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Identification and Characterization of Stem Cells in the Adult Mouse Spinal Cord

by

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Abstract

Multipotent stem cells that respond to the combination of epidermal growth factor and basic fibroblast growth factor have previously been isolated from the adult mammalian spinal cord. These cells meet the functional criteria of neural stem cells because they form spheres of undifferentiated cells that generate neurons, astrocytes, and oligodendrocytes and they expand their population to form subcloned spheres. The present study confirms that epidermal growth factor+basic fibroblast growth factor responsive stem cells exist in the adult mouse spinal cord, and tests stem cell responsiveness to epidermal growth factor, basic fibroblast growth factor, heparan sulfate and basic fibroblast growth factor+heparan sulfate. Only epidermal growth factor+basic fibroblast growth factor and basic fibroblast growth factor+heparan sulfate produce expandable spheres. Therefore these growth factor combinations are used subsequently to determine whether regional differences exist between stem cells from the cervical, thoracic and lumbar/sacral spinal cord. The properties investigated are: stem cell distribution and ability to renew, expansion, and generation of multiple phenotypes. The results show regional differences are present and are consistent with a hierarchical model that describes hematopoietic stem cells. Cells at the top of the hierarchy have a high capacity for renewal, while cells on successively lower levels have a reduced capacity for renewal. The findings of this study suggest that cervical, thoracic, and lumbar/sacral stem cells, correspond to progressively lower levels on the stem cell hierarchy. Primary and subcloned, as well as epidermal growth factor+basic fibroblast growth factor- and basic fibroblast growth factor+heparan sulfate-responsive stem cells also correspond to successive levels of this hierarchical model. Therefore, spinal cord stem cells may represent a heterogeneous population from various levels of the stem cell hierarchy.

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to Case

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Abbreviations Used

aCSF	artificial cerebrospinal fluid
AMCA	7-amino-4-methylcoumarin-3-acetic acid
anti-GFAP	anti-glial fibrillary acidic protein
anti-MAP-2	anti-microtubule-associated protein-2
BEHAB	brain-enriched hyaluronan-binding protein
bFGF	basic fibroblast growth factor
CNS	central nervous system
DIV	days *in vitro*
DMEM	Dulbecco's modified Eagle medium
E10	embryonic day 10
E12	embryonic day 12
E14	embryonic day 14
E15	embryonic day 15
E16	embryonic day 16
EGF	epidermal growth factor
FGF	fibroblast growth factor
FGFR1	fibroblast growth factor receptor 1
FGFR2	fibroblast growth factor receptor 2
FGFR3	fibroblast growth factor receptor 3
FGFR4	fibroblast growth factor receptor 4
GFAP	glial fibrillary acidic protein
HEPES	N-(hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
hs	heparan sulfate
LSD	least significant difference
MAP-2	microtubule-associated protein-2
NGS	normal goat serum
O-2A	oligodendrocyte-type 2 astrocyte
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
RA	retinoic acid
SVZ	subventricular zone

1 INTRODUCTION

1.1 Spinal Cord Formation

The central nervous system (CNS) is derived from a layer of ectoderm. This layer, along with the mesoderm and endoderm, gives rise to all of the cells in an organism (Alberts *et al.*, 1994). The ectoderm gives rise to two tissues, the epidermal ectoderm which becomes the outer layer of the skin, and the neural ectoderm which becomes the central nervous system (Carlson, 1988). Ectoderm is transformed into neural ectoderm in the absence of molecules that induce the epidermal ectoderm to differentiate (Wilson and Hemmati-Brivanlou, 1995; reviewed in Hemmati-Brivanlou and Melton, 1997). The spinal cord is believed to be distinguished from the rest of the developing CNS when a chemical signal, acts on neural ectoderm to establish spinal cord identity (Doniach, 1995; Kengaku and Okamoto, 1995; Lamb and Harland, 1995). The neural ectoderm is characterized by its high columnar cells that distinguish it from the remaining ectoderm (Carlson, 1988). This grouping of high columnar cells is termed the neural plate. During the process of primary neurulation, the neural plate bends upwards at the lateral margins until they meet, forming the neural tube (Sakai, 1989). Neural plate bending is facilitated by both rapid proliferation of cells and changes in cell shape (cubodial cells become narrowed at their apex and widened at their base) (Carlson, 1988). The caudal two-thirds of the neural tube become the spinal cord. Secondary neurulation occurs when a segment of solid tissue, the tail bud, joins the neural tube at its caudal aspect (Criley, 1969). A process of cavitation ensues and the tail bud acquires a lumen that is continuous with the neural canal. The tail bud contributes tissue to the lumbar/sacral region of the neural tube, overlapping with the neural tube proper such that the tail bud forms the ventral surface, and the neural tube forms the dorsal surface (Criley, 1969; Schoenwolf, 1978).

1.2 Spinal Cord Histogenesis

Neurons and glia are the two major classes of cells in the CNS that are produced during development by a layer of dividing cells lining the central canal (Sauer, 1935; Fujita, 1962; Nornes and Carry, 1978). This layer is called the ventricular zone. The cells in the ventricular zone give rise to CNS precursor cells: neuroblasts and glioblasts (Fujita, 1965; Martin and Langman, 1965).

Neurogenesis begins on embryonic day 10 (E10), in the mouse (Nornes and Carry, 1978) and one day later in the rat (Nornes and Das, 1974; Altman and Bayer, 1984). It is completed prenatally by embryonic day 15 (E15), and embryonic day 16 (E16), in the mouse and rat, respectively (Nornes and Carry, 1978; Altman and Bayer, 1984). Neurons are derived from neuroblasts which develop in the cervical region first, followed by the thoracic and lumbar/sacral regions, creating a rostrocaudal temporal gradient in spinal cord neuron production (Nornes and Das, 1974; Nornes and Carry, 1978). A ventral-to-dorsal gradient also exists in neuron production in which neurons first develop in the more ventral aspect of the developing spinal cord followed by neuron production in the dorsal spinal cord (Nornes and Das, 1974; Nornes and Carry, 1978). These cells migrate to their final positions along the processes of a transient population of glial cells termed radial glia (Rakic, 1972). Radial glial cell bodies are found in the ventricular zone and their processes extend laterally to the pial surface of the neural tube. The fate of this transient population of cells will be discussed below.

Gliogenesis refers to astrocyte and oligodendrocyte production in the CNS. It begins after the onset of neurogenesis (Fujita, 1965; Altman and Bayer, 1984) and proceeds in a rostral-to-caudal direction (Warf *et al.*, 1991). Differentiated glia are present in the rodent cervical spinal cord by E16 (Warf *et al.*, 1991). The number of spinal cord glia continues to increase up to 12 days postnatally (Sakla, 1965; Gilmore, 1971). However, low levels of glial proliferation have been observed in the spinal cords of young

adult mice (Kraus-Ruppert *et al.* 1975). Two hypotheses attempt to explain glial cell origins in the developing CNS. The first suggests that glia are derived from precursors lining the central canal (Fujita, 1965; Gilmore, 1971). Recent work in the E16 rodent cortex has shown utilizing retroviral-labeling techniques that the ventricular zone cells generate distinct astrocyte and oligodendrocyte clones (Luskin *et al.*, 1993), suggesting that a similar stiuation may occur in the spinal cord. Oligodendrocyte precursors have been found in the ventral neural tube (Warf *et al.*, 1991; Yu *et al.*, 1994), and some studies suggest that they migrate laterally and dorsally to populate the surrounding white matter (Warf *et al.*, 1991; Noll and Miller, 1993). However, a recent study using immunocytochemical labeling of quail/chick chimeras showed that oligodendrocytes are generated in both the ventral and dorsal neural tube and migrated in the ventrodorsal and the dorsoventral directions (Cameron-Curry and LeDouarin, 1995).

An alternative hypothesis proposes astrocytes and oligodendrocytes may be derived from radial glia (Hirano and Goldman, 1988). Radial glia transform into astrocytes when their processes detach from the ventricular and/or pial surface of the developing CNS in rodents and primates (Akers, 1977; Schmechel and Rakic, 1979). This work is supported by studies showing that cells with the morphological and antigenic characteristics of radial glia also express glial fibrillary acidic protein (GFAP) which is characteristic of astrocytes (Hirano and Goldman, 1988; Culican *et al.*, 1990; Hunter and Hatten, 1995). The radial glial origins of oligodendrocytes are not as well established. However, this hypothesis is supported by *in vitro* immunocytochemical studies showing that the cells labeled as radial glia eventually express an oligodendrocyte-specific antigen (Hirano and Goldman, 1988).

The production of spinal cord neurons and glia may be influenced by polypeptide growth factors and proteins. The spinal cord becomes distinct from the remaining CNS by the actions of a factor believed to be basic fibroblast growth factor (bFGF), on the neural tube (Kengaku and Okamoto, 1995; Lamb and Harland, 1995). When bFGF is applied to

animal caps from early gastrulae it induces the expression of spinal cord marker Hoxb-9 (Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995). Another factor that may be important in spinal cord histogenesis is brain-enriched hyaluronan-binding protein (BEHAB). During development BEHAB appears throughout the ventricular zone coincident with the onset of gliogenesis suggesting it may have a role in the production of astrocytes and/or oligodendrocytes (Jaworski *et al.*, 1995). Retinoic acid (RA) may also be important in oligodendrocyte development (Noll and Miller, 1994). RA is believed to inhibit oligodendrocyte differentiation, thus enabling the immature precursors to diffuse throughout the spinal cord prior to becoming terminally differentiatedoligodendrocytes (Noll and Miller, 1994). The previous examples have shown that proteins and polypeptides influence cell phenotype in the developing spinal cord. Proteins have also been shown to help identify spinal cord neurons. A relevant example is the homeobox gene product Islet-1, which is an early marker for motor neurons (Ericson *et al.*, 1992). In addition to the actions of polypeptides and proteins on cell phenotype, polypeptide growth factors also influence the proliferation of embryonic precursor cells (Kilpatrick and Bartlett, 1993, 1995; Reynolds and Weiss, 1996). These growth factors include bFGF and epidermal growth factor (EGF) (Kilpatrick and Bartlett, 1993, 1995; Reynolds and Weiss, 1996). Basic fibroblast growth factor (FGF) induces the proliferation of an E10 precursor cell from the mouse forebrain (Kilpatrick and Bartlett, 1993, 1995), while EGF enables forebrain stem cells and retinal precursor cells to divide (Anchan *et al.*, 1991; Reynolds and Weiss, 1996). These studies beg the question are bFGF and EGF involved in spinal cord histogenesis?

1.3 Epidermal Growth Factor

EGF belongs to a family of at least seven related polypeptides including transforming growth factor α, amphiregulin, and the pox virus growth factors (Shoyab *et*

al., 1989; Twardzik *et al.*, 1985; Marquardt *et al.*, 1984; Capenter and Wahl, 1990). These related polypeptides share sequence and structural homology. EGF is a polypeptide chain of 53 amino acids (Deuel, 1987) discovered in the submaxillary gland of the male mouse (Cohen, 1962). It has since been found in the mammalian telencephalon (Fallon *et al.*, 1984) and cerebrospinal fluid (Hirata *et al.*, 1982). EGF and its homologs competitively bind to the EGF receptor (Marquardt *et al.*, 1984; King *et al.*, 1986; Carpenter and Wahl, 1990). This receptor is a monomeric transmembrane glycoprotein (Ullrich *et al.*, 1984) which is induced to dimerize upon EGF binding (Yarden and Schlessinger, 1987). Dimerization enhances the receptor's tyrosine kinase activity (Schlessinger, 1988), thus enhancing the biological effect of EGF.

EGF is a potent mitogen for CNS cells; *in vitro* it enables proliferation of neuroepithelial progenitors (Anchan *et al.*, 1991; Reynolds *et al.*, 1992) and glia (Simpson *et al.*, 1982). It also acts *in vivo* to expand precursor cell populations in the murine forebrain (Craig *et al.*, 1996). Studies suggest that it may have additional roles, for example, in glial cell differentiation (Honegger and Guentert-Lauber, 1983) or neuronal survival (Morrison *et al.*, 1987; Casper *et al.*, 1991).

1.4 Fibroblast Growth Factor

The FGF family consists of at least nine related polypeptides (reviewed in Baird, 1994) and four fibroblast growth factor homologous factors (Smallwood *et al.*, 1996). Acidic and basic FGFs, the first FGFs characterized (Esch *et al.*, 1985; Gimenez-Gallego *et al.*, 1985), were originally isolated from the bovine brain and pituitary gland (Gospodarowicz, 1975; Thomas *et al.*, 1980). They are single chain polypeptides of 140 and 146 amino acids, respectively, and share a 55% sequence homology. FGFs bind to four high-affinity receptors -- transmembrane proteins with intracellular tyrosine kinase activity -- FGFR1, FGFR2, FGFR3, and FGFR4 (fibroblast growth factor receptors 1, 2,

3, 4), (reviewed in Basilico and Mosscaatelli, 1992). Basic FGF binds with high affinity to FGFR1 and FGFR2 (Dionne *et al.*, 1990). The FGFs also bind to a low-affinity receptor, the heparan sulfate (hs) proteoglycan, which is located in the extracellular matrix and on the cell membrane (Kiefer *et al.*, 1990: Ruoslahti and Yamaguchi, 1991). This low-affinity receptor enables FGF to bind to its high-affinity receptor, resulting in receptor dimerization and activation of intracellular receptor tyrosine kinases (Spivak-Kroizman *et al.*, 1994). A recent report found that hs activates FGFR4 independent of a growth factor via stimulating FGFR4 tyrosine phosphorylation in cultured myoblasts and fibroblasts. Heparan sulfate was unable to activate FGFR1, while its affect on FGFR2 remains unknown (Gao and Goldfarb, 1995).

Basic FGF is a mitogen for some CNS cells. It enhances proliferation of cultured precursor cells isolated from the embryonic retina (Lillien and Cepko, 1992) and spinal cord neuroblasts *in vitro* (Ray and Gage, 1994). Basic FGF also acts as a survival factor for cultured neurons from multiple CNS regions including the spinal cord (Unsicker *et al.*, 1987), the cortex and striatum (Walicke, 1988), and the hippocampus (Mattson *et al.*, 1989).

1.5 Combined and sequential effects of growth factors on CNS precursors

Growth factors often work individually to produce trophic and mitotic actions on cells *in vitro*. However, both combinations of growth factors and factors added sequentially can elicit a unique biological response. When oligodendrocyte-type 2 astrocyte (O-2A) progenitor cells are cultured in platelet-derived growth factor (PDGF) or bFGF alone they eventually differentiate into oligodendrocytes (Noble *et al.*, 1988; Bogler *et al.*, 1990). However, when PDGF and bFGF are used in combination O-2A cells fail to differentiate (Bogler *et al.*, 1990). Cattaneo and McKay (1990), showed that striatal

neuroblast proliferation in the rat embryo requires sequential treatment with bFGF followed by nerve growth factor. In addition, EGF and bFGF act sequentially on neural stem cells (Vescovi *et al.*, 1993; Reynolds and Weiss, 1996). EGF induces these stem cells to divide (Reynolds and Weiss, 1996), while bFGF acts on the EGF-generated cells to produce neuronal and neuronal/astroglial progenitor cells (Vescovi *et al.*, 1993). Stem cells also expand their population in response to combinations of growth factors. For example, EGF+bFGF enables stem cells isolated from the adult forebrain to proliferate *in vitro* (Weiss *et al.*, 1996a).

