF or of FGF2 with heparin.


A
E14.5 - High Density
FGF2
EGF
1400
1200
1000
800
600
400
200
0
B
E14.5 - Low Density
FGF2
EGF
80
70
60
50
40
30
20
10
0
C
P3 - Low Density
FGF2
EGF
140
120
100
80
60
40
20
0
1.0x10-12
1.0x10-11
1.0x10-10
1.0x10-9
1.0x10-8
1.0x10-7
Molarity
Number of neurospheres (mean ± S.E.M.)

at low densities is similar to the dose-dependent proliferation of neural stem cells isolated from the E14.5 GZ at low densities (Figure 3.3C). These results show that the dose-dependent proliferation of neural stem cells depends on cell density and not on the developmental stage at which stem cells are analyzed.

To further determine that the concentration of exogenous EGF or FGF2 does not select for progenitor cells that are differentially sensitive to varying growth factor concentrations in these serum-free culture conditions, we used microwell culture dishes (see Materials and Methods) and seeded E14.5 striatal GZ cells at clonal densities in various concentrations (0.6, 20, or 80 ng/ml) of either EGF or FGF2 separately. Microwells containing 1-3 cells (6 hours after plating) were followed for 7 days in order to assess colony formation. Single primary sphere colonies generated in these conditions were dissociated and replated in the identical growth factor concentration conditions to determine the self-renewal capacity of the colony forming cells. Individual primary colonies derived from single cells in FGF2 (0.6, 20, or 80 ng/ml) demonstrated the ability to generate new (secondary) colonies upon dissociation in either 0.6 ng/ml (6.6 ± 2.3 secondary colonies from each primary colony, n=12), 20 ng/ml (29.9 ± 7.9, n=12), or 80 ng/ml (73.9 ± 20.4, n=12) of FGF2. Similarly, individual colonies derived from single cells in EGF (0.6, 20, or 80 ng/ml) demonstrated the ability to generate secondary colonies upon dissociation in either 0.6 ng/ml (39.5 ± 9.9, n=8), 20 ng/ml (81.4 ± 24.2, n=11), or 80 ng/ml (83.4 ± 18.2, n=10) of EGF. Moreover, individual secondary colonies from each of these growth factor conditions demonstrated the ability to generate tertiary colonies (in the identical growth factor conditions) after a second passage. Individual secondary colonies derived from single primary colonies in FGF2 (0.6, 20, or 80 ng/ml) demonstrated the ability to generate tertiary colonies upon dissociation in either 0.6 ng/ml (3.0 ± 0.7, n=5), 20 ng/ml (21.5 ± 4.9, n=8), or 80 ng/ml (36.3 ± 16.8, n=6) of FGF2. Similarly, individual secondary colonies derived from single primary colonies in EGF (0.6, 20, or 80 ng/ml) demonstrated the ability to generate tertiary colonies upon dissociation in either 0.6 ng/ml

(12.3 ± 3.2, n=6), 20 ng/ml (35.8 ± 11.2, n=8), or 80 ng/ml (47.4 ± 15.3, n=8) of EGF. All primary, secondary and tertiary sphere colonies generated in 0.6 ng/ml FGF2 (and many, but not all in 0.6 ng/ml EGF) were smaller than the colonies generated in higher growth factor concentrations. Because the majority of the cells within colonies express EGF and FGF receptors (see above), this likely reflects the inability of all of the cells within the colonies to fully proliferate in these low concentration conditions and thus reducing the overall size (and number of stem cell progeny) of the colonies. These results show that single neural stem cells that clonally generate sphere colonies in varying concentrations of growth factors can self-renew.

In order to test that the self-renewing stem cells from different growth factor concentration remain multipotential, single secondary colonies generated from the primary colony cultures (microwell assay) in either EGF or FGF2 (from 0.6, 20, and 80 ng/ml conditions) were allowed to differentiate in the identical growth factor conditions on an adhesive substrate which facilitates differentiation of cells within the colonies (see below). Triple immunolabeling for the presence of neurons ($MAP2^+$ cells), astrocytes ($GFAP^+$ cells) and oligodendrocytes ($O4^+$ cells) demonstrated that in virtually all growth factor concentration conditions, secondary colonies contained neurons, astrocytes and oligodendrocytes (Figure 3.1C). These results are consistent with the immunostaining data (Table 3.1) from low cell density cultures (see below). However, no $O4^+$ oligodendrocytes were observed from secondary colonies generated in 0.6 ng/ml of FGF2 and plated in 0.6 ng/ml of FGF2 (but can form if serum is present; see below). This result is also consistent with the immunolabeling data (Table 3.1) obtained from low-density cultures in the same growth factor concentration conditions without serum. Therefore, neural stem cells proliferate in EGF and FGF2 to form clonally derived sphere colonies in a dose-dependent manner and retain their self-renewing and multilineage potential.

EGF and FGF2 elicits neural stem cell proliferation through independent signaling mechanisms

One possible explanation for the observed cell nonautonomous proliferative effects at high densities may be that in response to the application of exogenous growth factor, neural stem cells regulate their proliferation through the release of endogenous factors in an autocrine/paracrine manner. For example, Kilpatrick and Bartlett (1995) demonstrated that the proliferation of EGF-responsive E17 cortical precursors could be attenuated with the addition of inositol hexakisphosphate, which blocks the binding of FGF2 to the extracellular domain of its receptor, suggesting that the EGF-dependent proliferation of E17 cortical precursors was also dependent upon the endogenous release of FGF2. We asked whether neural stem cell proliferation in the presence of exogenous EGF or FGF2 (the only known critical mitogens for stem cell proliferation) was also dependent upon the endogenous release of either FGF2 or EGF, respectively.

We utilized E14.5 EGF-receptor null ($EGFR^{(-/-)}$) embryos (Figure 3.4A) (Sibilia and Wagner, 1995) and tested whether neural stem cell proliferation in FGF2 was attenuated in an $EGFR^{(-/-)}$ background. The number of sphere colonies generated from high density (50 cells/μl) cultures of E14.5 VZ from $EGFR^{(-/-)}$ mice in the presence of EGF was decreased by greater than 90% compared to littermate $EGFR^{(+/+)}$ controls ($p<0.05$), however the generation of large numbers of colonies in the presence of FGF2 was not affected in these mutants (Figure 3.4B). This result indicates that the endogenous release of EGF was not necessary for FGF-dependent neural stem cell proliferation, but that signaling through the EGFR was critical for EGF-dependent proliferation.

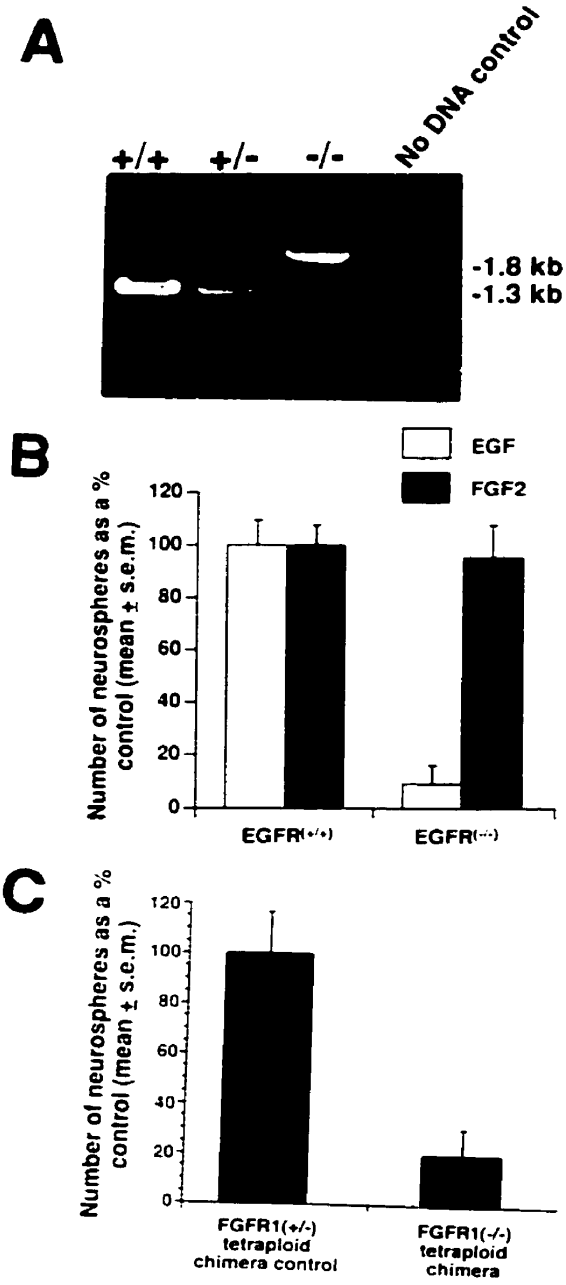
Neural tissue malformation and early embryonic lethality precluded an analysis of EGF- and FGF-dependent neural stem cell proliferation in $FGFR1^{(-/-)}$ E14.5 mouse GZ (Deng et al., 1994; Yamaguchi et al., 1994). However, utilizing tetraploid chimeric mutant mice ($ROSA26\text{-}FGFR1^{(-/-)}$) with a transient rescue of the early embryonic phenotype (due to the tetraploid dominance of extraembryonic tissue) (Ciruna et al., 1997), we observed a

large attenuation (approximately 80% decrease) in the number of neural stem cells proliferating in the presence of FGF2 from at E8.5 (Figure 3.4C). The lack of a complete attenuation in colony formation in the ROSA26-FGFR1$^{(-/-)}$ mutant may indicate that FGF2 may also produce a minor activation of other members of FGF receptor family at this early stage of development. Nonetheless, signaling through the FGFR1 is critical for most FGF2-dependent neural stem cell proliferation.

In order to more clearly define the role for endogenous growth factor release, we further tested if the secondary release of FGF2 upon EGF stimulation, and vice versa effected neural stem cell proliferation by blocking FGF2 signaling with an anti-FGF2 neutralizing antibody (Matsuzaki et al., 1989) which prevents the ligand from binding to its receptor, and blocking EGF signaling with a synthetic EGFR inhibitor (PD 153035) which selectively disables EGF signaling by binding to the tyrosine kinase domain of the EGFR (Fry et al., 1994). The number of colonies generated from high density (50 cells/μl) cultures of E14.5 GZ in the presence of FGF2 and anti-FGF2 antibody (1.25 μg/ml) was decreased by almost 100% ($p<0.05$), compared to the number of colonies generated in the presence of FGF2 and a non-specific control antibody (matched for species and isotype similarity) (Figure 3.5A). However, there was no difference in the number of colonies generated in the presence of EGF and the anti-FGF2 antibody, compared to controls in EGF (Figure 3.5A). The EGFR inhibitor (0.16 μM) did not block neural stem cell proliferation in the presence of FGF2, but was effective in eliminating neural stem cell proliferation in the presence of EGF (Figure 3.5B). These results show that EGF and FGF2 stimulate neural stem cell proliferation in high-density cultures through completely independent signaling mechanisms.

To determine if the cell nonautonomous effects that increased neural stem cell proliferation at high cell densities obscured any putative endogenous EGF or FGF2 signaling in response to exogenous growth factor, we tested the effects of blocking EGF

Figure 3.4 Neural stem cells proliferate independently of EGFR signaling in response to FGF2, but FGFR1 signaling is critical for FGF2-dependent neural stem cell proliferation. (A) The panel shows PCR analysis of genomic DNA isolated from E14.5 EGFR (+/+), (+/-) and (-/-) mice. The expected mutant (1.8 kb) and wildtype (1.3 kb) bands are indicated. (B) Neurospheres generated (mean as a percent control ± S.E.M.) from neural stem cells isolated from the E14.5 GZ of EGFR (-/-) (n=5) and EGFR (+/+) (n=6) embryos and plated at 50 cells/μl in the presence of EGF (20 ng/ml) (white bars) or FGF2 (10 ng/ml) with heparin (black bars). The 100% control represents the numbers of neurospheres generated from EGFR (+/+) embryos in either EGF or FGF2 and heparin. (C) Neurospheres generated (mean as a percent control ± S.E.M.) from neural stem cells isolated from the E8.5 anterior neural plate of ROSA26-FGFR1(+/-) tetraploid chimeric (controls; n=24) or ROSA26-FGFR1(-/-) tetraploid chimeric (n=25) embryos and plated at 10 cells/μl in the presence of FGF2 (10 ng/ml) with heparin.


A
+/+
+/-
-/-
No DNA control
-1.8 kb
-1.3 kb
B
EGF
FGF2
Number of neurospheres as a % control (mean ± s.e.m.)
120
100
80
60
40
20
0
EGFR(+/+)
EGFR(-/-)
C
Number of neurospheres as a % control (mean ± s.e.m.)
120
100
80
60
40
20
0
FGFR1(+/-) tetraploid chimera control
FGFR1(-/-) tetraploid chimera

signaling (EGFR inhibitor) or FGF2 signaling (anti-FGF2 antibody) in lower cell density cultures (10 cells/μl), where the influence of these cellular interactions on colony formation is less. A similar result was observed when the same experiment was performed at cell plating densities of 1 cell/μl, a condition that diminishes the cell nonautonomous effects even further (data not shown). Therefore, the results demonstrate that even at low cell density, secondary release of FGF2 in response to EGF (Figure 3.5C) or secondary release of EGF in response to FGF2 (Figure 3.5D) does not contribute to the numbers of neural stem cells that proliferate to form colonies in vitro. Thus, the facilitative cell nonautonomous effects at either high (50 cells/μl) or lower (10 cells/μl) cell densities are likely due to additional unknown endogenous factors and not due to endogenous EGF or FGF2 signaling. High cell density E14.5 GZ cultures sometimes results in a very small number of neural stem cells clonally proliferating to form neurospheres in the presence of serum-free media without exogenous growth factors (3.8 ± 1.2, n=6), which is never observed at lower cell densities. Thus, although the unknown mitogenic factor(s) that mediate this proliferation are not sufficient alone to account for the robust facilitative cell nonautonomous effects observed in the presence of exogenous EGF or FGF2, this does not preclude the possibility that these unknown factors may interact with the exogenous growth factors to substantially enhance neural stem cell proliferation.

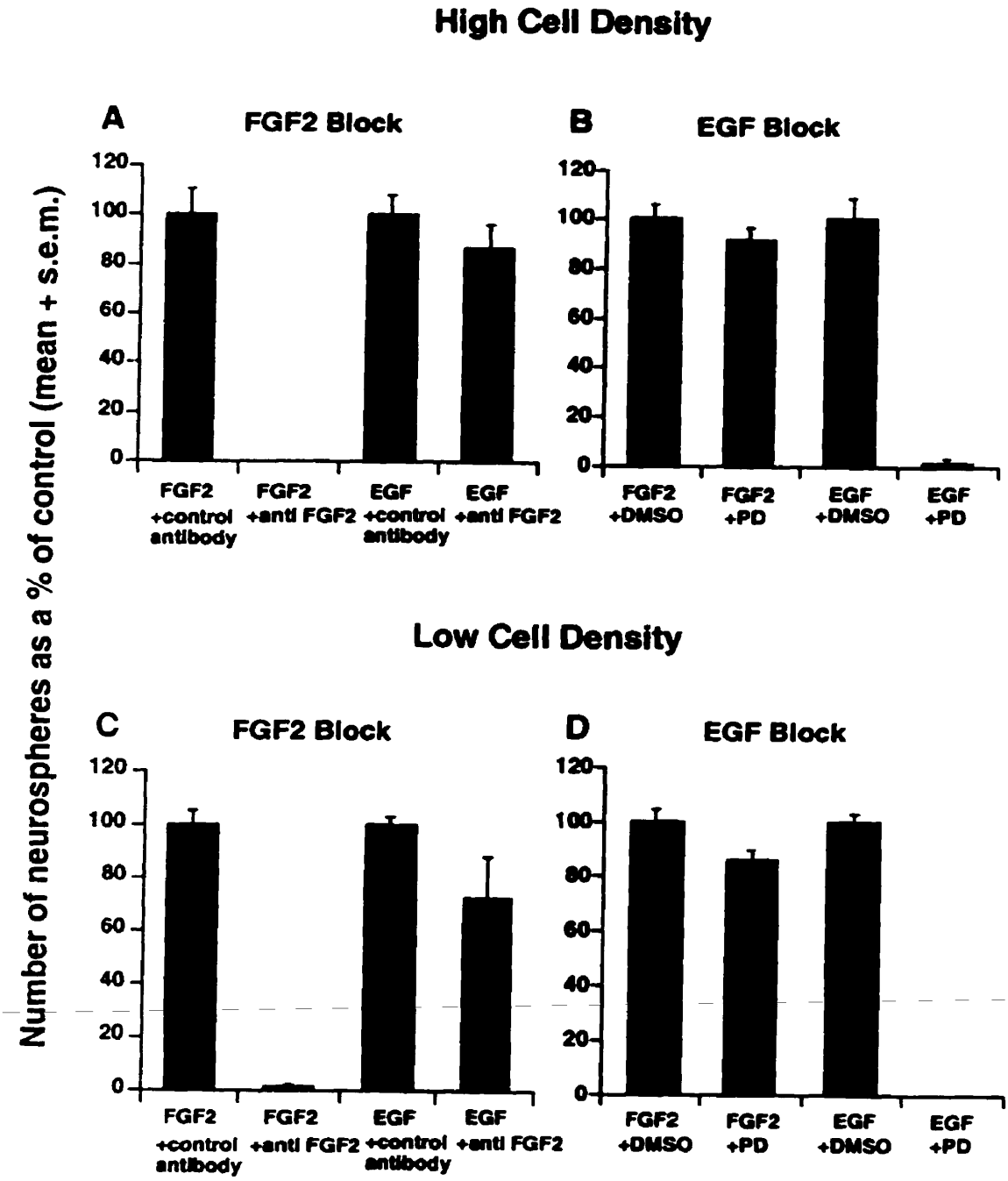
Growth factor concentration has little effect on the relative fates of neural stem cell progeny

It has been shown previously that the levels of growth factor receptors and their ligands are dynamic throughout development. For example, levels of FGF2 increase during embryogenesis (Powell et al., 1991; Nurcombe et al., 1993; Weise et al., 1993). The present findings that the numbers of neural stem cells isolated from the developing telencephalon increase during development and that growth factor concentration can

modulate neural stem cell proliferation even at one stage of development suggests that growth factor concentration may regulate neural stem cell proliferation in vivo. Having shown that neural stem cell proliferation is influenced by EGF and FGF2 concentration we investigated whether growth factor concentration had any influence on the fates of the stem cell-derived progeny.

Previous work has revealed that FGF2 influences the types of progeny generated from E10 cortical precursor cells in a concentration-dependent manner (Qian et al., 1997). When our neural stem cell-derived colonies are plated on an adhesive substrate, then the cells will adhere and in the presence of fetal bovine serum will differentiate into neurons, astrocytes and oligodendrocytes expressing phenotype-specific markers. Sphere colonies that were generated from the E14.5 striatal GZ in the presence of optimal concentrations of either EGF (20 ng/ml) or FGF2 (10 ng/ml), or low concentrations of either growth factor (0.6 ng/ml) demonstrated multipotentiality when cultured in the presence of media containing fetal bovine serum (data not shown). In order to address the effect of EGF and FGF2 on the generation of neurons and glia independent of any unknown factors present in serum, we isolated EGF- and FGF-responsive neural stem cells (at low cell densities) in either high or low concentrations of growth factor and then allowed for differentiation in the same serum-free conditions. The data show that independent EGF- and FGF-responsive primary neural stem cells both demonstrate multilineage potential, regardless of growth factor concentration (Table 3.1). These results are consistent with the results from the growth factor dose-dependent clonal analyses (see above). The proportion of neurons and oligodendrocytes decreased in the presence of lower concentrations of either EGF or FGF2, but the proportion of astrocytes (approximately 55%) remained constant. In low concentrations of FGF2 the percentage of $O4^+$ oligodendrocytes in the sampled cells was zero. However, since there is an obvious trend for a reduction in differentiated oligodendrocytes in low concentrations of both EGF and FGF2, we suggest that these conditions were likely not permissive for oligodendrocyte differentiation. Certainly, sphere

Figure 3.5 Endogenous release of EGF or FGF2 does not influence neural stem cell proliferation. The number of neurospheres generated from neural stem cells isolated from the E14.5 GZ in the presence of the anti-FGF2 antibody (1.25 μg/ml) is substantially reduced in the presence of FGF2 (10 ng/ml) with heparin compared to control (conAb and FGF2 + heparin), but not in the presence of EGF (20 ng/ml) compared to control (conAb and EGF), at 50 cells/μl (A) or 10 cells/μl (C) plating densities. The number of neurospheres generated in the presence of the EGFR inhibitor (0.16 μM PD) is also substantially decreased in the presence of EGF compared to control (DMSO + EGF), but not in the presence of FGF2 and heparin compared to control (DMSO + FGF2 + heparin), at 50 cells/μl (B) or 10 cells/μl (D). N=6 embryos for each growth factor condition group at both high and low cell densities.


High Cell Density
A
FGF2 Block
B
EGF Block
Number of neurospheres as a % of control (mean + s.e.m.)
120
100
80
60
40
20
0
FGF2 +control antibody
FGF2 +anti FGF2
EGF +control antibody
EGF +anti FGF2
FGF2 +DMSO
FGF2 +PD
EGF +DMSO
EGF +PD
Low Cell Density
C
FGF2 Block
D
EGF Block

colonies generated in low concentrations of FGF2 and allowed to differentiate in the presence of low concentrations of FGF2 and 10% FBS also produce a very small percentage of $O4^+$ cells (data not shown). Colonies plated in FGF2 generate more neurons compared to colonies plated in EGF (Table 3.1), which may suggest that FGF2-responsive stem cells are specified to produce more neurons than EGF-responsive stem cells. However, we can not rule out the perhaps more likely possibility that the growth factors were influencing the progeny of the stem cells (rather than the stem cells themselves) in a selective manner. Thus, although neural stem cell proliferation is regulated by growth factor concentration, both EGF- and FGF-responsive neural stem cells maintain their multilineage potential in the presence of varying growth factor concentrations.

