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EGF-responsive neural stem cells are a transient population in the developing mouse spinal cord

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Abstract

The adult mouse forebrain, which exhibits substantial ongoing cell genesis, contains self-renewing multipotent neural stem cells that respond to epidermal growth factor (EGF), but the adult spinal cord, which exhibits limited cell genesis, does not. Spinal cord development is a process characterized by defined periods of cell histogenesis. Thus, in the present study we asked whether EGF-responsive neural stem cells are present within the spinal cord during development. At embryonic day (E) 11, subsequent to the onset of neurogenesis, only fibroblast growth factor (FGF) receptors and FGF-2 (requiring heparan sulphate)-responsive stem cells are present in the spinal cord. Between E12 and 14, at the peak of spinal cord neurogenesis and the onset of gliogenesis, EGF receptors appear along with clonally derived highly expandable EGF-responsive neural stem cells. Following the cessation of cell histogenesis, the adult spinal cord is largely devoid of both EGF receptors and EGF-responsive stem cells. On the other hand, the FGF receptor1c subtype and multipotent FGF-2-responsive neural stem cells are present in early development and in the adult. The order of appearance of spinal cord neural stem cells and *in vitro* lineage analysis suggests that a more primitive FGF-2-responsive stem cell produces the EGF-responsive stem cell. These findings suggest that EGF-responsive neural stem cells appear transiently in the spinal cord, during the peak period of cell histogenesis, but are no longer present in the relatively quiescent adult structure.

Introduction

Neural stem cells of the mammalian central nervous system (CNS) are precursor cells, with the ability to proliferate, self-renew and give rise to neurons and glia (for review, see Gage, 2000; van der Kooy & Weiss, 2000; Weissman, 2000). Cells with these properties were first isolated from the forebrains of adult (Reynolds & Weiss, 1992) and embryonic (Kilpatrick & Bartlett, 1993) mice. Adult forebrain neural stem cells proliferate in culture in response to either epidermal growth factor (EGF; Reynolds & Weiss, 1992; Weiss *et al*., 1996a) or fibroblast growth factor-2 (FGF-2) combined with heparan sulphate (HS; Gritti *et al*., 1996). EGF-responsive neural stem cells of the adult forebrain reside in the mitotically active subependymal zone and appear to participate in the re-population of the structure (Morshead *et al*., 1994). Given that the postnatal/adult subependyma is the source of new neurons destined for the olfactory bulb (Luskin, 1993; Lois & Alvarez-Buylla, 1994), it is reasonable to suggest that the forebrain neural stem cells are required for ongoing adult histogenesis. In addition to the forebrain, the spinal cord is another adult CNS structure particularly enriched in neural stem cells (Weiss *et al*., 1996a). Surprisingly, adult spinal cord stem cells do not proliferate with EGF alone but do proliferate with combinations of either EGF and FGF-2 (Weiss *et al*., 1996a) or FGF-2 plus laminin (Shihabuddin *et al*., 1997, 2000). The adult spinal cord differs further from the forebrain in that there is no evidence for adult neurogenesis and only a small rate of glial cell turnover (Adrian & Walker, 1962; Fujita, 1965; Martin & Langman, 1965; Kraus-Ruppert *et al*., 1975; Frisen *et al*., 1995; Engel & Wolswijk, 1996; Horner *et al*., 2000).

Spinal cord histogenesis can be divided into at least three developmental periods. In the mouse, neurogenesis begins at embyonic day (E) 10 (E11 in rats) and is completed prenatally by E15 (E16 in rat) (Nornes & Das, 1974; Nornes & Carry, 1978; Altman & Bayer, 1984). Gliogenesis (astrocytes and oligodendrocytes) begins well after the onset of neurogenesis (Fujita, 1965; Altman & Bayer, 1984) and differentiated glia first appear at E16 and continue to be produced in significant quantities until postnatal day (P) 12 (Sakla, 1965; Gilmore, 1971; Warf *et al*., 1991). *In vitro* studies of the developing spinal cord have reported that FGF-2 (but not EGF) induces the proliferation of E10.5 spinal neuroepithelial cells with multilineage potential (when combined with a chick embryo extract; Kalyani *et al*., 1997) and E14 spinal neuroblasts (when cultured on laminin; Ray & Gage, 1994). On the other hand, EGF combined with FGF-2 could induce the proliferation of restricted progenitors derived from the E14 rat (astrocyte/oligodendrocyte-producing; Chandran *et al*., 1998) or embryonic human (astrocyte/neuron-producing; Quinn *et al*., 1999) spinal cord. Thus, a clear picture of the types of neural stem cells in the developing spinal cord has yet to emerge. Furthermore, recent studies of the embryonic forebrain provide conflicting arguments regarding the existence of a single neural stem cell responsive to either EGF or FGF-2 (Ciccolini & Svendsen, 1998) or two separate neural stem cells that are uniquely EGF-responsive or FGF-2-responsive (Tropepe *et al*., 1999). The objective of this study was to determine whether EGF-responsive neural stem cells exist at all

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in the developing spinal cord and, if so, what relationship they have to neural stem cells that are responsive to FGF-2.

Materials and methods

Primary culture and cell passaging

The cervical spinal cord was selected as the region of investigation, due to its previously described role as the origin of spinal cord histogenesis (Altman & Bayer, 1984; Warf *et al*., 1991) and following preliminary studies which found that adult stem cells from this region displayed the greatest degree of self-renewal (relative to thoracic and lumbar–sacral cord; C. E. Dunne and S. Weiss, unpublished observations). Adult/post-natal and embryonic animals were killed by cervical dislocation and decapitation, respectively. Cervical spinal cords were removed from embryonic days (E) 11–14, post-natal day (P) 2 or adult CD1 mice (Charles-River, Laval, QC, Canada) or B5/EGFP mice maintained on a CD1 background (kindly provided by Dr Andras Nagy, University of Toronto). The tissue was placed in phosphate-buffered saline (PBS) containing 0.6% of glucose, penicillin (50 U/mL) and streptomycin (50 mg/mL; both from Life Technologies, Gaithersburg, MD, USA). Tissue was mechanically dissociated with a fire-polished pipette in serum-free media composed of a 1 : 1 mixture of Dulbecco's modified Eagle's medium and F-12 nutrient (DMEM/F-12, Life Technologies). Cells were grown in 40 mL growth media in Corning T75 flasks, or in 2 mL of growth media in noncoated Nunc six-well culture dishes, at a concentration of 25 000 cells/mL. The growth media contained DMEM/F-12 nutrient (1 : 1), glucose, 0.6%; and, in mM, glutamine, 2; sodium bicarbonate, 3; and HEPES buffer, 5 mM, all from Sigma (St Louis, MO, USA) except glutamine (Life Technologies); insulin, 25 μg/mL; transferrin, 100 μg/mL; progesterone, 20 nM; putrescine, 60 μM; and selenium chloride, 30 nM; plus one of: (i) 20 ng/mL EGF (Chiron, Emeryville, CA, USA), (ii) 20 ng/mL FGF-2 (R & D Systems, Minneapolis, MN, USA) + 2 μg/mL HS (Sigma), or (iii) 20 ng/mL EGF + 20 ng/mL FGF-2. For adult cervical spinal cords, dissected tissue was placed in 95%O_2–5%CO_2 oxygenated artificial cerebrospinal fluid (aCSF, containing, in mM, NaCl, 124; KCl, 5; $MgCl_2$, 1.3; $CaCl_2$, 2; $NaHCO_3$, 26; D-glucose, 10; and penicillin–streptomycin solution (Life Technologies, 1 : 25); pH 7.35, ≈280 mosmol for further processing. The tissue was cut into smaller pieces (≈ 1 mm^3) and transferred into spinner flasks (Bellco Glass, Vineland, NJ, USA) with a magnetic stirrer filled with low Ca^{2+}, high Mg^{2+} aCSF (containing, in mM, NaCl, 124; KCl, 5; $MgCl_2$, 3.2; $CaCl_2$, 0.1; $NaHCO_3$, 26; D-glucose, 10; and penicillin–streptomycin 1 : 25 (pH 7.35, ≈280mosmol) and an enzyme mixture (1.33 mg/mL of trypsin, 0.67 mg/mL of hyaluronidase and 0.2 mg/mL of kynurenic acid). The stirring tissue suspension was aerated with 95%O_2–5%CO_2 at 32–35 °C for 90 min. Following this enzymatic incubation period the tissue was transferred to DMEM/F-12 (1 : 1, Life Technologies) medium containing 0.7 mg/mL of ovamucoid (Sigma) and triturated mechanically with a fired-narrowed Pasteur pipette. The dissociated cell suspension was centrifuged at 150 × ***g*** (400 r.p.m.) for 5 min, the pellet washed once and then plated (25 000 viable cells/mL) in noncoated six-well (2 mL volume) Nunc tissue culture dishes. For quantification of spheres derived in primary culture, the cells were cultured in 96-well plates, 5000 cells/200 μL per well, and at least eight wells per condition per experiment were analysed after 7 days *in vitro* (DIV).

