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BMP and FGF regulate the development of EGF-responsive neural progenitor cells

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SUMMARY

Temporal changes in progenitor cell responses to extrinsic signals play an important role in development, but little is known about the mechanisms that determine how these changes occur. In the rodent CNS, expression of epidermal growth factor receptors (EGFRs) increases during embryonic development, conferring mitotic responsiveness to EGF among multipotent stem cells. Here we show that cell-cell signaling controls this change. Whereas EGF-responsive stem cells develop on schedule in explant and aggregate cultures of embryonic cortex, co-culture with younger cortical cells delays their development. Exogenous BMP4 mimics the effect of younger cells, reversibly inhibiting changes in EGFR expression and responsiveness. Moreover, blocking endogenous BMP receptors in progenitors with a virus transducing dnBMPR1B accelerates changes in EGFR signaling. This involves a non-cell-autonomous mechanism, suggesting that BMP negatively regulates signal(s) that promote the development of EGF-responsive stem cells. FGF2 is a good candidate for such a signal, as we find that it antagonizes the inhibitory effects of younger cortical cells and exogenous BMP4. These findings suggest that a balance between antagonistic extrinsic signals regulates temporal changes in an intrinsic property of neural progenitor cells.

Key words: Cerebral cortex, Proliferation, EGF receptor, Stem cell, Rat

INTRODUCTION

Proliferation and differentiation occur in temporal and spatial patterns in the CNS. These patterns are generated as a result of intrinsic differences in progenitor cells and restricted expression of extrinsic signals (reviewed in Edlund and Jessell, 1999). One of the properties of progenitor cells that changes during development is their responsiveness to extrinsic signals. This determines whether cells respond to specific signals in their environment at distinct times, and influences their choice of response to signals that are pleiotropic. We recently reported that one of the molecular mechanisms for achieving differences in responsiveness to extrinsic signals involved quantitative changes in the expression of cell-surface receptors (Lillien, 1995; Lillien and Wancio, 1998; Burrows et al., 1997). A difference in the level of receptor expression has also been implicated in threshold-dependent differences in responses to decapentaplegic in *Drosophila* that contribute to spatial patterning (Lecuit and Cohen, 1998). What remains to be elucidated is how differences in such intrinsic properties of progenitor cells are controlled.

In the vertebrate forebrain, subsets of progenitor cells with distinct properties have been described (Levitt and Rakic, 1983; Luskin et al., 1988; Walsh and Cepko, 1988; Reynolds et al., 1992; Grove et al., 1993; Davis and Temple, 1994; Gage et al., 1995; Levison and Goldman, 1997; Mayer-Proschel et al., 1997). The representation of specific subsets that differ in their proliferative and phenotypic potentials varies during development (Levitt and Rakic, 1983; Williams and Price, 1995; Kilpatrick and Bartlett, 1995). Their relative representation tends to reflect the types of cells generated at specific stages of development. For example, at earlier embryonic stages, when more neurons are generated, progenitor cells that are restricted to a neuronal fate are more abundant, while at later embryonic stages, when more glia begin to develop, progenitor cells that are restricted to a glial fate are more abundant. It has been shown that progenitor cells that are more restricted in their proliferative and phenotypic potentials are derived from multipotent stem cells (Mayer-Proschel et al., 1997). Stem cells are normally represented in very small numbers, but it has been noted that multipotent stem cells also change during development (Burrows et al., 1997; Zhu et al., 1999). At earlier embryonic stages, stem cells have a bias for generating neuronal progeny, while later stem cells tend to generate more glia. Thus, the developmental change in cell type generation is initiated at the top of the progenitor cell hierarchy, in the stem-cell compartment.

Early and late embryonic multipotent stem cells also differ in their responsiveness to mitogens. Late embryonic and adult stem cells are responsive to EGF-family ligands (Reynolds and

Weiss, 1992). In contrast, at earlier stages of development, neural progenitor cells, including stem cells, are mitotically responsive to FGF2 but not to EGF (Kilpatrick and Bartlett, 1993, 1995; Ferri and Levitt, 1995; Ghosh and Greenberg, 1995; Gage et al., 1995; Ferri et al., 1996; Johe et al., 1996; Qian et al., 1997; Burrows et al., 1997). The acquisition of mitotic responsiveness to EGF is associated with the appearance of a subpopulation of progenitor cells that expresses relatively high levels of EGFRs (Burrows et al., 1997; Kornblum et al., 1997). Using a retrovirus to introduce extra copies of EGFRs into early progenitor cells, we showed that expression of a threshold number of EGFRs was required for mitotic responsiveness to EGF (Burrows et al., 1997). Early progenitor cells express lower levels of the EGFR and exhibit distinct responses to EGFR stimulation (Ferri and Levitt, 1995; Eagleson et al., 1996). Taken together, these findings suggest that differences in the level of EGFR expression determine how progenitor cells interpret an extrinsic signal at specific stages of development.