Neural stem cell proliferation is additive with the combination of EGF and FGF2 at low cell density

Neural stem cells present in the E14.5 GZ have EGF and FGF2 receptor signaling capacity, and either factor alone is sufficient to activate neural stem cell proliferation in serum-free, defined media. To determine directly the sensitivity of neural stem cell proliferation to EGF or FGF2, we assayed the numbers of colonies generated in the presence of EGF, FGF2 and the combination of both mitogens at very low cell plating densities. The numbers of neurospheres generated in the presence of FGF2 was greater than the number of neurospheres generated in the presence of EGF from 1 cell/μl to 10 cells/μl (Figure 3.6A). This observation is consistent with the previous finding that at a lower cell density (10 cells/μl) and optimal concentrations of EGF (20 ng/ml) and FGF2 (10 ng/ml) a greater number of colonies are generated in FGF2. Furthermore, from 1 cell/μl to 10 cells/μl the number of colonies generated in the combination of EGF and FGF2 was additive (Figure 3.6A). Conversely, the numbers of colonies generated in EGF or FGF2 at optimal concentrations and high cell plating density (50 cells/μl) were equivalent (Figure

Table 3.1. The relative proportions of neuronal and glial progeny from neural stem cells isolated in different growth factor concentrations. Neural stem cells isolated from the E14.5 GZ in different concentrations of EGF or FGF2 were cultured for 6 days in vitro and then transferred to coated coverslips and allowed to differentiate for a further 7 days in the same growth factor conditions. Coverslips were processed for MAP2 (neurons), GFAP (astrocytes) and O4 (oligodendrocytes) using immunocytochemistry.

Isolated and Differentiated in	% neurons (n)	% astrocytes (n)	% oligodendrocytes (n)
20 ng/ml EGF	6.1+0.7 (4)	56.3+6 (6)	2.6+0.3 (3)
0.6 ng/ml EGF	2.6+0.3 (6)	54.1+3.7 (6)	0.7+0.2 (6)
10 ng/ml FGF2	15.4+2 (5)	56.4+5.3 (6)	1.9+0.3 (5)
0.6 ng/ml FGF2	11.7+2.5 (6)	57.8+7.9 (4)	0.0+0.0 (6)

3.3A). We further examined the role of facilitative cell nonautonomous effects on neural stem cell proliferation over the lower range of cell densities (1 versus 10 cells/μl) by plating the same small absolute number of cells (2000) in different volumes. Neural stem cell proliferation was increased more than 10 fold in EGF and in FGF2 at the higher density when the same absolute numbers of cells were plated in different volumes (Figure 3.6B). Therefore, all of these data suggest that neural stem cells may have a differential sensitivity to EGF and FGF2, which can be revealed over relatively low cell densities, but that is obscured at high cell densities due to additional cell nonautonomous effects produced by the release of unknown endogenous growth factors.

One possible interpretation for the enhanced proliferation of neural stem cells in the presence of combined EGF and FGF2 compared to either factor alone over all of the lower cell densities (Figure 3.6A) is that EGF and FGF2 interact synergistically to elicit a stem cell to divide. To test this possibility, high and low growth factor concentrations were combined to determine if the number of E14.5 neural stem cell-derived colonies was greater than additive (i.e., substantially greater than the estimated number of colonies generated when adding the numbers of colonies seen when only a single high or low growth factor concentration was present). The combination of high and low concentrations of EGF and FGF2 did not appear to interact synergistically to promote neural stem cell proliferation (Figure 3.6C). These data argue that EGF and FGF2 can independently elicit the proliferation of EGF- and FGF-responsive stem cells.

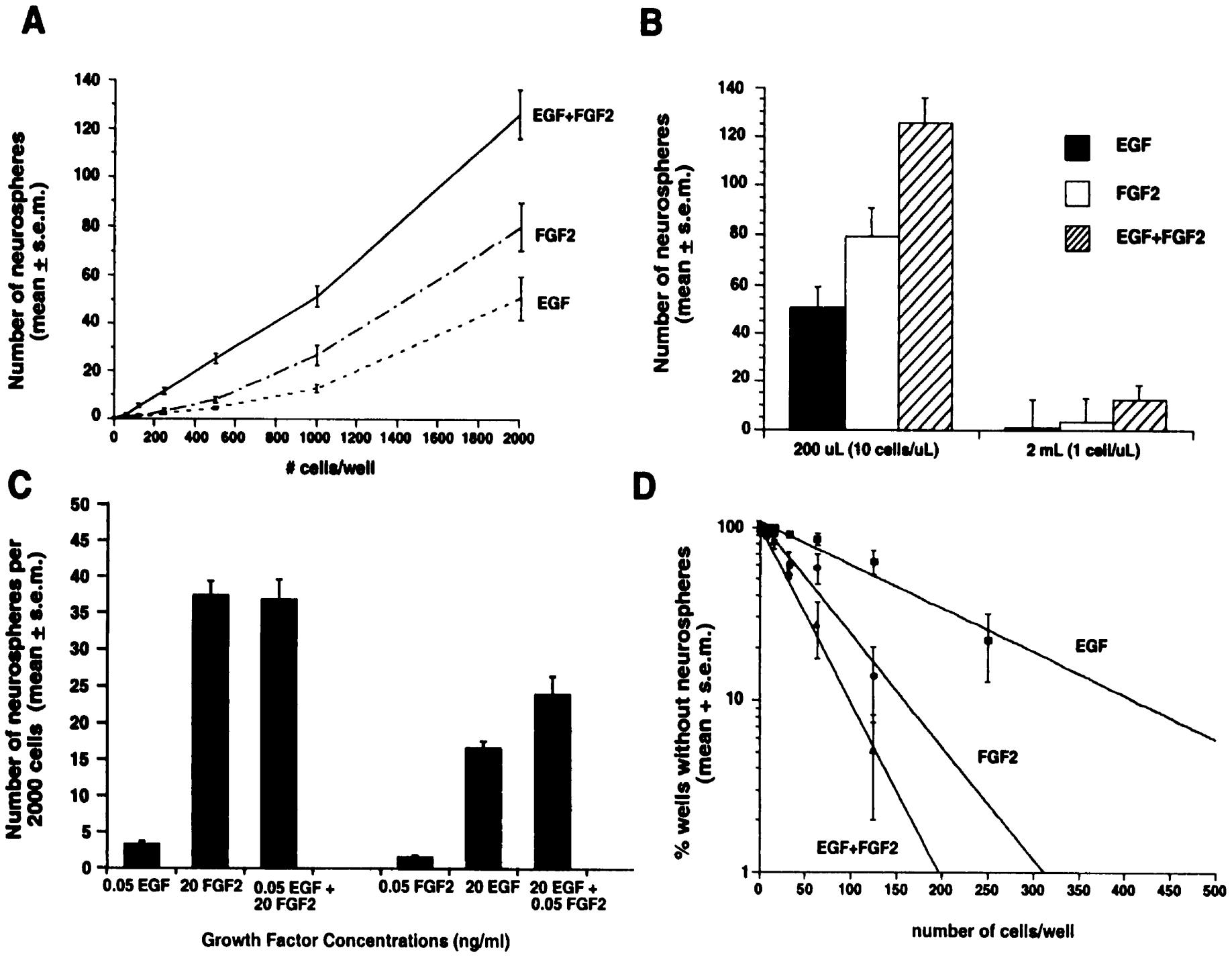
One possible explanation for the additivity in the number of colonies generated at low cell densities in the presence of the combined growth factors (and lack of synergy) is that there are separate populations of neural stem cells that are responsive to either EGF or FGF2. To further test the putative heterogeneity in the neural stem cell population we estimated the minimal frequency of neural stem cells at limiting dilutions. The lack of specific morphological or biochemical criteria for identifying neural stem cells in vivo

presents a major problem when attempting to determine the frequency of stem cells in the GZ. Currently, the best biochemical marker for neural precursor cells is the intermediate filament protein nestin (Lendahl et al., 1990), however it is not specific to neural stem cells and is expressed in other cell types including developing cardiac cells (Zimmerman et al., 1994) and neural progenitors. We took advantage of the neural stem cell colony assay, in which one colony represents the proliferation of a single self-renewing, multipotential neural stem cell (Reynolds and Weiss, 1996), and applied a limiting dilution analysis (Lefkovits and Waldmann, 1984; Sharrock et al., 1990; Bellows and Aubin, 1989) in order to determine the specific frequency of neural stem cells (amongst a population of VZ neural precursors) that proliferate in response to either EGF, FGF2 or the combination of EGF and FGF2. The linear relationship between the percentages of wells without colonies and the numbers of cells plated per well suggest that separate single neural stem cells are proliferating in EGF and FGF2 to give rise to colonies. When plating at limiting dilutions, the proportion of culture wells with no colonies (i.e. negative wells) is defined by the zero term of the Poisson distribution: $F_0=e^{-x}$. The number of cells required to allow for the isolation of one stem cell (x=1) can be calculated using $F_0=e^{-1}=0.37$ or 37%. Based on the Poisson distribution and the intersect at the 37% level, we estimated the minimal frequency of neural stem cells in the E14.5 VZ precursor population to be 0.6% in the presence of EGF, 1.3% in the presence of FGF2 and 2% (additive) in the presence of both EGF and FGF2 combined (Figure 3.6D). These data reveal that there are separate EGF- and FGF-sensitive populations of embryonic forebrain neural stem cells.

EGF-responsive neural stem cells are lineage descendents of FGF2-responsive neural stem cells

The above experiments revealed that EGFR and FGFR1 signaling is critical for EGF- and FGF2-dependent neural stem cell proliferation, respectively, and that FGF- and EGF-responsive neural stem cells may represent two separate subpopulations within the

Figure 3.6 At low cell densities, the frequency of E14.5 neural stem cells varies in EGF and FGF2 alone and proliferation is additive in the presence of EGF plus FGF2. (A) When decreasing numbers of cells were plated in 200 μl of media the number of neurospheres generated in the presence of both EGF (20 ng/ml) and FGF2 (10 ng/ml) with heparin was additive compared to the numbers generated in either growth factor alone at all cell densities tested (equal to and less than 10 cells/μl). (B) When the same absolute number of cells (2000 cells) was plated in different volumes, a substantial facilitative community effect was observed, and at both densities the number of neurospheres generated in both EGF and FGF2 was additive compared to other growth factors alone. (C) When cells are plated (10 cells/μl) in varying combined concentrations of growth factors, EGF and FGF2 do not interact synergistically to promote neural stem cell proliferation. (D) Cells plated at limiting dilution (in 200 μl volumes of media) revealed that the frequency in which at least one neural stem cell will proliferate to form a neurosphere (37% mark on the y-axis) varied in the presence of either EGF (20 ng/ml), FGF2 (10 ng/ml) with heparin, or both EGF and FGF2. N=6-7 embryos for each cell density and growth factor condition for all four experiments.


A
Number of neurospheres (mean ± s.e.m.)
140
120
100
80
60
40
20
0
0 200 400 600 800 1000 1200 1400 1600 1800 2000
cells/well
EGF+FGF2
FGF2
EGF
B
Number of neurospheres (mean ± s.e.m.)
140
120
100
80
60
40
20
0
EGF
FGF2
EGF+FGF2
200 uL (10 cells/uL)
2 mL (1 cell/uL)
C
Number of neurospheres per 2000 cells (mean ± s.e.m.)
50
45
40
35
30
25
20
15
10
5
0
0.05 EGF
20 FGF2
0.05 EGF + 20 FGF2
0.05 FGF2
20 EGF
20 EGF + 0.05 FGF2
Growth Factor Concentrations (ng/ml)
D
% wells without neurospheres (mean + s.e.m.)
100
10
1
0 50 100 150 200 250 300 350 400 450 500
number of cells/well
EGF
FGF2
EGF+FGF2

E14.5 GZ. Because both FGFR1$^{(-/-)}$ embryos and ROSA26-FGFR1$^{(-/-)}$ tetraploid chimeras die by approximately E9.5, in order to determine if a lineage relationship exists between FGF-responsive neural stem cells and EGF-responsive neural stem cells (which normally do not begin to emerge until E11.5-E13.5 in the striatal GZ) we generated diploid chimeras in which wildtype cells (CD1) allowed the embryos with cells harboring an FGFR1$^{(-/-)}$ mutation to survive until E14.5. We hypothesized that if the EGF-responsive neural stem cells arose independently of the FGF2-responsive neural stem cells during development, then an FGFR1 null mutation would not influence the numbers of EGF-responsive stem cells isolated, but would cause an attenuation of the FGF2-responsive stem cells (similar to what was observed from the E8.5 ROSA26-FGFR1$^{(-/-)}$ tetraploid chimera results).

The relative contribution (percent chimerism) of cells from either the ROSA26-FGFR1$^{(-/-)}$ lineage or the ROSA26-FGFR1$^{(+/-)}$ lineage in the diploid chimeras was estimated from histological sections on the basis of β-galactosidase (β-gal) staining (Figure 3.7A). Single hemispheres obtained from E14.5 telencephalons from ROSA26-FGFR1$^{(+/-)}$ diploid chimeras (controls, n=8) demonstrated a mean percent chimerism of 34.8% within the striatal GZ, while those obtained from ROSA26-FGFR1$^{(-/-)}$ diploid chimeras (n=8) demonstrated a mean percent chimerism of 12.4% within the striatal GZ.

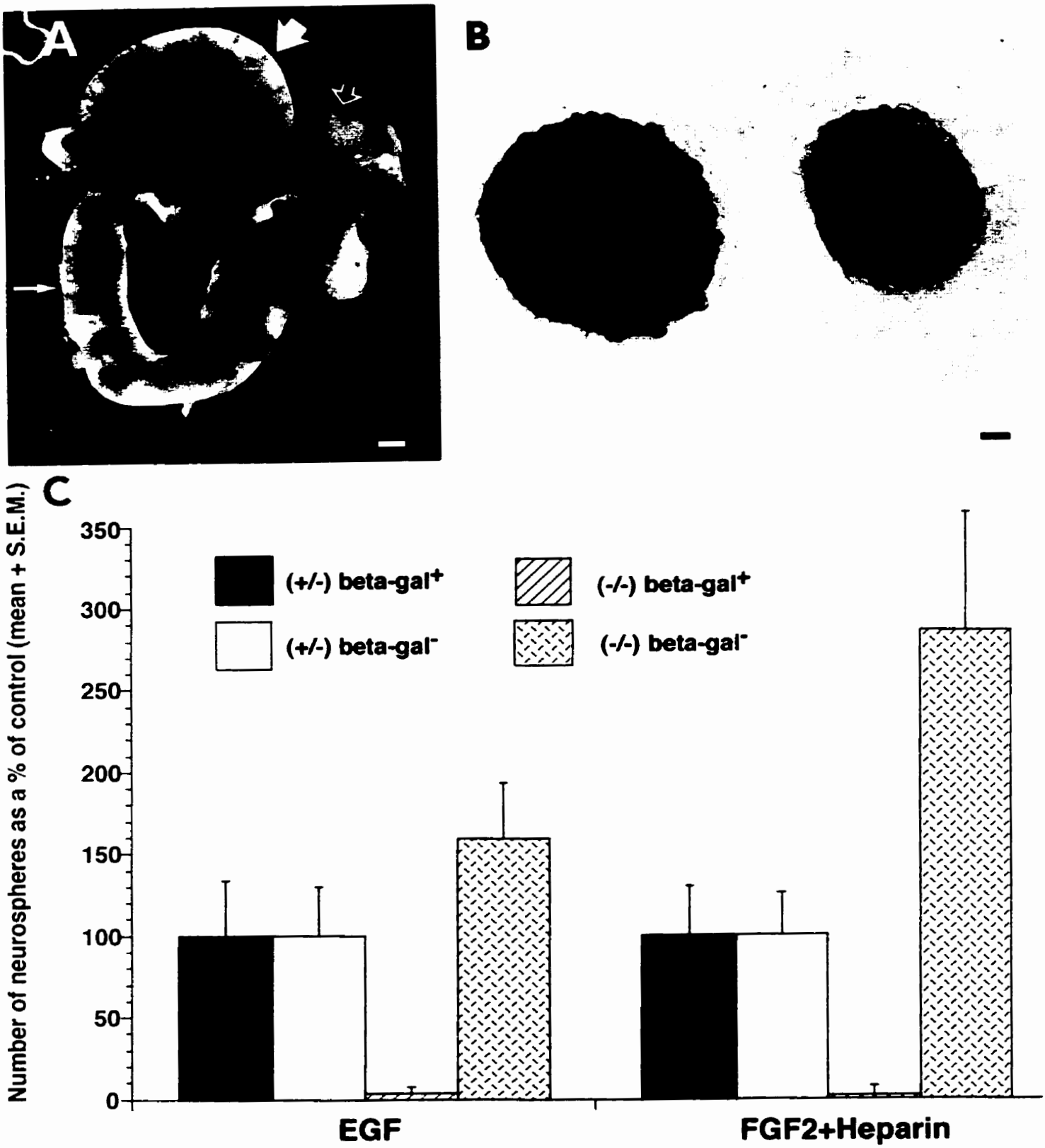
These estimates are consistent with previous chimerism data from diploid chimeric embryos analyzed between E9.5 and E10.5 showing that fewer mutant cells (compared to controls) contribute to anterior neural structures (Ciruna et al., 1997). Cells isolated from the striatal GZs of the opposite hemispheres were cultured in serum-free conditions in the presence of optimal concentrations of either FGF2 (10 ng/ml) or EGF (20 ng/ml). The numbers of β-gal^{+} colonies (containing all blue cells derived from either the ROSA26-FGFR1$^{(-/-)}$ stem cells or derived from the ROSA26-FGFR1$^{(+/-)}$ stem cells) and the numbers of β-gal^{-} colonies (containing all white cells derived from wildtype CD1 stem cells) were quantified after 7 days in culture (Figure 3.7B). The results show that the proliferation of both FGF- and EGF-responsive neural stem cells is decreased by greater than 95% in

E14.5 ROSA26-FGFR1$^{(-/-)}$ diploid chimeras (n=8) compared to ROSA26-FGFR1$^{(+/-)}$ diploid chimeric controls (n=8) (Figure 3.7C). The number of colonies generated in both EGF and FGF2 is enhanced by ~50% and by over two-fold, respectively, from wildtype CD1 derived neural stem cells (β-gal$^-$) in the ROSA26-FGFR1$^{(-/-)}$ diploid chimeras compared to the ROSA26-FGFR1$^{(+/-)}$ diploid chimeric controls (Figure 3.7C). This suggests that in these diploid chimeras, wildtype neural stem cells can partially compensate for the overall reduction in the numbers of neural stem cells in the GZ in vivo by increasing their symmetrical divisions prior to E14.5. However, since there is an overall 50% decrease in the total number of neurospheres generated from the ROSA26-FGFR1$^{(-/-)}$ diploid chimeras (ROSA and CD1 combined) compared to the ROSA26-FGFR1$^{(+/-)}$ diploid chimeric controls (data not shown), this increase in the number of sphere colonies in vitro may only represent a very minor compensatory effect. Thus, these results are consistent with the hypothesis that EGF-responsive neural stem cells are lineage descendents of the FGF-responsive neural stem cells during forebrain development.

Discussion

Simple neural lineage models depict self-renewing, multipotential stem cells giving rise to progenitor cells that are more restricted in their potential to generate differentiated neurons, astrocytes and oligodendrocytes depending on factors that influence either their proliferation, differentiation or survival (Gage et al., 1995; Temple and Qian, 1995; Johe et al., 1996; Weiss et al., 1996). The present data builds upon this fundamental notion to support an alternative model in which separate, lineage-related stem cells are also present in the E14.5 GZ (Figure 3.8). This conclusion is based on the evidence that: (1) only FGF-responsive neural stem cells are present at early developmental stages, but these cells can generate EGF-responsive stem cells when passaged in vitro and EGF-responsive stem cells can be isolated at later developmental stages in vivo; (2) at low cell densities

Figure 3.7 In ROSA26-FGFR1(-/-) diploid chimeras, both FGF2- and EGF-responsive neural stem cell proliferation is diminished from E14.5 striatal GZ cells harboring the FGFR1(-/-) mutation. (A) Lateral view of an E14.5 left hemisphere (rostral end to the left) stained with X-gal depicting the chimeric pattern of cells with either a transgenic ROSA26-FGFR1(+/-) genetic background (β-gal$^+$ cells appear blue with the X-gal histochemical reaction) or a wild type CD1 genetic background (non-staining white cells). Large, solid arrow pointing to the forebrain; open arrow pointing to the midbrain; small, solid arrow pointing to the spinal cord. (B) Neurospheres generated from single E14.5 striatal GZ stem cells after 7 days in vitro in the presence of 10 ng/ml FGF2, and subsequently processed for X-gal staining. Sphere colonies with a ROSA26-FGFR1(+/-) genetic background expressed β-gal and stained blue throughout with the histochemical reaction. The stem cell derived neurosphere with a wild type CD1 genetic background did not express β-gal and did not stain histochemically. Photographs of these two colonies were taken from a single culture. (C) Neural colonies generated from neural stem cells isolated from the E14.5 GZ of ROSA26-FGFR1(+/-) diploid chimeric mice (n=8) and ROSA26-FGFR1(-/-) diploid chimeric mice (n=8) and plated in duplicate at 10 cells/μl in the presence of EGF (20 ng/ml) or FGF2 (10 ng/ml) with heparin. The 100% control represents the numbers of neurospheres generated from ROSA26-FGFR1(+/-) diploid chimeric mice (controls). From these control mice, neurospheres were derived from stem cells with either a ROSA26-FGFR1(+/-) genetic background [black bars, (+/-) beta-gal$^+$] or from stem cells with a wild type CD1 genetic backgroud [white bars, (+/-) beta-gal$^-$] in either EGF or FGF2 and heparin. In ROSA26-FGFR1(-/-) diploid chimerias, neurospheres were derived from stem cells with either a ROSA26-FGFR1(-/-) genetic background [striped bars, (-/-) beta-gal$^+$] or from stem cells with a wild type CD1 genetic background [grey bars, (-/-) beta-gal$^-$]. Scale bars: A=10 mm, and B=100 μm.