Cell culture at clonal density

E14 spinal cord tissue was dissociated mechanically into single cells, and 150 cells were plated in single 35-mm culture wells (Sieber-Blum & Cohen, 1980). Two to three hours after plating, single cells were attached to the substratum. Cultures were followed daily, with the first cell divisions apparent after 5–7 DIV. In the absence of mitogen, no spheres were generated. In the presence of EGF, spheres were apparent after 12–14 DIV, with an average of 1.2 ± 0.7 (SEM) spheres per well (n = 96 wells in four independent primary cultures).

Renewal-expansion assay; single sphere dissociation

Single spheres were dissociated by taking a 10–100-μL aliquot of 7-DIV spheres and transferring the spheres into Nunclon 35-mm tissue culture dishes with culture medium. Under sterile conditions single spheres (300 μm in diameter) were transferred to 500-μL Eppendorf tubes containing 200 μL of medium, triturated 20–40 times and plated into a 96-well plate. The plates were scored 8 days later for the numbers of spheres derived from a single sphere.

Differentiation of single stem cell-generated spheres

Seven days *in vitro* after the primary culture or secondary culture, spheres were removed with a pipette, spun down at 150 × ***g*** (400 r.p.m), and resuspended in defined medium. The spheres were differentiated in single sphere cultures. Single isolated spheres were plated on poly L-ornithine-coated (15 μg/mL) glass coverslips in individual wells of 24-well Nunclon (1.0 mL per well) culture dishes. Embryonic and early postnatal spheres were differentiated in DMEM/F-12 medium with the hormone and salt mixture and 1% fetal bovine serum (FBS; Upstate Biotechnology, Lake Placid, NY, USA). Adult spheres were differentiated for 5 days in the mitogen(s) with which they were generated, followed by 5 days in DMEM/F-12 medium with the hormone and salt mixture (without serum). Medium was not changed for the rest of the experiment. Coverslips were processed 7 days later for indirect immunocytochemistry.

Antibodies

Primary antibodies for indirect immunocytochemistry included a mouse monoclonal antibody (IgG) to β-tubulin isotype III (Sigma; final concentration 1 : 1000), microtubule-associated protein-2 (MAP-2) (Boehringer/CHEMICON, Temecula, CA, USA; 1 : 1000) and nestin (Rat 401 from Developmental Studies Hybridoma Bank, Iowa City, IA, USA; 1 : 5), rabbit polyclonal antibody to glial fibrillary acidic protein (GFAP; Biomedical Technologies, Stoughton, MA, USA 1 : 400) and FGF receptor 1 (FGFR1; Santa Cruz, Santa Cruz, CA, USA; 1 : 100), sheep antiserum to EGF-receptor (EGFR; Biodesign, Saco, ME, USA, 1 : 100) and mouse monoclonal antibody (IgM) to O4 (Boehringer, 1 : 20). Secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) used were as follows: rhodamine-conjugated affinity-purified goat antibody to mouse IgG (1 : 200), AMCA-conjugated affinity-purified donkey antibody to mouse IgG (1 : 100), Cy3-conjugated affinity-purified donkey antibody to sheep IgG (1 : 500), fluorescein-conjugated affinity-purified goat or donkey antibody to rabbit IgG (1 : 100) and AMCA-conjugated affinity-purified goat antibody to mouse IgM (1 : 100).

Immunocytochemistry and cell counting

Indirect immunocytochemistry using secondary antibodies conjugated to rhodamine, fluorescein or AMCA was performed on single spheres 7 days after plating. Coverslips were fixed in 4% paraformaldehyde in PBS, pH = 7.2, for 30 min followed by 3 × 10-min washes in PBS. For simultaneous detection of neurons, astrocytes and oligodendrocytes, a triple-labelling protocol was used (Reynolds & Weiss, 1996; Weiss *et al*., 1996a). Cells were briefly permeabilized for 5 min (0.3% Triton X-100/PBS) after fixation followed by the addition of the neuron-specific mouse monoclonal antibody to either MAP-2 or β-tubulin (IgG) together with rabbit polyclonal antiserum

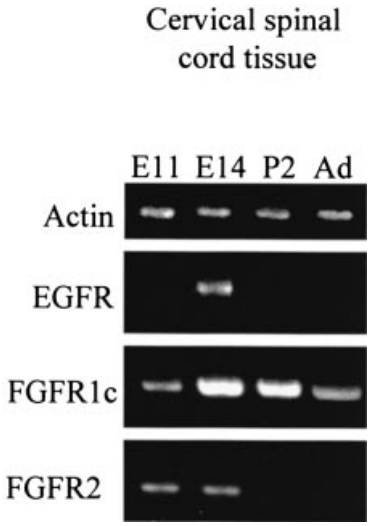

FIG. 1. Expression of EGF and FGF-2 receptors in the developing cervical spinal cord. The expression of receptor mRNA in E11, E14, P2 and adult cervical spinal cords was analysed with reverse transcription and the polymerase chain reaction (RT-PCR) using primers synthesized from mouse gene sequences, as described in Materials and methods. Significant quantities of EGFR were only detected at E14, whilst the FGFR1c (but not FGFR2) subtype of the FGF-2 receptor was present throughout development and into adulthood. This representative experiment was repeated three times with identical results.

to GFAP. Appropriate secondary antibodies were added followed by incubation with monoclonal antibody to O4 (IgM) and a goat antimouse IgM specific secondary (AMCA) was used to visualize the O4 antibody. Coverslips were rinsed, placed on glass slides with Fluorsave as mounting medium. Fluorescence was detected with a Nikon Optiphot photomicroscope.

For counting neuron numbers, immunoreactive cells for β-tubulin or MAP-2 were determined and tabulated relative to total cell number [fluorescent nuclei from Hoechst, bis-benzimide (1 μg/mL; Sigma) labelling]. In each experiment, at least 20 fields (20×) per coverslip, two coverslips per condition per experiment were counted. Each experiment was repeated 2–10 times.

To identify growth factor receptor expression on E14 cervical spinal cord cells, acutely isolated cells on coverslips were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Sheep anti-EGFR (Biodesign) and rabbit anti-FGFR1 (Santa Cruz), the primary antibodies, were diluted 1 : 100 and 1 : 200, respectively, in 10% normal donkey serum, 0.3% Triton X-100/PBS and applied overnight at 4 °C. After washes (3 × 5 min each), coverslips were incubated in donkey antisheep CY3 (1 : 500; Jackson Immunoresearch) and donkey antirabbit biotin (1 : 200; Jackson Immunoresearch) in 10% normal donkey serum, 0.3% Triton X-100/PBS for 30 mins. at 37 °C and then washed in PBS. The coverslips were incubated in streptavidin-FITC (1 : 1000; Jackson) and Hoechst and incubated at room temperature for 30 min. After a final series of three washes with PBS the coverslips were mounted in FluorSave Reagent on glass slides.