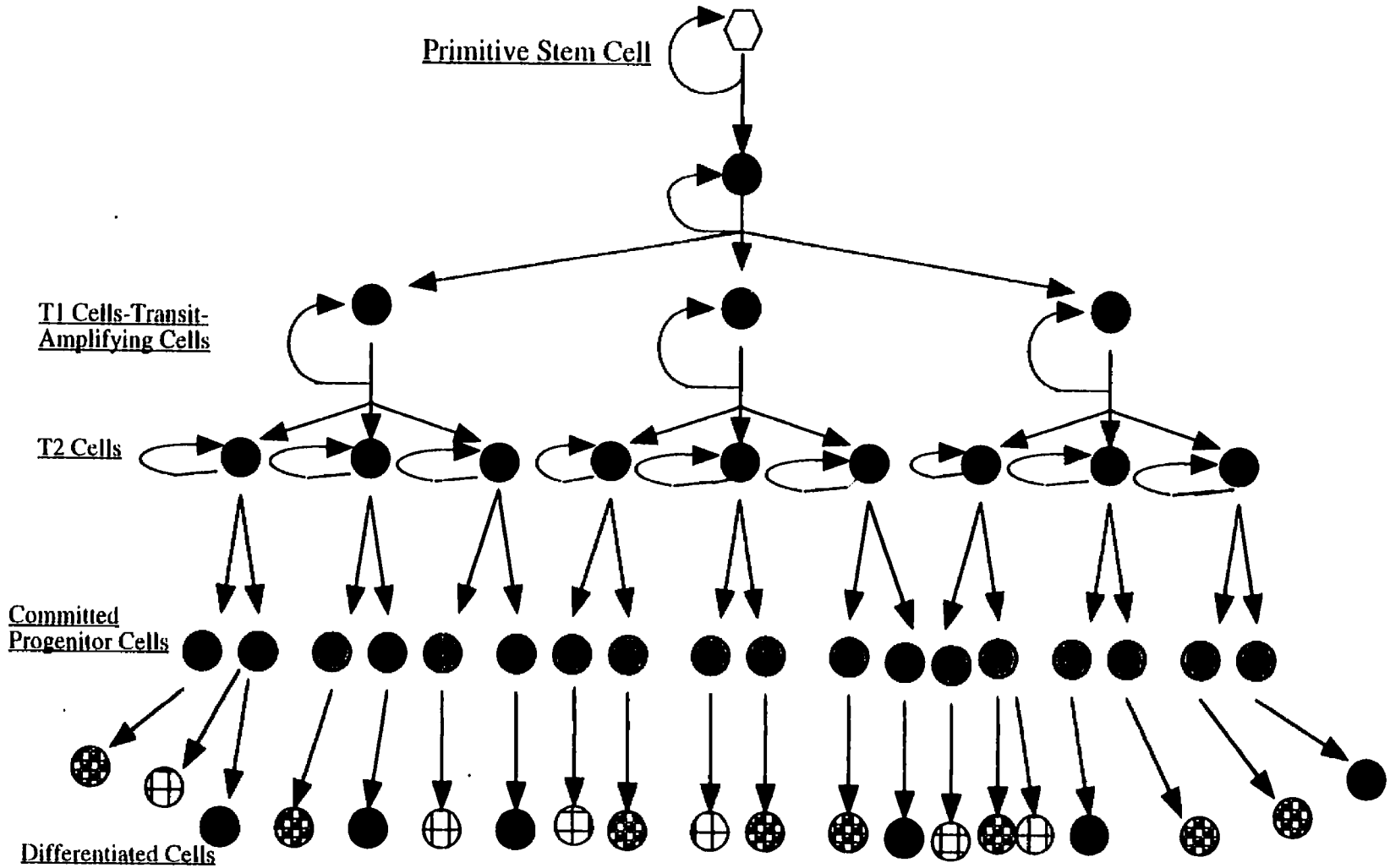
1.6 Stem Cells

Stem cells are defined functionally as "undifferentiated cells capable of (a) proliferation, (b) self-maintenance, (c) the production of a large number of differentiated functional progeny, (d) regenerating the tissue after injury, and (e) flexibility in the use of these options" (Potten and Loeffler, 1990). These cells have been found in various areas throughout the body including the intestinal epithelium and hematopoietic system.

Several studies have shown that stem cells differ with respect to their ability to self renew (Hodgson and Bradley, 1979; Harrison *et al.*, 1987). This may be related to their production of terminally-differentiated progeny via a temporary population of transit-amplifying cells (Figure 1) (Lajtha, 1979; Potten and Lajtha, 1982; Potten and Loeffler, 1990). Transit cells form a link between stem and differentiated cells. Like stem cells they can self renew, although this ability is limited, and like mature cells they eventually differentiate. Transit cells also lead to the generation of multiple differentiated phenotypes. Evidence for transit-amplifying cells has been found in the hematopoietic system and epidermal and intestinal epithelia (Potten and Hendry, 1973; Cheng and Leblond, 1974; Hellman *et al.*, 1978; Hall and Watt, 1989). The above studies suggest a model in which stem and transit cells are not distinct populations, but rather represent a hierarchy of cells

Figure 1. Model of CNS stem-cell hierarchy

This model shows the relationship between CNS stem cells, transit-amplifying cells, T2 cells, and committed cells. Cells in successive rows have a greater probability of differentiating and are less capable of unlimited renewal. The cell at the top is the most primitive stem cell; it renews itself and gives rise to transit-amplifying cells referred to as T1 cells. T1 cells are characterized by their ability to rapidly expand their population. They generate T2 cells which have a relatively-limited expansion potential. The bottom row represents committed progenitors that eventually become terminally differentiated, forming neurons, astrocytes and oligodendrocytes.


Primitive Stem Cell
T1 Cells-Transit-Amplifying Cells
T2 Cells
Committed Progenitor Cells
Differentiated Cells

(Figure 1), (Goodman *et al.*, 1977; Brown *et al.*, 1988; Potten and Loeffler, 1990). Cells at the top of the hierarchy are relatively quiescent (Becker *et al.*, 1965). They have the greatest self-renewal potential and the lowest probability of differentiating. They also generate cells that lead to the production of multiple differentiated phenotypes. These cells will be referred to as primitive stem cells. They, in turn, give rise to the rapidly proliferating transit- amplifying cells (Lajtha, 1979) that have an extensive capacity for expanding their population. Transit-amplifying cells in close association with primitive stem cells on the stem-cell hierarchy will be referred to as T1 cells (Figure 1). At successively-lower levels of the stem-cell hierarchy are the transit-amplifying cells that have increasingly-limited abilities to self renew and expand their population and a greater probability of differentiating than cells in levels above them. In addition, cells at successively-lower levels produce fewer differentiated phenotypes. Since cells at lower levels of this continuum have less self-renewal ability, expansion, and multilineage potential, they are distinct from T1 cells and will be referred to as T2 cells. Cells committed to a specific lineage that are undifferentiated will be called committed progenitor cells. The term precursor cells describes both stem cells and progenitor cells.

Transit cell capacity for dividing prior to differentiating is important, because it results in the production of many differentiated cells with relatively-few stem-cell divisions (Potten and Loeffler, 1990). This ability, coupled with a relatively short cell-cycle (Potten and Lajtha, 1982), enables transit-amplifying cells to rapidly expand their population, quickly generating differentiated cells. Such a rapid expansion is important in situations where many cells are needed, for example, after tissue injury. Without transit-amplifying cells, stem cells alone would probably be unable to generate the supply of differentiated cells required over an animal's lifetime. This is due to the finding that stem cells also have a limited ability to self renew (Siminovitch *et al.*, 1964). Siminovitch and colleagues found that when hematopoietic stem cells were serially transferred between animals their ability to

self renew decreased. Harrison (1972, 1973) has shown stem cells can self renew longer than an animal's lifespan, however, as animals age their stem cells have a reduced ability to self renew (Barrandon and Green, 1987). It may be possible that transit cells exist to ensure that differentiated cells can be produced throughout an animal's life without the unnecessary division of stem cells, reducing their self-renewal capability.

1.7 CNS stem cells

The existence of CNS stem cells was suggested when clonal analysis of a single cultured cell from the embryonic rat septum proliferated, giving rise to cells with the morphology of the original cell and two major CNS cell-types: neurons and astrocytes (Temple, 1989). The O-2A cell, isolated from cultures of neonatal rat optic nerve, also indicates the possibility that a CNS stem cell may exist. This is due to the finding that it proliferates or differentiates into oligodendrocytes or type-2 astrocytes, depending on the extrinsic signal received (Raff *et al.*, 1983; Raff, 1989; Williams and Price, 1992). Subsequently, Reynolds *et al.*, (1992) discovered an EGF-responsive precursor cell with the ability to self renew and proliferate, in cell cultures of embryonic day 14 (E14) mouse striatum. This cell formed cell clusters, termed "spheres," that generated neurons, astrocytes, and oligodendrocytes (Reynolds and Weiss, 1996). A cell with similar properties was isolated from the embryonic day 12 (E12)-E14 rat ventricular zone when cultured in media conditioned by astrocytes and meningeal cells (Davis and Temple, 1994). However, to demonstrate the presence of a stem cell one must show its ability to self renew over many generations and maintain its multilineage potential (Potten and Loeffler, 1990). In subsequent experiments Reynolds and Weiss (1996) demonstrated that populations of the EGF-responsive cell could be propagated for at least ten generations without effecting its ability to expand its population or its multilineage potential. These data suggest that this precursor is a stem cell (reviewed in Weiss *et al.*, 1996b). Therefore, a neural stem cell can

be defined as a cell capable of expanding its population while maintaining its ability to generate multiple neural phenotypes (Figure 2) (Weiss *et al.*, 1996a).

Identifying stem cells in embryonic tissue is problematic, because by definition stem cells give rise to differentiated cells while maintaining their population. Hall and Watt (1989) suggest that a stem cell "is any cell with a high capacity for self-renewal, extending throughout adult life." Most embryonic stem cells do not exist throughout adult life (Price, 1995). Therefore, true stem cells can only be isolated from adult organisims.

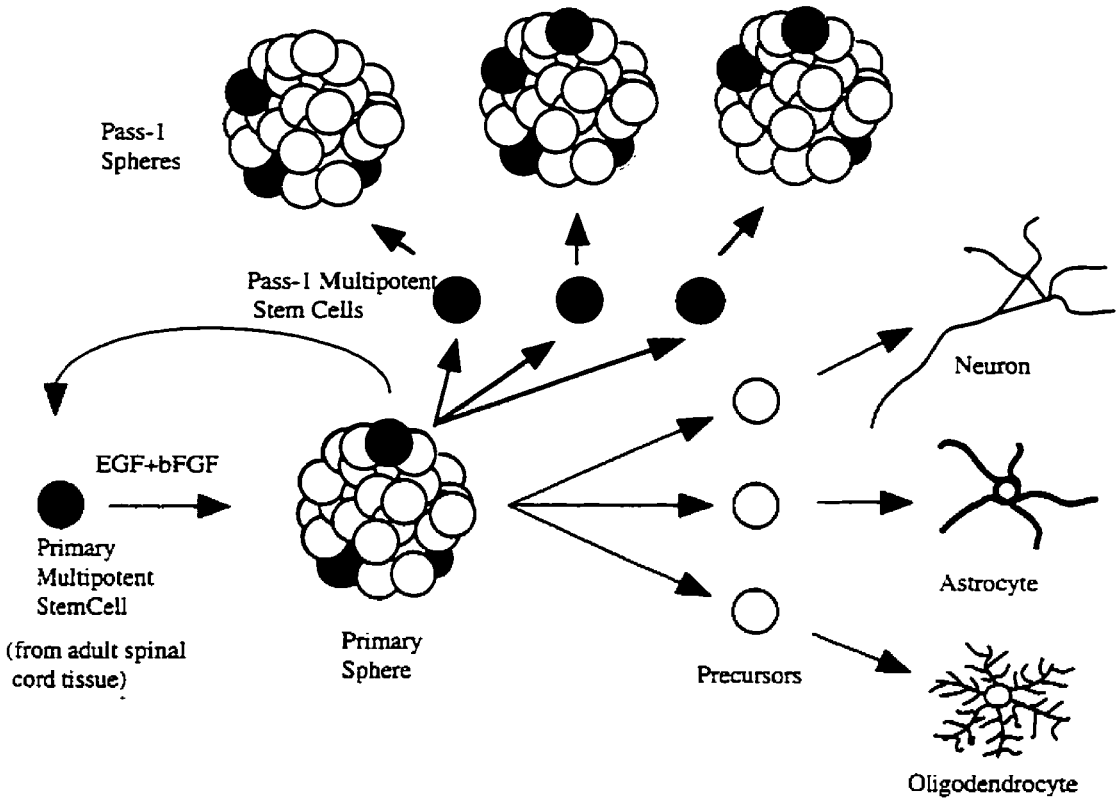
1.7.1 Adult CNS stem cells

Studies on the adult demonstrate the presence of cell turnover and new differentiation in the forebrain, suggesting a stem cell may exist in the adult CNS. Smart (1961) showed evidence of cell turnover and the presence of undifferentiated cells in brains of adult rodents. These cells were located in the subventricular zone (SVZ), an area adjacent to the lateral ventricles. Recent work on the adult SVZ suggests it contains cells that generate newly differentiated cells. This was demonstrated when SVZ cells labeled with tritiated thymidine *in vivo* gave rise to neurons and glia in tissue cultured *in vitro* (Lois and Alvarez-Buylla, 1993). Additional evidence indicates transgenic adult SVZ cells from an adult donor migrate to the olfactory bulb when transplanted into the SVZ of a normal adult host. Here they were identified as neurons by staining with the neuronal marker neuron-specific enolase (Lois and Alvarez-Buylla, 1994).

A stem cell was discovered in the adult CNS when striatal cells from the forebrain were cultured in the presence of EGF (Reynolds and Weiss, 1992; reviewed in Weiss *et al.*, 1996b). This cell generated spheres and was able to self-renew, proliferate, and generate neurons, astrocytes, and oligodendrocytes *in vitro* (Weiss *et al.*, 1996a).

Figure 2. Renewal/expansion and differentiation of CNS stem cells

This schematic shows the renewal/expansion and differentiation potential of an EGF+bFGF-responsive stem cell. The stem cell generates an adult spinal cord sphere containing stem cells (black circles) that upon subcloning form spheres. The primary stem cell originated in the adult spinal cord. This cell gives rise to a primary sphere. Stem cells within the primary sphere are called pass-1 stem cells. These pass-1 stem cells produce pass-1 spheres. Stem-cell renewal occurs when at least one subcloned sphere is formed, while expansion refers to the production of two or more subcloned spheres. This primary stem cell can also produce cells that eventually become neurons, astrocytes, and oligodendrocytes.


Pass-1
Spheres
Pass-1 Multipotent
Stem Cells
Neuron
EGF+bFGF
Primary
Multipotent
StemCell
(from adult spinal
cord tissue)
Primary
Sphere
Astrocyte
Precursors
Oligodendrocyte

Evidence suggests the adult CNS stem cell is present in the SVZ (Morshead *et al.*, 1994). This study compared sphere production from several brain regions, and found that spheres were only produced by tissue that contained the SVZ. This study also demonstrated the presence of a relatively-quiescent population of SVZ cells in addition to the rapidly-proliferating group of cells previously identified there (Morshead and van der Kooy, 1992). Morshead *et al.*, (1994) suggest that the *in vivo* source of spheres is a relatively-quiescent population of SVZ stem cells. They used high doses of tritiated thymidine to kill the rapidly-proliferating cells. The absence of BrdU incorporation into SVZ cells 0.5 days after treatment with tritiated thymidine suggested the rapidly-proliferating cells had been killed. However, when these SVZs were cultured, the number of spheres produced equaled the number of spheres produced by control animals (Morshead *et al.*, 1994). The presence of cell turnover demonstrated in the adult forebrain begs the question, are dividing cells present in other regions of the adult CNS?