A
B
C
Number of neurospheres as a % of control (mean + S.E.M.)
350
300
250
200
150
100
50
0
(+/-) beta-gal+
(+/-) beta-gal-
(-/-) beta-gal+
(-/-) beta-gal-
EGF
FGF2+Heparin

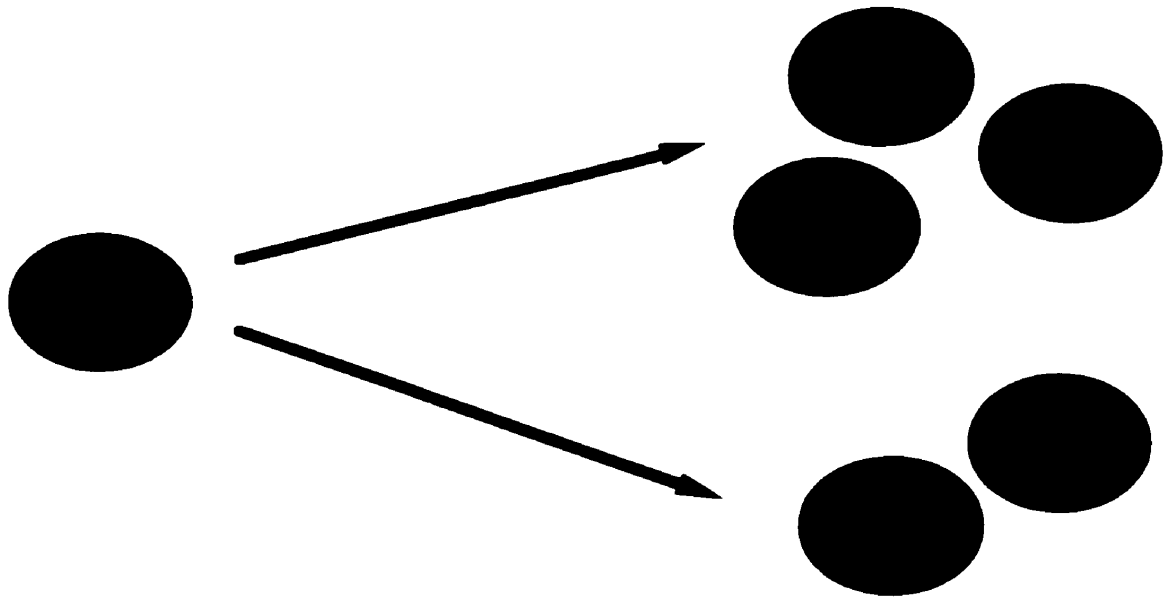
greater numbers of neural stem cells proliferate in response to FGF2 than to EGF; (3) neural stem cell proliferation is additive in the presence of both mitogens; (4) there is a differential frequency of EGF- and FGF-responsive stem cells at limiting dilutions, suggesting that these two stem cell populations are separate; and (5) a mutation in the FGFR1 gene which prevents the expansion of the FGF2-dependent neural stem cells during telencephalic development also prevents the emergence of the EGF-responsive neural stem cells. Unknown endogenous factors (other than EGF or FGF2) can facilitate the proliferation of FGF- and especially EGF-responsive subpopulations to form neurospheres, and at higher cell densities serve to obscure the differences between the EGF- and FGF-responsive stem cells. Despite the presence of separate EGF- and FGF-responsive stem cells, both types are self-renewing and capable of generating neurons, astrocytes and oligodendrocytes.

A neural stem cell that proliferates in response to FGF2 emerges earliest in embryonic forebrain development

The expression of FGFR1 within the E8.5 neural plate indicates that neuroepithelial cells at this early stage of development are competent to proliferate in response to FGF2, the primary ligand for this FGFR subtype (Ornitz and Leder, 1992; Johnson and Williams, 1993). Using the in vitro clonal neurosphere assay for neural stem cells, we showed that only FGF-responsive neural stem cells are present as early as E8.5 within the anterior neural plate, near the onset of neural development in the post-gastrulating mouse embryo. The substantial attenuation of E8.5 neural stem cell proliferation observed in mice lacking a functional FGFR1 (ROSA26-FGFR1$^{(-/-)}$ tetraploid chimeras) suggests that the mitogenic signal provided by FGF2 is primarily mediated by FGFR1. However, FGF2 may also signal through other FGFR family members to cause a small percentage of the FGF2-dependent neural stem cells to proliferate at this early stage of neural development. For example, FGFR2 has been shown to be expressed in the early embryonic GZ (Orr-Urtreger

et al., 1991). The sphere colonies generated in FGF2 can be subcloned in vitro in the presence of EGF or FGF2, suggesting that the FGF-responsive neural stem cells are the lineage precursors to the EGF-responsive neural stem cells. Our evidence from the ROSA26-FGFR1$^{(-/-)}$ mouse diploid chimeric analyses demonstrates that this lineage relationship between FGF-responsive and EGF-responsive neural stem cells also exists in vivo. Since both EGF- and FGF-responsive neural stem cells that contained the FGFR1$^{(-/-)}$ mutation were significantly diminished from the E14.5 striatal GZ, our data strongly suggest that the ontogeny of the EGF-responsive neural stem cell is not independent of the FGF2-responsive neural stem cell, but rather that the FGF2-responsive neural stem cell gives rise to the EGF-responsive neural stem cell during forebrain development. It is possible that the FGF-responsive stem cell population secretes an unknown factor that is necessary to promote the development of an EGF-responsive stem cell population. However, this is unlikely since we would expect that in the diploid chimeric mice, the FGF-responsive stem cells with the wildtype CD1 background would be able to provide this unknown factor to allow the ROSA26-FGFR1$^{(-/-)}$ (or ROSA26-FGFR1$^{(+/-)}$ control) cells to proliferate in response to EGF at E14.5. The neural stem cell-derived colonies in the present study also demonstrate multilineage potential (generating neurons, astrocytes and oligodendrocytes), confirming their stem cell status. There is a ~7-fold increase in the number of neural stem cells (in similar cell density cultures) isolated between E8.5 (anterior neural plate) and E14.5 (striatal GZ) in the present study. The estimated frequency of colony-forming stem cells as a percentage of the total viable GZ cells cultured in vitro increases from ~0.3% at E8.5 to at least 2% at E14.5. Since only the striatal GZ was used to determine stem cell frequency at E14.5 and not the cortical GZ (the other major derivative of the anterior neural plate), the magnitude of the increase in neural stem cell frequency may be underestimated. Nonetheless, these results reveal that the neural stem cell population substantially expands through symmetrical divisions within the GZ throughout the early stages of forebrain development. These initial observations provide a basis for

Figure 3.8 Model of EGF- and FGF2-dependent proliferation of neural stem cells during telencephalic development. The FGF2-responsive neural stem cells at E8.5 are the lineage precursors to the EGF-responsive stem cells, and both of the relatively separate EGF- and FGF2-responsive subpopulations are present in the E14.5 GZ. Self-renewal capacity and multilineage potential is maintained in both subpopulations.



E8.5
Anterior Neural Plate

E14.5
Telencephalic Germinal Zone

FGF2-responsive neural stem cell

EGF-responsive neural stem cell

further examining when and to what extent symmetrical stem cell divisions occur during this time period.

Between E13.5 and E15.5 there is an increase in the EGF responsiveness of neural stem cells isolated from the cortical GZ and striatal GZ. An early FGF2 responsiveness of neural precursors has been observed in multipotential precursors from the E10 telencephalon (Kilpatrick and Bartlett, 1993) and neural stem cells from the E10 spinal cord (Kalyani et al., 1997). Different regions of the developing forebrain are known to have a differential pattern of maturation. There is evidence for caudorostral and ventrodorsal gradients of maturation in the forebrain, with the most rostral (olfactory bulbs) and most dorsal (cortex) regions developing last (Smart and Smart, 1982). The absence of substantial EGF-responsiveness of neural stem cells may reflect the "immaturity" of this telencephalic region. The stronger expression of the EGFR in ventral forebrain regions at early stages is consistent with this possibility (Eagleson et al., 1996). One possible mechanism for the increased responsiveness to EGF may involve the upregulation of EGFR expression. In accordance with this interpretation, Burrows et al. (1997) have shown that very few stem cells present in the E12-E15 rat cortical germinal zone normally proliferate in response to EGF, which is ontologically similar to the E10-E13 mouse cortical germinal zone. However, when the number of EGFRs is increased in these cells using a retroviral construct then neural stem cell proliferation in response to EGF is enhanced at these early ages. This suggests that a critical ratio of ligand:receptor was necessary to elicit EGF-dependent proliferation of neural stem cells that were present, but relatively unresponsive (Burrows et al., 1997). If only a single stem cell population exists, then an enhanced EGF response in the EGFR-transfected cells could be interpreted as the precocious response of a single stem cell to EGF. However, it is equally plausible that if separate EGF- and FGF-responsive stem cell populations were present in the GZ, both types of stem cells can be transfected with the EGFR construct, enhancing their responsiveness to EGF. Indeed, one novel prediction from the present results is that if both

EGF- and FGF-responsive stem cells can be transfected with the EGFR construct, then the additive effects on neurosphere formation at cell low densities in the presence of EGF and FGF2 combined would disappear.

The FGFR1$^{(-/-)}$ mutation is embryonic lethal (between E7.5 and E9.5) and mutant mice demonstrate severe axial patterning defects (Deng et al., 1994; Yamaguchi et al., 1994). Embryos with a less severe phenotype at E8.5 displayed relatively small and disorganized neural folds (Yamaguchi et al., 1994). This suggests that although cells of the dorsal midline ectoderm are specified to become neural, the FGFR1$^{(-/-)}$ neural stem cells present at this early stage of development may be unable to proliferate in response to FGF2 to expand the population, which may be a necessary antecedent to neurogenesis. Our present results reveal that FGFR1 is critical not only for FGF2-dependent proliferation of neural stem cells at E8.5, but also for the expansion of this population throughout forebrain development. The EGFR$^{(-/-)}$ lethal phenotype, on the other hand, was dependent on genetic background, with the age of death ranging from implantation to postnatal day 20 (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995). Epithelial proliferation and differentiation were compromised in many tissues. Neural development in early stages of embryogenesis was relatively spared, but by E18 there appeared to be reduced cortical size in both the cerebrum and cerebellum, with a concomitant GZ expansion (Threadgill et al., 1995). The EGFR$^{(-/-)}$ mutation appeared to affect multiple mechanisms (including cell survival, proliferation, differentiation and migration) regulating neuronal and glial cell types (Sibilia et al., 1998). The present results show that the EGF-induced proliferation of neural stem cells isolated from the EGFR$^{(-/-)}$ forebrain GZ is drastically reduced, but that FGF-responsiveness of neural stem cells in these mutant mice is not affected. Since early neural development is apparently normal in these mutants, these results indicate that EGF-responsiveness of neural stem cells is not necessary at early stages, but may subsequently be required for the production of progenitor cells at later stages of development (as judged by the late neural phenotype in the EGFR$^{(-/-)}$ mice). Furthermore, it will be important to

determine if the progenitor cells produced by forebrain stem cells are dependent upon EGF for the proper production of neurons and glia from older embryonic and early postnatal periods, when the EGF-dependent neurodegeneration is most prevalent (Sibilia et al., 1998).

Separate EGF- and FGF-responsive neural stem cell populations co-exist at later stages of forebrain development

There are two possible mechanisms by which FGF2 and EGF can influence neural stem cell proliferation at different stages of development. The first is that the neural stem cell possesses only FGFR1 signaling at E8.5, but by E14.5-15.5 has developed both EGFR and FGFR1 signaling capacity, either of which alone may be sufficient to activate the neural stem cell. It is conceivable that the differences in EGF- and FGF2-dependent proliferation may actually reflect the ability of these mitogens to regulate later progenitor cell proliferation within a stem cell lineage (sphere colony), rather than differentially influencing two separate stem cell populations to divide. These differences, however, would still support the notion that neural stem cell lineages can be differentially regulated by EGF and FGF2. The second possibility is that the FGF-responsive neural stem cell at E8.5 is the precursor in a lineage to the EGF-responsive stem cell, but that both of the separate EGF- and FGF2-responsive neural stem cells are present in the E14.5-15.5 GZ (Figure 3.8). Indeed, this hypothesis is strongly supported by our analysis of the ROSA26-FGFR1$^{(-/-)}$ diploid chimeras. Although EGFR and FGFR expression appears to be ubiquitous among neural stem cells and their progeny in primary sphere colonies, the variation in receptor expression within individual cells provides at least some initial correlative evidence to suggest that cells which express mostly EGFR or mostly FGFR are maintained as differentially responsive cells, and perhaps some of which may be stem cells with the capacity to self-renew. It is clear from the present results that neural stem cell proliferation in EGF or FGF2 is concentration-dependent, and others have shown that stem

cell proliferation in the presence of EGF also depends on the levels of EGFR (Burrows et al., 1997). The fact that high levels of EGFR are required for some neural stem cells to divide (Burrows et al., 1997) offers the possibility that cells expressing high levels of one receptor proliferate in the presence of ligand in a concentration-dependent manner, but that low levels of the other receptor render the cells unresponsive regardless of ligand concentration.

Previous reported estimates of the frequency of FGF2-responsive multipotential precursor cells in the embryonic forebrain ranged from approximately 4% (at E10) to 5% (at E17) (Kilpatrick and Bartlett, 1995). On the other hand, the frequency of E12 and E14 cortical GZ cells isolated in conditioned media with the characteristics of stem cells was approximately 7% (Davis and Temple, 1994). Limiting dilution analyses revealed that the minimal frequency of neural stem cells in the E14.5 GZ is 0.6% in the presence of EGF, 1.3% in the presence of FGF2 and 2% in the combination of EGF and FGF2, and indicates that stem cells are differentially recruited on the basis of what growth factor is present. The higher frequencies reported by others may have included progenitor cells as well as true stem cells. Alternatively, our estimated frequencies of stem cells using limiting dilution may be low because of the present finding that cell nonautonomous effects of cell density, that are not due to the endogenous release of EGF or FGF2, more than proportionally increase the number of stem cells. Thus, cell-plating density is a critical variable when determining maximal stem cell frequency. Nonetheless, all of these estimates reveal that the stem cell pool makes up only a small percentage of the total GZ precursor population. The present data suggest a large increase in the absolute number of forebrain stem cells between E8.5 and E14.5-15.5, but recent estimates of the numbers of stem cells in the adult forebrain subependyma (Morshead et al., 1998) suggest that the stem cell population may not substantially increase from later embryogenesis to adulthood.

Neural stem cell proliferation is differentially sensitive to EGF, FGF2 and separate cell nonautonomous effects

Neural stem cell proliferation also may be secondarily modulated via cell-cell communication in response to a primary mitogenic signal. The present results reveal that growth factor concentration regulates the proliferation of neural stem cells in a cell density-dependent manner. The secretion of diffusable factors by responsive cells may subserve such cell nonautonomous effects. Furthermore, the concentration of the factor(s) will be greater when many secreting cells are in close proximity than when only a few dispersed cells are present. Therefore, the density of cells plated in a volume of media is critical for enabling endogenous secreted factor(s) to influence neural stem cell proliferation in our serum-free conditions. Others (Hulspas et al., 1997) have demonstrated that the proliferation of neural stem cells is density-dependent even at cell densities ranging from 50-200 cells/μl. Furthermore, Hulspas et al. (1997) showed that by mixing cells of a ROSA26 genetic background with cells of a Balb/c genetic background that virtually all of their stem cell colonies at such high cell densities were clonal (i.e. did not contain a mixture of cells with both genotypes). This was also true in our results with ROSA26-FGFR1$^{(-/-)}$ and ROSA26-FGFR1$^{(+/-)}$ diploid chimeras. When striatal GZ tissue, containing a mixture of ROSA and CD1 cells, was dissociated and cultured at low cell density in vitro, the neural colonies that were generated were either composed of entirely β-gal^{+} (blue) cells or entirely CD1 (white) cells and no mixed clones were observed.

In the present study, we demonstrated that there is a facilitative effect of increased density on the proliferation of both FGF- and EGF-responsive neural stem cell subpopulations, but which may have a greater influence EGF-responsive stem cell proliferation. We have also shown that neural stem cell proliferation (at both high and low cell densities and in the presence of optimal concentrations of exogenous EGF or FGF2) is independent of the endogenous secretion of FGF2 or EGF by neural precursors in response to the primary exogenous mitogenic signal. Therefore, the nature of the observed

cell nonautonomous effects remains to be determined. Proliferation of neuroepithelial cells has been shown to depend on the production of IGF-1, acting itself as a survival factor (Drago et al., 1991). Cellular interactions mediated by secreted factors also may regulate receptor levels. For example, KGF (FGF7) is a potent inducer of TGFα in keratinocytes and causes activation and down modulation of the EGFR (Dlugosz et al., 1994), influencing keratinocyte responsiveness indirectly by activating an EGFR-mediated signal for proliferation. Thus, mitogenic signals from other members of the FGF growth factor family or the upregulation of survival factors, such as IGF-1, may mediate the cell nonautonomous effects, which potentiate the FGF- and EGF-responsive neural stem cell proliferation. The source of these factors also remains undetermined. Even at high cell densities, pure populations of oligodendrocyte precursors do not divide in the absence of exogenous growth factors, suggesting that mitogenic signals for these precursors originate from cell types other than the precursors themselves (Barres et al., 1992). In the present in vitro assay, primary cultured neural stem cells are part of a heterogeneous population of GZ cells including postmitotic progenitors. Therefore, it will be of interest to determine if the cell density-dependent proliferation is mediated by stem cell interactions exclusively, by progenitor/postmitotic cells, or by a mixture of all of these cell types.

The different neural stem cells maintain multipotentiality irrespective of growth factor conditions

The independent EGF- and FGF-responsive neural stem cells isolated here both demonstrate multilineage potential. Consistent with previous results on expanding E10 cortical precursors (Qian et al., 1997), the proportion of $O4^+$ oligodendroglial progeny increases, with both increasing FGF2 and EGF concentrations in the present study. However, two of the present results are notably different from these previous findings. First, the proportion of $GFAP^+$ astrocytes was similar in all growth factor conditions assayed (Table 3.1). Qian et al. (1997) reported that the glial cells generated in cortical stem

cell clones were predominantly of the oligodendrocyte lineage (98%), and that only in the presence of astrocyte-meningeal cell-conditioned media (plus FGF2) would the percentage of glial-containing clones that contain astrocytes increase substantially. The present results demonstrate that neural stem cells isolated from the striatal GZ generate all three major neural cell types. One possible explanation for these differences is that there are temporal (E10 vs. E14.5) and spatial (cortical GZ vs. striatal GZ) restrictions on the competence of stem cells to generate neurons and glia in response to growth factor concentration. Second, the proportion of MAP2$^+$ neurons decreased with decreasing concentration of both FGF2 and EGF (Table 3.1). This result stands in contrast to the previous finding that the percentage of βtubulin III$^+$ neurons present in cortical stem cell clones increased to >90% in low concentrations of FGF2 (Qian et al., 1997). Low FGF2 concentrations may delay neuronal differentiation of progenitors rather than act on the stem cells directly. Thus, the increase in βtubulin III$^+$ cells (Qian et al., 1997) may represent a selective increase in the proportion of immature neurons which then results in a corresponding decrease in the proportion of more differentiated (MAP2$^+$) neurons (Table 3.1). It is possible that the instructive capacity of EGF and FGF2 may only be revealed when a very small number of cells (i.e. small number of cells/clone) are present in a well, as was the case in the study reported by Qian et al. (1997). Perhaps the same cell nonautonomous effects that facilitate neural stem cell proliferation from a primary culture are also present within sphere colonies (i.e. a relatively greater number of cells/clone) to ensure the multipotentiality of the stem cell by maintaining relatively similar proportions of various differentiated progeny, even under various growth factor conditions. The presence of EGFR and FGFR1 on all neural stem cell-derived cells within colonies indicates that the degree to which neuronal and glial fate may be altered also depends on the ability of EGF or FGF2 to act in a inductive manner on the progeny of neural stem cells (rather than on the stem cells themselves) to promote progenitor cell survival, differentiation or proliferation. Although it remains formally possible that EGF and FGF2 can differentially instruct stem cells to generate neurons and

glia (Johe et al., 1996), the cells isolated by Qian et al. (1997) may be a later precursor in the stem cell lineage, that may or may not be multipotential, compared to the stem cell isolated in the neural stem cell colony forming assay which is multipotential and self-renewing. Indeed, we would suggest that the true neural stem cells (EGF- or FGF-responsive) always retain their undifferentiated multipotential state throughout life and that inductive environmental signals influence the differentiation of the progeny of the stem cells (although not the stem cells themselves). For example, BDNF (Ahmed et al., 1995) and IGF-1 (Arsenijevic and Weiss, 1998) have been shown to enhance the differentiation of neurons from EGF-derived sphere colonies, while bone morphogenetic proteins (BMPs) (Gross et al., 1996) promote astrocyte differentiation from EGF-derived neurospheres.