Immunohistochemistry

To observe the expression of EGFR *in vivo*, indirect immunohistochemistry was carried out on E14 cervical spinal cord tissue sections. Timed-pregnant CD-1 mice were killed on gestational day 14 and embryos were fixed with cold 4% paraformaldehyde in PBS overnight at 4 °C. The tissue was cryoprotected in changes of cold 10% and 20% sucrose in PBS for 2 h each, followed by 30% sucrose overnight at 4 °C. Tissue was embedded in O.C.T. compound (Sakura Finetek, Torrance, CA, USA) and cut into transverse sections, 12 μm thick, and mounted on gelatinized slides. Before immunolabelling, sections were postfixed with cold acetone (4 °C) for 10 min, followed by extensive washes in PBS. Sheep anti-EGFR, the first primary antiserum, was diluted 1 : 100 in 10% normal donkey serum, 0.3% Triton X-100/PBS and applied overnight at 4 °C. After washes, sections were incubated in donkey antisheep CY3 (1 : 500; Jackson Immunoresearch) in 10% normal donkey serum, 0.3% Triton X-100/PBS and Hoechst 33258 (Sigma) in PBS for 30 mins. at 37 °C. The slides were washed in PBS and coverslipped in FluorSave Reagent (Calbiochem, San Diego, CA, USA).

TABLE 1. E14 cervical spinal cord EGF-generated spheres can be derived in clonal density primary culture

	EGF	FGF-2 + HS
Clonal frequency (number per 35 mm well)	1.2 ± 0.7	0
Multipotentiality (number that contain N, A and O)	40 out of 89	n.d.
Renewal/expansion (number that produce more than one sphere)	55 out of 72	n.d.

Clonal density culture analysis was performed as described in Materials and methods. N, A and O, neurons, astrocytes and oligodendrocytes; n.d., not determined.

Reverse transcription and PCR

Total RNA was extracted from spinal cords and spheres using the acid guanidinium thiocyanate-phenol-chloroform extraction method, using Trizol reagent (Life Technologies). After annealing 1 μg of total RNA samples and 0.5 μg oligodT (65 °C), they were reverse transcribed with 200 U of Superscript reverse transcriptase (Life Technologies) plus, in mM, dNTPs, 0.5; Tris pH 8.3, 50; KCl, 75; $MgCl_2$, 3; and DTT, 10. The reaction was carried out for 90 min at 42 °C, and the final product was diluted with distilled water to a final volume of 50 μL.

For EGF receptors (accession no. X59698) primers used were (5′): ctgctgccacaaccaatgtgctgc and (3′): ggccactggcaggatgtgaaggtc. The amplification product was 438 bp (positions 872–1261). For FGFR1c (accession no. U36456), the primers used were (5′): caaaccaaaccg-taggcctgtagc and (3′): ggagacattccgtagatgaagcacca. The amplification product was 567 bp (positions 231–748). For FGFR2, we used the primers used in the report of Orr-Urtreger *et al.* (1993) – (5′): aacggtcaccacaccggc and (3′): ctgcagaactgtcaaca. The amplification product was 336 bp. For beta-Actin (accession no. X03672), primers used were (5′): cgtgggccgccctaggcacca and (3′): ttggccttagggtt-caggggg. They produced an amplification product of 243 bp (positions 182–424).

Polymerase chain reaction analyses were performed in a final volume of 50 μL containing 25 pmol of each primer, 5 μL of the reverse transcription product, 10 mM tris-HCl pH 8.3, 50 mM KCl, 2.5 mM MgCl2, 0.4 mM of each dNTP, 5% DMSO and 2.5 units of taq DNA polymerase. After denaturation, amplification cycles (35 cycles for EGF receptors, 30 cycles for FGFR1c and FGFR2 and 28 for actin) were: 94 °C for 45 s, annealing (55 °C for EGF and FGFR1c receptors, 57 °C for FGFR2 and 58 °C for actin) for 45 s and 72 °C for 1 min. Final products were analysed on a 1.5% agarose gel. The presence of contaminants was checked with template- or reverse transcription-free PCR reactions. As positive controls we used cDNAs from placenta and fibroblasts for EGF receptors, total

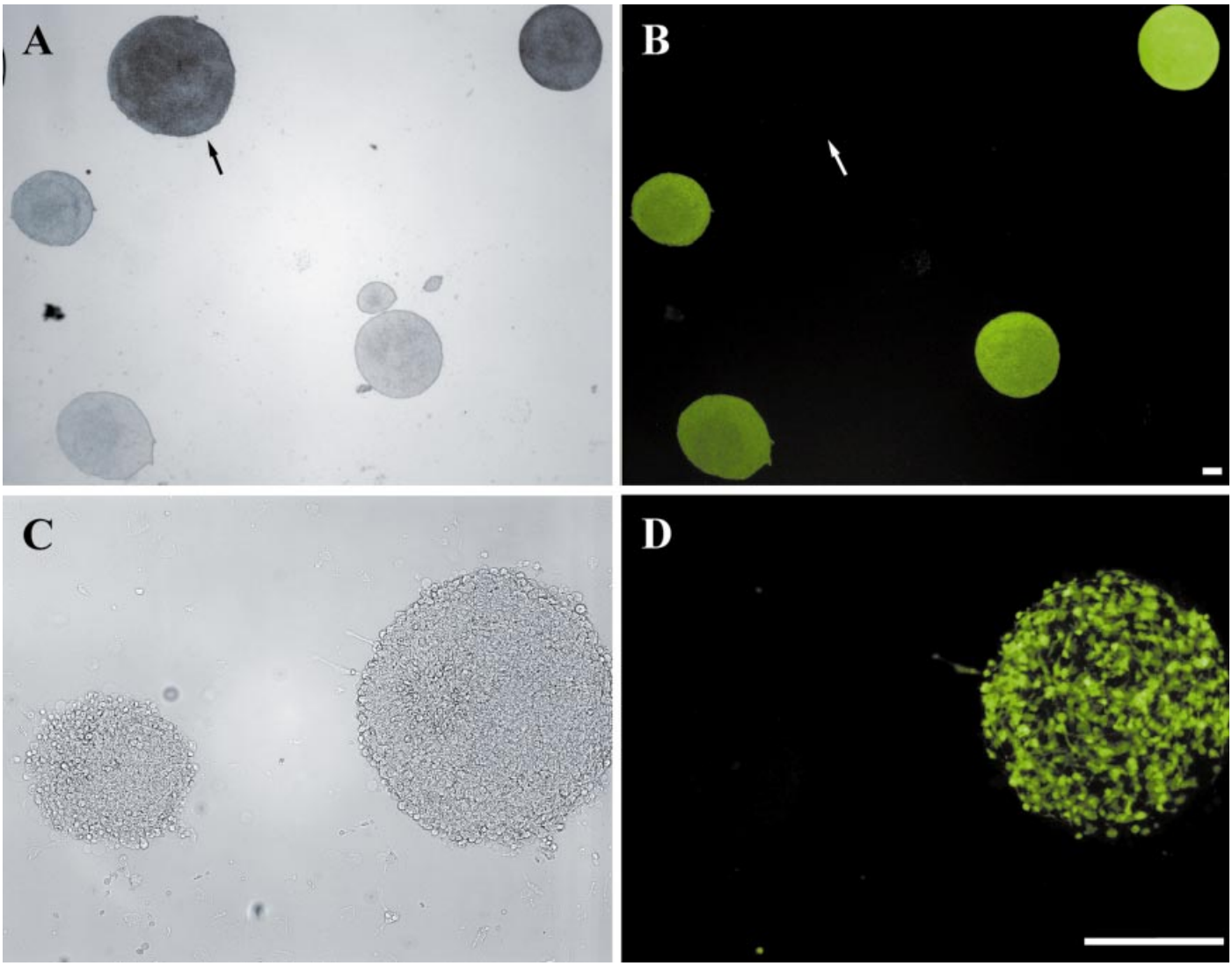

FIG. 2. EGF-generated spheres from mixed cultures of E14 spinal cord cells from CD-1 and EGFP mice. Cells from CD-1 mice and EGFP mice were mixed by 1 : 9, respectively, and cultured as described in the methods. (A and C) Phase-contrast photomicrographs. (B and D) Fluorescent photomicrographs. Arrows are indicating an EGFP-negative sphere. A larger sphere in panels C and D appears chimeric and may be formed by cells from both origins. Scale bars, 100 μm (A and B), 500 μm (B and D).

embryos (E11) and P28 cerebellum for FGFR2 and adult brain and salivary glands for FGFR1c. The specificity of PCR products was checked by sequencing purified fragments.