1.7.2 Cell turnover in the adult spinal cord

The adult spinal cord lacks a SVZ, however, during development undifferentiated cells are present in the ventricular layer lining the central canal (Fujita, 1965; Martin and Langman, 1965), raising the possibility that stem cells are present there. Several *in vivo* and *in vitro* studies, show that glial cell turnover occurs in the uninjured adult spinal cord (Adrian and Walker, 1962; Kraus-Ruppert *et al.*, 1975; Engel and Wolswijk, 1996). Adrian and Walker (1962) also showed that this turnover occurs in the spinal cord's ventricular layer in response to injury. Gilmore and Leiting (1980) showed that the ventricular zone cells divide in response to injury, even though the central canal escapes the lesion. That adult spinal cord ventricular zone cells divide in response to injury is also suggested by increased immunoreactivity to the intermediate filament protein nestin 2 to 14 days postlesion (Frisen *et al.*, 1995). Frisen and colleagues suggest that nestin-positive

cells proliferate and migrate from the central canal to the site of injury where they contribute to the glial scar. The presence of dividing cells in the adult spinal cord (Adrian and Walker, 1962; Kraus-Ruppert *et al.*, 1975; Engel and Wolswijk, 1996) suggests the possibility that a spinal cord stem cell may exist.

Recent evidence has shown spinal cord tissue generates spheres that grow in cultures supplemented with EGF+bFGF (Weiss *et al.*, 1996a). Since a molecular marker for neural stem cells is unavailable, their presence was inferred using renewal/expansion and differentiation assays. The renewal/expansion assay demonstrates the sphere's ability to give rise to more spheres. This assay uses single-sphere dissociation techniques to show dissociated stem cells from a primary sphere can generate pass-1 spheres (Figure 2). Expansion refers to the number of pass-1 spheres produced. This number is related to the number of stem cells present in the primary sphere. Renewal simply refers to the ability to generate passaged spheres. In the following report, renewal will be substituted for self renewal to determine the position of a stem cell on the stem-cell hierarchy. Therefore, a relatively-primitive stem cell is able to generate spheres for more generations than a cell from a lower level of the stem-cell hierarchy (Figure 1). This substitution is necessary because with current procedures it is impossible to determine whether an adult neural stem cell has self renewed. The differentiation assay demonstrates the presence of multiple terminally-differentiated progeny in spheres by utilizing indirect immuocytochemistry. To prove that a single cell can both generate a sphere and be multipotent, Weiss and colleagues cultured an individual spinal cord cell and showed that it both generated a sphere and gave rise to neurons, astrocytes, and oligodendrocytes.

1.7.3 Unanswered questions about spinal cord stem cells

Although stem cells have been identified in parts of the adult spinal cord (Weiss *et al.*, 1996a), little is known about their distribution, growth factor requirements, and

regional characteristics. Therefore the following study poses several questions regarding these issues. First, what is the distribution of spinal cord stem cells in the rostrocaudal plane? Distribution is important as it may have a relationship to stem cell function. For example, in the adult forebrain, dividing cells appear to be distributed in a decreasing rostrocaudal gradient, between the SVZ surrounding the rostral lateral ventricle and the fourth ventricle (Morshead *et al.*, 1994). When tritiated thymidine was injected into the lateral ventricle the dividing cells migrated towards the olfactory bulb, where they were labeled as neurons (Lois and Alvarez-Buylla, 1994). Therefore, localized populations of spinal cord stem cells, like the dividing cells in the lateral ventricle , may perform specialized functions *in vivo*. The second issue addressed in this study is, what are the growth requirements of spinal cord stem cells? Growth factor responsiveness is imporant as it may also be related to the biological function of stem cells. Experiments comparing stem cells from the lateral ventricles with third and fourth ventricle stem cells found only lateral ventricle stem cells were stimulated to divide by EGF (Weiss *et al.*, 1996a). However, the combination of EGF+bFGF stimulated division in stem cells from all ventricles tested (Weiss *et al.*, 1996a). Previous work on spinal cord stem cells has demonstrated that they also respond to EGF+bFGF, however, few additional factors have been tested (Weiss *et al.*, 1996a). If these cells do respond to other growth factors, will the various groups of growth factor-responsive stem cells have the same properties? This leads to the third question, are there regional and/or growth factor differences in the stem cell properties renewal, expansion, and multipotency? Experimental evidence from other systems suggests stem-cell properties can be described by their position on a stem-cell hierarchy (Figure 1) (Goodman *et al.*, 1977; Magli *et al.*, 1982). This model proposes cells exist in a continuum; those at the top have a high capacity for self renewal and a low probability of differentiating, those at the bottom have the reverse properties (Hall and Watt, 1989). Do spinal cord stem cells fit this model? If so, does spinal cord region or

growth factor condition affect the position these stem cells hold on this continuum?

1.8 Statement of Hypothesis

Stem cells with different growth requirements are unevenly distributed along the rostrocaudal axis of the spinal cord. These cells may exist in a hierarchy relationship.

1.9 Specific Questions

i) What are the growth requirements and rostrocaudal distribution of spinal cord stem cells?

Work on neural stem cells isolated from the SVZ of the adult mouse has shown these cells form spheres in response to EGF+bFGF and bFGF+hs (Gritti *et al.*, 1996; Weiss *et al.*, 1996a). Weiss *et al.* (1996a) have demonstrated that the adult spinal cord also contains an EGF+bFGF-responsive stem cell. This cell fails to respond to EGF or bFGF alone but the effect of bFGF+hs has not been tested. Therefore, the present study confirms that spinal cord stem cells respond to EGF+bFGF and tests the effect of bFGF+hs, and hs on these cells. In addition, it retests the stem cell response to EGF, and bFGF. Once the growth factor conditions inducing stem cells to form expandable spheres have been established, these conditions will be used to determine the rostrocaudal distribution of spinal cord stem cells. A study of the adult rodent brain suggests that stem cells are distributed unevenly around the lateral ventricles (Morshead *et al.*, 1994). This is an example of uneven stem cell distribution in the CNS which raises the question are spinal cord stem cells also distributed in this way? The experiments in this study are performed *in vitro* on cervical, thoracic, and lumbar/sacral tissue. Regional differences among stem-cell distribution *in vivo* is related to the number of spheres produced by each region *in vitro*. These spheres were quantified after 8 days *in vitro* (DIV).

ii) Are spinal cord stem cells heterogeneous?

Stem cells are considered heterogeneous if they vary in terms of renewal, ability to expand their population, and/or their potential to generate multiple differentiated cell-types. Stem cells from the embryonic mouse striatum are heterogeneous in terms of their ability to expand their population (Reynolds and Weiss, 1996). In addition, renewal among stem cells of the hematopoietic system is observed to be heterogeneous (Nakahata and Ogawa, 1982). These findings raise the possibility that adult spinal cord stem cells may be heterogeneous. In this study, stem-cell heterogeneity was measured in terms of the following properties: 1) ability to expand their population and 2) multipotentiality. In all experiments performed here, spheres were tested with growth factors producing expandable spheres as determined in (i).

iii) Does the spinal cord contain a relatively primitive stem cell and if so is it localized to a specific region?

Stem-cell theory suggests that these cells exist in a hierarchy (Figure 1) (Hall and Watt, 1989), with a relatively quiescent self-renewing cell located at the top giving rise to a highly-proliferative cell population and eventually differentiated cells at the bottom (Potten and Loeffler, 1990). Relatively-quiescent cells have been found in the adult mammalian forebrain (Morshead *et al.*, 1994). This discovery suggests that such quiescent cells may also be present in the spinal cord. To test this possibility in the present study, subcloning experiments were performed where spheres from each spinal cord region were propagated until subcloned spheres failed to produce further spheres. The most-primitive stem cell was defined as the one that could be subcloned, that is renewed, over the most generations.

2 MATERIALS AND METHODS

2.1 Primary cell culture of adult spinal cord

Adult mice (male and female CD1 Charles River) were killed by cervical dislocation and their vertebral columns were removed between the second cervical vertebra rostrally and the fourth coccygeal vertebra caudally. Using the first and last ribs as landmarks, spinal columns were cut into three sections: cervical (tissue rostral to the first rib), thoracic (tissue spanning the length of the rib cage), and lumbar/sacral (tissue caudal to the twelfth rib). The spinal cord was removed and placed in 95% O_2/5% CO_2 - aerated artificial cerebrospinal fluid (aCSF) containing 124 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, (all from BDH), 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM D-glucose (all from Sigma), and 1:50 penicillin-streptomycin solution (Life Technologies), (pH 7.35) for further dissection. The tissue was cut into smaller pieces and transferred to spinner flasks (Bellco Glass, one region/flask) with a magnetic stirrer filled with low Ca^{2+}, high Mg^{2+} aCSF (124 mM NaCl, 5mM KCl, 3.2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM D-glucose, and 1:25 penicillin-streptomycin solution (pH 7.35)), and an enzyme mixture (1.33mg/ml trypsin, 0.67mg/ml hyaluronidase, and 0.2 mg/ml kynurenic acid (all from Sigma)). The stirring tissue suspension was aerated with 95% O_2/5% CO_2 at 32-35°C for 90 minutes. After this enzymatic incubation period, the tissue was transferred to 1:1 Dulbecco's modified Eagle medium (DMEM)/F-12 (Life Technologies) medium containing 0.7 mg/ml ovamucoid (Sigma) and triturated mechanically with a fire-narrowed Pasteur pipette. The dissociated cell suspension was centrifuged at 400 rotations per minute (rpm) for 5 minutes, and the pellet was resuspended and then plated in noncoated 6-well (2 ml volume) Nunc tissue-culture dishes in defined media composed of 1:1 DMEM/F-12, including N-(hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) buffer (5 mM), glucose (0.6%), sodium bicarbonate (3 mM), and glutamine (2 mM) (all from Sigma except

glutamine [Gibco]). A defined hormone and salt mixture composed of insulin (25 mg/ml), transferrin (100 mg/ml), progesterone (20 nM), putrescine (60 mM), and sodium selenite (30nM) was used to replace serum. To the above medium EGF, or bFGF, (human recombinant; R&D Systems) or both were added at 20 ng/ml or bFGF+hs (hs at 2 μg/ml). Primary stem-cell proliferation was detected after 8 days *in vitro* and characterized by the formation of spheres of undifferentiated cells (Reynolds and Weiss, 1992).

2.2 Differentiation of EGF+bFGF- and bFGF+hs-generated spheres

Eight-to-ten days after the primary or pass-1 culture, single spheres were removed with a pipette and plated on poly-L-ornithine-coated (Sigma, 15 mg/ml) glass coverslips in individual wells of 24-well Nunclon culture dishes in (1.0 ml/well) DMEM/F-12 medium with the hormone and salt mixture and EGF+bFGF or bFGF+hs consistent with the primary or pass-1 culture environment. Spheres were incubated at 37°C and processed 21 days later for indirect immunocytochemistry.

2.3 Antibodies

The following primary antibodies were used: mouse monoclonal anti-microtubule-associated protein-2 (anti-MAP-2; IgG, 1:1000, Boehringer) mouse monoclonal anti-O4 (IgM, 1:20, Boehringer) to identify neurons and oligodendrocytes, respectively. Rabbit polyclonal anti-glial fibrillary acidic protein antisera (anti-GFAP; IgG, 1:300) labeled astrocytes (BTI).

Secondary antibodies were raised in goat, against mouse or rabbit immunoglobulins and conjugated to the fluorophore rhodamine isothiocyanate (1:200), fluroescein isothiocyanate (1:100), or 7-amino-4-methylcoumarin-3-acetic acid (AMCA, 1:100) (all from Jackson Immunochemicals).

2.4 Immunocytochemistry

Indirect immunocytochemistry was carried out on spheres attached to glass coverslips after 21 DIV. Spheres were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2, for 20 minutes, followed by three 5 minute washes in PBS. Cells were then permeabilized for 5 minutes with 0.3% Triton X-100 in PBS followed by two 5-minute washes in PBS. The addition of a neuron-specific monoclonal antibody to microtubule-associated protein-2 (MAP-2) (IgG) together with polyclonal antiserum to GFAP both in 10% normal goat serum (NGS)/PBS and a 2-hour incubation at 37°C followed. Three PBS rinses (5 minutes each) preceded the addition of appropriate secondary antibodies (in PBS) and a 30-minute incubation. Cells were rinsed three times in PBS (5 minutes each) followed by incubation (2 hours) with monoclonal antibody to O4 (IgM) in 10% NGS and a goat anti-mouse IgM specific secondary (AMCA) was used to visualize the O4 antibody. Coverslips received two 5-minute washes in PBS and were rinsed with water, placed on glass slides, and mounted on coverslips, using Fluorsave (Calbiochem) as the mounting medium. Cells were visualized on a Zeiss microscope and photographed using Kodak T-Max 400 ASA film.

2.5 Measurement of sphere diameter

Sphere diameters were measured eight-to-eleven days after culture, using a glass disc of concentric rings fitted on the microscope's eyepiece. Measurements were made in 100-μm categories, and when 'spheres' were asymmetrical, their widest dimension was used.

2.6 Renewal/expansion assay: Dissociation and propagation of spinal cord spheres for clonal analyses.

i) To test whether growth factor stimulated stem cells exhibit expansion, individual spinal cord spheres were dissociated (Figure 2). Single spheres between 100 and 400 μm in diameter were dissociated by taking a 15-μl aliquot of 8-to-10 DIV sphere and transferring them into Nunclon 35-mm tissue culture dishes with growth factor containing medium consistent with the primary culture environment. Under sterile conditions, single spheres were transferred to 500-μl Eppendorf tubes containing 200 μl of medium, triturated 90 times, and plated onto a 96-well plate and incubated at 37°C. The plates were scored 13 days later for the number of spheres derived from a single sphere.

ii) To determine whether spinal cord stem cells exhibit renewal and expansion over successive generations, individual spheres were serially subcloned. Spheres were dissociated (see above) and deposited into 300 μl of media and growth factors, consistent with the primary or passaged environment, in 24-well plates. Plates were observed at 8-day intervals until 32 DIV, during this time any spheres present were counted and a selection of 200-300 μm spheres were dissociated and replated. This process was repeated until stem cells failed to produce spheres.

2.7 Statistical Analysis

Analysis of variance, followed by Tukey's honestly significant difference post hoc test, was used to determine statistical significance in the results section, except where otherwise indicated. In addition, when discussing the number of experiments performed, 'N' refers to the number of cell cultures made, while 'n' refers to the number of spheres tested per culture.