The presence of different classes of stem cells during development raises the possibility that the early specification of forebrain compartments may be partly due to the establishment of different stem cell populations. Although in the present study both EGF- and FGF-responsive stem cells retain their multilineage potential in defined serum-free conditions, it is possible that there are unique intrinsic components in each of the separate stem cell lineages. The interaction between such distinct intracellular components and selective exogenous factors (e.g. BDNF, IGF-1, BMPs) may act to regulate the production of specific types or numbers of neuronal and glial progeny in a temporally- and spatially-dependent manner.

Chapter IV

Neural Stem Cell Regionalization

This chapter will be submitted for publication:

Tropepe, V., Hitoshi, S., Ekker, M., van der Kooy, D. (2000). Neural stem cell lineages are regionally specified in the develooping brain, but this regionalization is not irreversible and can be altered by local induction.

Summary

Regional patterning in the developing mammalian brain is partially regulated by restricted gene expression patterns within the germinal zone, which is composed of stem cells and their progenitor cell progeny. Using an in vitro colony-forming stem cell assay, we demonstrate that under defined conditions, the behavioral properties of neural stem cells isolated from the embryonic neocortex, basal ganglia and midbrain primordia are conserved, but that these stem cells express molecular markers of regional identity, independent of their in vivo environment. We tested whether isolated stem cell colonies were committed to their identity of origin by utilizing mice harboring a zebrafish *dlx4/dlx6lacZ*-transgene, which is specifically expressed in the subventricular zone and mantle region of the E14.5 ventral forebrain. We show that although none of the isolated stem cell colonies expressed this specific regional marker (transgene), cues exclusively from the ventral forebrain in a co-culture paradigm could induce transgene expression in neural stem cell colonies derived from both the dorsal and ventral forebrain as well as from the midbrain. Thus, neural stem cells and their progeny are regionally specified in the developing brain, but this regional identity can be altered by local inductive cues.

Introduction

The vertebrate brain is parceled into specialized regions during development. A prevailing model proposes that regional identity is established through the formation of transient segment-like structures. These structures are called prosomeres in the forebrain, mesomere for the midbrain and rhombomeres in the hindbrain and are defined by morphological, hodological and gene expression criteria (Rubenstein et al., 1994; Lumsden and Krumlauf, 1996). In mammals, the cerebral cortex and striatum (composed of the lateral and medial ganglionic eminences) comprise the main dorsal and ventral anatomical

derivatives, respectively, of the second most anterior prosomeric segment within the forebrain (Puelles, 1995). Early expression of regulatory genes prefigures compartmental histogenesis in these specialized regions. For instance, members of the *Emx* family of homeobox genes, such as *Emx-1* and *Emx-2*, are expressed within the cortical primordium (Simeone et al., 1992), while members of the *Dlx* family of homeobox genes, such as *Dlx1* and *Dlx-2*, are expressed within the striatal primordium (Bulfone et al., 1993). In these regions, gene expression is correlated with precursor cells within the germinal zone, which is composed of stem cells and their progenitor cell descendents. Thus, non-overlapping patterns of gene expression along the dorsoventral axis may enable proliferating precursor cells within the germinal zone of these compartments to be specified to a restricted cortical or striatal cell fate. Indeed, there is evidence for clonal restriction of some (Fishell et al., 1993), but not all (Anderson et al., 1997a) progenitor cell migration within these dorsal and ventral forebrain territories, and this pattern of lineage restriction is also observed between rhombomeres in the developing hindbrain (Fraser et al., 1990).

Regional patterning is regulated by inductive signals from adjacent tissues, such as the prechordal plate and notochord (Lumsden and Krumlauf, 1996). Neural stem cells, which are considered the lineage precursors to all neuronal and glial cells in the mammalian nervous system (Weiss et al., 1996; Gage, 2000), are mitotically active at the onset of forebrain neurogenesis and are present in the neural plate prior to the overt segregation of dorsal and ventral forebrain compartments (Tropepe et al., 1999; Martens et al., 2000). Hence, neural stem cells together with their progeny are poised to receive inductive signals that establish a regional identity in vivo. It is not known, however, whether neural stem cells possess intrinsic regional identity, which, for example, can be maintained in the absence of their in vivo environment, or whether neural stem cells in the developing brain generally lack positional information. The latter possibility may indicate that the acquisition of regional specification in the mammalian brain occurs primarily at the level of the progenitor cells in a lineage-independent manner. In the present study, we investigated

whether mouse neural stem cells isolated from different germinal zone regions along the dorsoventral and anteroposterior axes of the brain displayed region-specific characteristics.

Materials and Methods

Isolation of forebrain and midbrain neural stem cells

Timed-pregnant CD1 mice (Charles River) or timed-pregnant *zfdlx4/6lacZ* transgenic mice on a CD1 genetic background (Zerucha et al., 2000) at the specified gestational ages were killed via cervical dislocation. The uteri were aseptically removed and transferred to petri dishes containing sterile Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS; GIBCO) with 30% glucose (Sigma) and 2% penicillin/streptomycin (5000 IU; GIBCO). Each embryo from the uterine sac was dissected and transferred to a new sterile petri dish containing fresh PBS in order to rinse away excess blood. Under a dissecting microscope (Zeiss) and using sterile, fine forceps the amniotic sac was gently removed and the embryos were again transferred to a new petri dish containing fresh PBS. Head primordia were excised and transferred into fresh PBS with a wide-bore, fire-polished Pasteur pipette. E14.5 or E18.5 brains were removed after the removal of the epidermis and calvarium using iris scissors. Brains derived from *zfdlx4/6lacZ* transgenic mice were bisected and one hemisphere was processed for β-galactosidase histochemistry (see below). Tissue from the dorsal cortex, the ventral striatum, and the dorsal and ventral portions of the midbrain of the opposite hemisphere was dissected. For cortical and midbrain dissections, the meninges were also removed prior to culturing. The neuroepithelium was washed in PBS to remove loosely adherent cells. Unless specified, the ventral forebrain dissected tissue included both ventricular and subventricular zones. Tissues dissected from these regions were transferred to serum-free media (see below) and mechanically dissociated into a cell suspension with a small-bore, fire-polished Pasteur

pipette. Cell viability was assessed using trypan blue exclusion (0.4%; GIBCO) and typically ranged from 80% to 85%.

Cell culture

Cortical, striatal and midbrain cells were cultured in a neural stem cell colony-forming assay (Reynolds et al., 1992). Cells were plated at 10 cells/μl in 24-well (0.5 ml/well) uncoated plates (Nunclon) in serum-free media composed of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM; GIBCO) and F-12 nutrient (GIBCO) including 0.6% glucose (Sigma), 2 mM glutamine (GIBCO), 3 mM sodium bicarbonate (Sigma), and 5 mM HEPES buffer (Sigma). A defined hormone and salt mixture (Sigma) that included insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (20 nM), putrescine (60 μM), and selenium chloride (30 nM) was used instead of serum. Cells were plated in serum-free media containing 10 ng/ml FGF-2 (human recombinant; Sigma) and 2 μg/ml heparin (Upstate Biotech), or 20 ng/ml EGF (mouse submaxillary; Upstate Biotech). In order to assess self-renewal, primary sphere colonies (selecting mainly floating colonies after 7 days in vitro) were subcloned by mechanically dissociating a single sphere colony in 0.2 ml of serum-free media, in identical growth factor conditions as the primary culture, and plated in uncoated 96-well (0.2 ml/well) plates (Nunclon). Stem cell self-renewal was assessed by identifying new sphere colonies after a further 6-7 days in vitro.

Immunocytochemistry

Seven days after primary culture, single sphere colonies were transferred to individual wells of a 24-well culture plate (Nunclon) (0.5 ml/well) previously coated with MATRIGEL basement membrane matrix (15.1 mg/ml stock solution diluted 1:25 in serum-free media; Becton-Dickinson). The media contained 1% fetal bovine serum (Gibco) and was not changed for the rest of the culture period. Wells were processed 7 days later using immunocytochemistry, at which time cells within the sphere colonies spread considerably

throughout the MATRIGEL substrate. Colony cells were fixed in 4% paraformaldehyde (in PBS, pH 7.2) for 20 minutes at room temperature followed by 3 (5 minutes each) washes in PBS (pH 7.2). Cells were then permeabilized for 5 minutes in PBS containing 0.3% Triton-X, rinsed for 5 min (2x) in PBS and blocked for 1 hour in PBS containing 10% normal goat serum (NGS). After blocking, cells were incubated in anti-MAP-2 mouse monoclonal (IgG) (1:1000; Boehringer Mannheim) and anti-GFAP rabbit polyclonal (IgG) (1:400; Chemicon) antibodies diluted in PBS containing 10% NGS overnight at 4°C. Cells were then rinsed in PBS three times (5 minutes each) and subsequently incubated in FITC goat anti-rabbit (1:200; Jackson ImmunoResearch) and TRITC goat anti-mouse (1:200; Jackson ImmunoResearch) secondary antibodies at 37°C for 30 min. Cells were rinsed three times (5 minutes each) in PBS. Separately cultured sphere colonies (from similar primary culture conditions) were used for oligodendrocye immunocytochemistry in order to confirm previous findings that sphere colonies generated from neocortical and GE neural stem cells contained neurons, astrocytes and oligodendrocytes and were therefore multipotential (Tropepe et al., 1999) and to determine the multipotentiality of midbrain derived stem cells under similar conditions. Cells were incubated in anti-O4 mouse monoclonal (IgM) antibody (1:40; Boehringer Mannheim) in PBS containing 10% NGS at 4°C overnight. The next day, wells were rinsed three times (5 minutes each) and subsequently incubated in FITC goat anti-mouse-IgM (1:200; Jackson ImmunoResearch) secondary antibody in PBS containing 10% NGS at 37°C for 30 minutes. Cultures were counter labeled with the nuclear stain Hoechst 33258 (0.015 mg/ml stock solution diluted to 0.001 mg/ml; Boehringer-Mannheim) for 5 min at room temperature. After rinsing three times (5 minutes each), Fluor-mount was added to each well. Fluorescence was visualized using a Nikon Microphot microscope. Secondary antibody-only controls were processed simultaneously using the identical protocol except dilution solutions were devoid of primary antibodies. All secondary antibody controls were negative. Numbers of cells were

quantified by counting 3-4 random standardized areas (using an ocular grid) at 20X objective magnification in each culture.

RT-PCR

Total RNA was isolated using the RNeasy extraction kit (Qiagen) and 1 μg of total RNA was used to synthesize cDNA with oligo-$d(T)_{12-18}$ primers and MuMLV reverse transcriptase (Superscript II; Boehringer-Mannheim) at 50°C for 1 hour. The PCR reaction mixture (20 μl) consisted of 1 μl cDNA, 16 pmol 5' primer, 16 pmol 3' primer, 0.2 mM dNTP, 2 μl PCR reaction buffer and 0.8 U of Taq polymerase (Promega). cDNA was amplified in a thermal cycler (Perkin-Elmer). For all primer pairs denaturation for 30 sec at 94°C, annealing for 30 sec and extension at 72°C was used. The sense and antisense primers, Mg^{2+} concentration, annealing temperature, extension time and number of PCR cycles were used for the following genes. *Emx1*: sense 5'-CGAGAAGAACCACTACGTGG-3', antisense 5'-AGGTGACATCGATGTCCTCC-3', 1.5 mM Mg^{2+}, 56°C, 40 sec, 40 cycles. *Otx1*: sense 5'-TCACAGCTGGACGTGCTCGA-3', antisense 5'-GCGGCGGTTCTTGAACCAAA-3', 1.65 mM Mg^{2+}, 58°C, 40 sec, 35 cycles. *Dlx2:* sense 5'-ACACCGCCGCGTACACCTCCTA-3', antisense 5'-CTCGCCGCTTTTCCACATCTTCTT-3', 1.2 mM Mg^{2+}, 56°C, 40 sec, 40 cycles. *En1:* sense 5'-GACAGTGGCGGTGGTAGTG-3', antisense 5'-GAGGAGCCTGGAGGTGGC-3', 1.2 mM Mg^{2+}, 56°C, 40 sec, 40 cycles. As a control, cDNA amplification of the *GAPDH* gene (glyceraldehyde-3-phosphate dehydrogenase) was simultaneously run in each PCR experiment. Primers for *GAPDH:* sense 5'-ACCACAGTCCATGCCATCAC-3', antisense 5'-TCCACCACCCTGTTGCTGTA-3' and PCR reaction conditions were similar to conditions used for Emx1 amplification (see above). Amplified products were electrophoresed in 2% agarose gel containing ethidium bromide (25 μg/ml) and bands were visualized with UV light (DualLite Transilluminator, Fisher Biotech).

Co-cultures

CD1 embryos at 14.5 days of gestation were isolated and their brains dissected in PBS as described above. In order to use intact striatal tissue as a substrate for sphere colony co-cultures, E14.5 brains were placed (rostral end facing up) in a 35 mm petri dish and covered with a 1% solution of low melting point agarose (Sigma) at ~37°C and then immediately placed at 4°C to harden the agarose solution. Brains were then sectioned on a vibratome in ice cold 0.9% saline. Approximately two to three 400 μm thick coronal sections were cut from each brain. Sections used in the analysis included cortex, striatum, septum and preoptic areas rostrally (surrounding the lateral ventricles) and anterior thalamus caudally (surrounding third ventricle). Sections that were more rostral (including only cortex and septum and no or very little striatal tissue) were either excluded or used as a control for the absence of striatum. Individual sections were placed flat on a Transwell-Col filter insert (Corning) measuring 24 mm in diameter (3.0 μm pore size) in 6-well plates and incubated in 1 ml of serum-free media (as above) for 1 hour. A similar procedure was used for cortical (dorsal cortex) tissue substrates and midbrain (presumptive tectum) tissue substrates except that the slices were horizontally cut and placed ventricular zone side up on the filters. Two to three individual sphere colonies (derived from E14.5 transgenic cortical, striatal or midbrain tissue) were placed on the slice tissue (in the region of the striatum for coronal slices) with a pipette under an inverted microscope at 4X objective magnification. Co-cultures were incubated at 37°C and 5% CO_2 undisturbed for a maximum of 5 days. Although cell death was minimized with this protocol, it remained evident after 5 days (necrotic tissue was often light brown in appearance) but was easily distinguished from positive β-gal staining. Co-cultures were then processed for β-gal expression histochemically (see below).

β-Galactosidase histochemistry

X-gal staining was used to detect the expression of the *LacZ* transgene in single hemispheres of *zfdlx4/6lacZ* transgenic brains, primary *zfdlx4/6lacZ* sphere colonies generated from cortical, striatal or midbrain tissues, primary monolayer cultures from these same tissues, or 5-day co-cultures of E14.5 CD1 forebrain and midbrain tissue and *zfdlx4/6lacZ* primary sphere colonies (as above). Tissues were rinsed in 100 mM sodium phosphate buffer (pH 7.4), and then fixed in 0.2% gluteraldehyde, 2 mM $MgCl_2$, 5 mM EGTA and 100 mM sodium phosphate (pH 7.3) at room temperature for 5 min. (sphere colonies, monolayer cultures) or 15 min. (hemispheres, and co-cultures). Tissue samples were then washed (3x) in wash buffer containing 0.02% NP-40, 0.01% deoxycholate, 2 mM $MgCl_2$ and 100 mM sodium phosphate (pH 7.3) for 5-15 min. each. Tissues were stained in 1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 0.02% NP-40, 0.01% deoxycholate, 2 mM $MgCl_2$ and 100 mM sodium phosphate buffer (pH 7.3) at 37°C overnight. Tissues were then rinsed with wash buffer and stored at 4°C. For cryosections, brain hemispheres were postfixed overnight in 4% paraformaldehyde at 4°C, subsequently cryoprotected with 20% sucrose, cryosectioned at 14 μm.

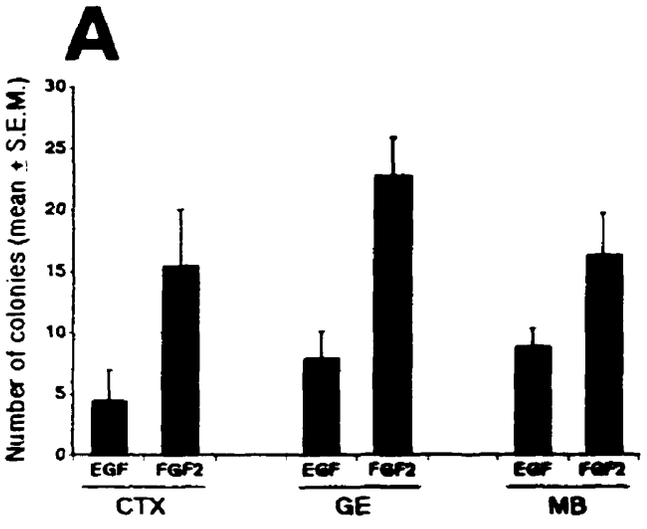
Results

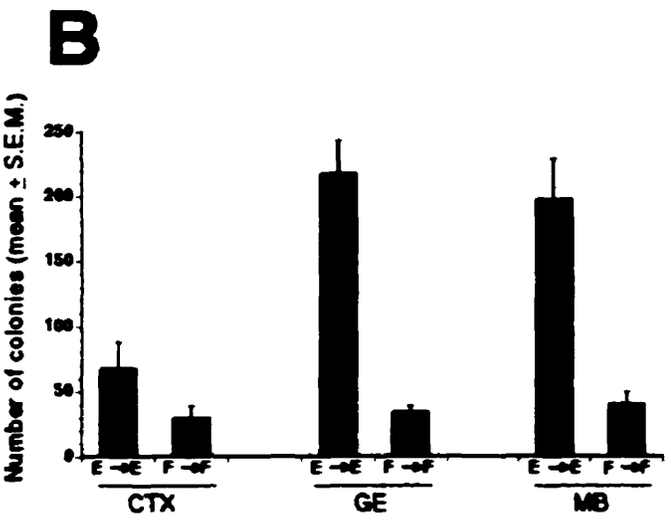
Between neural compartments: Neural stem cells isolated from forebrain and midbrain primordia demonstrate comparable behavioral characteristics

Although the neurotransmitter phenotypes of neurons and general cytoarchitecture of the cortex, striatum and midbrain are quite distinct, neural stem cells within these developing regions do not appear to be restricted in their capacity to generate neurotransmitter-specific neurons. For example, isolated embryonic striatal stem cell colonies (or adult striatal stem cell colonies) in vitro can generate dopaminergic neurons, which are not normally found in the mature striatum (Daadi and Weiss, 1999). Thus, the

differentiation of specific types of neurons appears to be mediated primarily by local extrinsic factors and is not an intrinsic property of stem cells in different regions. We reasoned that although neural stem cell progeny in vivo can be influenced to differentiate in a region specific manner, neural stem cells themselves may, in fact, display similar behavioral characteristics when isolated in identical conditions independent of their local environment. Neural stem cell behavior can be operationally defined (and empirically tested) as the ability to proliferate and produce progenitor cells, self-renewal capacity and neural multilineage potential. We compared the behavior of cortical, striatum and midbrain derived neural stem cells isolated from mouse germinal zone dissections at E14.5. We have previously shown that at this stage of development, co-existing populations of FGF-responsive and EGF-responsive neural stem cells divide to produce clonal colonies of undifferentiated cells (Tropepe et al., 1999). The majority of these progeny will differentiate into the three main types of nerve cells (neurons, astrocytes and oligodendrocytes) while a small proportion of stem cell descendents maintain the ability to generate new clonal colonies. Thus, the original colony-forming cell is considered to have self-renewal and multilineage potential (Potten and Loeffler, 1990; Weiss et al., 1996; Morrison et al., 1997) in response to these growth factors. Both EGF-responsive and FGF-responsive neural colonies were generated from cortical, striatal and midbrain cultures (Fig. 4.1A). Consistent with previous findings (Tropepe et al., 1999) there was a greater number of FGF-responsive stem cells compared to EGF-responsive stem cells from cortical and striatal germinal zone tissues. Furthermore, this growth factor-dependent bias in colony formation was similarly observed from midbrain derived neural stem cells (Fig. 4.1A) suggesting that distinct EGF- and FGF-responsive neural stem cell populations are distributed not only along dorsoventral compartments of the forebrain, but also along the anteroposterior axis between the forebrain and midbrain.