Statistical analysis

Analysis of significant differences were checked using ANOVA followed by unpaired *t*-test to compare mean values from different experiments (e.g. values obtained from adult cells vs. embryonic cells) and paired *t*-test to compare values within experiments (e.g. number of spheres obtained in EGF vs. number of spheres obtained in FGF-2 + HS). Values expressed as percentage (e.g. number of neurons) were analysed using the χ^2-test.

Results

EGF-responsive, expandable, multipotent stem cells are present during the peak period of spinal cord histogenesis

We first asked whether EGF receptors (and consequently EGF-responsive stem cells) are present in the developing spinal cord during peak periods of cell histogenesis. Reverse transcription of total RNA from E11, E14, P2 and adult cervical spinal cords, followed by the polymerase chain reaction (RT-PCR), was used to examine expression of the EGF receptor and the two FGF-2-preferring receptors, FGFR2 and FGFR1c (Fig. 1). Significant expression of FGFR1c receptors is present throughout development and into adulthood, as previously reported (Weise *et al*., 1993; Grothe & Meisinger, 1995; Stapf *et al*., 1997). The FGFR2 receptor subtype was detected at E11 and E14 but not postnatally. On the other hand, significant (detectable by 35 cycles in PCR; see Materials and methods) EGF receptor expression was detected only at E14, the peak period of spinal cord histogenesis.

Given the discrete expression of the EGF receptor, we focused our initial experiments on examining dissociated E14 cervical spinal cords for the presence of growth factor-responsive neural stem cells *in vitro*, using our sphere-forming assay (Reynolds & Weiss, 1996; Weiss *et al*., 1996a). Although we have previously demonstrated that spheres of the embryonic forebrain germinal zone are clonally derived (Reynolds & Weiss, 1996), we sought to establish the clonal

nature of spinal cord spheres. This was deemed particularly critical for spheres that would be generated in the presence of EGF, given that EGF-responsive neural stem cells have yet to be identified in spinal cord tissue. This was done in two ways. First, we cultured E14 cervical spinal cord cells at clonal density (150 cells in a 35-mm dish; Sieber-Blum & Cohen, 1980) in the presence of either EGF or FGF-2 + heparan sulphate (HS, 2 μg/mL; Table 1). After 10–14 DIV, in the presence of EGF an average of one sphere was generated in each 35-mm well (1.2 ± 0.7, $n = 4$ experiments, 24 replicates per experiment). Of 89 such EGF-generated spheres examined for phenotype, 40 contained neurons, astrocytes and oligodendrocytes, whilst the remainder produced only glial cells (astrocytes and oligodendrocytes or astrocytes alone). Of 72 such EGF-generated spheres examined for renewal/expansion, that being the ability to generate more than one secondary sphere after single-sphere dissociation, 55 spheres were expandable. These findings suggest that the EGF-generated spheres were indeed clonally derived from individual precursor cells. On the other hand, in the presence of FGF-2 + HS, no spheres were formed when primary E14 cervical spinal cord cells were plated at clonal density. These results suggest that additional factors are required for FGF-2-responsive stem cells to proliferate, that such factors are probably released by other cells, and effective concentrations are reached when higher densities are used for primary culture (see the Discussion for citations of other studies which support this hypothesis).

The second approach used to confirm the clonal nature of EGF-generated spheres was to coculture E14 cervical spinal cords from CD-1 mice with those from E14 CD-1 mice containing enhanced green fluorescent protein (EGFP) in all cells (Hadjantonakis *et al.*, 1998), at a 1 : 9 ratio of wild type to EGFP in the presence of EGF. At high-density plating (10 000 cells per 35-mm dish), virtually all spheres contained either all EGFP cells or all wild-type cells (Fig. 2A and B). A small number of spheres contained cells that were either EGFP-positive or EGFP-negative (Fig. 2C and D). In three experiments, these mixed spheres comprised 10.5 ± 3.0 of the wild-type population, or $\approx 1\%$ of the total population. It is unclear whether these small numbers of chimeric spheres are true chimeras of two individual precursors or examples where transgene expression from a single cell became variable or chimeric.

Taken together with the clonal density cultures, these findings unambiguously demonstrate that at least 99% of EGF-generated spheres from the E14 cervical spinal cord are clonally derived from individual precursor cells and that multipotent and expandable EGF-responsive neural stem cells are present in the embryonic spinal cord.

EGF- and FGF-2-responsive spinal cord stem cells have distinct developmental and phenotypic properties

Before comparing the properties of EGF- or FGF-2 + HS-responsive spinal cord stem cells, we sought to confirm that we were utilizing the optimal concentrations of mitogens for these studies. When 5000 E14 cervical spinal cord cells were plated in individual wells of a 96-well plate, a dose-dependent increase in sphere formation was apparent with both EGF and FGF-2 + HS (Fig. 3A). Whilst EGF was 50–60-fold more potent than FGF-2, both factors produced their maximum efficacy at 10–20 ng/mL. Thus, for all subsequent comparisons, 20 ng/mL of each mitogen were used. We then determined, for each developmental period examined, the numbers of spheres generated from 5000 plated cells in the presence of either EGF or FGF-2 + HS (Fig. 3B). At E11, a very small number of spheres (< 5 per 5000 cells or $< 0.1\%$) were formed in the presence of EGF. This increased dramatically between E12 and 14, with a peak response at E14 of close to 2.5% of plated cells generating spheres. At P2 and in adult tissue, again a very small number of spheres (similar to E11) were generated in response to EGF. The numbers of spheres generated in response to FGF-2 + HS was more consistent, ranging between 0.4 and 2.0% of plated cells. At virtually all stages (other than E14), FGF-2 + HS produced at least twice as many spheres as did EGF. Peak production was at E13, with close to 2% of plated cells generating spheres.

In a previous study of E14 striatal EGF-responsive cells that formed spheres, 75–80% of stem cell-generated spheres produced more than two secondary spheres (i.e. were expandable) and contained precursors to neurons, astrocytes and oligodendrocytes (i.e. were multipotent) (Reynolds & Weiss, 1996). Hence, the phenotypic properties (expansion and multipotency) of a sphere serve to define the stem-like nature of the cell responsible for its generation. Thus, in the next series of experiments, we examined the expansion and multipotency (Table 2) of single EGF- or FGF-2 + HS-generated spheres from primary cultures of the developing spinal cord. We chose spheres of equivalent size (300 μm) at the various developmental periods. For the expansion assay, single primary spheres generated in either EGF or FGF-2 + HS were mechanically dissociated and re-plated (in the same growth factor in which they were generated) in a single well and the percentage of primary spheres that produced more than one secondary sphere was determined after one week (Table 2). For the multipotent phenotype assay, single spheres were plated on poly L-ornithine-coated coverslips in differentiating conditions (as described in Materials and methods) and subsequently examined with triple-label immunocytochemistry for neurons, astrocytes and oligodendrocytes (Table 2). Primary spheres generated in the presence of EGF, from E11, E14 and adult spinal cords, had remarkably different properties. The vast majority of E14 EGF-generated spheres expanded ($72 \pm 8\%$) and were multipotent ($82 \pm 16\%$). On the other hand, $< 50\%$ of E11 and adult EGF-generated spheres were multipotent and were little (E11, $6 \pm 3\%$) or not at all (adult) expandable. The FGF-2 + HS-generated spheres that expanded and were multipotent (and thus were most stem cell-like in nature/origin) to the greatest extent were those derived from E14 or adult spinal cords. However, at all stages of development the FGF-2-generated spheres were multipotent. Taken together, these findings suggest that at E14, highly expandable multipotent stem cells that respond to either EGF or FGF-2 are present in the spinal cord, whilst in the adult only FGF-2-responsive stem cells are present.