3 RESULTS

3.1 Characterization and growth factor dependence of adult spinal cord stem cells

3.1.1 The adult spinal cord contains stem cells that proliferate in response to EGF+bFGF and bFGF+hs

Previous studies have shown that adult CNS stem cells proliferate in response to various growth factors. Reynolds and Weiss (1992) isolated the first such stem cell in cultures of dissociated adult striatum, stimulated with EGF. Subsequently a bFGF responsive stem cell was discovered in striatal cultures (Gritti *et al.*, 1996). Recent *in vitro* evidence demonstrates the presence of an EGF+bFGF-responsive stem cell in some regions of the murine spinal cord (Weiss *et al.*, 1996a). The present study asks whether spinal cord spheres are derived from stem cells and whether EGF and bFGF, individually or in combination stimulate adult spinal cord stem cells to divide. The first defining characteristic of neural stem cells is their ability to expand their population. This is demonstrated when a dissociated primary sphere produces pass-1 spheres *in vitro.*

Equal lengths of tissue from cervical, thoracic, and lumbar/sacral spinal cord regions were cultured in defined media supplemented with EGF, bFGF, hs, EGF+bFGF, or bFGF+hs. All growth factors tested with the exception of EGF produced primary spheres. Therefore only bFGF, hs, EGF+bFGF, and bFGF+hs spheres were further tested in the expansion assay. After 8-10 DIV individual spheres were dissociated and plated in growth factor conditions identical to those used in primary culture. Table 1 shows that EGF+bFGF and bFGF+hs primary culture spheres from all spinal cord regions tested produced multiple pass-1 spheres. Basic FGF or hs alone failed to do so although they

Table 1. Only EGF+bFGF and bFGF+heparan sulfate produce expandable spheres

Spinal Cord Region	# primary spheres producing two or more secondary spheres/total # spheres dissociated				
	EGF	bFGF*	hs*	EGF+bFGF	bFGF+hs
Cervical	N.S.	0/4	0/5	40/40	37/38
Thoracic	N.S.	0/4	0/5	51/51	30/30
Lumbar/Sacral	N.S.	0/4	0/5	45/45	13/14

* approximately 15% yielded 1 secondary sphere
N.S., no sphere, N≥2 cell cultures, n≥2spheres

would occasionally produce a single pass-1 sphere. Since the growth factor combinations EGF+bFGF and bFGF+hs were the only conditions tested enabling stem cells to expand, these growth factor combinations were used exclusively in all subsequent experiments.

The second defining characteristic of stem cells is their ability to generate multiple types of differentiated progeny. Reynolds and Weiss (1996) showed that EGF responsive stem cell generated spheres produce neurons, astrocytes, and oligodendrocytes *in vitro*. To determine whether spinal cord spheres were indeed produced by multipotent stem cells, I asked whether the three neural phenotypes could be produced. Primary EGF+bFGF spheres from cervical, thoracic, and lumbar/sacral regions were plated on poly-L-ornithine -coated coverslips in defined media supplemented with EGF+bFGF and incubated for 21 days. The spheres were subsequently processed using indirect immunocytochemistry, probing for the presence of neurons, astrocytes, and oligodendrocytes using antibodies to microtubule-associated protein-2, glial fibrillary acidic protein, and O4, respectively (Figure 3). All primary EGF+bFGF-generated spheres produced the three major CNS cell types. These results will be described in 3.1.5.

3.1.2 Stem cells are distributed in the spinal cord in an increasing rostral-to-caudal gradient

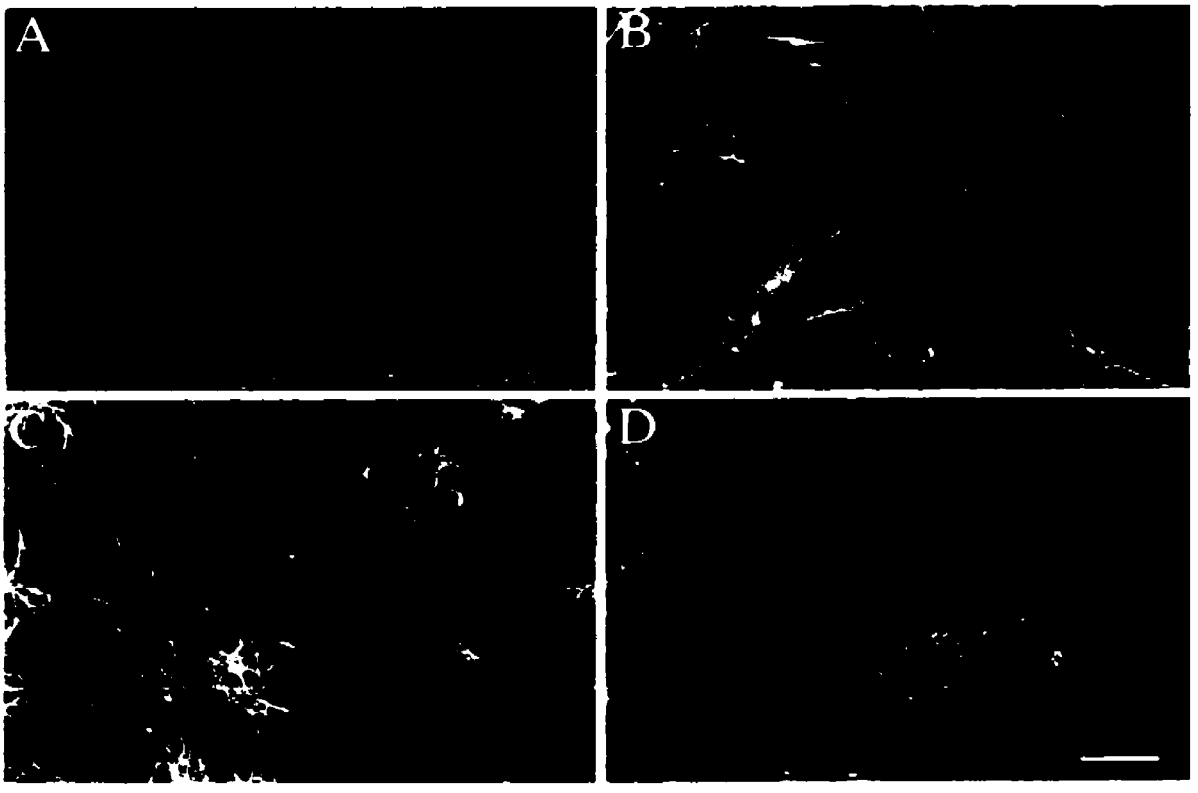
The data presented above demonstrates that:

1) Spheres produced by spinal cord tissue produce spheres in response to EGF+bFGF and bFGF+hs.
2) Spinal cord spheres are multipotent.

These findings demonstrate that spinal cord spheres contain stem cells. Previous experiments have also shown that EGF+bFGF-responsive stem cells are present in the adult murine spinal cord (Weiss *et al.* 1996a). Weiss *et al.* (1996a) also showed EGF+bFGF-responsive stem cells are present in an increasing gradient from the thoracic to

Figure 3. Spinal cord stem cells produce neurons, astrocytes, and oligodendrocytes in response to EGF+bFGF

EGF+bFGF-generated (8-10 DIV) spheres were cultured in defined medium for 21 DIV in the presence of EGF+bFGF. Subsequently, spheres were fixed and processed for immunocytochemistry with antibodies against MAP-2 and O4 and antisera against GFAP. Phase-contrast micrograph of cells from a cervical sphere (A) and labeled with MAP-2 (B), GFAP (C), and O4 (D). Bar, 42 μm.


A
B
C
D

the lumbar/sacral spinal cord, based on spheres produced per 5000 cells cultured. The presence of bFGF+hs-responsive stem cells identified in the spinal cord in the present study raises two questions: a) what is the regional distribution of bFGF+hs stem cells along the spinal cord's rostral-to-caudal axis and b) does this represent a novel stem-cell population distinct from the EGF+bFGF-responsive stem cells? To determine the regional distribution of spinal cord stem cells, equal lengths of cervical, thoracic, and lumbar/sacral tissue were cultured. They were then plated in defined media, supplemented with EGF+bFGF or bFGF+hs, and incubated for 8 days. The lumbar/sacral segment yielded a 4-5-fold greater number of spheres versus cervical or thoracic tissue, in either growth factor condition (Figure 4, $p<0.001$). In addition, there was no difference in the number of spheres produced by EGF+bFGF versus bFGF+hs for any spinal cord region, suggesting that the stem cells responsive to these growth-factor combinations may belong to the same population.

3.1.3 Spinal cord spheres are not uniform in size

Lumbar/sacral spinal cord tissue generates more spheres in EGF+bFGF or bFGF+hs than the same length of tissue from other spinal cord regions. Besides these regional differences in sphere number, after 10-11 DIV, spheres were found to grow to a variety of sizes. This observation led me to ask whether mean sphere size varies with spinal cord region and/or growth factor condition? To determine average sphere sizes, cervical, thoracic, and lumbar/sacral primary culture spheres were measured after 10-11 DIV.

The data showed that sphere sizes vary with spinal cord region and growth factor condition (Figure 5). Thoracic spheres cultured in EGF+bFGF had the largest mean diameter of all spheres tested ($p<0.001$). Spheres with the smallest mean diameter were the bFGF+hs-generated lumbar/sacral spheres ($p<0.001$). However, the EGF+bFGF

Figure 4. EGF+bFGF and bFGF+hs stimulate spinal cord stem cells to form spheres in an increasing rostral-to-caudal gradient.

EGF+bFGF- and bFGF+hs-generated spheres from equal lengths of cervical, thoracic ,and lumbar/sacral tissue were counted after 8 DIV. The number of spheres produced increased in tissue from progressively more caudal spinal cord regions but did not vary with growth factor condition. Lumbar/sacral tissue produces significantly more spheres than comparative regions in EGF+bFGF and bFGF+hs ($p<0.001$, N=6 cell cultures).

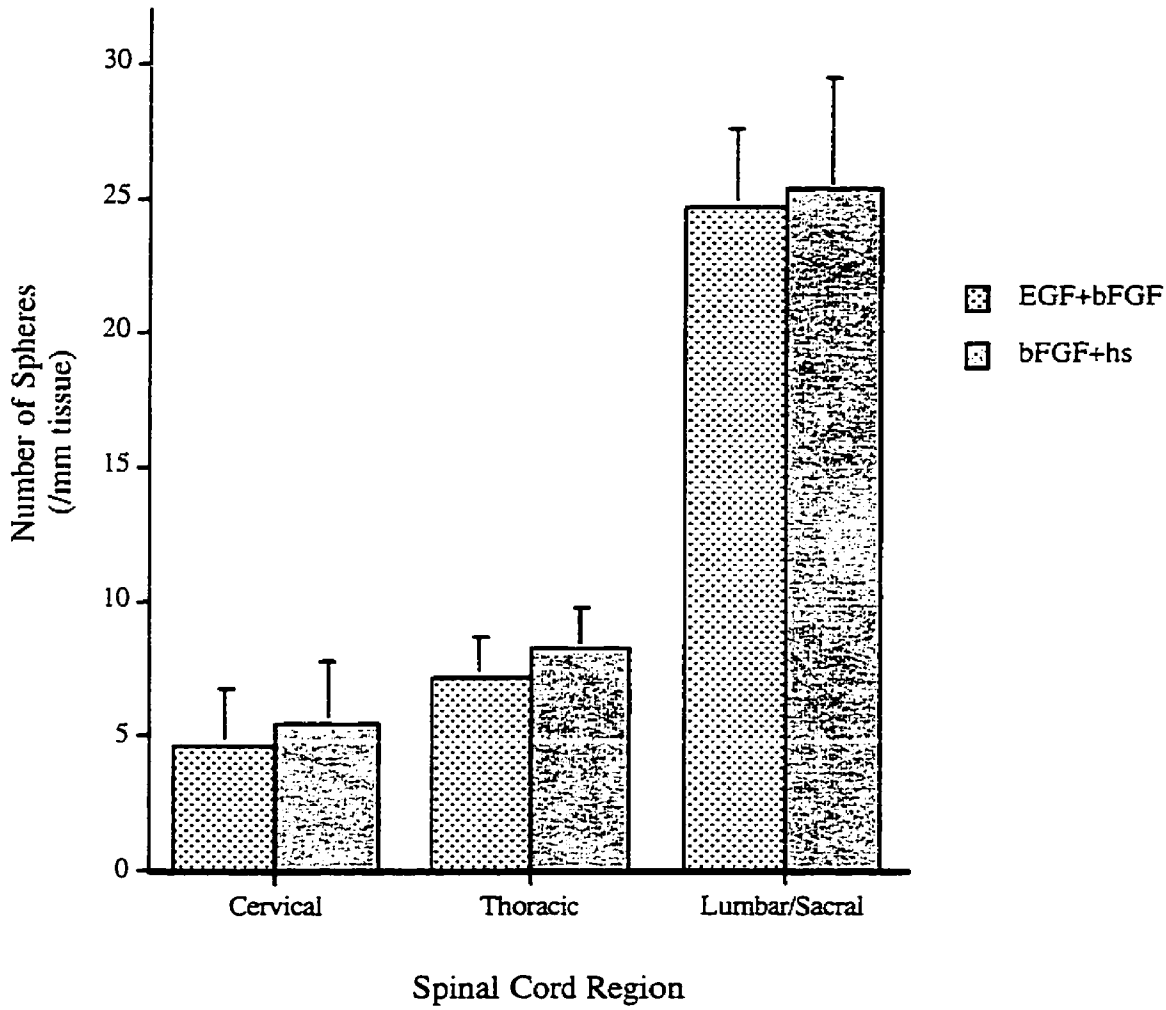
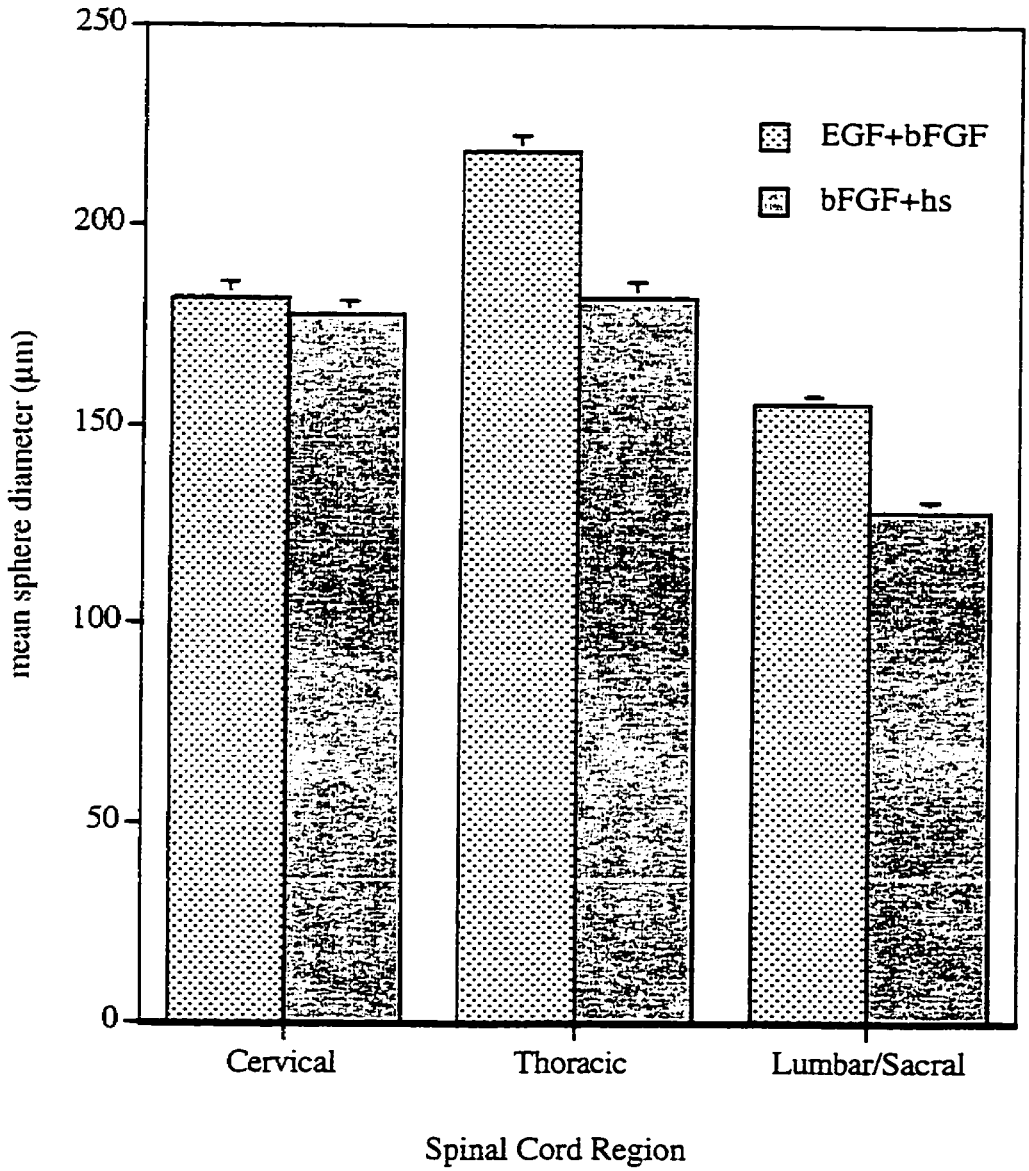

30
25
20
15
10
5
0
Number of Spheres
(/mm tissue)
Cervical
Thoracic
Lumbar/Sacral
Spinal Cord Region
EGF+bFGF
bFGF+hs

Figure 5. Stem cell-generated sphere sizes vary with spinal cord region

Spinal cord stem cell-generated spheres, cultured in defined media and EGF+bFGF or bFGF+hs, were measured after 10 to 11 DIV. The largest spheres were found in thoracic EGF+bFGF cultures, these spheres were significantly larger than spheres from all other conditions ($p<0.001$). Lumbar/sacral stem cells cultured in EGF+bFGF or bFGF+hs produced the smallest spheres ($p<0.001$) of the three spinal cord regions; bFGF+hs produced the smallest lumbar/sacral spheres (all spheres in five cell cultures were measured).