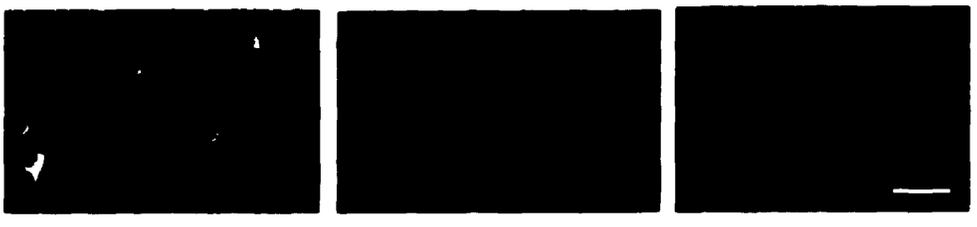
Figure 4.1. Neural stem cells isolated from different regions of the E14.5 mouse brain display comparable behavioral characteristics in vitro. (A) EGF-responsive and FGF-responsive neural stem cells isolated from the cortical, striatal and midbrain germinal zones (VZ+SVZ) and cultured in serum-free media clonally proliferated to form sphere colonies after 7 days of culture. Data represent 2-6 separate experiments and the means were derived from a total of n=12 embryos per group. (B) To assess the self-renewal capacity of the original colony-forming neural stem cell, single primary cortical (n=7 EGF, n=9 FGF2), striatal (n=11 EGF, n=12 FGF2) and midbrain (n=12 EGF, n=12 FGF2) neural stem cell colonies were manually dissociated and re-cultured in identical growth factor conditions and the numbers of secondary colonies from each single primary colony was quantified after 7 days in culture. Data are representative of at least 2 separate experiments for the region-specific sphere colonies sampled. (C) Immunolabeling for neurons (anti-MAP2$^+$), astrocytes (anti-GFAP$^+$) and oligodendrocytes (anti-O4$^+$) derived from single neural stem cell colonies cultured for 7 days in serum-free media and subsequently allowed to differentiate for a further 7 days on an artifical extracellular matrix substrate in media containing 1% FBS. Ctx, cortex; STR, striatum; MB, midbrain. Scale bar: 20 μm.





C

	% MAP2 (n)	% GFAP (n)	% O4 (n)
CTX	15.4 +/- 2.3 (6)	36.8 +/- 3.5 (3)	7.2 +/- 2.4 (3)
GE	14.9 +/- 3.1 (6)	58.9 +/- 6.7 (3)	3.4 +/- 0.9 (3)
MB	6.4 +/- 0.9 (11)	55.3 +/- 3.4 (5)	5.3 +/- 1.9 (6)



Single FGF-generated and EGF-generated primary colonies from all three regions were capable of producing new secondary colonies after 7 days under similar growth factor conditions (Fig. 4.1B). The number of new secondary colonies generated from the subcloning of a single primary colony can be considered an estimate of the extent to which the initial primary colony-forming stem cell underwent symmetric (expansionary) divisions (Reynolds and Weiss, 1996). EGF-responsive stem cells from all three germinal regions demonstrate a greater capacity for symmetric division than FGF-responsive stem cells isolated from the same germinal region (Fig. 4.1B).

To assess neural stem cell multipotentiality, single FGF-responsive colonies were cultured for 7 days under conditions known to promote neuronal and glial differentiation of stem cell progeny. Cultures were processed for immunocytochemistry to detect the pan-neuronal protein MAP2, the astrocyte-specific intermediate filament protein GFAP, and the oligodendrocyte-specific sulfatide antigen O4. Colony-forming stem cells derived from cortex, striatum and midbrain demonstrated neural multilineage potential (Fig. 4.1C). Overall, the proportion of neuronal and glial cells that differentiated under these conditions was comparable among the region-specific stem cell colonies. However, the percentage of $MAP2^+$ neurons observed from the midbrain stem cell colonies (6.4%) was significantly less than the percentage of $MAP2^+$ neurons derived from neocortical stem cell colonies (15.4%) ($t = 4.35$, $p<0.05$). A similar, but not statistically significant, difference was also observed for the percentage of $MAP2^+$ neurons differentiating from midbrain (6.4%) and striatal stem cell colonies (14.9%). Thus, these data suggest that under our in vitro differentiation conditions midbrain stem cells may have a relatively attenuated intrinsic ability to generate neurons compared to forebrain stem cells. However, differentiation of stem cell progeny is sensitive to substrate and growth factor composition in vitro (Ahmed et al., 1995; Qian et al., 1997; Tropepe et al., 1999). Thus, a more likely explanation may be that forebrain and midbrain stem cell progeny are differentially responsive to environmental

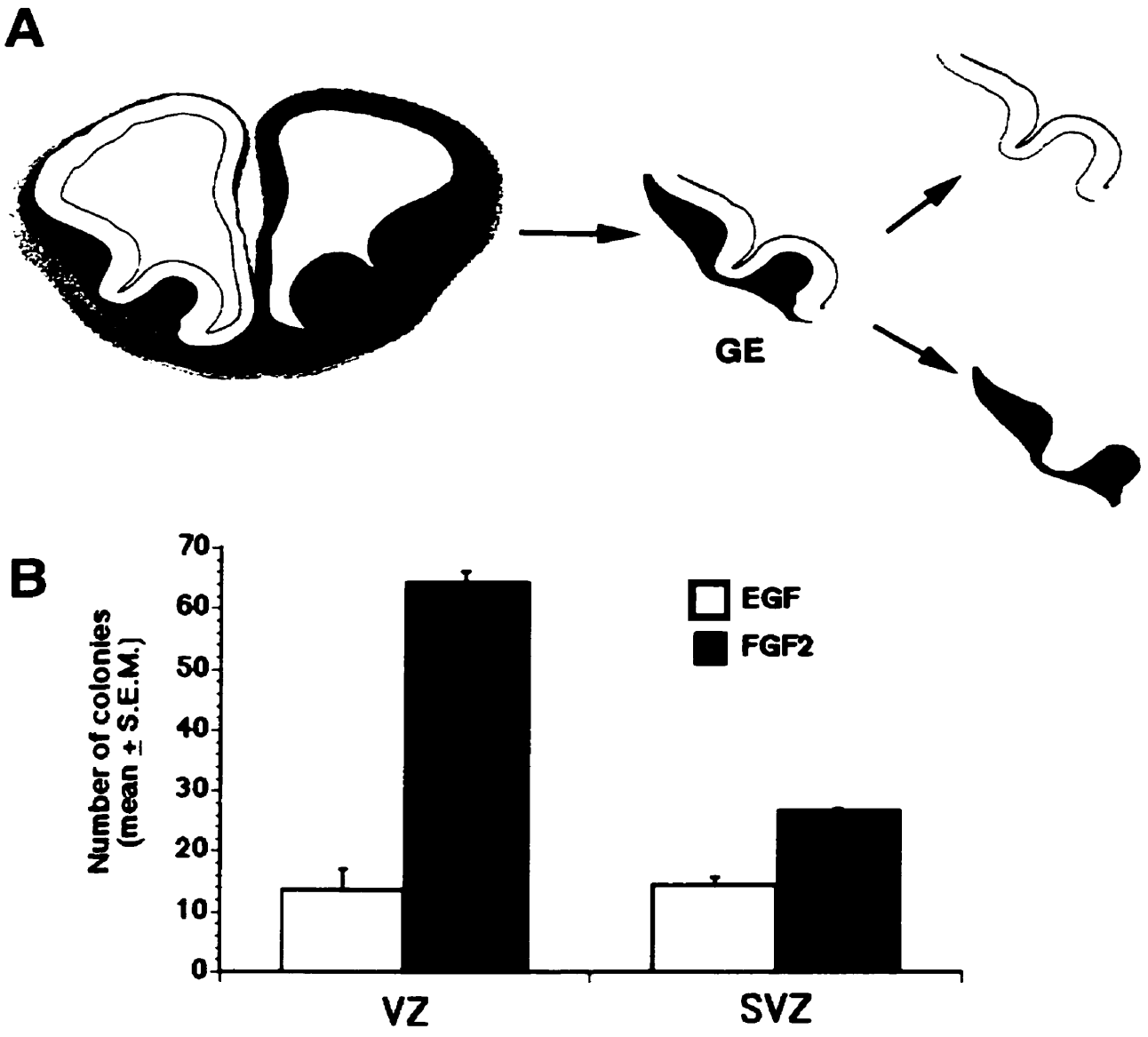
conditions. Although implied, whether this bias is in the progeny is strictly inherited from their stem cell precursors remains to be formally demonstrated.

Taken together, these data indicate that the behavior of EGF- and FGF-responsive neural stem cells is generally conserved between dorsal forebrain (cortex), ventral forebrain (striatum) and midbrain neural compartments. However, behavioral resemblance alone does not reveal whether these stem cell populations have unique positional identity that is established during the course of neural patterning. It is possible that the regulation of stem cell behavior (as defined above) may be independent of a stem cell's position in the developing brain. Indeed, positional information may partly regulate the types of progeny generated by neural stem cells in different brain regions in vivo (or under defined conditions in vitro), even though neural stem cell behavior is generally conserved between neural compartments.

Within neural compartments: EGF- and FGF-responsive stem cell populations are differentially distributed in the ventral forebrain germinal zone

Given that neural stem cells are spatially distributed between germinal compartments in the brain, we explored whether EGF- and FGF-responsive stem cells were similarly distributed among two distinct proliferative zones (ventricular zone – VZ, and subventricular zone – SVZ) within a single neural compartment. Our previous observations (Tropepe et al., 1999; Martens et al., 2000) suggested that the emergence of an EGF-sensitive stem cell population between E11.5 and E13.5, from an earlier established FGF-sensitive stem cell population in the VZ, within the striatum correlated with the development of SVZ. Thus, to determine whether EGF-responsive stem cells were preferentially localized to the SVZ, we used coronal slices of the E14.5 forebrain (~500 μ m) to facilitate dissection of the VZ from the SVZ within the striatal germinal zone (lateral and medial ganglionic eminences were both included) (Fig. 4.2A). Although the separation

Figure 4.2. EGF-responsive and FGF-responsive neural stem cells are differentially spatially distributed in the ventral forebrain germinal zone. (A) Schematic representation of the microdissection procedure. Coronal sections (~500 μm thick) were obtained from E14.5 brains and the VZ (including some SVZ tissue) was dissected away from the majority of the SVZ (including some mantle zone tissue as well) in the ventral forebrain and cells were cultured in either EGF or FGF2 at relatively low cell densities. Figure adapted from Altman, J and Bayer, S.A. (1995). Atlas of prenatal rat brain development. CRC Press. Boca Raton. (B) Primary colonies generated after 7 days of culture in serum-free media from EGF-responsive and FGF-responsive neural stem cells localized to the VZ or SVZ of the ventral forebrain. The data represent 3 separate experiments with n=12 embryos sampled. STR, striatum.


A
GE
B
Number of colonies
(mean ± S.E.M.)
70
60
50
40
30
20
10
0
EGF
FGF2
VZ
SVZ

of these two proliferative zones within the germinal zone is not complete, great care was taken during the dissection to control for the depth of the VZ and the contours of the striatum. The cell suspensions of the VZ and SVZ tissues were plated at the same densities. The numbers of E14.5 FGF-generated colonies was greater than the numbers of EGF-generated colonies both in isolated VZ tissue ($t = 13.9$. $p<0.05$) and SVZ tissue ($t = 2.7$, $p<0.05$) (Fig. 4.2B). However, there were more than twice as many FGF-generated colonies from the isolated VZ tissue compared to the SVZ tissue ($t = 27.8$, $p<0.05$), whereas, similar numbers of EGF-generated colonies were observed from both the VZ and SVZ tissues ($t = 0.1$, $p>0.05$) (Fig. 4.2B). This differential distribution of FGF- and EGF-responsive stem cells in the VZ and SVZ also persisted into late embryonic stages. At E18.5, the numbers of EGF-generated colonies in the VZ (32.1 ± 4.2, $n = 10$) and SVZ (25.0 ± 4.8, $n = 10$) were not statistically different ($t = 1.1$, $p>0.05$); whereas, significantly more FGF-derived colonies were generated from the VZ (34.5 ± 5.4, $n = 10$) than the SVZ (7.7 ± 0.9, $n = 10$) ($t = 4.9$, $p<0.05$). These data suggest that the FGF-responsive stem cell population is localized primarily to the VZ of the GE germinal compartment, whereas EGF-responsive stem cells are approximately equally distributed throughout the striatal germinal compartment during initial embryonic forebrain development. Furthermore, by late neurogenesis, the population of EGF-responsive stem cells within the SVZ is approximately three-fold greater than the FGF-responsive population, suggesting the independent developmental regulation of these two types of neural stem cells. Thus, neural stem cells are differentially spatially organized along the ventriculopial axis within a single histogenic compartment.

Forebrain and midbrain stem cell colonies express unique molecular markers of regional identity

The above experiments showed that neural stem cell populations (with similar self-renewal capacity, multilineage potential and growth factor responsiveness) are localized along the dorsoventral and anteroposterior axes (between histogenic compartments) within the brain. Moreover, neural stem cell populations can be differentially distributed within a single histogenic compartment (between the striatal VZ and SVZ). To determine if forebrain and midbrain neural stem cell colonies have unique regional identity, we assayed for the expression of region-specific regulatory genes *Emx1*, *Dlx2* and *En-1* in isolated forebrain and midbrain stem cell colonies using RT-PCR. During development *Emx1* is expressed in the cortex (Simeone et al., 1992) and *Dlx2* is expressed in the striatum (Bulfone et al., 1993). On the other hand, *En1* expression is localized to the posterior midbrain and anterior hindbrain region (isthmus) (Davis and Joyner, 1988). FGF-responsive stem cell colonies were generated from the cortical, striatal and midbrain germinal zones from E14.5 embryos and age-matched primary tissue isolates served as positive controls for gene expression. Isolated stem cell colonies demonstrated region-specific gene expression patterns (Fig. 4.3). E14.5 cortical colonies express *Emx1*, but not *Dlx2* or *En1*. Striatal colonies express *Dlx2*, but not *Emx1* or *En1*. Midbrain colonies express *En1*, but do not express *Emx* or *Dlx* genes. A similar expression profile was observed for EGF-generated stem cell colonies isolated from the same germinal regions (data not shown). We also determined the expression of *Otx1*, which is distributed throughout the dorsal and ventral forebrain and midbrain compartments at early stages of neural development, but declines after E15.5 (Boncinelli et al., 1993). The results show that by E14.5, *Otx1* expression is present in isolated forebrain derived neural colonies (both cortical and striatal), but is not expressed in midbrain derived colonies (Fig. 4.3). Thus, at this later stage of development, *Otx1* expression molecularly defines a boundary between neural stem cell lineages of the forebrain and midbrain.

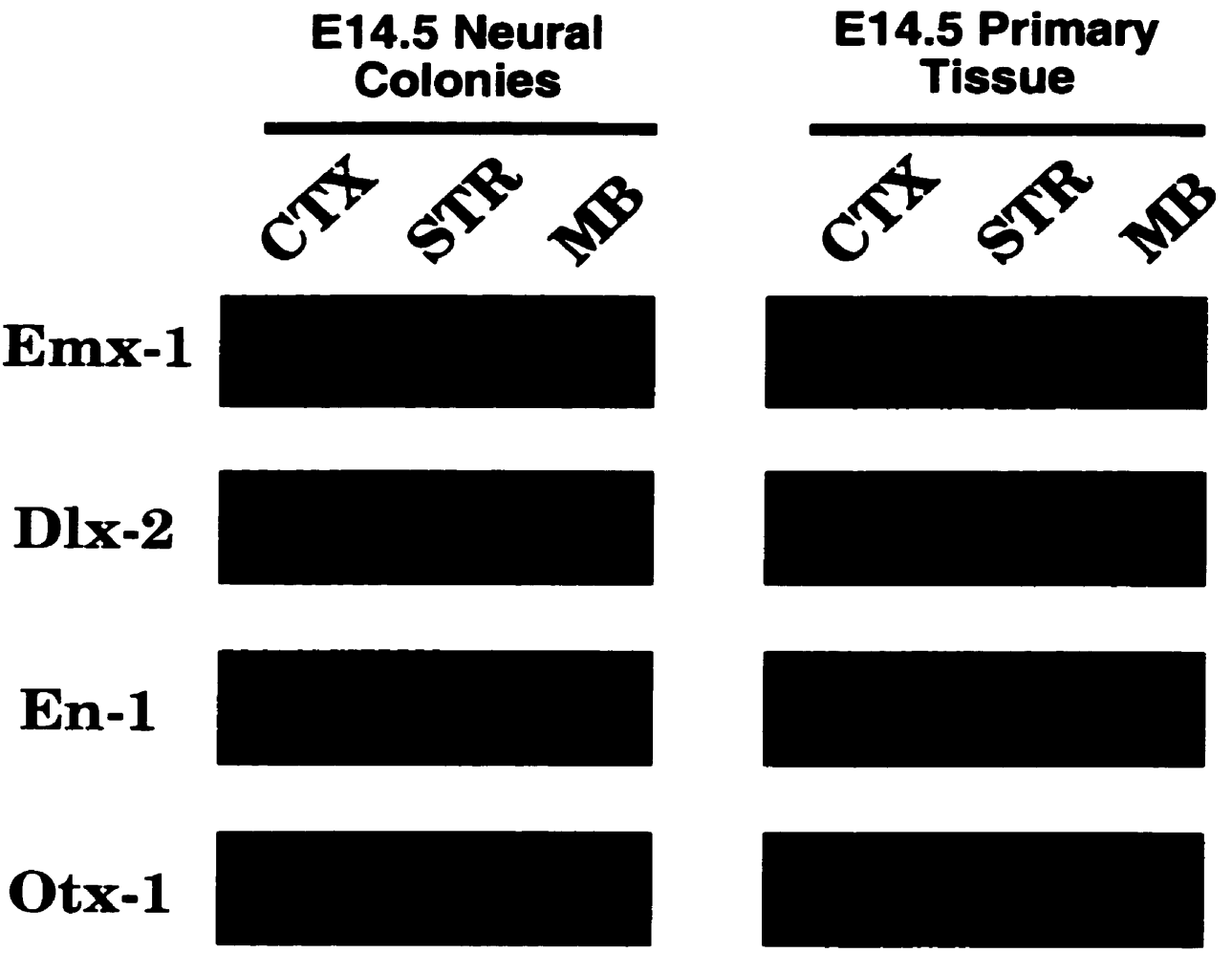
These findings suggest neural stem cells along the dorsoventral as well as anteroposterior axes have intrinsic regional identity that is maintained when isolated in

vitro. Moreover, this region-specific positional information (in the form of gene expression) is distinct from the region-nonspecific or relatively comparable neural stem cell behavior.

Relatively late markers of ventral forebrain regional identity are not expressed by isolated striatal stem cell colonies

Clonal neural stem cell derived colonies are composed of undifferentiated cells. The expression of *Dlx2* in striatal derived stem cell colonies is consistent with the fact the in vivo, *Dlx2* is localized to the VZ and SVZ where stem cells and their early progenitor cell progeny reside (Eisenstat et al., 1999). As the progenitor cells migrate away from the germinal zone into the overlying mantle region they begin to express other genes of the *Dlx* family, such as *Dlx5* and in more mature cells *Dlx6* (Eisenstat et al., 1999). To test if isolated neural stem cell colonies express relatively late markers of regional identity, we utilized *zfdlx4/dlx6lacZ*-transgenic mice (Zerucha et al., 2000). Transgene expression in the E14.5 ventral forebrain resembles the expression of endogenous *Dlx5* and *Dlx6*, but is thought to more closely mimic endogenous *Dlx5* expression (Zerucha et al., 2000). We confirmed that the *zfdlx4/dlx6*-enhancer-driven *lacZ* expression is exclusively confined to a majority of cells in the SVZ and mantle region of the ventral forebrain, including the striatum and septum, but not the VZ (in 14 μm sections of the E14.5 forebrain, data not shown) (Zerucha et al., 2000). Thus, expression of this specific reporter construct acted as a molecular marker for precursor cells within striatal stem cell colonies that progressed through to later stages of differentiation, essentially recapitulating the in vivo differentiation sequence. However, neural stem cell colonies isolated from the striatum (either VZ or SVZ) did not express the transgene when assayed histochemically. Neocortical and midbrain neural stem cell colonies were similarly negative for transgene expression. Thus, cells within striatal derived stem cell colonies do not express genes that are normally restricted to more mature striatal cells. There are two possible explanations for these results. First, cells within a neural stem cell colony may require further differentiation in order to express late

Figure 4.3. Isolated E14.5 neural stem cell colonies express region-specific genes. Gene expression analysis was determined using RT-PCR. RNA was isolated from neural stem cell colonies derived from cortical, striatal or midbrain germinal zone dissections generated after 7 days of culture. Primers were designed to detect Emx1 (229 bp), Dlx2 (310 bp), En1 (567 bp) and Otx1 (128 bp). To normalize for the amount of cDNA present in the sample, the cDNA for GAPDH (452 bp) was amplified and band intensity was comparable to the band intensities of the other markers (not shown). Ctx, cortex; STR, striatum; MB, midbrain. Data are representative of 3 separate experiments.


E14.5 Neural Colonies
E14.5 Primary Tissue
CTX
STR
MB
CTX
STR
MB
Emx-1
Dlx-2
En-1
Otx-1

regional markers. Second, the expression of this specific transgene (and other late markers of differentiation) in stem cell progeny may depend not on the degree of differentiation, but rather on regional cues provided by the cells within the striatal environment.

To distinguish between these two possibilities, we tested whether further differentiation of neural colony-derived progenitor cells was sufficient for transgene expression by culturing cortex, striatum and midbrain derived stem cell colonies in conditions that promoted neuronal and glial differentiation (as above). After 7 days of differentiation, cells were assayed for transgene expression histochemically. Approximately 200 cells were randomly sampled for each differentiated sphere colony. β-gal$^+$ cells were extremely rare (<0.3% averaged over all of the sphere colonies assessed) in differentiated striatal stem cell colonies isolated from either the VZ (n=69 colonies) or the SVZ (n=83 colonies). The majority of differentiated striatal sphere colonies did not contain β-gal expressing cells. Furthermore, no transgene expressing cells were observed from cortical colonies (n=36 colonies) or midbrain colonies (n=23). Thus, differentiation alone is not sufficient for transgene expression (a later marker of regional identity) in stem cell derived neuronal and glial progeny, even though it is sufficient, for example, for neurotransmitter-specific neuronal differentiation – a late event in neuronal maturation (Daadi and Weiss, 1999). These data suggest that although the expression of early markers of regional identity may be intrinsic to isolated neural stem cells (and inherited by their early progeny) the expression of late regional markers in these progeny may require the induction and/or maintenance by specific regional cues provided by other cells.