The additive nature of EGF and FGF-2 generation of spheres and growth factor receptor expression on acutely dissociated nestin-positive cells suggests the presence of two (or more) populations of neural precursor cells in the E14 cervical spinal cord

Given the presence of neural stem cells at E14 that respond to either EGF or FGF-2 + HS, we next asked whether these might represent distinct precursor populations. This was done in two ways. First, we examined the sphere generation in the presence of saturating concentrations of both mitogens (Table 3). When 5000 E14 cervical spinal cord cells were exposed to 20 ng/mL of EGF alone, 120 ± 25 spheres were generated. Under the same experimental conditions, 20 ng/mL of FGF-2 + HS generated 60 ± 8 spheres. When EGF was coincubated with FGF-2 + HS, 207 ± 12 spheres were generated. The total expected if the separate actions of EGF and FGF-2 + HS are combined was 184 ± 6 spheres. Thus, the numbers of spheres generated by EGF and FGF-2 + HS was additive, suggesting that they are probably derived from two distinct populations of neural precursor cells.

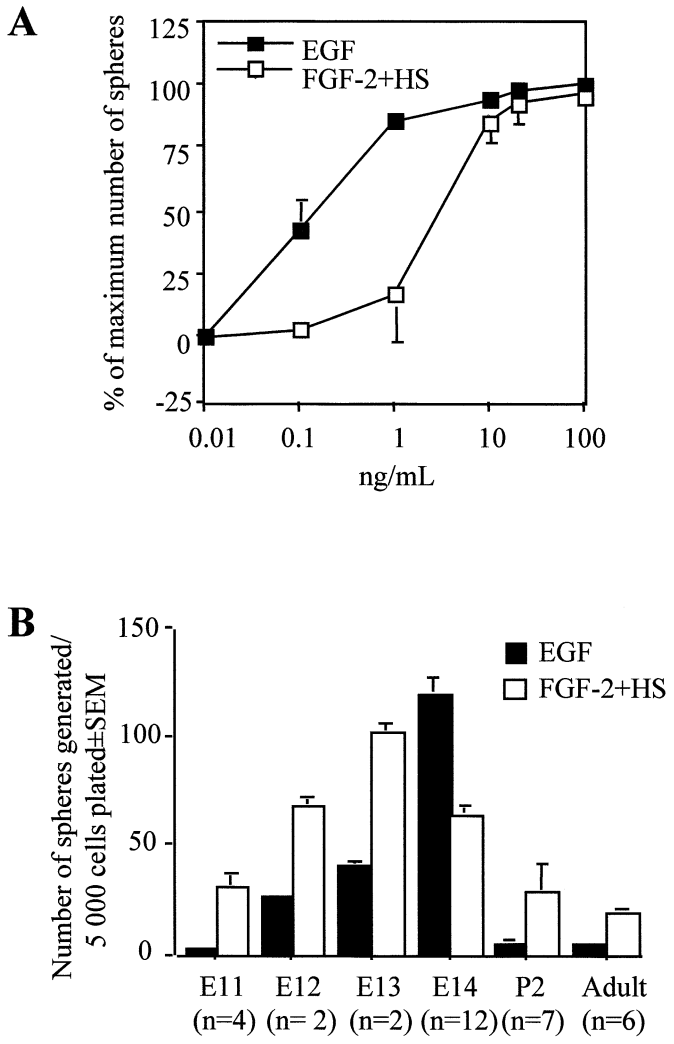

FIG. 3. FGF-2- and EGF-responsive spinal cord stem cells have different developmental profiles. (A) EGF and FGF-2 induce dose-dependent increases in sphere formation from E14 cervical spinal cord cells. Data are expressed as the percentage of maximal sphere number derived from 5000 cells plated in individual wells of a 96-well plate and are the mean ± SEM of three experiments. (B) Significant numbers of EGF-generated spheres are produced from E12–14 cervical spinal cord cells *in vitro*, whilst FGF-2-generated spheres are produced from cells derived throughout development and into adulthood. Cervical spinal cords were isolated from embryonic, postnatal and adult mice, and the number of spheres generated after 7 DIV from 5000 cells plated in 200 μL was analysed. Data are the mean ± SEM of 2–12 individual experiments, with at least eight replicates within each condition and developmental period.

TABLE 2. Expansion and phenotype properties of EGF- and FGF-2-generated spheres

Developmental period	Expansion†		Multipotent phenotype‡	
	EGF	FGF-2	EGF	FGF-2
E11	6 ± 3	19 ± 4*	46 ± 9	81 ± 5
E14	72 ± 8*	75 ± 2	82 ± 16*	88 ± 10
Adult	0	62 ± 4	40 ± 8	89 ± 10

Data are the mean percentage ± SEM of 3–5 different culture experiments, with a total of 21–96 spheres examined per condition. †% of primary spheres that give rise to more than 1 secondary sphere; ‡% spheres containing neurons, astrocytes and oligodendrocytes. *$P < 0.005$ vs. the other developmental periods analysed with the same growth factor.

TABLE 3. Numbers of spheres produced by saturating concentrations of EGF and FGF-2 are additive

Culture condition	Number of primary spheres
(1) EGF (20 ng/mL)	120 ± 15
(2) FGF-2 (20 ng/mL) + HS	60 ± 8
(3) Expected if adding (1) and (2)	184 ± 6
(4) EGF + FGF-2 + HS	207 ± 12

The numbers of spheres generated after 7 DIV from 5000 cells plated in 200 μL were analysed. Primary cultures and sphere generation were carried out as described in Materials and methods. Data are the means of at least three independent culture conditions, with at least eight replicates within each condition.

In the embryonic brain, EGF receptors have been largely localized to the germinal zone, and more specifically to the subventricular zone (Misumi & Kawano, 1998), with expression beginning during the later stages of neurogenesis and onset of gliogenesis (Kornblum *et al*., 1997). Whilst FGFR1 is expressed in the embryonic spinal cord, expression of EGF receptors has not been reported. Although our RT-PCR analysis provides evidence for EGFR expression in the E14 spinal cord (Fig. 1), we sought to further characterize the nature of this expression, both with respect to the intact cervical spinal cord and in relation to precursor cells that express FGFR1 *in vitro*. In the E14 cervical spinal cord, indirect immunocytochemistry with EGFR antiserum showed highly enriched receptor expression in the ventral aspect of the ventricular zone (Fig. 4A and B), an expression pattern not unlike that previously reported for FGFR1. These results support the contention that EGFR is expressed in embryonic spinal cord precursors. To further support this hypothesis and determine the frequency of EGFR-expressing precursors, we performed triple label immunocytochemistry on acutely dissociated cells of the E14 cervical spinal cord with antibodies directed against EGFR, FGFR1 and nestin (Fig. 4C–F). Virtually all nestin-expressing cells (Fig. 4F) also expressed EGFR (Fig. 4C). The nestin/EGFR-expressing cells were comprised of two populations, those that coexpressed FGFR1 and those that did not (76.7 ± 3.7% and 19.2 ± 4.1%, respectively, of the total nestin-immunoreactive population, $n = 3$; Fig. 5E). A very small number of the nestin-immunoreactive cells expressed only FGFR1 (3.3 ± 0.7%), and even fewer expressed neither EGFR nor FGFR1 (0.9 ± 0.5%).

One cannot correlate receptor expression with the existence of distinct multipotent neural stem cell populations (see Discussion). However, given that within the 95% of nestin-immunoreactive cells which express EGFR, some express FGFR1 and others do not, and taken together with the additive actions of EGF and FGF-2, two or more populations of multipotent neural stem cells may be present in the E14 cervical spinal cord.