250
200
150
100
50
0
mean sphere diameter (µm)
EGF+bFGF
bFGF+hs
Cervical
Thoracic
Lumbar/Sacral
Spinal Cord Region

generated lumbar/sacral spheres were smaller than those from other spinal cord regions ($p<0.001$). Cervical sphere diameters were between thoracic and lumbar/sacral sphere diameters ($p<0.001$) in EGF+bFGF or bFGF+hs. In addition, there was no significant difference between sphere diameters of cervical EGF+bFGF versus bFGF+hs spheres.

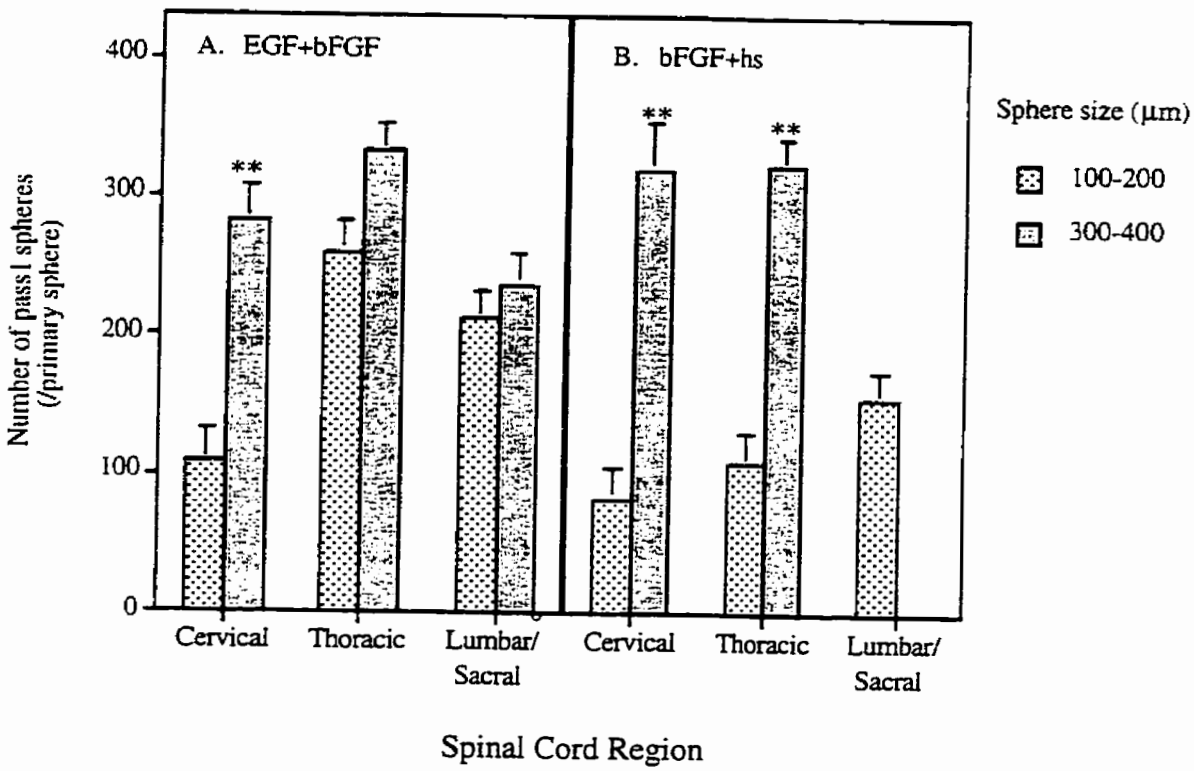
3.1.4 Stem-cell expansion is related to sphere size

Primary-culture spheres grew to a variety of sizes in all conditions tested. This observation led to the question, is sphere size related to stem-cell expansion? Stem-cell expansion was determined by finding the relative numbers of pass-1 spheres generated by a single primary sphere. This is an effective measure of expansion because the number of stem cells in a primary sphere is related to the number of pass-1 spheres produced by the primary sphere (Figure 2) (Weiss *et al.*, 1996a). Small (100-200 μm) and large (300-400 μm) primary spheres were cultured in EGF+bFGF and bFGF+hs for 8-10 DIV. At this time they were passaged and incubated for 13 days. EGF+bFGF-generated primary spheres were always passaged into EGF+bFGF, while bFGF+hs spheres were always passaged into bFGF+hs. After the incubation period, pass-1 spheres were counted.

I predicted that small primary spheres would generate fewer pass-1 spheres than large primary spheres. Some results fulfilled this prediction (Figure 6). For example, the mean number of pass-1 spheres per primary sphere was significantly different between small and large cervical spheres. This was true for both EGF+bFGF ($p<0.001$), and bFGF+hs ($p<0.001$). It was also true in the thoracic region for bFGF+hs ($p<0,001$). However, contrary to the prediction, the mean number of pass-1 spheres per primary sphere was not significantly different between small and large thoracic spheres cultured in EGF+bFGF. This was also true for EGF+bFGF-generated lumbar/sacral spheres. Unfortunately results for small versus large lumbar/sacral bFGF+hs spheres are

Figure 6. The expansion potential of small-versus-large spheres varies with growth-factor combination and spinal cord region

Small and large primary spheres were dissociated and plated in individual wells of 96-well plates in culture media supplemented with (A) EGF+bFGF or (B) bFGF+hs. The number of pass-1 spheres generated by each primary sphere was quantified after 13 DIV. The number of pass-1 spheres produced by small and large spheres was related to growth factor and spinal cord region. ** denotes a significant difference from the comparative size for the specified region ($p<0.001$, N=5 cultures, $n\geq2$ spheres).


A. EGF+bFGF
B. bFGF+hs
Number of passl spheres (/primary sphere)
400
300
200
100
0
**
**
**
Cervical
Thoracic
Lumbar/ Sacral
Cervical
Thoracic
Lumbar/ Sacral
Spinal Cord Region
Sphere size (μm)
100-200
300-400

unavailable as culture sof lumbar/sacral cells stimulated with bFGF+hs do not produce large spheres. In addition, the mean number of pass-1 spheres per small primary sphere was significantly different between EGF+bFGF- and bFGF+hs-generated thoracic spheres ($p<0.001$). The results have shown spinal cord stem cells may vary in terms of their expandability and that this heterogeneity is growth factor related.

3.1.5 Influence of EGF+bFGF-versus-bFGF+hs on neural phenotype for primary and pass-1 spheres

The potential to generate multiple differentiated cell types, in addition to expandability, are the defining characteristics of stem cells. Furthermore, the hierarchical model of stem cell division illustrated in Figure 2, suggests that stem cells become more differentiated in succeeding generations. Therefore, the following experiments ask: a) are stem cells heterogeneous with respect to the ability to produce multiple neural phenotypes, and if so, is this ability growth factor related b) does the lineage potential of spinal cord stem cells change from primary to pass-1 spheres? To determine stem cell-derived neural phenotypes, primary and pass-1 spheres from the three spinal cord regions were plated on poly-L-ornithine-coated coverslips and incubated for 21 days. The spheres were then processed for indirect immunocytochemistry to detect neurons, astrocytes, and oligodendrocytes with antibodies or antisera to MAP-2, GFAP, and O4, respectively.

EGF+bFGF-treated primary and pass-1 stem cells frequently generated the three neural phenotypes (Table 2). All primary spheres tested were tripotent regardless of stem-cell origin. Pass-1 stem cells generated the three phenotypes in over 92% of spheres assayed, depending on spinal cord region.

Some stem cells treated with bFGF+hs were restricted in their ability to produce neurons, astrocytes, and oligodendrocytes. This ability was related to stem-cell generation

Table 2. Stem-cell ability to generate multiple differentiated progeny is related to growth-factor combination

	# spheres producing given phenotype/total # spheres tested			
Spinal Cord Region	EGF+bFGF		bFGF+hs	
	Primary	Pass 1	Primary	Pass 1
Cervical				
Ne,A,O	22/22	59/60	16/17	9/17
Ne,O	-	-	1/17	7/17
A,O	-	1/60	-	-
Ne	-	-	-	1/17
Thoracic				
Ne,A,O	28/28	76/82	15/19	6/15
Ne,O	-	1/82	4/19	9/15
A,O	-	3/82	-	-
Ne	-	2/82	-	-
Lumbar/Sacral				
Ne,A,O	16/16	50/50	5/11	-
Ne,O	-	-	4/11	8/13
A,O	-	-	-	-
Ne	-	-	2/11	5/13

Ne-neuron, A-astrocyte, O-oligodendrocyte
$N \geq 4$ cell cutures, $n \geq 2$ spheres

– (primary versus pass-1), and site of stem cell-origin in the spinal cord (Table 2). Stem cells become increasingly restricted in terms of lineage potential as sphere generation increases ($p<0.05$ for all regions combined). This is illustrated in Table 2, which shows that the number of primary spheres expressing antigens for neurons, astrocytes, and oligodendrocytes is approximately two-fold greater than pass-1 spheres from the same region. However, lineage restriction increases significantly in primary spheres as stem cells that produce these spheres are derived from progressively-caudal spinal cord regions. Primary cervical stem cells were tripotent in significantly more spheres tested compared with lumbar/sacral stem cells ($p<0.05$) (Table 2). No difference was found in neural phenotypes generated by small-versus-large spheres cultured in EGF+bFGF or bFGF+hs (data not shown).

In summary, bFGF+hs cervical spheres are the most multipotent relative to lumbar/sacral spheres in primary culture (Table 2). EGF+bFGF experiments did not reveal any differences in stem-cell ability to produce the three neural phenotypes as over 92% of all spheres tested were tripotent.

3.2 Isolation of a relatively-primitive spinal cord stem cell and identification and characterization of distinct stem-cell populations

3.2.1 The effect of subcloning on time to generate spheres

The results have identified regional and/or growth-factor differences in spinal cord stem cells a) with respect to their ability to produce the three neural phenotypes (cervical stem cells are tripotent more often then stem cells from other regions), b) with respect to sphere size (thoracic EGF+bFGF-responsive stem cells generate the largest spheres), and c) the greatest number are present in the lumbar/sacral segment. These differences suggest that spinal cord stem cells may come from various levels of the stem-cell hierarchy (Figure 1) and that a relatively primitive spinal cord stem cell exists. However, before performing subcloning studies to address this issue, it was important to determine the length of time required to form spheres from passaged cells. This was necessary because primary and pass-1 stem cells have different origins. Primary stem cells occur *in vivo*, while pass-1 stem cells are a product of tissue-culture conditions. This suggests they may have different properties, including the time required to form spheres. In addition, the timing of sphere formation is important for later comparisons. Please review Figure 2 for a clear understanding of the subcloning experiments. Primary spheres form within 8 days regardless of spinal cord region and growth-factor condition (Figure 4). Preliminary results suggest EGF+bFGF pass-1 spheres are also generated in 8 days, but that bFGF+hs pass-1 spheres require more time in culture to form. To determine any regional or growth-factor differences on time to generate spheres, passaged cells were observed, and any spheres present were counted at 8-day intervals up to 32 DIV. In all experiments, EGF+bFGF-generated spheres were always passaged into EGF+bFGF, and bFGF+hs-generated spheres were always passaged into bFGF+hs.

The time required to generate pass-1 spheres varies with growth-factor combination. Most EGF+bFGF-stimulated stem cells produce pass-1 spheres by 8 DIV

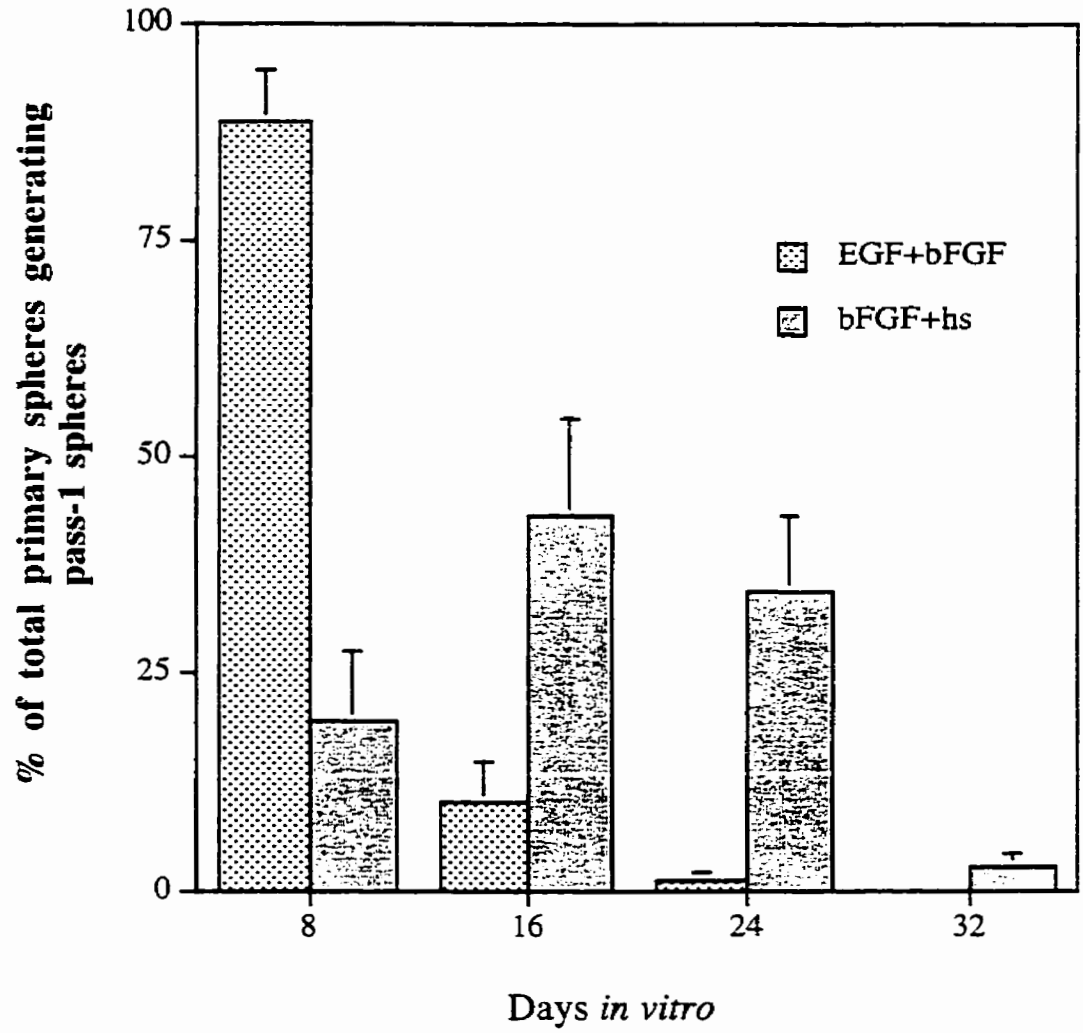
(Figure 7), and significantly fewer require 16 or 24 DIV to generate spheres ($p<0.01$). However, sphere production is evenly distributed over 8, 16, and 24 DIV when pass-1 stem cells are stimulated with bFGF+hs. It is important to note that although approximately 20% of bFGF+hs pass-1 spheres were produced by 8 DIV, these spheres were at maximum 100 μm in diameter, only half the size of EGF+bFGF spheres produced by this time. The data show some primary and passaged stem cells differ in their time requirements to produce spheres. In addition, most pass-2 and pass-3 EGF+bFGF-stimulated stem cells require even more time in culture to produce spheres (data not shown). This suggests further differences may exist between primary and subcloned stem cells.