Progenitor cells that express high levels of EGFR in the late embryonic cortex are lineal descendants of early progenitors that express low levels of EGFR (Burrows et al., 1997). In principle, temporal regulation of changes in EGFR expression and signaling could reflect either a cell-autonomous mechanism or a response to extrinsic signals. Although cell-autonomous mechanisms have been implicated in the regulation of some differences between cortical progenitor cells (Chenn and McConnell, 1995; Zhong et al., 1996; Qian et al., 1998), extrinsic signals have been shown to modulate EGFR expression in a variety of cells. For example, retinoids reduce the transcription of EGFRs in squamous carcinoma cells (Grandis et al., 1996), EGF-family ligands increase EGFR transcription in liver epithelial cells (Earp et al., 1986), NGF reduces cell-surface expression (Brown and Carpenter, 1991; Seedorf et al., 1995) and transcription (Shibutani et al., 1998) of EGFRs in PC12 cells, and SHH produced by *Drosophila* photoreceptors induces EGFR expression in lamina precursor cells (Huang et al., 1998). These findings raised the possibility that the change in EGFR expression in cortical progenitor cells could be modulated by extrinsic signals.

In the present study, we show that the developmental change in EGFR expression and responsiveness in cortical progenitors occurs on schedule in explant and aggregate cultures, but not in monolayer cultures, suggesting that cell-cell signaling is involved in its regulation. Our findings further suggest that a balance between positive and negative extrinsic signals contributes to the temporal pattern of changes in this property of cortical progenitor cells, and implicate BMP and FGF family members as candidate signals.

MATERIALS AND METHODS

Animals

Timed-pregnant Sprague-Dawley rats were obtained from Charles River. Embryonic stages were confirmed by crown-rump length and examination of external features (Angulo y Gonzales, 1932; Long and Burlingame, 1938).

Viruses

A replication-incompetent retrovirus that co-expresses dominant-negative BMP receptor 1B (dnBMPR1B) and β-geo (*lacZ* fused to neomycinphosphotransferase; Friedrich and Soriano, 1991) was made by sub-cloning the coding sequence for mouse dnBMPR1B (also known as Alk6) (ten Dijke et al., 1994) into the pLIG viral vector (Lillien, 1995), to generate pLIG-dnBMPR1B. The cDNA for dnBMPR1B was provided by Drs ten Dijke and Niswander. It contains an amino acid substitution (lysine to arginine) in the ATP-binding site, which reduces kinase activity and inhibits BMP signaling (Zou and Niswander, 1996; Zou et al., 1997). The virus co-expresses β-geo, facilitating the identification of infected cells with β-galactosidase (β-gal) antibody. High titer viral stocks (approximately 9×10^6 cfu/ml, unconcentrated) were made in psi-2 cells (Cepko et al., 1993). The biological activity of the virus was confirmed using explants of E15 rat cortex. We observed a decline in neuronal differentiation and failure to migrate, as reported for a dnBMPR1A virus (Li et al., 1998). For a control virus, we used a construct that expresses the histochemical marker β-galactosidase (Burrows et al., 1997). Similar effects were observed with multiple viral stocks from two clones of dnBMPR1B producer cells.

Cultures

Explants of E12 and E15 rat cortex were prepared as described (Burrows et al., 1997). Briefly, the dorsolateral region of the cortex was dissected in Hanks' BSS (Gibco-BRL) at room temperature, without removing pial or meningeal membranes. Explants were placed ventricular surface down on filters (Nucleopore, 0.2 um pore, 12 mm diameter) floating in 1.5 ml of culture medium in 35 mm dishes. Culture medium consisted of DMEM:F12 (1:1; Gibco-BRL) with N2 supplements made from individual components (Sigma) (Bottenstein and Sato, 1979), penicillin/streptomycin (Gibco-BRL) and insulin (Sigma; 25 μg/ml). Explants were infected by adding 20-30 μl of medium containing virus to the tops of the filters 30 minutes after explants were added. The virus infects dividing progenitor cells, integrates stably and can be used to follow the fate of progenitor cells. Growth factors were added daily to the culture medium in the dishes at the concentrations indicated, beginning 1 day after infection. Recombinant BMP4, BMP2, BMP6, EGF, TGFα, HB-EGF, FGF2, FGF6, FGF8, SHH, activin A, TGFβs 1-3, NT3, and LIF were obtained from R&D. Explants were cultured at 37°C in 6% CO_2 for 4-7 days.