The combined actions of FGF-2 and EGF in inducing the formation of adult spinal cord spheres is due to sequential actions of first FGF-2 and then EGF

The results of this study show that both EGF- and FGF-2 + HS-responsive neural stem cells are present in embryonic spinal cord, whilst the adult spinal cord contains only FGF-2 + HS-responsive stem cells. However, we previously found that although neither EGF nor FGF-2 (without HS) alone could induce adult spinal cord neural stem cell proliferation, a combination of EGF + FGF-2 could (Weiss *et al*., 1996a). This may be because: (i) FGF-2 up-regulates EGF

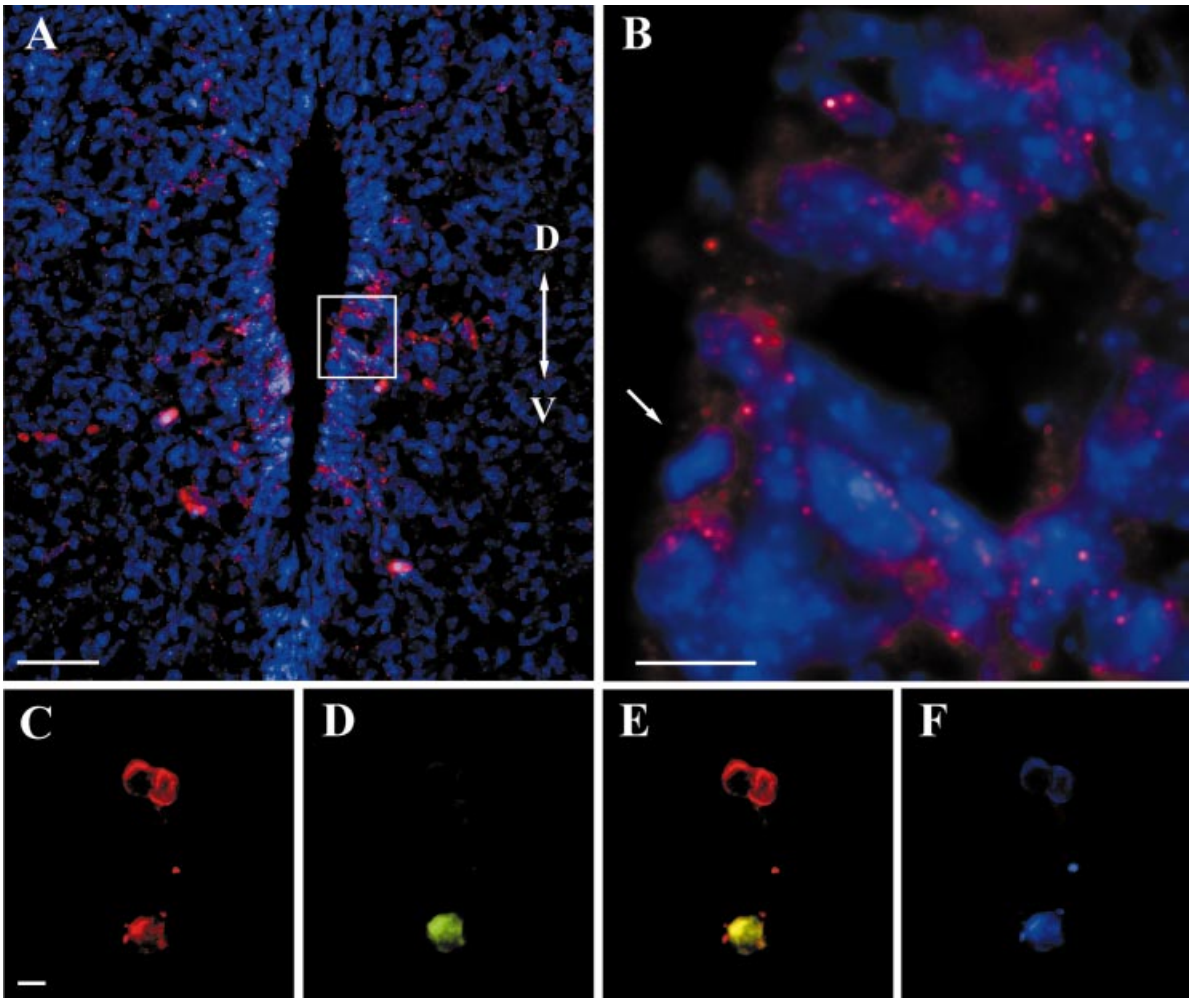

FIG. 4. EGFR and FGFR1 expression in the E14 cervical spinal cord. (A and B) Identification of the EGFR-expressing cells in the germinal zone of E14 spinal cord. EGFR immunohistochemistry was detected by Cy3 (red) fluorescence on 12-μm transversal sections. D, dorsal; V, ventral. An arrow in panel B (higher magnification picture of the square in panel A) is indicating a dividing cell. (C–F) Two distinct types of progenitor cells are shown: nestin + EGFR and nestin + EGFR + FGFR1. E14 spinal cords were dissociated into single-cell suspension and plated onto poly L-ornithine coated coverslips in the culture medium without mitogens. Two hours after the plating, cells were fixed and processed for the triple-labelling immunocytochemistry (red, EGFR; green, FGFR1; blue, nestin). Scale bars, 50 μm (A), 10 μm (B–F).

receptor expression to levels sufficient for ligand activation or (ii) FGF-2 alone is sufficient to induce the production of secondary stem cells that can respond to EGF. To examine the latter of these two possibilities, we dissociated adult cervical spinal cords and exposed cells to either EGF or FGF-2 for 12, 24, 48 or 72 h. Following washout, cells were switched to the other mitogen and incubated for an additional 9 DIV. To control for washout, FGF-2 was switched to HS and virtually no spheres were observed ($n = 3$), confirming the success of washing the first mitogen. A constant 10-day exposure to EGF + FGF-2 throughout (with appropriate washes to mimic the switch experiments) served as control. The results are presented in Fig. 5A. Two important observations were made. First, only cultures that were exposed to FGF-2 followed by EGF generated spheres in numbers approaching those generated with EGF + FGF-2. Second, 48 h was required for significant sphere production; a 24-h preincubation with FGF-2 was insufficient. After 72 h of FGF-2, sequential addition of EGF yielded close to 80% of the numbers of spheres generated by EGF + FGF-2. Virtually all the spheres generated by sequential addition of FGF-2 and EGF were expandable and multipotent ($n = 18$ spheres from three cultures, 100% being expandable, yielding 54 ± 4 spheres per single dissociated sphere, 88% containing neurons, astrocytes and oligodendrocytes), as observed for adult spheres generated by EGF + FGF-2 (Weiss *et al.*, 1996a) or by FGF-2 + HS (Table 2 in this study).

If FGF-2 induces the division of an adult stem cell and one of the progeny becomes EGF-responsive, we would expect a correlation between the numbers of FGF-2-induced stem cell doublets and EGF's ability to generate spheres (in a temporal fashion as in Fig. 5A). Thus, to test whether the time course of sequential actions correlates with the mitotic activity of FGF-2, we counted the numbers of doublets (two cells joined by an apparent mitotic cleft) in our cultures, in control and FGF-2 after 12–72 h (Fig. 5B). A small increase in doublets was observed at 24 h, but the most significant numbers of doublets were observed at 48 and 72 h in FGF-2, corresponding to 2- and 2.5-fold increases over control, respectively. There was an excellent correlation between the numbers of doublets vs. time in FGF-2 and the numbers of spheres generated after sequential incubation of FGF-2 to EGF ($r^2 = 0.97$). In related studies, we grew individual adult FGF-2 + HS-generated spheres for seven days, dissociated single spheres and subcultured them into EGF alone. Of