3.2.2 Thoracic EGF+bFGF-stimulated stem cells have the highest expansion potential

Studies in the hematopoietic system suggest that stem cells at different levels of the stem-cell hierarchy vary regarding their ability to expand their population (Hellman *et al.*, 1978; Nakahata and Ogawa, 1982). Relatively-primitive stem cells near the top (Figure 1) have a large capacity for self renewal and a small capacity for expansion compared to the T1 transit cells they produce (Potten and Lajtha, 1982). The differences among expansion potentials for primitive cells-versus-transit cells generated several questions. First, does the spinal cord contain stem cells with varying abilities to expand? Second, are these stem cells localized to specific regions? Third, how does the expansion potential change in successive generations? To answer these questions, spinal cord 200-300-μm spheres from cervical, thoracic, and lumbar/sacral regions were subcloned in EGF+bFGF or bFGF+hs

Figure 7. Pass-1 spheres grow more quickly in EGF+bFGF than in bFGF+hs

Pass-1 EGF+bFGF and bFGF+hs spheres were observed at 8-day intervals until 32 DIV. Data points represent the percentage of primary spheres generating pass-1 spheres over 100 μm in diameter that are present for the first time by 8, 16, 24, or 32 DIV, compared with the total number of dissociated spheres yielding spheres. Most dissociated EGF+bFGF spheres formed pass-1 spheres by 8 DIV. This represents a significant difference over both EGF+bFGF spheres produced at other time points and time required to form bFGF+hs spheres ($p<0.01$) which are evenly distributed over 8, 16, 24, and 32 DIV. Data are from the three spinal cord regions combined. (N=8 cell cultures, $n\geq2$ spheres).


100
75
50
25
0
% of total primary spheres generating pass-1 spheres
EGF+bFGF
bFGF+hs
8
16
24
32
Days *in vitro*

for two successive passages. The number of passaged spheres produced was counted after up to 32 DIV. Primary spheres generated in EGF+bFGF were always subcloned in EGF+bFGF, while bFGF+hs generated primary spheres were always subcloned in bFGF+hs. It is important to note that all spheres were passaged after 8 DIV except bFGF+hs pass-1 spheres. The latter spheres were not large enough for passaging until 16 DIV. The results were expressed in terms of expansion potential, that is, the number of spheres produced from a dissociated sphere.

The results indicate spinal cord spheres expand to varying degrees (Table 3). However, regional differences in expansion are limited to pass-1 spheres cultured in EGF+bFGF. The highest expansion occurred in primary spheres from the thoracic region. These spheres produced significantly more pass-1 spheres than comparative regions ($p<0.05$). While the expansion potential of EGF+bFGF-generated spheres varied among some spinal cord regions at pass 1, comparative bFGF+hs stem-cell expansion exhibited no regional differences (Table 3). However, expansion potential did change between primary and pass-1 spheres. Primary spheres generated at least 5-fold more pass-1 spheres than the number of spheres at pass 2 generated by a pass-1 sphere (Table 3). This represents a statistically-significant difference ($p<0.05$).

3.2.3 Cervical EGF+bFGF spheres are subcloned for more passages than comparative regions

Studies in the hematopoietic system have shown that stem cells can differ in their abilities to self renew (Goodman *et al.*, 1977; Harrison *et al.*, 1987). This has led to the proposal that stem cells and transit cells exist in a continuum from cells with a high capacity for self renewal and a low differentiation capacity to cells with the reverse properties (Potten and Loeffler, 1990). Since a defining feature of stem cells is their ability to self renew over extended periods, one can assume that such a cell would be closer to the top of

Table 3. The expansion potential of stem cell-generated spheres decreases with passage

EGF+bFGF	mean # of spheres generated from a sphere + S.E.M.		
Pass	Cervical	Thoracic	Lumbar/Sacral
Primary -- Pass 1	168±43	310±42	196±23
Pass 1 -- Pass 2	33±10	17±8	23±16
bFGF+hs			
Pass			
Primary -- Pass 1	106±23	120±33	114±27
Pass 1 -- Pass 2	5±3	2±1	6±3

EGF+bFGF - pass-1 and pass-2 spheres generated from spheres passaged at 8 DIV

bFGF+hs - pass-1 spheres generated by spheres passaged at 8 DIV
pass-2 spheres generated by spheres passaged at 16 DIV

N=8 cell cultures, n≥2 spheres

the stem-cell hierarchy (Figure 1), or more primitive, than stem cells with more-limited self-renewal potential. Unfortunately, self renewal cannot accurately be tested without appropriate stem-cell markers. However, renewal can be determined. Renewal is defined as the ability of a passaged sphere to form spheres. The stem-cell-hierarchy suggests stem cells that can be renewed over more generations are closer to the top than those with a relatively-limited renewal potential (Figure 1). Therefore, stem cells with a high capacity for renewal can be identified as relatively-primitive stem cells. This has led me to ask, does a relatively primitive spinal cord stem cell exist and if so how is it distributed along the rostrocaudal axis? Previous results showed primary and pass-1 stem cells varied in terms of time required to form spheres (Figure 7) and expansion potential (Table 3). The purpose of this experiment was to determine the maximum number of generations stem cells from the three spinal cord regions could be subcloned, regardless of expansion or time required to generate spheres. Spinal cord spheres, 200-300-μm in diameter, from cervical, thoracic, and lumbar/sacral regions were subcloned for successive passages in EGF+bFGF and bFGF+hs. Primary spheres generated in EGF+bFGF were always subcloned in EGF+bFGF, and bFGF+hs was always used to subclone bFGF+hs-generated spheres. It is important to note that all primary spheres were passaged after 8 DIV. However, while the size of passaged spheres used was kept consistent, due to the results described in section 3.2.1, spheres in subsequent generations may have been passaged after 8, 16, 24, or 32 DIV. The data is expressed as the renewal-frequency, a ratio of passaged spheres producing spheres to the total number of spheres passaged. The inconsistencies in age among passaged spheres should not affect the interpretation of renewal-frequency results, since the purpose was to determine regional differences among stem cell ability to renew regardless of the effect of time to form spheres. For clarification it is important to note, subcloned primary spheres produce pass-1 spheres, subcloned pass-1 spheres produce pass-2 spheres, and so on.

The results to this subcloning experiment showed stem cells could be passaged for a maximum of 3 generations. Almost all attempts to produce fourth-generation spheres from pass-3 spheres were unsuccessful. However, one exception was a cervical EGF+bFGF-generated pass-3 sphere that successfully produced pass-4 spheres (data not shown). Only EGF+bFGF-stimulated stem cells from the cervical and thoracic regions could be passaged through the maximum number of generations (Figure 8). Cervical EGF+bFGF-stimulated stem cells are more reliably passaged (have a higher renewal frequency) at pass 3 than comparative thoracic stem cells ($p<0.05$). The renewal frequency decreases significantly over successive passages. The average renewal frequency is highest in pass 1 and decreases in pass 2 and pass 3 (growth factor and region combined, $p<0.05$ for all comparisons). Growth factor combination has no effect on this decrease for thoracic and lumbar/sacral stem cells. In contrast, the renewal frequency of cervical EGF+bFGF-stimulated stem cells remains constant from pass 1 to pass 3.

3.2.4 Re-examining properties of primary-versus-passaged cervical spheres

The data presented earlier showed differences exist between primary and pass-1 stem cells (Tables 2, 3, Figure 8).

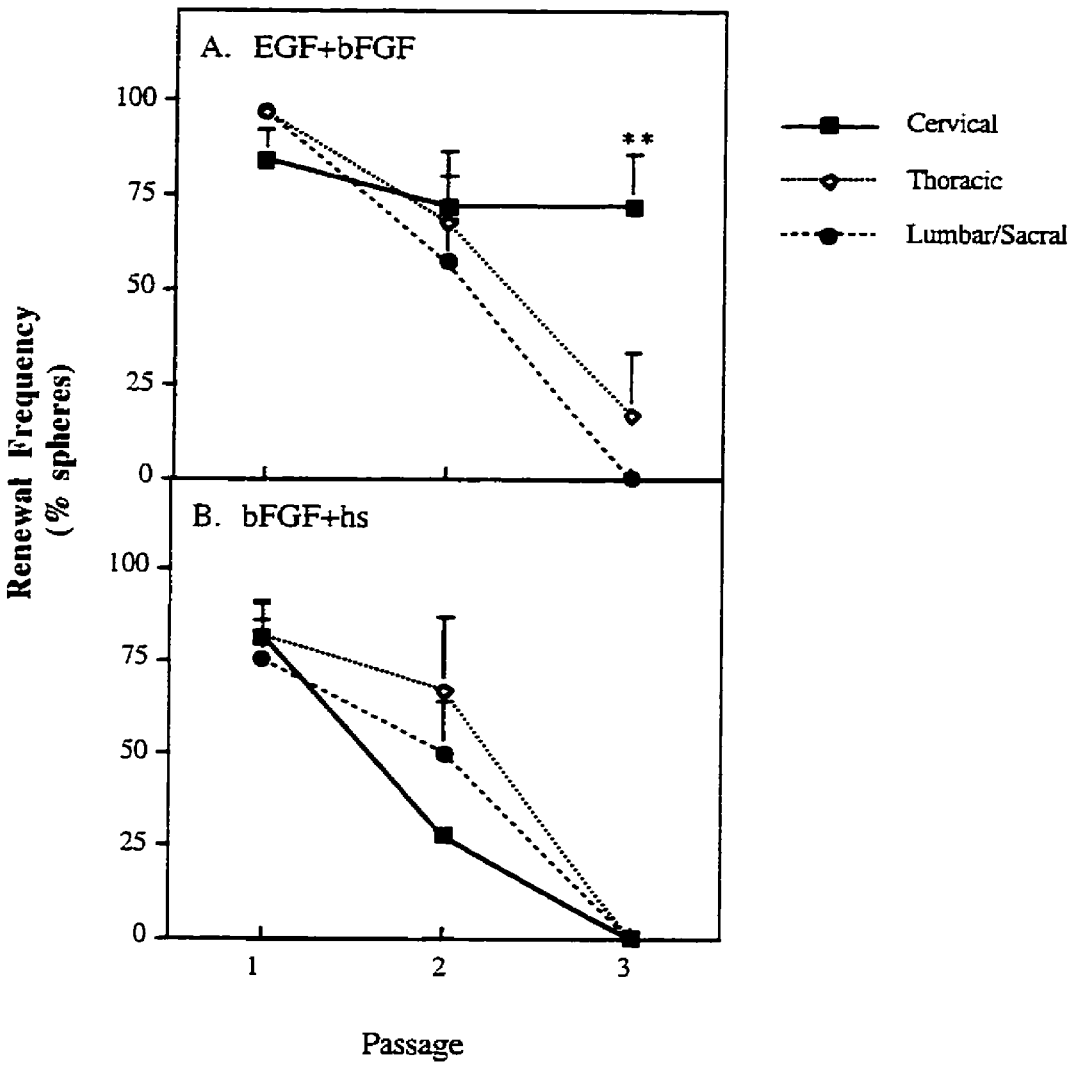
(a) Primary stem cells are more expandable (Table 3).

(b) Primary stem cells have a greater probability to renew (Figure 8).

(c) Primary stem cells have a greater probability of maintaining their multilineage potential (Table 2).

Figure 8. The renewal frequency of subcloned spheres varies with spinal cord region and growth-factor condition over successive passages

Subcloned spheres were observed at 8-day intervals until 32 DIV to determine whether passaged spheres generated spheres. Passage 1, 2, 3 (x-axis) refers to pass-1 spheres generated by primary spheres, pass-2 spheres generated by pass-1 spheres, and pass-3 spheres generated by pass-2 spheres, respectively. The renewal frequency represents the percentage of spheres passaged that actually formed spheres. Renewal frequencies for EGF+bFGF spheres (A) and bFGF+hs spheres (B) are shown. Cervical pass-3 EGF+bFGF spheres have a higher renewal frequency (**$p<0.05$) than thoracic or lumbar/sacral spheres (A). As a consequence of subcloning stem cells, fewer spheres were available for passaging on each successive generation. However, for any data point $N \geq 3$ cultures, $n \geq 1$ sphere.


A. EGF+bFGF
B. bFGF+hs
Renewal Frequency
(% spheres)
100
75
50
25
0
**
1
2
3
Passage
Cervical
Thoracic
Lumbar/Sacral

Some of these differences are growth-factor specific. For example, multilineage potential between primary and pass-1 spheres only decreases in bFGF+hs. There is also a difference among pass-1 stem cells, namely the time required to generate spheres (Figure 7). The pass-1 data show a large number of bFGF+hs-stimulated stem cells form spheres more slowly than EGF+bFGF stimulated stem cells. Due to these new considerations, a unifying series of studies was undertaken whereby time to generate spheres and growth factor requirements were normalized for a clear comparison of the properties demonstrated by primary and passaged spheres. The following experiments used cervical spheres only because they were reliably passaged for the most generations (Figure 8). Cervical pass-1 spheres generated after 8 and 16 DIV in EGF+bFGF and 16 DIV in bFGF+hs were compared for renewal frequency, stem cell expansion, and neural phenotype. All comparisons used pass-1 spheres 200-300 μm in diameter. In these experiments, EGF+bFGF spheres were always plated under this growth factor condition; bFGF+hs spheres were always plated in the presence of bFGF+hs. For the expansion assay, spheres were counted after 32 DIV. In Table 4, primary-culture data from previous results was included for comparison purposes.