Specific cues from the ventral forebrain induce the expression of a ventral-specific transgene in both forebrain and midbrain stem cell colonies.

To determine if late markers of regional identity can be induced by local cues we used two versions of a co-culture paradigm. In the first experiment, cortical or striatal stem

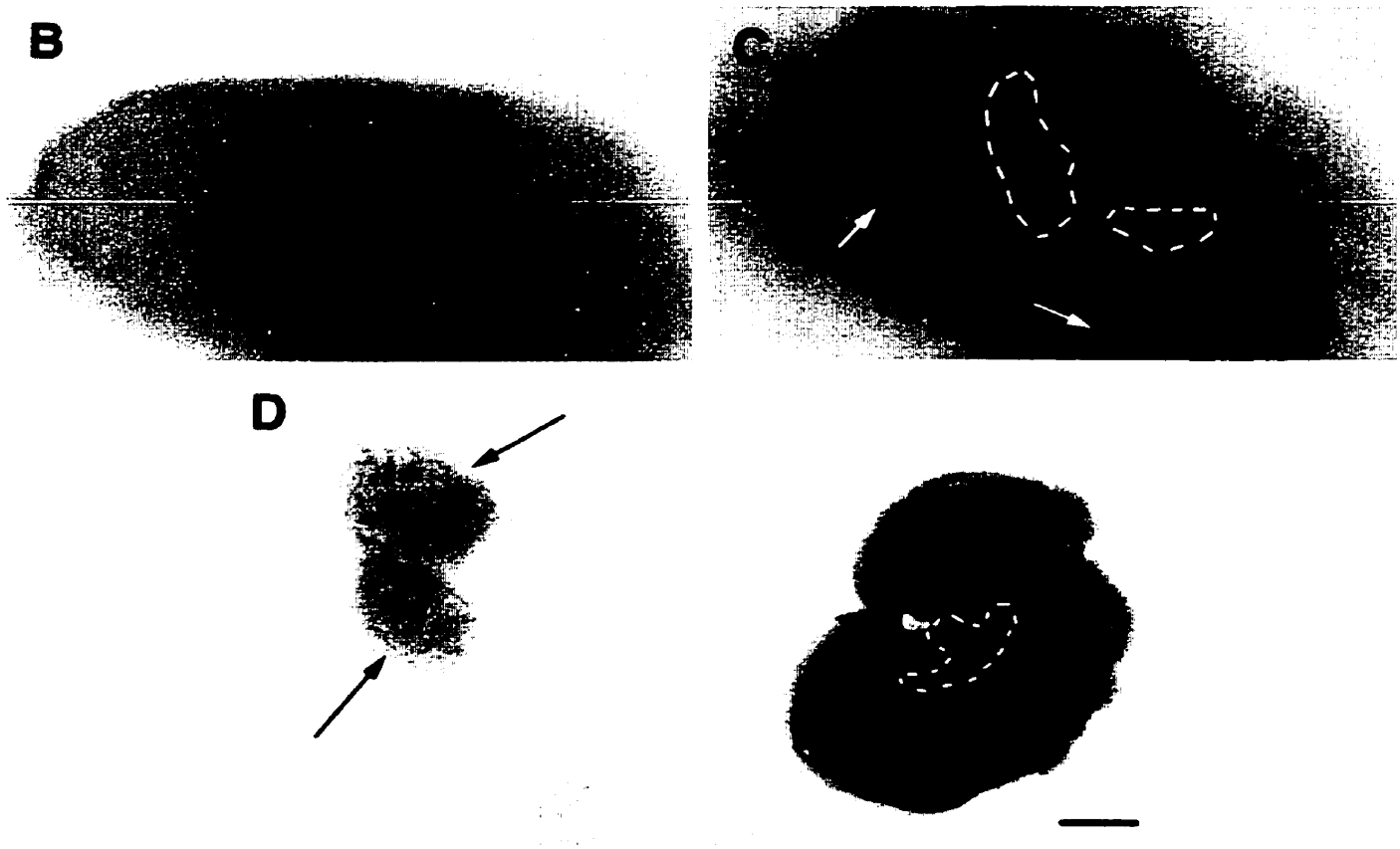
cell colony derived cells from transgenic mice were dissociated and co-cultured with host CD1 cells from a primary (non-sphere forming) striatal dissection (1:1 ratio) at relatively high cell densities (50 cells/μl each) in similar differentiation conditions as above. Marker gene expression was not induced in colony cells derived from either the neocortex or the striatum (n=6-12 co-cultures per group). However, given that these co-culture conditions do not compensate for the extensive contact-mediated cellular interactions that may be necessary for regional specification, we employed a more sensitive co-culture slice paradigm where tissues remained relatively intact and viable over a 5-day period. Coronal 400 μm sections from E14.5 CD1 forebrain were used as a substrate on 6-well filter inserts. Using a pipette, 1-3 striatum derived colonies were deposited on the slice tissue in the vicinity of the striatal germinal zone/mantle region. After 5 days of co-culturing, robust transgene expression was detected histochemically in 82% of the co-cultured colonies (Fig. 4.4A) in the germinal zone region of the striatum (lateral to the ventricular lumen) and the septum (medial to the ventricular lumen) (Fig. 4.4C). Some distortion of the slice morphology is evident after 5 days and β-gal$^+$ cells were often observed very close to the midline between the hemispheres (Fig. 4.4C). In contrast, horizontal slice cultures of cortex or midbrain (or rostral coronal slices that do not contain striatal tissue) were not sufficient substrates for inducing transgene expression in striatal stem cell colonies (Fig. 4.4B). Thus, the striatal primordium acts as a specific inductive cue for the expression of a relatively late molecular marker of regional identity in isolated ventral forebrain stem cell lineages.

In order to define more precisely the source of the inductive signal, we dissected away the VZ tissue (which included some SVZ) from the remaining SVZ/mantle region of the striatum in 400 μm E14.5 coronal sections and cultured the separate VZ tissues on the same filter situated at a distance from the remaining brain section. We reasoned that since the progeny of neural stem cells (derived from the germinal zone) within the clonally derived colonies did not express the transgene in isolation, the source of the inductive

Figure 4.4. Region-specific gene expression in isolated forebrain and midbrain neural stem cell colonies is not irreversible. (A) One-three neural stem cell colonies (cortical, striatal or midbrain) derived from E14.5 *zfdlx4/dlx6lacZ* transgenic mice were co-cultured with E14.5 CD1 tissue (cortical, striatal or midbrain) and the numbers of co-cultures that had detectable transgene expression (β-galactosidase$^+$ with X-gal histochemistry) were tabulated. (B) Transgene expression was not induced in cortical, striatal and midbrain colonies when placed on CD1 cortical tissue (depicted here) or midbrain tissue. (C) After 5 days in serum-free media, robust transgene expression could be observed only when sphere colonies were placed in the region of the ventral forebrain (dashed outline). Some necrosis was also evident (arrows) in the host tissue after 5 days in vitro. (D) Transgene expression was not observed (0/4 co-cultures) in isolated E14.5 striatal VZ tissue (arrows), but was expressed (4/4 co-coultures) in the remaining striatal tissue composed of SVZ and mantle regions (dotted lines on right). Data represent 3 separate experiments. Scale bar: 1 mm.

A

Colony	Host tissue	# Co-cultures	X-gal+	% X-gal+
GE	GE	11	9	82
CTX	GE	13	11	85
MB	GE	4	3	75
GE	CTX	12	0	0
CTX	CTX	8	0	0
MB	CTX	4	0	0
GE	MB	6	0	0
CTX	MB	6	0	0
MB	MB	2	0	0



signal in the co-culture paradigm likely arose from the striatal mantle region, where transgene expression is observed in vivo. Transgene expression from striatum derived stem cell colonies was not observed when colonies were co-cultured with isolated VZ tissue (Fig. 4.4D). In contrast, robust transgene expression was induced in co-cultured GE derived stem cell colonies when placed in the SVZ/mantle region of the ventral forebrain (Fig. 4.4D). These findings suggest that the once neural stem cell progeny (born in the germinal zone) migrate into the overlying mantle zone in the ventral forebrain, local cues regulate the expression of different sets of regionally restricted genes in a cell nonautonomous manner.

We next sought to determine if dorsal forebrain and midbrain stem cell lineages, which normally do not express early or late markers for ventral forebrain identity but instead are specified to their region of origin, could be induced to express the ventral forebrain *zfdlx4/dlx6lacZ* transgene marker. Cortical and midbrain derived stem cell colonies were similarly co-cultured on CD1 striatal primordia (coronal slices) or on CD1 cortical or midbrain primordia (horizontal slices) under the same culture conditions. After 5 days, both cortical (85%) and midbrain (75%) colonies (Fig. 4.4A) had strong transgene expression in an analogous pattern when exposed to the striatal environment. In contrast, no transgene expression was observed when these colonies were co-cultured with either cortical or midbrain substrates. These data indicate that local cues from ventral forebrain tissue can specifically induce the expression of a relatively late-expressing ventral forebrain gene in isolated neural stem cell lineages from both the dorsal forebrain and the more posterior midbrain, even though these neural stem cell colonies do not express *Dlx2* (an early ventral regional marker). Thus, neural stem cell lineage specification along the dorsoventral and anteroposterior axes is not irreversible and region-specific gene expression, especially later markers of regional identity, depend on inductive signals provided by the local environment.

Discussion

Histogenesis of distinct forebrain and midbrain compartments is regulated by the generation of neuronal and glial progenitor cells from neural stem cells localized to the germinal zone. In principle, the pattern of regional histogenesis can occur in two ways. First, common (region independent) neural stem cells may divide to produce progenitor cells, which are then induced to manifest a region-specific pattern of gene expression and morphology according to local cues (whether soluble or contact mediated). In this case, neural stem cells are not intrinsically specified and their progeny subsequently acquire their regional identity. Second, neural stem cells may harbor a region-specific identity (established prior to the onset of neurogenesis) that is inherited by their progenitor cell progeny. Consistent with this latter possibility, our data demonstrate that neural stem cell derived colonies in vitro (formed clonally from single neural stem cells) express molecular markers of regional identity. Although these findings do not allow us to conclude unequivacally that the very few neural stem cells themselves within each colony express regional markers, the fact each colony is clonally derived from a single stem cell suggests that regionalization is intrinsic to the colony forming stem cells within E14.5 forebrain and midbrain compartments.

Previous reports demonstrated that the early expression of transcription factors in the anterior neural plate (E8.5) already defines a regionalization pattern that persists after the onset of neurogenesis (Shimamura et al., 1995). Furthermore, when precursor cells derived from the early neural tube (E9.5-E10.5) are isolated in vitro, they maintain their regional specification (Nakagawa et al., 1996). Our present results are among the first to demonstrate that the E14.5 embryonic forebrain neural stem cells (from dorsal or ventral compartments) isolated from their in vivo environment generate clonal colonies that express a forebrain-specific regional marker *(Otx1)*, whereas neural stem cells isolated from the

midbrain instead express a midbrain-hindbrain-specific regional marker (*En1*). These data reveal that neural stem cells in the E14.5 mammalian brain manifest a regional identity along the anteroposterior axis during development. In a very recent study, Zappone et al. (2000) demonstrated that mouse E14.5 cortical neural stem cell colonies expressed a telencephalic restricted *Sox2* transgene, but that this transgene was not expressed by spinal cord derived neural stem cell colonies. Thus, neural stem cell regionalization may be regulated throughout the entire developing CNS. Moreover, neural stem cell regionalization during development is not restricted to the anteroposterior axis. We show that even within the forebrain, neural stem cell colonies derived from the dorsal compartment (cortex) express *Emx1*, but not *Dlx2*, and neural stem cell colonies derived from the ventral compartment (striatum) express *Dlx2*, but not *Emx1*. Thus, neural stem cells maintain a distinct dorsoventral identity within the forebrain, suggesting that neural stem cell regionalization can be regulated within distinct histogenic compartments rather than between broad CNS domains only.

The expression of different *Dlx* genes in the ventral forebrain is correlated with the sequence of cellular differentiation (Liu et al., 1997; Eisenstat et al., 1999). *Dlx1* and *Dlx2* are expressed in progenitor cells primarily within the VZ and SVZ of the developing striatum. In the process of differentiation and migration out of the germinal zone, progenitor cells begin to express first *Dlx5* and then *Dlx6* once they reside in the mantle zone and differentiate into mature projection neurons and interneurons, some of which migrate to the cortex. Although *Dlx2* is expressed in striatum derived neural stem cell colonies, extremely few colony cells express the *zfdlx4/dlx6lacZ* transgene, which normally mimics the endogenous expression of *Dlx5* and *Dlx6* in vivo (Zerucha et al., 2000), after an additional 7 days in differentiation conditions in vitro. These findings suggest that the expression of early regional ventral forebrain markers (*Dlx2*) may be intrinsic to isolated striatal neural stem cells (and inherited by their early progenitor cell progeny), but that expression of these genes may not be sufficient for cells within the

colony to acquire a mature regional phenotype (*Dlx5/6*). It remains formally possible that in the absence of the specific in vivo ventral forebrain environment the expression of late regional markers may require more time during in vitro differentiation. However, our findings suggest that a late regional identity may instead require a region-specific inductive cue that is not present within the neural stem cell colonies. When isolated neural stem cell colonies were co-cultured on E14.5 brain tissue slices, transgene expression in striatum derived colonies was induced within 5 days, but only when the colonies were localized to the ventral forebrain. Both cortical and midbrain tissue was not sufficient to induce transgene expression. Thus, the full expression sequence of *Dlx* genes and regional maturation may require further induction or maintenance of an extrinsic signal (present in vivo, but not in vitro), as opposed to the notion that the full sequence of *Dlx* expression is simply triggered (intrinsically) once progenitor cells express *Dlx1* or *Dlx2* (Eisenstat et al., 1999). The fact that cortical and midbrain neural stem cell colonies could be induced to express a relatively late marker of ventral forebrain regional identity (transgene) without expressing *Dlx2* is consistent with the idea that the sequence of *Dlx* gene expression may be primarily regulated by extrinsic cues.

These studies also reveal the more precise tissue source of the specific ventral forebrain inductive signal on neural stem cell colonies. I showed that the ventral VZ is not the source of the inducing signal for late regional gene expression. Rather, the overlying striatal SVZ/mantle zone tissue was sufficient for inducing transgene expression. These findings indicate that the inductive signal is localized to differentiated regions of the ventral forebrain. Interestingly, differentiation of very few neural stem cell derived progeny in the absence of the in vivo co-culture environment showed transgene expression. Thus, neuronal and glial differentiation per se may not regulate the positional information of these cells (at least outside of their natural environment).

The nature of the specific ventral forebrain inductive signal on neural stem cell colonies is not known. *Sonic hedgehog* (Shh) has been shown to be critical for inducing a

ventral forebrain regional identity (Chiang et al., 1996; Nakagawa et al., 1996; Shimamura and Rubenstein, 1997; Kohtz et al., 1998). However, Kohtz et al. (1998) demonstrated that *Dlx2* induction by Shh was limited by an early period of competence in telencephalic explants, which was lost after E12.5 in rat (~E10.5 in mouse). I demonstrated that the *zfdlx4/dlx6lacZ* transgene (which mimics *Dlx5* and *Dlx6* in vivo) could be induced in striatum, cortical or midbrain neural stem cell colonies (the latter two of which do not express *Dlx2*) by mouse ventral forebrain tissue at E14.5. One possibility is that transgene expression (or late regional markers in general) is regulated independently of early regional marker gene expression (e.g. *Dlx2*), both of which can be induced by Shh or other ventral forebrain derived factors. This would explain why neural stem cell colonies derived from cortex and midbrain could be induced to express the transgene, independent of early *Dlx2* expression. Alternatively, upregulation of *Dlx1* and *Dlx2* may be required prior to transgene expression in all of the co-cultured neural stem cell colonies (which our techniques did not allow us to detect). If the latter model is correct, then one prediction would be that late regional gene expression in forebrain or midbrain neural stem cell colonies derived from *Dlx1/Dlx2* double knockouts (Anderson et al., 1997b) would be attenuated.

Several studies have demonstrated that regional specification of forebrain precursors is mediated by extrinsic cues. For instance, when ventral (striatal) precursors isolated from the embryonic rodent forebrain ectopically integrate into dorsal (cortical) or more posterior (midbrain) tissues after transplantation in vivo, cells differentiate in a region-specific manner (Brustle et al., 1995; Campbell et al., 1995; Fishell, 1995). These findings indicate that precursor cells derived from any one region can alter their morphological, biochemical and hodological properties according their specific environment. Here I demonstrate that ventral forebrain regional identity can be induced in dorsal forebrain derived or midbrain derived neural stem cell colonies, suggesting that

neural stem cell lineages may be specified but not committed to any one compartmental identity.

Is the regulation of neural stem cell behavior distinct from the regulation of neural stem cell regional identity? The fundamental behavior of mammalian neural stem cells, which can be operationally defined as the ability to generate neuronal and glial progenitor cells, self-renewal capacity and neural multilineage potential, is similar in stem cells localized throughout the developing brain. Superimposed upon this fundamental behavior, is a unique pattern of spatial organization both between and within histogenic compartments. During forebrain development, FGF-responsive neural stem cells can give rise to separate EGF-responsive neural stem cells (Tropepe et al., 1999). We demonstrated that the relative proportion of co-existing FGF-responsive and EGF-responsive neural stem cells in dorsal and ventral forebrain compartments is similar to that of the midbrain compartment. These findings reveal that the mechanism for the formation of these separate neural stem cell populations may be the same in all regions of the developing brain. The emergence of an EGF-responsive neural stem cell subpopulation is coincident with the development of the SVZ within the striatal germinal zone (Tropepe et al., 1999). Here I show that at E14.5, neural stem cells in the striatum are differentially distributed along the ventriculopial axis and this spatial organization persists into later stages of neurogenesis. FGF-responsive neural stem cells are preferentially localized to the VZ and EGF-responsive neural stem cells are distributed throughout the VZ and SVZ. Furthermore, EGF-responsive neural stem cells are more abundant in the SVZ at later stages (E18.5) compared to the FGF-responsive population. Thus, in addition to producing the first neuronal progenitor cells by dividing asymmetrically before the formation of the SVZ, FGF-responsive neural stem cells in the VZ also divide asymmetrically to produce EGF-responsive neural stem cells that become progressively localized to the SVZ. The significance for this differential distribution is currently not well understood. A null mutation in the EGF-receptor gene can preferentially affect the proliferation of EGF-

responsive neural stem cells and is associated with glial degeneration in the postnatal cortex (Sibilia et al., 1998; Tropepe et al., 1999). In contrast, a FGF-receptor-1 null mutation diminishes FGF-responsive neural stem cell behavior at very early stages and prevents the emergence of the EGF-responsive neural stem cell population at later stages (Tropepe et al., 1999). It is possible that the extrinsic signals (e.g. mitogens) regulating neural stem cell behavior in vivo may change over time. Thus, the recruitment of different classes of stem cells may be under the control of specific signals in any one compartment.

A null mutation in the *Mash1* gene in mouse affects the formation of the SVZ in the striatal compartment as well as specific neuronal subtypes that are generated from the SVZ (Casarosa et al., 1999). In these animals, *Notch* signaling is attenuated affecting the generation of neuronal progenitor cells in the SVZ, particularly from the medial ganglionic eminence. Given that *Notch* signaling has recently been shown to affect neural stem cell behavior (Nakamura et al., 2000), neural stem cell behavior may similarly be affected by the loss of *Mash1* signaling (perhaps more prominently in EGF-responsive stem cells). This raises the interesting question of whether regional identity is also affected in these mutant animals or whether stem cell regionalization and stem cell behavior are distinctly regulated. In light of the present results, one prediction would be that striatal neural stem cells from the *Mash1* null mutants would maintain their ventral forebrain regional identity, even though the generation of neuronal progeny from neural stem cells (mediated in part by *Notch* signaling) is compromised. On the other hand, can regional neural stem cell identity be altered without affecting neural stem cell behavior? Although it is still uncertain whether neural stem cells themselves (together with their progeny) change their regional identity, our co-culture experiments suggest that fundamental neural stem cell behavior would be conserved even if neural stem cell position between histogenic compartments is altered.

In conclusion, these findings reveal that neural stem cell lineages from different compartments in the developing mammalian brain behave in a remarkably similar manner, but have a distinct spatial identity. Within a single histogenic compartment (e.g. the

striatum), relatively separate neural stem cell populations, based on their growth factor responsiveness, are differentially segregated along the ventriculopial axis. Between histogenic compartments (along the dorsoventral or anteroposterior axes), isolated neural stem cell lineages have a region-specific pattern of gene expression. Furthermore, additional regional identity in neural stem cell progeny, which is indicative of a more differentiated phenotype in a single histogenic compartment, can be induced by local cues in a cell nonautonomous manner. Finally, regional specification of neural stem cell lineages between dorsal and ventral or anterior and posterior histogenic compartments may not be irreversible. Thus, the fates of neural stem cell lineages are not committed to specific histogenic compartments, but rather some phenotypic plasticity is inherent to all neural stem cell lineages.

Chapter V

General Discussion

In this thesis, I have revealed putative mechanisms that regulate the formation of neural stem cells during mammalian development. I propose a pattern of neural stem cell formation that occurs in at least three stages. The first stage involves the acquisition of a pluripotent primitive neural stem cell identity from the embryonic epiblast. The second stage involves the restriction of multilineage potential and the diversification of growth factor responsiveness resulting in stem cell heterogeneity. Finally, the last stage involves the establishment of a unique (but not a committed) regional "address" for neural stem cell lineages - a pattern that correlates with the development of specialized functional regions in the brain.