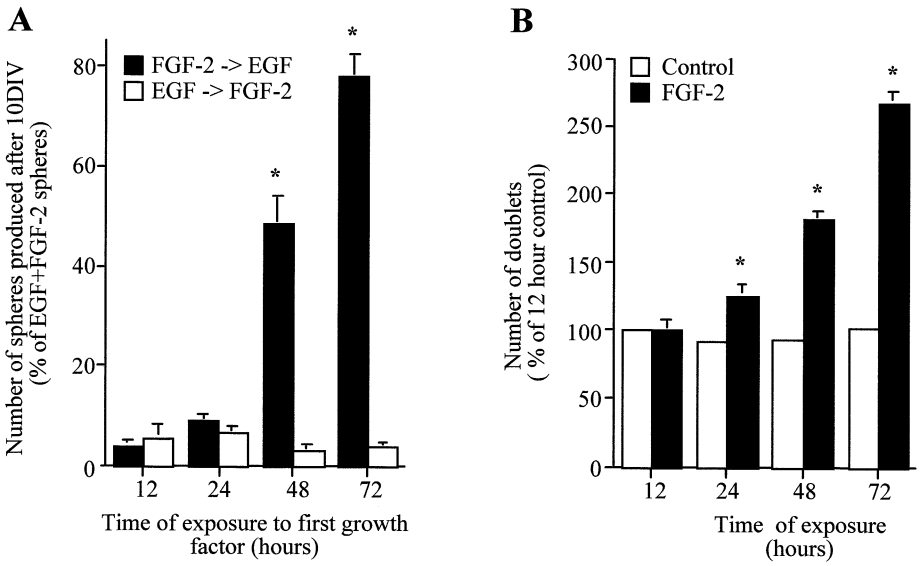

FIG. 5. FGF-2 and EGF can act sequentially to generate spheres from adult spinal cord neural stem cells. (A) The sphere-producing actions of combined EGF + FGF-2 can be mimicked by FGF-2 and EGF in sequence, but not by EGF and then FGF-2. Isolated adult cervical spinal cord cells were exposed to either EGF or FGF-2 for the indicated times, followed by a washout and exposure to the other growth factor for a total of 10 days. The numbers of spheres generated after 10 DIV were counted and expressed as a percentage of those generated after 10 days of combined EGF + FGF-2 throughout. Data are the means ± SEM of three independent culture experiments, with six replicates within each condition in each experiment. *$P < 0.01$ when compared to 12- or 24-h conditions. (B) FGF-2 induces the increased formation of doublets in cultures of adult spinal cord cells. Isolated adult cervical spinal cord cells were exposed to control media or FGF-2-containing media for the indicated times. The number of cell doublets were counted at each period and normalized (%) to 12 h with control media. Data are the means ± SEM of three independent culture experiments, with six replicates within each condition in each experiment. *$P < 0.01$ when compared to control conditions.

32 such individual spheres, 80 ± 4% produced more than two secondary spheres after subcloning in EGF. Virtually identical results were obtained with E14 FGF-2 + HS-responsive neural stem cells being subcultured into EGF, and in both cases (E14 and adult) the secondary EGF-generated spheres were >80% expandable and multipotent themselves (data not shown). Taken together with our results illustrated in Fig. 5, these data suggest that adult FGF-2-responsive spinal neural stem cells produce secondary neural stem cells *in vitro* that proliferate in response to EGF.

Discussion

Two principal conclusions may be drawn from our experimental results. First, an EGF-responsive multipotent expandable neural stem cell appears in the spinal cord between E12-14, the peak period of spinal cord histogenesis, and is no longer present in the adult. Second, a population of multipotent neural stem cells that are FGF-2-responsive are present throughout development and into adulthood. Although our results suggest that FGF-2-responsive neural stem cells may give rise to EGF-responsive stem cells, the data are not conclusive in this regard. These conclusions are discussed below and illustrated in a summary model in Fig. 6.

At least two neural stem cell populations, EGF- and FGF-2-responsive, are found in the developing spinal cord

Previous studies of early embryonic (Kalyani *et al*., 1997) or adult (Weiss *et al*., 1996a) spinal cord failed to identify EGF-responsive neural stem cells *in vitro*. We hypothesized that this may have been due to the developmental periods examined and thus we explored the ontogeny of both EGF receptors and putative EGF-responsive neural stem cells. We found that EGF receptors and highly expandable and multipotent EGF-responsive neural stem cells are present in the E14 spinal cord. This developmental period represents the overlap between neurogenesis and gliogenesis in the spinal cord, and the most intensive period of cytogenesis (Altman & Bayer, 1984). Similar EGF-responsive stem cells are present in the forebrain ventricular/subventricular zone, a structure that is always mitotically active and generating new cells (Smart, 1961; Morshead & van der Kooy, 1992; Lois & Alvarez-Buylla, 1993, 1994), during early development and in the adult (reviewed in Weiss *et al*., 1996b). Given that this study reports the first identification of multipotent spinal cord cells responsive to EGF, we demonstrated that these cells could be derived in primary cultures plated at clonal density (< 15 cells/cm^2; Table 1). This is similar to the clonal identification of EGF-responsive stem cells in the embryonic striatum (Reynolds & Weiss, 1996). Thus, regardless of the region of CNS origin, EGF can induce the clonal proliferation of embryonic multipotent neural stem cells in completely defined experimental conditions. Surprisingly, under these very low density primary culture conditions, FGF-2 + HS did not induce sphere formation. We surveyed the literature and found that there are no reports of primary clones of FGF-2-responsive multipotent cells in defined culture conditions. In fact, FGF-2 actions on primary neuroepithelial precursors appear to require additional undefined factors that may be found in embryo extracts (Kalyani *et al*., 1997) or serum (Kilpatrick & Bartlett, 1995; Shihabuddin *et al*., 1997). High-cell-density, primary FGF-2-responsive, cells formed spheres in this study, further suggesting that unidentified factors secreted by other CNS cells support neural stem cell proliferation in concert with FGF-2. Interestingly, a recent study reports the identification of such a novel factor, a glycosylated form of cystatin, which is required for FGF-2 actions on hippocampal neural stem cells (Taupin *et al*., 2000).

Unique developmental profiles are the first line of evidence supporting our hypothesis that there are distinct populations of neural stem cells in the spinal cord (see illustrations in Fig. 6). The first stem cells to appear are FGF-2-responsive (Kalyani *et al*., 1997; this

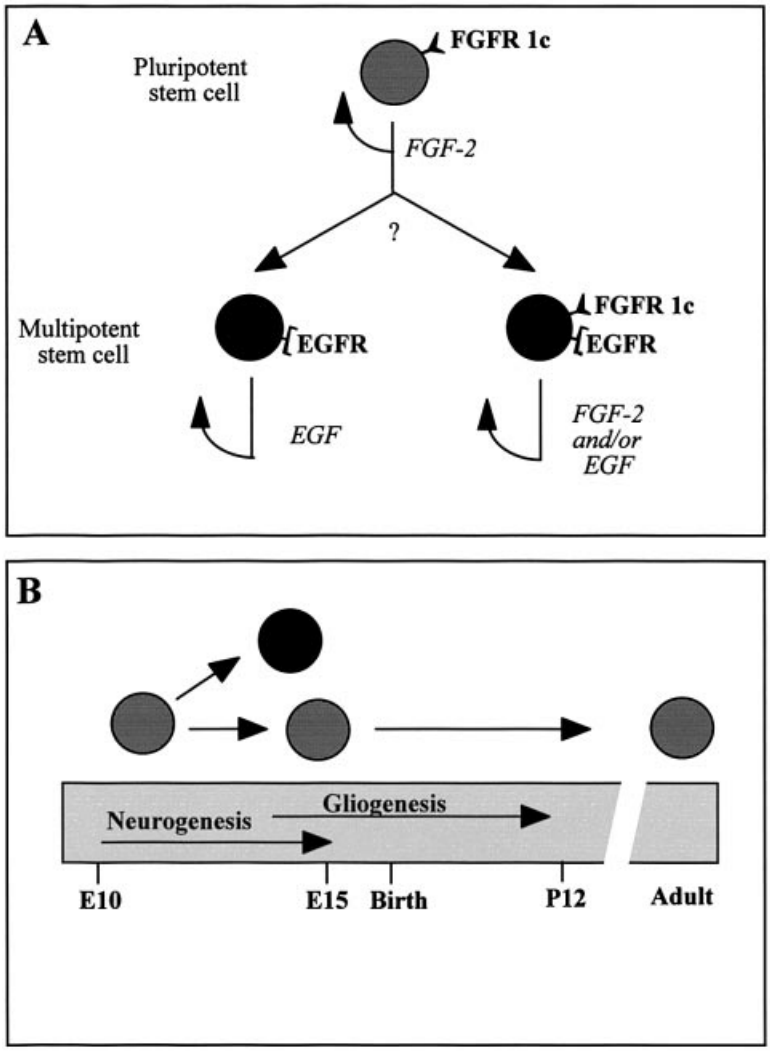

FIG. 6. Model for the ontogeny and possible lineage relationships between FGF-2- and EGF-responsive neural stem cells in the mouse spinal cord. (A) Primary (pluripotent) FGF-2-responsive stem cells give rise to secondary EGF-responsive multipotent stem cells. Our data support the contention that, both during development and in adulthood, FGF-2-responsive neural stem cells expressing the FGFR1c receptor subtype can be precursors to a separate population of secondary stem cells that express either EGFR alone or both EGFR and FGFR1c receptors. (B) Distinct neural stem cell populations are present at different spinal cord developmental periods. Pluripotent FGF-2-responsive stem cells are present throughout development and into adulthood. During spinal cord histogenesis, secondary populations of stem cells with extensive expansion properties, in particular the EGF-preferring type, are generated to subserve the peak periods of cell production and the relative generation of the neuronal and glial cell phenotypes.