Expansion potential was the major difference between primary and pass-1 stem cells. The results showed expansion decreased significantly in the number of passaged spheres produced by primary-versus-pass 1spheres cultured in EGF+bFGF ($p<0.001$) and bFGF+hs ($p<0.01$, t-test). Stem cell expansion was the only property affected by time required to generate spheres. The 8 DIV pass-1 EGF+bFGF-stimulated stem cells generated a two-fold increase in the number of pass-2 spheres produced compared with 16 DIV stem cells ($p<0.05$, least significant difference [LSD]). All pass-1 stem cell properties tested varied with growth factor condition. The renewal frequency of bFGF+hs-generated pass-1 spheres represented a significant decrease ($p<0.05$, LSD) compared with pass-1

Table 4. Some properties of cervical stem cells vary with sphere age, generation, and/or growth factor combination

	EGF+bFGF			bFGF+hs	
Property	**8 DIV** primary-pass1	**8 DIV** pass 1-pass 2	**16 DIV** pass 1-pass 2	**8 DIV** primary-pass 1	**16 DIV** pass 1-pass 2
Renewal Frequency (% passaged spheres generating spheres ± S.E.M., N≥4, n≥3).	84±8	63±13	56±12	81±10	21±5
Stem Cell Expansion (mean # passaged spheres produced by a sphere ± S.E.M., N=4, n≥3)	168±43	55±10	26±6	106±23	2±1
Neural Phenotype # of primary and pass-1 spheres producing phenotypes listed/total # spheres tested.					
Ne,A,O	22/22	66/66	47/49	16/17	0/36
Ne,O	-	-	-	1/17	29/36
Ne,A	-	-	2/49	-	-
Ne	-	-	-	-	7/36
N≥2, n≥4					

Ne-neuron, A-astrocyte, O-oligodendrocyte

spheres (8 and 16 DIV) cultured in EGF+bFGF. Expansion potential also decreased between EGF+bFGF-and bFGF+hs-generated pass-1 spheres. The stem-cell-expansion results showed the number of pass-2 spheres (generated by pass-1 spheres) is over 13 times greater in EGF+bFGF groups compared with bFGF+hs ($p<0.05$, LSD). In addition, the multilineage potential was related to growth-factor condition. The multilineage potential of EGF+bFGF-stimulated stem cells remained the same for pass-1 8 or 16 DIV spheres. Over 95% of EGF+bFGF spheres tested generated the three neural phenotypes. However bFGF+hs-generated pass-1 spheres failed to produce the three cell types. This represents a significant decrease in tripotency compared with primary bFGF+hs-generated spheres ($p<0.01$, t-test). It is unlikely that this result occurred because pass-1 bFGF+hs spheres required 16 DIV to form since the EGF+bFGF data showed that time to form spheres did not significantly affect lineage potential.

4 DISCUSSION

This study had three major findings. First, regional differences exist among spinal cord stem cells. Each region contains stem cells with properties characteristic of different levels on the stem-cell hierarchy (Figure 1). Second, primary and passaged stem cells are different, and this difference is manifested in properties of primary-versus-passaged spheres. Third, EGF+bFGF and bFGF+hs have dissimilar effects on the properties of spinal cord stem cells.

4.1 Spinal cord stem cells represent a heterogeneous population

The results of this study demonstrate that stem cells from different spinal cord regions have different properties. Starting with the cervical region and proceeding to the thoracic and lumbar/sacral regions, these differences include:

1) Differences in the number of times stem cells can be passaged. Cervical stem cells can be reliably passaged for the most generations. In EGF+bFGF they are passaged for three generations while maintaining a relatively-high renewal frequency (Figure 8).
2) Cervical stem cells are the most reliably tripotent. When cultured in either EGF+bFGF or bFGF+hs, cervical stem cells have the greatest probability of generating the three neural cell types (Table 2).
3) Thoracic stem cells generate the largest spheres (Figure 5).
4) Primary thoracic stem cells have the greatest expansion potential in EGF+bFGF (Table 3).
5) Lumbar/sacral stem cells represent the majority of spinal cord stem cells (Figure 4).

6) Lumbar/sacral stem cells generate the smallest spheres and are unable to renew beyond two generations (Figure 8).

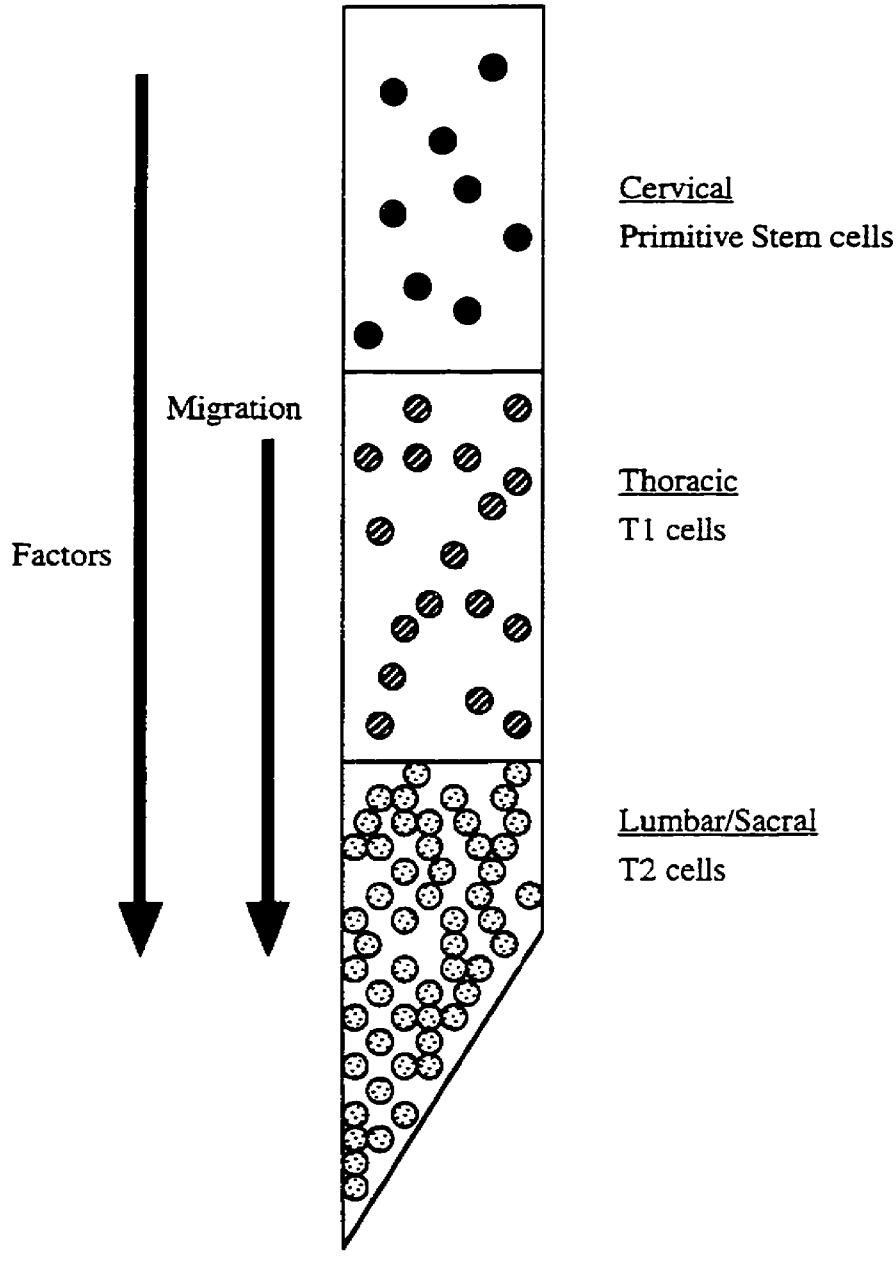
The variety of properties expressed among spinal cord stem cells imply that stem cells from the three regions studied are inherently different. Stem cells with heterogeneous properties often exist in a hierarchical relationship (Figure 1).

Stem cells from various levels of this hierarchy seem to be present in the adult spinal cord (Figure 1). The results suggest the cervical region may contain stem cells that are relatively primitive, thus located near the top of the hierarchy. Thoracic and lumbar/sacral stem cells may represent cells from successively-lower levels on this continuum.

Subcloning experiments imply that a relatively-primitive stem cell exists in the cervical spinal cord. Functionally, the defining feature of a primitive neural stem cell is its ability to renew over the largest number of generations thus placing it at the top of the stem-cell hierarchy. Cervical EGF+bFGF-stimulated stem cells can renew for three generations. Some thoracic EGF+bFGF-stimulated stem cells can also renew for three generations. However, cervical stem cells have a significantly-greater renewal frequency than thoracic stem cells, indicating that the cervical stem cells are more likely to renew (Figure 8). A second line of evidence is that relatively-primitive stem cells give rise to multiple differentiated phenotypes (Nakahata and Ogawa, 1982; Weiss *et al.*, 1996b). Primary cervical stem cells were in fact more likely to maintain their multilineage potential than comparative regions. The differentiation study showed that when bFGF+hs-generated spheres were tested for the presence of neurons, astrocytes, and oligodendrocytes, the probability of finding all three cell types present in a sphere decreased significantly from the cervical to the lumbar/sacral spinal cord (Table 2). Therefore, based on pass-3 renewal frequency and multilineage potential, relatively-primitive stem cells are present in the cervical spinal cord (Figure 9). Three characteristics of the data suggest a T1 transit-

Figure 9. Model of stem-cell populations in the spinal cord according to the stem-cell hierarchy

Stem cells represent successively lower levels of the stem-cell hierarchy from the rostral to caudal spinal cord. The cervical spinal cord contains a relatively-primitive stem cell, while thoracic and lumbar/sacral regions are enriched with T1 and T2 cells from lower positions on the hierarchy. These regional differences may represent a lineage relationship, the product of cell migration during development, or they may have been induced by chemical gradients acting on spinal cord stem cells to varying degrees.


Migration
Factors
Cervical
Primitive Stem cells
Thoracic
T1 cells
Lumbar/Sacral
T2 cells

amplifying cell is present in the thoracic region. First, transit cells are characterized by their ability to expand their population. Primary thoracic EGF+bFGF-stimulated stem cells expanded their population significantly more than cervical and lumbar/sacral stem cells. Second, since T1 cells have a restricted ability to renew compared to primitive stem cells, I would predict that a region rich in T1 cells would exhibit low-renewal frequencies. The data confirm this, with the thoracic region having a lower pass-3 renewal frequency than the cervical region. One would also predict that a cell with extensive expansion potential would generate relatively-large spheres, and the data illustrate that primary thoracic stem cells generated the largest spheres when stimulated with EGF+bFGF. This is consistent with the model's prediction for T1 cells. Together, these data suggest that there is a population of T1 transit-amplifying cells in the thoracic spinal cord (Figure 9). Lumbar/sacral stem cells appear dominated by a population of T2 transit cells. They are only able to renew for two generations in EGF+bFGF, in contrast to cervical and thoracic stem cells which renew for three generations. In addition, lumbar/sacral stem cells are also the least likely to generate the three neural phenotypes. Primary bFGF+hs-stimulated lumbar/sacral stem cells are only tripotent in 45% of spheres tested (Table 2). Cervical and thoracic stem cells produced the three cell types in 94% and 79% of spheres tested. Therefore, lumbar/sacral stem cells have the least ability to renew and the most restricted lineage potential compared to other spinal cord stem cells. This implies lumbar/sacral tissue contains cells downstream from both primitive stem cells and T1 cells on the stem-cell hierarchy. Therefore, lumbar/sacral stem cells may include a population of T2 cells (Figure 9). Since T2 cells are the progeny of rapidly-expanding T1 cells, one would predict a large number of stem cells in the lumbar/sacral region. This prediction is fulfilled as lumbar/sacral tissue contains the most-enriched population of stem cells in the spinal cord (Figures 4, 9).

A study in the hematopoietic system shows that stem cells distinguished by rate of proliferation are unevenly distributed in the bone marrow, with lower concentrations of cells in the center of the marrow and higher concentrations adjacent to the bone (Lord *et al.*, 1975). Furthermore, the cells next to the bone surface proliferate at a higher rate than the deeper stem cells, suggesting these two groups of stem cells may be from different levels of the stem-cell hierarchy. The hematopoietic study, therefore, provides an analogous example in which stem cells from different levels of the stem-cell hierarchy may be spatially distinct, consistent with the findings in the adult spinal cord.

What accounts for the finding that primitive stem cells are found in the cervical spinal cord? One model accounting for regional differences is the lineage model of stem-cell development (Dzierzak and Medvinsky, 1995). This model predicts that some stem cells from rostral spinal cord regions migrate caudally during development. Cervical stem cells would therefore produce thoracic stem cells, and thoracic stem cells would then produce lumbar/sacral stem cells. Hypothetically, after a cell divides, one daughter (cell A) may remain stationary, while the other (cell B) migrates a short distance in the caudal direction. When cell B divides, the process repeats, and one daughter will migrate while the other remains stationary. In the proposed migration process, cervical stem cells would divide and one daughter would move small increments in a caudal direction. Eventually stem cells originating in the cervical spinal cord would travel into the thoracic region. This process of cells dividing and one daughter moving in a caudal direction would continue until a thoracic daughter enters the lumbar/sacral region. Each time a cell divides, its progeny may occupy successively lower levels on the stem-cell hierarchy (Nakahata and Ogawa, 1982; Potten and Loeffler, 1990). Therefore, stem cells in rostral regions will be relatively primitive compared with stem cells from caudal regions. This would account for the primitive, T1, and T2 cells isolated in the cervical, thoracic, and lumbar/sacral regions respectively. For example, the lumbar/sacral region contains the largest population of

spinal cord stem cells. The adjacent thoracic region contains T1 cells which by definition rapidly expand their population (Lajtha, 1979). Lumbar/sacral stem cells may therefore be derived from T1 thoracic cells that were undergoing expansion while migrating caudally in the spinal cord. This would explain the large population of T2 cells present in the lumbar/sacral spinal cord. Several studies show that cells migrate short distances along the rostrocaudal axis of the developing neural tube (Leber *et al.*, 1990; Artinger *et al.*, 1995; Leber and Sanes, 1995). Leber and Sanes (1995) demonstrated that cells migrate rostrocaudally in the spinal cord area of the early neural tube. Furthermore, evidence that migrating precursor cells divide has been found for neuronal progenitors destined for the olfactory bulb (Menezes and Luskin, 1994; Rousselot *et al.*, 1995).

A second possible model explaining regional differences among spinal stem cells is the local development model (Dzierzak and Medvinsky, 1995). Similar to the lineage model, this model suggests that stem cells within each region derive from stem cells present during early development. The lineage model suggests regional populations of stem cells have distinct characteristics due to caudal migration of more-primitive stem cells (Figure 9). In contrast to the lineage model, the local development model suggests that adult stem cells are derived from non-migrating populations of cells that have been present throughout the neural tube since early development. More specifically, adult cervical stem cells maybe derived from a population of non-migrating stem cells in the cervical region of the neural tube. Adult thoracic stem cells derive from non-migrating stem cells in the early thoracic region of the neural tube and so on for lumbar/sacral stem cells. However, the stem-cell hierarchy still applies to cells in the context of this model. The data presented here indicate that cervical tissue contains relatively-primitive stem cells, while thoracic tissue and lumbar/sacral tissue contain T1 and T2 populations, respectively (see preceding discussion). Therefore, the position of an adult stem cell on the stem-cell hierarchy is related to the position its ancestors held on this hierarchy. Adult cervical stem cells are

relatively primitive, therefore they were derived from ancestors located near the top of the stem-cell hierarchy. The thoracic spinal cord contains T1 cells, therefore they are the progeny of cells from lower levels on the stem-cell hierarchy. Lumbar/sacral tissue contains T2 cells, therefore lumbar/sacral ancestors are derived from a relatively-low level on the stem-cell hierarchy.