In this final chapter, I discuss my working model of neural stem cell ontogeny with respect to its implications for neural development and for cellular homeostasis in the adult CNS. Furthermore, I speculate on a possible, and arguably simplistic, stem cell phylogeny by comparing what is known about stem cells in different metazoan phyla in the hope of drawing attention to the utility of the comparative approach for advancing our knowledge of neural stem cell biology.

Neural stem cell ontogeny

Once the primordium of the embryo proper is established (i.e., the segregation of ES cells in the ICM from extraembryonic tissue), the formation of the neural stem cell lineage is initially under inhibitory control. I suggest that in isolation at relatively low cell densities, ES cells have an autonomous tendency to differentiate into neural cells, but that this tendency is partially mitigated by intercellular signals (stronger at higher densities) that inhibit neural differentiation. In vivo, where cell density (and neural inhibition) is maximal, neural stem cell differentiation is highly dependent on the suppression of neural inhibition, which is initially mediated by surrounding tissues and subsequently by specialized regions of the embryo, such as the organizer. This notion is consistent with what is currently known about the acquisition of neural fate in vertebrate embryos. Thus, my data support

the default hypothesis of neural specification. I have shown that molecules, such as BMP4 (a TGFβ family member of extracellular proteins) can actively suppress the formation of ES derived neural colonies. Furthermore, functionally blocking TGFβ-related signaling by targeted null mutation of the *Smad4* gene or extracellular anatagonism of BMP proteins using the antagonist mCer-l or noggin can enhance neural colony formation. In vivo, where cells are in close contact, the action of such neural inhibitory molecules within the epiblast is suppressed by the same antagonists derived from the AVE (Beddington and Robertson, 1999) or the organizer (Bachiller et al., 2000). The default mechanism, which is purported to play a major role in the formation of neural tissue in the epiblast, may function by allowing individual cells to adopt a neural stem cell identity. However, the type of neural stem cell that is formed appears to be intermediate between an ES cell and a CNS derived neural stem cell, suggesting that the neural stem cell lineage may first be established from a primitive neural stem cell.

My results suggest that the primitive neural stem cell is the lineage precursor to more definitive neural stem cells in the developing mammalian CNS. First, primary neural colony formation (clonally derived from a single ES cell) requires both FGF and LIF. While subsequent self-renewal of colony-forming stem cells requires only FGF, other factors such as LIF can facilitate this process. However, under these conditions EGF-responsiveness is not achieved. FGF-responsiveness, but not EGF-responsiveness, of subcloned ES derived primitive neural stem cell colonies is comparable to the FGF-responsive, but not EGF-responsive, neural stem cells isolated from as early as the E8.5 anterior neural plate. Here, neural stem cells are initially only FGF-responsive, but subsequently give rise to both FGF- and separate EGF-responsive subpopulations that are partially segregated within the germinal zone of the developing brain. At these stages, LIF alone is not sufficient for, but can facilitate neural colony formation. Thus, the change in growth factor responsiveness over time may reflect the various stages of the neural stem cell lineage. This is supported by a second piece of evidence, which demonstrated that

neural colonies also have a distinct profile of neural (and non-neural) gene expression. Neural genes, such as *Emx2* and *HoxB1*, are both expressed in ES derived neural colonies, whereas these genes are separately expressed from distinct neocortical or midbrain/hindbrain derived neural stem cell colonies, respectively, which is consistent with the regional in vivo expression pattern (Rubenstein et al., 1998). Furthermore, other neural forebrain genes such as *Otx1* and *Six3* are not expressed in ES derived neural colonies, but are expressed in E14.5 forebrain (both dorsal and ventral compartments) derived colonies. These data indicate that overt regionalization and anteroposterior identity are not likely to be present in ES derived neural stem cells, but that regional identity is acquired and maintained at later stages possibly through the influence of patterning signals from non-neural as well as neural tissues. Finally, ES derived primitive neural stem cells display a much broader potential to give rise to neural and non-neural tissues. ES derived neural stem cell colonies contain neurons, astrocytes and oligodendrocytes as well as undifferentiated cells that express the neural precursor marker nestin. However, the expression of at least one non-neural gene (GATA-4) in ES derived neural colonies, but not forebrain neural colonies, and the apparent ubiquitous colonization of ES colony derived cells in chimeric embryos, which is observed infrequently from forebrain derived colonies (Clarke et al., 2000), indicate that the primitive neural stem cells are pluripotent. Furthermore, these data suggest that the pluripotency is dramatically attenuated once primitive neural stem cells have developed into definitive neural stem cells by E8.5. However, the molecular mechanisms regulating the subsequent restriction in neural stem cell lineage potential and change in growth factor sensitivity remains to be determined.

Are there parallels to this pattern of stem cell development in other mammalian tissues? The development of hematopoietic stem cells provides some surprising insight. It is commonly believed that hematopoiesis during mouse embryogenesis begins at ~E8 in the yolk sac blood islands (Moore and Metcalf, 1970). Hematopoietic precursor cells then migrate first to the liver, then spleen and finally to the bone marrow where they reside into

adulthood (Morrison et al., 1994). The ability to repopulate the entire hematopoietic lineages in irradiated adult animals is a definitive criterion of hematopoietic stem cell activity. After E11.5, all of these tissues have repopulation ability, suggesting that hematopoietic stem cells are present in these tissues and have undergone migratory dispersion from their initial yolk sac origin. Muller et al. (1994) demonstrated that cells from the aorta-gonad-mesonephros (AGM) region of the younger E10.5 embryo, a novel identified site of embryonic hematopoiesis, effectively repopulated irradiated adult recipient mice. However, other age-matched (E10.5) tissues, such as the liver and yolk sac, were incapable of repopulating. Thus, a somewhat counter-intuitive model emerged whereby early hematopoietic precursors from the yolk sac were, in fact, more restricted in their potential to give rise to the various blood lineages under repopulation conditions and that the true hematopoietic stem cell activity was acquired at later stages (Muller et al., 1994). These early stem cells may reflect primitive hematopoietic stem cells, which subsequently mature into definitive hematopoietic stem cells. Alternatively, two separate populations of stem cells may develop independently. Comparable transplant experiments in the nervous system using neural stem cells have been performed, but in contrast to the blood system, neural stem cell derived progeny undergo mostly glial differentiation in vivo and functional recovery due to re-instatement of neuronal circuitry has not been established. However, partial functional recovery after in vivo transplantation of neural stem cell derived oligodendrocytes (selected in vitro) has been observed (Brustle et al., 1999). Such paradigms are considered definitive "functional" assays for specific cell-type neural replacement therapy. Nonetheless, the ability of enriched stem cell populations, without prior selection of progenitor cell progeny, to directly orchestrate the cell-type specific differentiation in vivo has not been demonstrated. The problem here is an inability to prospectively identify neural stem cells in a population. In this regard, some advancements are being made to be able to sort, using flow cytometry, neural crest derived stem cells (see below) that express the low affinity neurotrophin receptor p75 (Morrison et al., 1999). To

summarize, it would seem that although lineage potentials vary, at least one similarity between the ontogeny of the hematopoietic stem cell and that of the neural stem cell might be considered. This involves the general progression during development from a primitive (transient during early development) type of stem cell to a more definitive (later development and lasting throughout life) type of stem cell.

Current evidence suggests that mammalian tissues may contain a heterogeneous population of stem cells well into adulthood. Hematopoietic stem cells may either be long-term repopulating stem cells or short term repopulating stem cells. Both have multilineage potential and persist throughout life, but differ with respect to their long-term reconstitution potential (Trevisan and Iscove, 1995). Thorgeirsson (1996) has argued that the adult liver contains both unipotential stem cells that give rise to hepatocytes and multipotent oval cells that give rise to bile duct epithelium (and other epithelial types such as acinar cells of the exocrine pancreas) as well as hepatocytes. There is evidence that osteoprogenitors in fetal calvarium may be a heterogeneous population based on the expression of different molecular markers (Aubin, 1998), but it remains to be determined whether these different progenitors arise from distinct stem cell classes. I have demonstrated that the embryonic CNS during mid-neurogenesis is composed of separate FGF-responsive and EGF-responsive neural stem cell subpopulations that co-exist throughout the CNS and are lineage related. Furthermore, these stem cells have distinct cell cycle kinetics (Martens et al., 2000) and appear to be spatially segregated, in part, within the germinal zone. The reason for such an organization is currently not clear. Both types of neural stem cells have self-renewal and neural multilineage potential in vitro and express similar region-specific patterning genes within a single histogenic compartment. Also, the growth factor dependent bias (more FGF-responsive stem cells than EGF-responsive stem cells) is present in several regions of the embryonic CNS at relatively early stages and it remains controversial whether this difference persists into adulthood (Craig et al., 1996; Gritti et al., 1999). It is possible that since the EGF-responsive stem cells emerge later during development, they

may be more actively involved in gliogenesis in vivo, which persists beyond neuronogenesis into early postnatal stages. Indirect evidence comes from studies using the EGF-receptor null embryos, which demonstrate that glial, but not neuronal differentiation is specifically impaired in the developing neocortex (Sibilia et al., 1998). Given that EGF-responsive stem cells are distributed further away from the ventricular lumen, another possibility is that discrete sources of signaling molecules in more differentiated regions during development may preferentially influence this subpopulation compared to the FGF-responsive population, which appears to be localized closer to the lumen. In late embryonic stages, EGF-responsive stem cells are estimated to divide more frequently than FGF-responsive stem cells (Martens et al., 2000). Furthermore, adult EGF-responsive stem cells in the forebrain subependyma undergo enormous expansionary (symmetric) divisions after EGF infusion into the lateral ventricles compared with symmetric divisions induced by infusion of FGF (Craig et al., 1996). It is conceivable that the EGF-responsive stem cells may participate more actively in the adult homeostasis of neuronal production in the forebrain, while the FGF-responsive stem cells remain more quiescent. Consistent with this possibility, in TGFα null mice the number of dividing neuronal progenitor cells is diminished by ~50%, even though the ability of EGF-responsive stem cells to form colonies in vitro is undiminished in the presence of exogenous EGF (Tropepe et al., 1997). TGFα is a member of the EGF family of growth factors that is preferentially expressed in the forebrain subependyma, whereas there is little or no FGF expression (Seroogy et al., 1993) and the presence of FGF-responsive stem cells in this region (Gritti et al., 1996) does not seem to compensate for the loss in progenitor cell proliferation. Thus, the lack of EGF receptor signaling may have attenuated neural stem cell division in vivo resulting in a substantial loss of olfactory interneurons (Tropepe et al., 1997). In contrast, few EGF-responsive neural stem cells are present in the adult spinal cord compared to FGF-responsive stem cells (Weiss et al., 1996). If FGF-responsive stem cells are similarly more quiescent in the spinal cord as they may be in the forebrain, then the presence of fewer

EGF-responsive stem cells may account for the rare turnover of precursor cells surrounding the central canal compared to the forebrain. In fact, infusion of EGF alone into the spinal central canal was ineffective at promoting precursor cell proliferation in vivo (compared to saline control) even though FGF infusion resulted in a significant increase in proliferating cells in this region (Martens and van der Kooy, 1999). Therefore, these observations suggest that the behavior of these two neural stem cell subpopulations may be differentially regulated in a temporally and spatially dependent manner, but their full contribution during development and to cellular homeostasis in the adult CNS remain to be determined. Stem cells have been implicated in mediating neuronogenesis in the adult rodent hippocampus (Kempermann et al., 1997; Gould et al., 1999a) and primate neocortex (Gould et al., 1999b). The relationship of these anatomical loci to learning and memory provide an interesting avenue for investigating the role of neural stem cells in brain function.

Other ontological phenomena may be more specific to tissues that bear strict anteroposterior patterning during development, such as the CNS or perhaps the gut, which ultimately translates into regional specialization in the adult. Transplants of different regions of intestinal epithelium from the duodenum to the colon subcutaneously resulted in persistent regional differences in cell types present in the ectopic site (Rubin et al., 1992). In chapters I and IV, I reviewed some the evidence for regional specification along the anteroposterior and dorsoventral axes of the brain. Two of the main findings from the results in chapter IV that I believe are novel and interesting are: (1) that neural stem cell lineages maintain their regional identity even when isolated from their natural in vivo environment; and (2) that this specification is not irreversible.

In vivo gene expression (Rubenstein et al., 1998) and progenitor cell migration patterns (Halliday and Cepko, 1992; Walsh and Cepko, 1992; Fishell et al., 1993) identify clear boundaries that separate the development of different forebrain compartments. Several studies have shown that progenitor cells that are specified to a ventral forebrain identity can

take on a new host region-specific identity when allowed to integrate into the neocortex (dorsal forebrain), as revealed by molecular, morphological or hodological criteria (Brustle et al., 1995; Campbell et al., 1995; Fishell, 1995). This apparent plasticity in dorsal versus ventral cell identity may be related to the mode of rodent forebrain development. Others have demonstrated that early and late neocortical progenitors in the ferret (with a comparatively protracted rate of neocortical development) display a striking commitment in their ability to integrate into only homotypic layers when transplanted to host neocortical tissue (Frantz and McConnell, 1996). An interesting question that was not addressed by these studies, however, is whether the progenitor cells that were used had an intrinsic regional identity at the time of the transplant, or whether the local environment was required to constantly maintain their identity. In other words, did these dorsal and ventral forebrain cells maintain their regional identity away from their original or even heterotopic environment? To begin to address this issue, I showed for the first time that isolated stem cells along the anteroposterior and dorsoventral axes can generate a colony of stem/progenitor cells in vitro that maintains the molecular identity of origin. I infer that this specification is intrinsic to the stem cells, which is then inherited by their progenitor cell progeny in the absence of the in vivo environment. However, local environmental cues can certainly influence regional marker gene expression, suggesting that these stem cell lineages are not committed to their region of origin, at least in terms of gene expression patterns, which is consist with the aforementioned transplant studies. What is not clear is whether the observed plasticity is a function of the stem cell progeny exclusively, or whether stem cells themselves can alter their regional identity when influenced by local cues. The lack of a specific neural stem cell marker does not permit a conclusive answer to this question at this time. However, one experiment that is in progress could potentially address this important issue. Briefly, embryonic midbrain neural stem cell colonies derived from mice ubiquitously expressing the GFP transgene could be dissociated into a cell suspension and injected into the embryonic forebrain lateral ventricle. The test is whether midbrain stem

cells (that express midbrain-specific genes, but not forebrain-specific genes), that randomly integrate into either the dorsal forebrain (neocortical) or ventral forebrain (striatum) germinal zones after several days, could generate new colonies (isolated GFP^+ colonies in vitro) that now express forebrain-specific markers (using RT-PCR). If my model of reversible regional stem cell identity is correct, then it is predicted that these midbrain stem cell derived colonies will in fact express forebrain-specific genes and may down-regulate midbrain-specific genes. Alternatively, if neural stem cells themselves are committed to their identity of origin (and hence the observed plasticity is intrinsic to progenitor cells), then midbrain stem cells conditioned by a forebrain environment will generate neural colonies that maintain midbrain-specific gene expression when clonally isolated in vitro.

Recent transplant studies investigating neural plasticity using neural stem cells provide rather conflicting results. Ader and colleagues (2000) demonstrated that when embryonic ventral forebrain (or spinal cord) derived neural stem cell colonies were transplanted to the early post-natal eye, the vast majority of cells adopted an oligodendrocyte phenotype expressing myelin along the nerve fibers of the ganglion cells. Very few or no neural stem cell colony derived cells were observed to integrate into other retinal cell layers or differentiate into retinal neurons. Thus, the post-natal retinal environment, at best, facilitated the differentiation of oligodendrocytes, a cell type normally generated by forebrain stem cells, but not retinal neurons. However, retinal stem cells isolated from the embryonic and adult pigmented ciliary margin of the eye can in fact generate several retinal cell types in vitro (photoreceptor neurons, bipolar neurons, and Muller glia) based on molecular and morphological criteria, but oligodendrocytes were not observed (Tropepe et al., 2000). Given that oligodendrocytes are not normally produced from retinal precursors during development, these studies suggest that there may be a certain degree of phenotypic restriction when comparing the forebrain versus the eye. This can occur in at least two ways. First, retinal stem cells may be restricted to produce only retinal cell types (as mentioned above) and forebrain stem cells may be restricted to produce

only forebrain cell types (e.g. pyramidal neurons, astrocytes and oligodendrocytes). Second, the postnatal retinal environment may not be competent to induce tissue-specific change in phenotype of the transplanted forebrain stem cell-derived progeny. The corollary experiment where retinal stem cell colonies are transplanted to an ectopic forebrain site may confirm whether this is also true for the forebrain environment, and this experiment is currently underway in the lab. Other evidence strongly rejects this notion of restriction of regional stem cell identity in the CNS (Bjornsen et al., 1999). These authors observed that embryonic or adult forebrain derived neural stem cell colonies that have sequentially been subcloned for many generations in vitro could rescue lethally irradiated mice by generating new hematopoietic cell lineages in the bone marrow. Other studies also revealed that tissue-specific stem cells may have a common inherent potential to produce lineages of other tissues (Eglitis and Mezey, 1997; Gussoni et al., 1999). More recently, Clarke et al. (2000) demonstrated that adult neural stem cell colonies could contribute to all embryonic tissues except, interestingly, the hematopoietic lineages. As discussed in Chapter II, the relatively infrequent contribution of the neural stem cell colonies to non-neural tissues in the mouse chimera paradigm is consistent with my observation that non-neural lineage potential becomes restricted through the development of primitive neural stem cells to definitive neural stem cells. However, it is clear that environmental cues can substantially influence the ability of neural stem cell lineages to adopt specific phenotypes, a phenomenon that was evident from my results of Chapter IV on neural stem cell specification. One caveat, however, is that since populations of stem cells and their progenitors are assayed simultaneously in these ectopic transplant experiments, it is uncertain whether such dramatic plasticity revealed in the neural colony transplants (into blood or blastocysts) is intrinsic to the stem cell progeny - in their ability to readily change their identity in response to the local environment or niche - or whether the plasticity is intrinsic to the neural stem cells. One intriguing possibility is that if stem cells from all tissues retain the potential to generate all types of cells, then this ability may be a vestige from ancestral stem cells during

the course of metazoan evolution (see below). Nonetheless, our knowledge of how exogenous factors could potentially influence the "choice" of a neural stem cell to generate a specific progenitor cell type remains limited.

Progress in the biology of the neural crest has perhaps been the most instrumental in elucidating how growth factors can influence the fate of multipotent precursor cells. The neural crest is a transient migratory population of precursor cells that originate from the dorsal tips of the neural folds just prior to complete neural tube closure, and give rise to a variety of cell types including neurons of the PNS, Schwann cells, melanocytes, cartilage and smooth muscle cells (Le Douarin, 1982; Bronner-Fraser and Fraser, 1989). It has been demonstrated that the neural crest contains a population of stem cells that can produce alternative neural fates by instructive signals (Stemple and Anderson, 1992). For example, GGF can promote crest stem cells to produce Schwann cells, BMP2/4 can promote autonomic neuronal differentiation, and TGFβ1-3 can promote smooth muscle cell differentiation (Shah et al., 1996). Because these crest stem cells can only be isolated as late as the early postnatal period (Morrison et al., 1999), but not from the adult, their self-renewal is evidently limited and therefore they may represent a transient renewing multipotent precursor population according to some operational stem cell definitions (Gage, 2000). A similar conclusion for an "instructive" influence of growth factors has been drawn from analyses of CNS neural stem cells. Johe et al. (1996) demonstrated that PDGF could influence neural stem cells to produce neurons, whereas CNTF promoted astrocyte differentiation. A prevailing problem, however, is that in these studies selective effects of survival or proliferation on progenitor cells can not be dealt with adequately. What is required is a specific stem cell marker that can be used to monitor how these factors influence a single stem cell to divide asymmetrically to produce one stem cell and one unique progenitor cell. In chapter III, I demonstrated that neural stem cell derived progeny are strongly influenced by their differentiation culture environment. So far, the most promising advance has been to first purify the stem cell population by flow cytometry

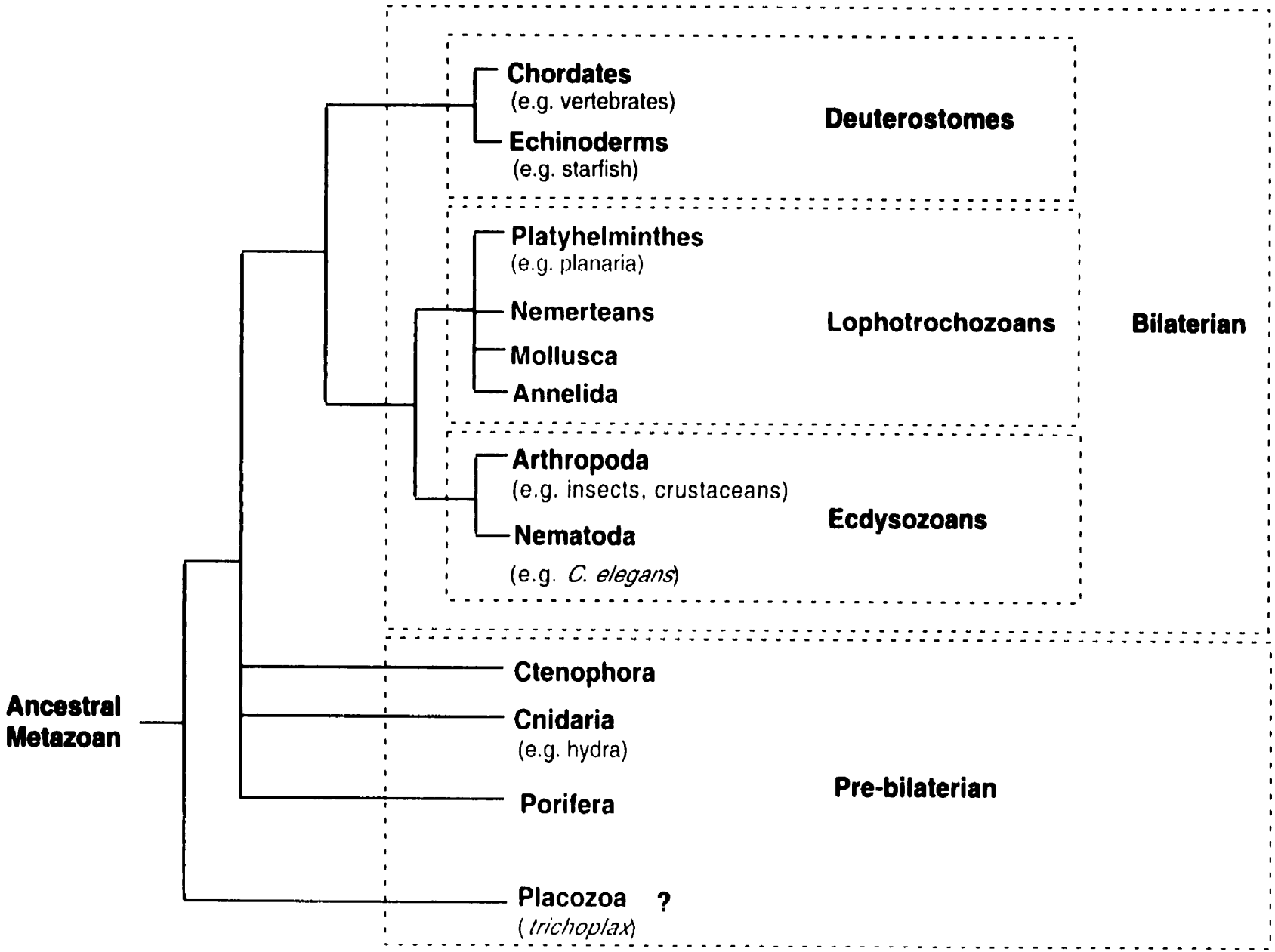
(Morrison et al., 1999). Thus, subsequent exposure of these cells (most of which have multilineage potential and appear to be a homogeneous stem cell population) to the various growth factors may reveal quantitative differences that could be used to argue the "instructive" versus "selective" effects of these factors. Nonetheless, it is clear from these examples that the identification of a unique neural stem cell marker (if one exists) would make an enormous impact on neural stem cell biology.