study). Highly expandable, multipotent EGF-responsive stem cells (and significant expression of the EGFR) appear transiently between E12 and 14. This type of EGF-responsive cell is not found in the adult spinal cord. On the other hand, FGF-2-responsive stem cells are present throughout development and in the adult cord. These findings contrast with those obtained in studies of the telencephalon (Tropepe *et al*., 1999) where FGF-2-responsive neural stem cells are the first to appear, yet both EGF- and FGF-responsive cells persist in adult mice. Because the FGFR1c subtype of FGF-2-preferring receptors is expressed throughout development *in vivo*, in isolated E10.5 rat spinal cord neuroepithelial cells (Kalyani *et al*., 1999) and in spheres *in vitro* (data not shown), we propose that it is characteristic of FGF-2-responsive spinal cord stem cells, as is the case for telencephalic FGF-2-responsive stem cells (Tropepe *et al*., 1999).

The second line of evidence supporting the existence of at least two populations of neural stem cells is the additivity of sphere number responses to saturating concentrations of EGF and FGF-2. At E14, when the most expandable and multipotent embryonic EGF- and FGF-2-responsive neural stem cells are present, the numbers of primary spheres generated by FGF-2 + HS and EGF are additive (Table 3), suggesting that they are derived from separate stem cells. However, we cannot entirely rule out the possibility that both FGFR1c and EGF receptors are present on all neural stem cells and that it is the synergistic actions of FGF-2 and EGF that allows for recruitment of more stem cells. Thus, by virtue of the two receptor systems working in synergy, one would recruit a greater number of neural stem cells when combining EGF and FGF-2 + HS than with either factor alone (this would imply that the additive nature of the EGF and FGF-2 + HS was thus coincidental). Such a possibility is supported by the observation that $\approx 75\%$ of the nestin-positive precursors from the E14 spinal cord express both FGFR1 and EGFR. Regardless of whether the EGF-responsive spinal cord neural stem cells express FGF receptors, these cells are clearly distinct from those FGF-2-responsive neural stem cells in early development (E11) and in the adult, which are incapable of self-renewing or expanding in the presence of EGF.

The appearance of the EGF-responsive neural stem cell in the spinal cord, during the most demanding period of cell production and the overlap between neurogenesis and gliogenesis, may be due to two of its properties. First, our data shows that the E14 EGF-responsive stem cell is highly expandable and thus could serve for enhanced cell production. Second, in comparison to FGF-2-responsive precursors, EGF-responsive precursors consistently produce a greater number of glial cells (Kilpatrick & Bartlett, 1993, 1995; Vescovi *et al*., 1993; Ray & Gage, 1994; Reynolds & Weiss, 1996). Moreover, EGFR expression is prominent in the ventral aspect of the germinal zone (Fig. 5) which is known to contain oligodendrocyte progenitors (Pringle & Richardson, 1993; Noll & Miller, 1993; Hall *et al*., 1996).

It thus seems reasonable to suggest that, during development, which neural stem cells are present may be dictated by the relative requirement for neurons or glia. The temporal appearance of FGF-2 (principally neuron-producing)- vs. EGF (principally glial producing)-responsive stem cells in the spinal cord support such a conclusion. In addition, recent studies show that growth factor concentration (Qian *et al*., 1997), receptor expression levels (Burrows *et al*., 1997) and other extrinsic signals (Gross *et al*., 1996; Johe *et al*., 1997; Dutton *et al*., 1999; Rowitch *et al*., 1999) may further regulate the phenotypes of the progeny of these multipotential neural stem cells. Taken together, these findings add to a growing body of evidence suggesting that multipotential neural stem cells, through intrinsic and extrinsic mechanisms, operate throughout development in the generation of neurons and glia.

FGF-2-responsive neural stem cells may be the source of EGF-responsive stem cells

FGF-2-responsive stem cells are present prior to the EGF-responsive stem cells during spinal cord development (Kalyani *et al*., 1997; this study). This prompted us and colleagues (Rao, 1999) to hypothesize that the early-appearing FGF-2-responsive stem cells are the precursors to the EGF-responsive stem cells. Two lines of evidence in this study support this hypothesis. First, FGF-2-responsive neural stem cells from both E14 and adult spinal cord produce secondary EGF-responsive stem cells *in vitro*. Second, the combined actions of FGF-2 + EGF in stimulating adult spinal cord stem cells to proliferate (Weiss *et al*., 1996a) appears due to the sequential actions of FGF-2 and EGF and probably requires the FGF-2-responsive cell to divide. However, we have not entirely ruled out the alternative explanation – that FGF-2 enhances EGF receptor expression on adult spinal cord stem cells. In line with this possibility, it has been reported for embryonic striatal (Ciccolini & Svendsen, 1998) and cortical (Lillien & Raphael, 2000) precursors that treatment with FGF-2 induces the acquisition of EGF responsiveness (receptor expression). On the other hand, in previous studies of fibroblasts and PC12 cells, where both receptors are present, FGF-2 reduced both high and low affinity binding sites for EGF (Maher, 1993), and in a separate study FGF-2 pretreatment reduced the mitogenic actions of EGF on astrocytes (Huff & Schreier, 1989).

Although the exact nature of all the EGF-responsive cells is uncertain, several issues argue against similar mechanisms operating here. First, in the Ciccolini & Svendsen (1998) study, the EGF responsiveness of E14 striatal precursors occurred in the absence of FGF-2, and FGF-2 acted to enhance EGF responsiveness. Adult spinal cord stem cells studied here did not develop EGF responsiveness without FGF-2 treatment. Second, 24 h of FGF-2 treatment was sufficient to promote embryonic striatal precursor responses to EGF, whilst we found that no less than a 48-h pretreatment was required for spinal cord precursors. Third, the responses of E14 striatal cells to saturating concentrations of EGF and FGF-2 were never additive whilst, in our study, spinal cord stem cells always showed additive responses to the mitogens. The fact that stem cells from different brain regions may behave differently in culture is not surprising, given evidence for their differential responses to mitotic signals (Weiss *et al*., 1996a) and ability to produce distinct neuronal phenotypes (Kalyani *et al*., 1997; Shihabuddin *et al*., 1997).

Conclusions

Our findings, in concert with some previous observations and proposals (Rao, 1999; Tropepe *et al*., 1999), are summarized in Fig. 6. Pluripotent FGF-2-responsive neural stem cells arise early in development. Between E12 and 14, these FGF-2-responsive neural stem cells give rise to a secondary population of neural stem cells that are EGF-preferring and express the EGFR and/or both the EGFR and the FGFR1c. Our data supports the contention that this population of EGF-preferring neural stem cells is transient and distinct from the FGF-2-preferring neural stem cells present throughout development and in the adult. We conclude that during spinal cord development and into adulthood separate populations of EGF- and FGF-2-preferring neural stem cells subserve unique roles in spinal cord cell histogenesis.

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Abbreviations

aCSF, artificial cerebrospinal fluid; CNS, central nervous system; DIV, days *in vitro*; DMEM, Dulbecco's modified Eagle's medium; E, embryonic day; EGF, epidermal growth factor; EGFP, enhanced green fluorescent protein; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; FGF-2, fibroblast growth factor-2; FGFR1, fibroblast growth factor receptor 1; GFAP, glial fibrillary acidic protein; HS, heparan sulphate; MAP-2, microtubule-associated protein-2; P, postnatal day; PBS, phosphate-buffered saline; RT-PCR, reverse trasnscription–polymerase chain reaction.

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