Why would local development lead to regional differences among spinal cord stem cells? One possible explanation is that spinal cord stem cells were exposed to chemical gradients during development (Figure 9). In this way cells in distinct regions could be exposed to different concentrations of factors, or to different factors entirely. It may be that exposure to specific factors induces a stem cell to occupy a given level of the stem-cell hierarchy. This discussion will focus on some examples of signal molecules in development and how this may be related to stem-cell differences among spinal cord regions.

Chemical gradients are believed to play a role in anteroposterior neural patterning (Lamb and Harland, 1995; Doniach, 1995). Neuralization occurs in *Xenopus* by inhibition of epidermal induction via signals such as noggin (Smith and Harland, 1992; Zimmerman *et al.*, 1996), and chordin (Holley *et al.*, 1995; Piccolo *et al.*, 1996). Following neuralization an additional signal, possibly bFGF (Lamb and Harland, 1995), is proposed to establish forebrain/midbrain/hindbrain/spinal cord identity by acting on the neural ectoderm in a chemical gradient (Doniach, 1995). Therefore, spinal cord stem cells from the cervical region may be primitive relative to thoracic and lumbar/sacral cells due to their exposure to chemical gradients during development. This would be possible if a factor stimulating stem cell division was present in a concentration gradient over the rostrocaudal axis of the spinal cord. In the above scenario lumbar/sacral stem cells would undergo the most cell division, therefore progressing the farthest down the stem-cell hierarchy. Thoracic and cervical cells would divide less frequently and maintain higher positions on

the hierarchy. This would account for the finding that the cervical spinal cord is enriched with primitive stem cells, while thoracic and lumbar/sacral regions contain T1 and T2 cells, respectively. The limb bud is another example of how signaling molecules influence regional development (Duboule, 1994; Maden, 1994; Niswander *et al.*, 1994). Two factors ensuring the correct anterior-posterior axis formation are RA and the sonic hedgehog gene product; they influence development at the posterior surface of the limb bud (Thaller and Eichele, 1987; Marigo *et al.*, 1996). When they are expressed at the anterior margin a mirror image duplication of that part of the limb results (Tickle *et al.*, 1982; Riddle *et al.*, 1993). Therefore, the presence or absence of a chemical signal controls cell fate in the limb bud.

Presently the data are insufficient to determine if either model accounts for the regional differences among spinal cord stem cells. The one exception is the discrepancy between the expansion potential of cervical and thoracic stem cells, which suggests these regions do not share a lineage relationship (Table 3).

4.2 Stem cells forming primary and pass-1 spheres are distinct

The differences between primary and pass-1 spheres suggest they are produced by stem cells with dissimilar characteristics. The first half of this discussion will focus on differences in expansion between primary and pass-1 spheres. Predictions made using the stem-cell hierarchy (Figure 1) suggest that the expandability of pass-1 spheres is greater than that of primary spheres. I will produce two explanations of why this prediction is not fulfilled in the data. The latter part of the discussion will examine how stem cells producing the two groups of spheres are related on the stem-cell hierarchy (Figure 1). Two lines of evidence suggesting that stem cells generating primary spheres are closer to the top of the hierarchy compared with those generating pass-1 spheres will be discussed. These data suggest primary and pass-1 spheres are different since they are indeed generated by

stem cells with distinct properties. This discussion will focus on cervical spheres. Since they were the most-reliably passaged spheres, they were chosen as the focus of the final study that compared properties of primary and passaged spheres (Table 4).

The stem-cell hierarchy (Figure 1) makes two predictions about daughter stem cells. First, it indicates that relatively-primitive stem cells give rise to transit-amplifying cells that rapidly expand their population (Goodman *et al.*, 1977; Lajtha, 1979; Potten and Loeffler, 1990). Second, daughter cells from successively-lower levels are relatively restricted in their ability to generate multiple differentiated phenotypes (Barrandon and Green, 1987; Grigoriadis *et al.*, 1988; Hall and Watt, 1989). The first prediction suggests that the number of spheres produced in successive passages should increase. However, the data presented in this study show the number of spheres produced in successive passages decreases (Table 4). There are at least two explanations for this result. They involve the properties of cells within the primary sphere, and the *in vitro*-versus-*in vivo* growth environment. Expansion in the sphere population may decrease over successive generations because the maximum stem-cell expansion occurs in the primary sphere. For this discussion it is important to note that each sphere is presumably generated by a stem cell. Therefore, the number of spheres produced in pass 1 is related to the number of stem cells in a primary sphere. In turn, the number of spheres produced in pass 2 is related to the number of stem cells present in the pass-1 sphere and so on. The results of this study show that pass-1 spheres produce significantly fewer pass-2 spheres compared with the number of pass-1 spheres generated by a primary sphere (Table 4, $p<0.05$). It is possible that the primary stem cell does produce a transit-amplifying cell. By definition, the transit-amplifying cell has only a limited capacity for self renewal (Lajtha, 1979; Potten and Loeffler, 1990). This cell may expand in the primary sphere to such a degree that the large number of progeny it gives rise to are no longer transit-amplifying cells and, therefore, do not possess the ability to extensively expand their population. Work in the hematopoietic

system supports this finding (Siminovitch *et al.*, 1964; Hellman *et al.*, 1978). Hellman *et al.* (1978), using serial transfer of stem cells into irradiated mice, showed that the ratio of stem cells at the end of the transfer period to the number originally injected decreases over three generations to approximately one-tenth of the original value. Together, the spinal cord data and the hematopoietic studies suggest the expansion potential of primary spheres is relatively extensive compared to pass-1 spheres. Expansion in the sphere population may also decrease over successive passages due to the culture conditions. The *in vitro* growth environment may prevent increasing sphere expansion over successive generations due to the absence of a factor or factors present *in vivo* and required for this expansion process.

The finding that expansion declines with passage provides the first line of evidence suggesting primary spheres are different from passaged spheres. This result implies primary and pass-1 spheres are derived from stem cells on successively-lower levels of the stem-cell hierarchy (Figure 1).

The second line of evidence suggesting subcloned spheres are from lower levels on the stem-cell hierarchy is the time to form passaged spheres. Primary spheres were always produced after 8 DIV. Pass-1 spheres cultured in EGF+bFGF were generated in 8 or 16 DIV. In contrast, pass-1 spheres cultured in bFGF+hs required 16 DIV to grow to an adequate size for passaging (Figure 7). Length of time required for spheres to grow was related to multipotency and expansion. When the time required for sphere formation increased, multipotency and expansion decreased. My data revealed a significant decrease in expansion of pass-1 spheres generated after 16 DIV compared with those generated after 8 DIV (Table 4, $p<0.05$, LSD). This decrease occurred in EGF+bFGF and bFGF+hs. In addition, the multilineage potential of bFGF+hs-generated 16 DIV spheres was significantly less than bFGF+hs-generated 8 DIV primary spheres (Table 4, $p<0.05$). Although the EGF+bFGF 8-versus-16 DIV data do not represent a significant change in

tripotency of pass-1 spheres, there are fewer pass-1 (16 DIV) spheres that are tripotent compared with 8 DIV pass-1 and primary spheres. This demonstrates that lineage restriction represents a lower level of the stem-cell hierarchy. Late-forming spheres are therefore at a lower level of the stem-cell hierarchy than 8 DIV pass-1 spheres.

4.3 EGF+bFGF and bFGF+hs actions on spinal cord stem cells

The finding that both EGF+bFGF and bFGF+hs stimulate stem cells to divide raises the following question, do these growth factor combinations act on the same population of cells? In this section I will discuss two results suggesting that the above factors stimulate the same population and describe why they may act on this population in different ways. This will be followed by predictions of how each growth-factor combination would affect stem-cell properties. Two lines of indirect evidence suggest that EGF+bFGF and bFGF+hs act on the same stem-cell population. First, the stem-cell-distribution study (Figure 4) implied that each spinal cord region contains the same number of EGF+bFGF- and bFGF+hs-responsive stem cells. Both growth factor combinations produced the same number of stem cell-generated spheres for a given spinal cord region. Equal lengths of tissue were cultured together and were found to contain the same number of stem cells. Therefore, it is possible that these stem cells come from the same population. It would be unlikely to find the same number of EGF+bFGF- and bFGF+hs-responsive stem cells in *every* spinal cord region should they represent different stem-cell populations. Reports in the hematopoietic system indicate that different factors stimulate overlapping populations of precursor cells to divide (Leary *et al.*, 1987; Messner *et al.*, 1987), suggesting that this may also be possible for neural stem cells. Second, EGF+bFGF+hs were used together to stimulate stem-cell division. If EGF+bFGF- and bFGF+hs-responsive stem cells represented two separate populations, one would expect that the combined effects of these growth factors would produce a 2-fold increase in sphere

production. The results showed the effects were non-additive, that is, there was no increase in sphere production compared with either growth-factor combination used alone (J. Hewson and S. Weiss, personal communication).

If both growth-factor combinations are acting on the same population of stem cells, the results imply these factors are stimulating stem cells in different ways. Stem cells from various spinal cord regions manifest different properties when cultured in EGF+bFGF versus bFGF+hs. For example, bFGF+hs-stimulated stem cells are (1) relatively lineage restricted compared with EGF+bFGF-stimulated stem cells (Table 2) and (2) pass-1 stem cells are less expandable then comparative EGF+bFGF stem cells (Table 4). What accounts for the different effects of these growth factors? EGF is known to be a powerful mitogen for both neurons and non-neuronal cells (Simpson *et al.*, 1982; Carpenter and Wahl, 1990; Anchan *et al.*, 1991). Stem cells from the embryonic and adult mouse striatum divide in response to EGF (Reynolds and Weiss, 1992, 1996). EGF also stimulates populations of embryonic stem cells to self renew for 10 generations (Reynolds and Weiss, 1996). In addition, it has been suggested that stem cells do not necessarily move to lower levels of the stem-cell hierarchy with every cell division (Potten *et al.*, 1987; Potten and Loeffler, 1990). Together, these findings suggest EGF enables daughter cells to maintain stem cells properties to a greater degree then cells cultured in its absence.

The notion that EGF enables stem-cell progeny to maintain stem-cell characteristics makes it possible to predict the effect of EGF+bFGF and bFGF+hs on stem-cell properties, including renewal frequency, expansion potential, and multilineage potential. The first prediction is that renewal frequency of EGF+bFGF-stimulated stem cells should be higher than for stem cells treated with bFGF+hs. Only cervical stem cells producing pass-2 or pass-3 spheres support the prediction (Figure 8, Table 4). Renewal frequencies for stem cells from other regions do not change with growth-factor condition. The EGF model may support this result. Potten and Loeffler (1990) suggested the probability of

remaining on one level of the stem-cell hierarchy over several divisions is higher for cells near the top. Previous results suggest cervical stem cells are close to the top of this continuum. The renewal-frequency finding provides support for this argument.

Second, the EGF model predicts that stem-cell expansion would be enhanced in EGF+bFGF compared with bFGF+hs. The results are ambiguous, since primary stem cells expand to the same degree in either condition, unless they are from the thoracic region, while passaged cells (for example pass 1cells forming pass 2 spheres), expand more in EGF+bFGF (Tables 3, 4, Figure 6). This implies that expansion potential does not become growth factor-dependent until stem cells are subcloned. Therefore, the stem-cell property enabling expansion is the same among primary EGF+bFGF- and bFGF+hs-stimulated stem cells from all regions except thoracic. This discrepancy suggests thoracic stem cells may possess a responsiveness to EGF+bFGF not present in stem cells from other regions.

Third, the EGF model predicts EGF-stimulated stem cells will preferentially maintain their multilineage potential. Both primary and pass-1 results support this prediction. EGF+bFGF stimulated lumbar/sacral stem cells from primary culture generate the three neural cell types significantly more often than comparative bFGF+hs-stimulated stem cells (Table 2) ($p<0.05$, t-test). Table 4 shows that pass-1 cervical spheres are tripotent in EGF+bFGF but not in bFGF+hs. These results suggest EGF or the combination of EGF+bFGF, but not bFGF+hs, enable expression of the three neural phenotypes. Since maintenance of multilineage potential (Hall and Watt, 1989) is a characteristic of primitive stem cells the results imply tripotent stem cells treated with EGF+bFGF are from a relatively-high level on the stem-cell hierarchy.

In summary the results suggest EGF+bFGF-stimulated stem cells maintain primitive stem-cell properties more than bFGF+hs-stimulated stem cells. However, this was often related to spinal cord region and generation. The implication is that while growth

factors can influence stem-cell properties, they may act in combination with intrinsic qualities of a cell.

5 CONCLUSIONS AND FUTURE DIRECTIONS

Previous work in our lab has shown that neural stem cells are present in the adult forebrain and spinal cord (Reynolds and Weiss, 1992; Weiss *et al.*, 1996a). The spinal cord stem cells are of great importance because of their implication in treating spinal cord injury. However, before such clinical applications are possible, it is necessary to understand the spinal cord stem-cell properties. This work represents the first in-depth study of characteristics of an adult CNS stem cell. The important results are that regional differences exist between spinal cord stem cells; stem-cell properties change with subcloning; and stimulation with different growth factors affects the properties manifested in stem cells. Together the results imply that these stem cells are organized in a hierarchy, similar to stem-cell organization described for other systems (Hall and Watt, 1989). Knowledge of this organization enables one to make predictions regarding properties of stem cells after subcloning. The ability to localize stem cells on this hierarchy, both before and after subcloning, allows researchers to make appropriate choices regarding which stem cells are most appropriate for their purposes. For example, to induce a specific neuronal phenotype one may prefer to study a relatively-primitive stem cell that would presumably have the greatest multilineage potential. However, there are many unanswered questions regarding the characteristics and functions of spinal cord stem cells. What is the biological significance of the presence of stem cells in the adult spinal cord? Do other factors stimulate spinal cord stem cells to divide? Can specific factors influence stem-cell properties such as renewal and expansion? Is it possible to specifically induce a differentiated phenotype? Are there specific markers for neural stem cells? Determining the properties of stem cells and factors that control them will hopefully enable *in vivo* stem-cell manipulation as a viable, non-invasive treatment for spinal cord injury.

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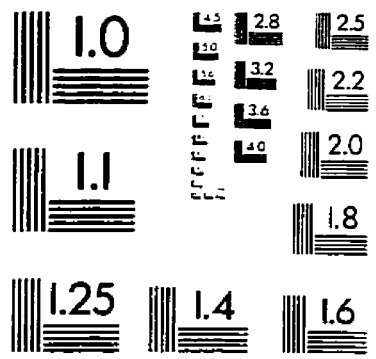
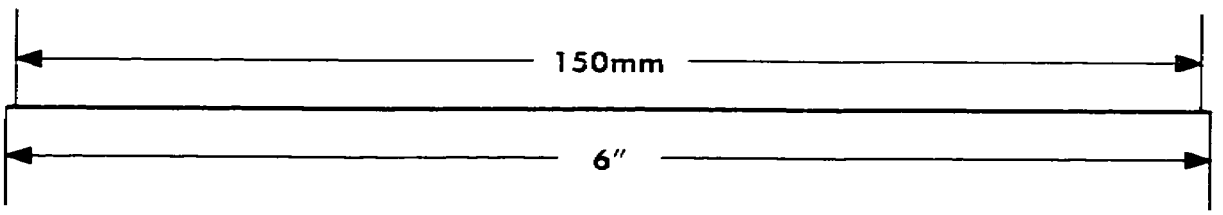
IMAGE EVALUATION
TEST TARGET (QA-3)





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