Toward a stem cell phylogeny

All metazoans harbour a diverse assemblage of specialized cell types that form the basis for a variety of simple or complex functional organ systems. Some animals contain unique cell types that are not representative of all animal phyla. For instance, the stinging cells, or nematocytes, of cnidarians are not present in any other phylum (Bode, 1996). On the other hand, with the exception of the phylum porifera (sponges), all extant animals have nerve cells. The existence of a common specialized cell type, such as a neuron, in all animals that have evolved independently of the sponges may indicate that porifera underwent a secondary loss of nerve cells through the course of their evolution. Alternatively, nerve cell specialization may have evolved after the bifurcation of the sponges and all other animals from their last common ancestor. From an embryological perspective, the generation of common specialized cell types, such as nerve cells, among most metazoans would seem to suggest that there is a common developmental strategy for generating a diverse repertoire of cell types. During mammalian embryogenesis, for instance, single primordial stem cells (e.g. the ES cells of the ICM) produce all cell lineages of the embryo, including tissue-specific stem cells. The continued presence of stem cells in adult tissues and their contribution to regeneration and cellular homeostasis suggests that the cellular and molecular events underlying mammalian embryogenesis may be recapitulated during regeneration. To speculate further, it is possible that a similar developmental strategy is common to all metazoan phyla (Figure 5.1) and that the origin of the metazoa was

Figure 5.1. Metazoan phylogeny. Schemtaic representation of descendent extant phyla from ancestral metazoans. Only some phyla have been included in each of the superphyla (lophotrochozoa, ecdysozoa, deuterstomata) for clarity and branching lines depict lineal relationships and do not accurately indicate the time of phyletic divergence. Figure is adapted from Adoutte et al., 1999. Please note that this tripartite organization for the metazoan radiation is used as a convenient reference for the relationships of the extant phyla mentioned in the text and is by no means a definitive phylogeny.

Metazoan Radiation



contingent upon the multiplication and diversification of a single ancestral stem cell. Below I offer several pieces of evidence for a plausible scenario linking the putative stem cell origin of multicellular animals and a role for these stem cells during development, regeneration and adult cellular homeostasis.

Origins

To begin to address this issue, it is important to first establish whether there is any evidence that the transition from single-celled organisms to multicellular animals can, in principle, have occurred through the multiplication and specialization of a single stem cell. The morphology and behavior of two extant basal eukaryotes provide relevant examples that support this possibility.

First, choanoflagellates, which are single-celled protists, are characterized by a specialized collar of microvilli that surround a flagellum (Margulis et al., 1991). This structure allows these single-celled organisms to absorb food (bacteria) that is filtered through the pumping action of the flagellum. Porifera feed by a very similar filtering action of specialized cells called choanocytes, named as a result of their striking structural and functional similarity to their single-celled organismal counterparts (Ruppert and Barnes, 1994). Thus, the structure and function of a single-celled organism can assume a similar role as a specialized cell within a multicellular animal, and sponges are the closest living relatives to these basal eukaryotes and represent a basal position in metazoan phylogeny (Knoll and Carroll, 1999). One possibility is that during the course of the evolution from a choanoflagellate-like cell, cell division produced multiple cells endowing this putative ancestral multicellular animal with novel cell lineage relationships, cell type specializations within the cluster and environmental adaptability, which would be favorably selected. Interestingly, the placozoan species trichoplax contain cells, many of which have ciliated "hoods" resembling poriferan choanocytes, that can rebuild tissues through mitosis after dissociation (Rassat and Ruthmann, 1979; Ruthmann and Terwelp, 1979). Trichoplax is

the most primitive metazoan known and may closely resemble the first multicellular animals, but cell lineage in these species has not been determined. In keeping with my hypothesis, then, one would predict that a stem cell exists in basal metazoan species that, through cell division, can account for the putative diversification of cell types from a single ancestral stem cell. If the ancestral choanoflagellate-like cell were a stem cell, then that ancestral stem cell also may have been capable of regeneration – a property that is common among the vast majority of stem cells found in extant metazoans. In this regard, the asexual reproduction in protozoans, such as *Bursaria* (reviewed in Sanchez Alvarado, 2000), is strikingly similar to regeneration in these same organisms – each half of a severed cell regenerates into a fully formed protozoan. However, it is clear that regeneration in a single celled organism is quite different from the contemporary view of regeneration in animals. In single celled organisms, parts of a severed cell are capable of producing whole individuals. Nonetheless, regeneration may be one of the most primordial of attributes and a common ancestral origin of regeneration in all metazoans may be intimately linked with a common ancestral stem cell origin for the metazoa.

Second, amoeboid protists, such as *Dictyostelium discoideum*, exist as single-celled organisms under favorable conditions, but in contrast to the metazoa, these organisms can achieve multicellularity by cell aggregation in response to nutrient depletion. The slug that is formed as a result of cellular aggregation has clear anteroposterior organization, including a few "specialized" cell types, such as pre-spore cells (posterior) and pre-stalk cells (anterior) (Brown and Firtel, 1999). If separated, however, pre-stalk or pre-spore cells can regenerate the missing cell types. Although this broad range of cellular plasticity exists in all of the slug cells, a small proportion of cells, the so-called ALC, seem to contribute most to the regenerating process, essentially acting like stem cells (Abe et al., 1994). Thus, slug formation in *Dictyostelium* represents a putative scenario of the diversification of a single stem cell. Since all single-celled amoebae have the potential of becoming any cell type within the organized slug, the partial segregation of stem-like and

more differentiated cell types parallels the organization of many metazoan tissues that are formed through stem cell proliferation. Taken together, these two examples reveal possible links between single-celled organisms, which independently perform all necessary "organismal" functions and multicellular organisms that parcel different functions to specialized cells. This transition, I would argue, occurs as a result of multiplication and diversification of a single ancestral stem cell.

Metazoan Life history

One stem cell criterion that has been previously mentioned is longevity - the ability of the stem cell population to last throughout life. In order to account for a phylogeny of stem cells, one must also consider the evidence for the presence of adult stem cells in representative species for all of the extant metazoan phyla (Figure 5.1). Comparative data on stem cell biology in this regard is sparse, but I will provide a few relevant examples.

One example of a pre-bilaterian animal has already been mentioned. The porifera (sponges) contain a small population of totipotent stem cells, called archaeocytes, lodged in the body wall that can replace cells that have been lost (Ruppert and Barnes, 1994). These animals do not have any organs, per se, but contain several specialized cell types. Normally the archaeocytes are involved in ingesting and digesting food and transporting nutrients to other cells. These functions bare a resemblance not only to the choanocytes, which can be generated from these stem cells during regeneration, but also to the single-celled choanoflagellates. Evidence for a direct lineage relationship between the archaeocytes and all other poriferan cells during development is necessary to further validate the notion that stem cells are the ultimate lineage precursors in this species. The cnidaria, like the porifera, arose very early in metazoan evolution and represent a second group of pre-bilaterian animals (Knoll and Carroll, 1999). Hydra, a member of the cnidarian phylum, consists of several stem cell lineages (epithelial and interstitial) and have robust regenerative capacity (Bode, 1996). Both epithelial and interstitial stem cells exhibit self-renewing and multipotentiality

throughout life. Interestingly, epithelial stem cells in hydra normally have a basic functional role in protection and osmoregularity in addition to their stem cell characteristics (Bode, 1996). Thus, in these relatively simple pre-bilaterian animals, stem cells may at least partially assume a specialized function that is independent of their "stemness." In fact, a recent example of such a dual role for stem cells comes from the adult mammalian forebrain neural stem cells that appear to demonstrate astrocyte-like characteristics (Doestch et al., 1999). One can similarly infer such a role for adult mammalian retinal stem cells that reside in the pigmented ciliary margin of the eye (and are pigmented) and display stem cell characteristics in vitro (Tropepe et al., 2000). These mammalian retinal stem cells resemble other vertebrate retinal stem cells in both anatomical localization and multilineage potential (Johns, 1977; Wetts et al., 1989), but differ with respect to their frequency of division. Adult in vivo retinal neuronogenesis in teleost and amphibian species is relatively constant, whereas mammalian stem cells are quiescent. The absence of observable retinal stem cell activity in vivo may indicate that their stemness is actively repressed but they persist by virtue of the fact that they may have an independent function. However, further experiments are required in several representative phyla in order to confirm the possibility of a dual role for stem cell function that is evolutionarily conserved.

Animals belonging to the ecdysozoa, a number of diverse phyla represented by the fact that animals molt their outer cuticle (Adoutte et al., 1999), are capable of adult regeneration (e.g. limb regeneration) and, in some cases, contain putative adult stem cells involved in cellular homeostasis. Arthropod species, such as decapod crustaceans (Schmidt and Harzsch, 1999) and insects (Cayre et al., 1994) have been shown to exhibit adult neurogenesis in the central olfactory pathway and dorsal cortex, respectively, of the brain. Neural stem cells are thought to mediate adult arthropod neurogenesis (Schmidt and Harzsch, 1999). Neural stem-like cells are similarly thought to actively participate during the formation of the insect peripheral nervous system during development (Doe et al., 1998), but it remains unclear whether these stem cells are the same population identified in the adult

insect brain (mushroom body). Also, the presence of stem cells in many other ecdysozoan species, such as the onychophora or the gastrotrichs is not known, although regeneration is known to occur in these species. In contrast, there is very little evidence for adult stem cells in the other major ecdysozoan group, the nematodes. During the development of *Caenorhabditis elegans*, the P-blastomeres undergo a series of asymmetirc cell divisions to produce the somatic founder cell populations AB, MS, E and C as well as the germline precursor P_4 (Sulston et al., 1983). Thus, these stem-like cells are self-renewing and multipotential during development. In adult stages, however, stem cells are either terminally quiescent or all cell lineages undergo terminal differentiation, and thus stem cells are absent. Furthermore, there have been no reported cases of regeneration in these animals (of which I am aware). Thus, with regards to the apparent absence of adult stem cells in this extensively characterized species, and the apparent absence of regeneration, the nematodes may be a highly derived group of animals that underwent secondary loss of stem cell mediated development and regeneration over the course of their evolution. Whether this is true for all nematodes or is indicative of a handful of favorite lab animals remains to be determined.

Members of the lophotrochozoa, such as the planarian species within the platyhelminthes phylum, represent the simplest bilaterian triploblastic (three germ layer) animals (Adoutte et al., 1999). From planarian regeneration studies it was suggested that totipotent somatic stem cells, called neoblasts, existed in the mesenchymal space throughout the body to supply appropriate cells for regeneration (Baguna et al., 1989; Agata et al., 1999). In these studies, it was postulated that the neoblasts primarily contributed to the regeneration blastema (remnant stump left over from the severed tissue), which may indicate that stem cell activity is absent or not activated in non-neoblast cells. A molecular marker for this type of segregation was found in the form of a vasa-like RNA helicase gene, which is expressed in the neoblast chromatid bodies (unique morphological features of somatic stem cells) as well as the germline cells (Shibata et al., 1999). It is presently uncertain whether other members of the lophotrochozoa have stem cells in the adult that contribute to the

process of regeneration. Cells with stem-like properties have been observed during the development of molluscan and annelid development. For instance, one of the macromeres that is established during cleavage stage is induced to become the stem cell of the left and right mesodermal bands, called the mesentoblast (van den Biggelarr et al., 1997). However, it is not clear whether these or other putative stem cells persist in adult stages and contribute to the process of regeneration or cellular homeostasis. Again, the fact that adult regeneration does occur in many lophotrochozoan phyla is consistent with the persistence of stem cells into adulthood.

Actually, many groups of lophotrochozoa (including platyhelmithes, annelids and nemerteans) as well as invertebrate deuterostomes (such as echinoderms and the hemichordates) contain species that present another intriguing problem with respect to the existence of stem cells in adult stages. Several species within these groups undergo what is known as maximal indirect development. Indirect developers give rise to a rather simple ciliated larva that may bear little or no resemblance to the adult body form (Peterson et al., 1997). During metamorphosis, many larval structures are lost and the juvenile develops from a small patch of cells, called set-aside cells, that remain pluripotent and relatively undifferentiated throughout embryogenesis (Peterson et al., 1997). Davidson and colleagues have argued that the invention of set-aside cells and their utilization of novel genetic regulatory mechanisms, such as *Hox* gene expression, allowed for the evolution of the bilaterean body plan (Davidson et al., 1995; Peterson et al., 2000). Their hypothesis for modern invertebrates generally assumes that at early embryonic stages, animals that undergo maximal indirect development will generate a higher proportion of larval-specific cells, whereas animals that undergo direct development (larva that share several features with the adult body form) contain less larval-specific cells and a greater proportion of set-aside cells. These authors also argue that indirect development (segregation of larval and set-aside cells) is the ancestral state and that several transitions toward a direct development pattern must have occurred. For the purpose of the present discussion, the mode of

metazoan development that best represents the ancestral state is an esoteric point that is beyond the scope of this thesis. Regardless of mode, it may be inaccurate to posit that set-aside stem cells were an invention for the bilaterians. An alternative view is that stem cells during early stages of development may either produce cells that form transient structures that are subsequently lost (indirect development) or structures that prefigure the adult anatomy (direct development). Afterall, stem cells with similar behaviors to that of set-aside cells are found in abundance in pre-bilaterian animals.

Finally, the deuterstomes are comprised of the echinoderm, hemichordate and chordate phyla. Regeneration is known to occur in the hemichordates and in echinoderms, but stem cells have not been clearly identified. The chordates are subdivided into the urochordates (ascidians), cephalochordates (amphioxus) and the vertebrates. As representative of vertebrates, stem cells in mammals (primarily rodents) has been extensively investigated and, as previously described, stem cells play a major role during regeneration and cellular homeostasis. There is also evidence for whole-body stem cell mediated regeneration in the botryllid ascidians (Rinkevich et al., 1995). Whether undifferentiated stem cells are the source of regeneration in other non-vertebrate chordates or within other deuterostome phyla remain to be determined.

Model

I propose that the origin of the metazoa occurred through the multiplication and diversification of a stem cell. Stem cell lineage selection in the context of evolution has recently been considered (van der Kooy and Weiss, 2000; Weissman, 2000). Although a stem cell-based phylogeny is admittedly a reductionist approach and akin to a "selfish stem cell," it is nonetheless comparable to alternative methods that have traditionally involved the comparison of single or few anatomical structures, early embryonic cleavage pattern, and more recently DNA sequence analyses of a handful of genes.

The hypothesis for a stem cell phylogeny can be summarized as follows. First, the single-celled pre-metazoan ancestor was a stem cell. Second, the single-celled stem ancestor divided to produce a multicellular organism composed of stem cells that can be differentially specified, but that retain multilineage potential. Specialization of cell types (e.g. observed during slug formation in *Dictyostelium*) is directly correlated with their ability to adapt to different microenvironments. Third, the formation of multiple distinct cell types in simple metazoans (e.g., placozoa, porifera) is a consequence of stem cell asymmetric division during development. Although all of the cells within the proposed ancestral metazoan would retain stem cell properties, stem cell function becomes progressively restricted to only small subsets of cells, while the other cells remain committed to a specific cell fate, as is observed in extant basal metazoans. In contrast to the model put forth by Davidson and colleagues, set-aside cells (as described in maximally indirect developing species) are the primordial stem cells and not a subsequent invention that prefigured the emergence of a new bilaterian form of development. I would argue that metazoan lineages that develop directly simply by-pass the production of non-retained larval structures. Alternatively, species that maintain a maximally indirect mode of development have evolved independent mechanisms for generating many larval tissues (from set-aside stem cells), perhaps for the purpose of niche adaptation of the larvae, which are then discarded in the juvenile. This would also suggest that once the stem cells have generated larval structures, they remain relatively quiescent until the onset of juvenile development. Interestingly, larval structures are not all lost when these animals undergo metamorphosis. For example, the larval gut gives rise to the juvenile gut (van den Biggelaar et al., 1997). Thus, stem cells generate several cell types at the onset of development, some are then lost and yet others are generated at different times. Finally, in many invertebrate as well as several vertebrate species (e.g. especially in mammals), the stem cells of development persist into adulthood where they may be ubiquitous as in the hydra, or may be sporadically displaced and differentially active during regeneration, as in

the planaria. A direct test of this would be to label individual cells in very early stages of development using modern indelible labeling techniques (e.g. retroviral reporter gene expression constructs) and observing their contribution during adult regeneration. Furthermore, in addition to regeneration and cellular homeostasis, stem cells may have a functional role in physiological homeostasis (e.g. the poriferan archaeocytes, mammalian forebrain subependymal astrocyte). Although this hypothesis may be adequate as a starting point for understanding stem cell phylogeny, it clearly requires further consideration on the putative mechanisms that define the important transitions discussed above.

In addition to gaining a better understanding for the role of stem cells in the evolution of metazoan development, investigating stem cell biology in the pre-bilataria, lophotrochozoa, ecdysozoa and deuterostomes is advantageous for three main reasons. First, many of the species within these taxa have a relatively simple body plan with manageable numbers of cells for lineage analyses offering highly resolved spatial and temporal information on how stem cells behave during regeneration in the adult. Thus, the use of simple experimental paradigms can provide an abundance of information on the basic function of stem cells in metazoan development and homeostasis. Second, just as broad-ranging similarities in embryological stages helped to define the relationship between species sharing a common body organization over the last two hundred years, a deeper knowledge of the mechanisms of embryology will further resolve our understanding of these relationships in the next two hundred years. Lineage relationships between stem cells and their diverse progeny within any one species will be infinitely useful in comparing how other species achieve a similar cell type diversity. For instance, do all vertebrates have neural stem cells? Are relative restrictions in tissue-specific stem cell potentials in mammals or other classes of animals coincident with an increase in overall body size or complexity in organization? Finally, one can test whether a default mechanism for neural fate specification (neural stem cell, neuronal or other neural cell types), which is proposed to account for the establishment of a neural fate in vertebrates, occurs in other metazoan phyla. As an

extension of the stem cell phylogeny proposed in this chapter, one may infer that if default neuralization is, in fact, a universal mechanism for generating neural cell types (and other cell types by initially inhibiting neural differentiation), then it follows that the metazoan radiation may have originated from a neural stem-like cell. Such a simplistic and speculative notion is, of course, meaningless without some initial hint of evidence or at least rational deduction. Well, I have no evidence to offer, but I am struck by the complex behavior of some single celled protists, such as the *Paramecium*, that effectively sense and respond to their microenvironments just as neurons do. My intention here is not to draw direct parallels with single celled protists and mammalian neural stem cells. However, I believe in the possibility that the evolutionary history for metazoans originated from a single nerve-like cell that could have thrived as an individual organism, multipled to produce new and distinct cell types, and retained this potential at later stages of life. Further investigations of stem cells in species of diverse phyla will help to sustain or refute this claim. Regardless, comparative stem cell biology is poised to make a significant impact on our understanding of the development and evolution of metazoan diversity.

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