Aggregate cultures were prepared from E12 and E15 explants 1 day after infection to allow time for expression of virally transduced genes in progenitor cells. Explants were dissociated in trypsin (0.1%; Sigma) for 15 min, 35°C, and triturated in DNase and egg-white trypsin inhibitor (0.1 mg/ml each; Sigma). 1.5×10^5 cells from explants exposed to virus were mixed 1:1 with cells from uninfected E15 or E12 explants in 0.5 ml of culture medium in a 15 ml conical tube and centrifuged as described (Watanabe and Raff, 1991). After exposing explants to virus, only a small population of progenitor cells is infected. For example, aggregates made with 1.5×10^5 cells from E15 explants exposed to virus included approximately 50-100 virally marked cells. Pelleted cells were left in centrifuge tubes at 37°C for 3-4 hours. Pellets of aggregated cells were gently dislodged from the tubes, placed on filters as described for explants, and cultured for 3-7 days. Aggregates were dissociated, stained for EGFR expression and assayed for mitotic responsiveness (neurosphere formation), as described for explants.

Cultures of 'neurospheres' were prepared by dissociating explants or aggregates as described above. 5×10^4 cells were cultured per well in 24-well plates (Corning) in 0.5 ml of DMEM-F12 serum-free medium containing EGF (1-10 ng/ml; R&D) or FGF2 (5 ng/ml; R&D). 200 μl of medium containing fresh growth factor was added every fourth day. The number of neurospheres per well was counted after 10 days.

Immunocytochemistry

To test mitotic responsiveness in the neurosphere assay, explants and

Table 1. EGFR expression and responsiveness to EGF

Age	Preparation	Time in culture	Neurospheres per well		EGF $R^+/\beta\text{–gal}^+$
			EGF 1 ng/ml	EGF 10 ng/ml	
E15	Explant	4 days	544±59	900±86	2.7±0.3
E15	Monolayer	4 days	0*	0‡	0*
E15	Aggregate	4 days	337±37	734±117	3.9±1.0
E12	Explant	4 days	2.9±1‡	5.4±1.4‡	0‡
E12	Explant	7-8 days	453±105	696±131	6.6±1.4

Explants of E15 or E12 rat cortex were cultured for 4-8 days. To mark a small population of dividing progenitor cells, explants were infected with a virus transducing β-galactosidase ('control virus') when placed in culture. Explants were dissociated at the times indicated ('time in culture'), and stained with antibodies against β-gal and EGFR to assess their expression of high levels of EGFR ('EGFR+/β-gal+'), or cultured for 10 days in EGF (1-10 ng/ml) to assess their ability to divide in response to EGF to form a neurosphere ('neurospheres per well'). In some cases, explants were dissociated 1 day after infection and grown as a monolayer culture or pelleted to form an aggregate, as described in Materials and Methods. For assays of mitotic responsiveness, 5×10^4 cells were cultured per well. The development of high levels of EGFR expression and mitotic responsiveness to EGF occurred on schedule in explant and aggregate cultures, but not in monolayer cultures. For statistical analysis, E15 explants were compared with E15 aggregates, E15 monolayers and E12 explants cultured for 4 or 7 days. The significance of these differences was determined using an unpaired Students *t*-test.

*$P<0.005$

‡$P<0.0005$.

aggregates had to be dissociated. Staining to assess EGFR expression among these cells was therefore performed after dissociation. Suspensions of dissociated cells were plated on poly-D-lysine-coated slides in culture medium for 3-4 hours at 37°C to allow recovery of surface proteins. Cells were fixed for 10 minutes in 10% formalin (Sigma) or 4% paraformaldehyde in 3% PIPES, rinsed in PBS, blocked in PBS+10%FCS+0.1% triton and stained with a cocktail of rabbit anti-β-gal (5-Prime, 3-Prime) and sheep anti-EGFR (UBI) for 1 hour at room temperature. Antibodies were visualized with donkey anti-sheep Cy3 and donkey anti-rabbit Cy2 (Jackson ImmunoResearch). For analysis of PCNA cyclin expression, cells were treated with methanol (4°C) for 10 min after fixation in 4% paraformaldehyde, then rinsed and blocked as described above. Cells were stained with a cocktail of rabbit anti-β-gal and mouse anti-PCNA cyclin (Sigma), followed by donkey anti-rabbit Cy3 and donkey anti-mouse Cy2 (Jackson ImmunoResearch). Staining was analyzed using a Leica DMR fluorescence microscope coupled to a Sensys digital camera. Images were captured using IPLab Spectrum 3.2 and Photoshop 4.0 software.

Neurosphere formation by infected progenitors

To determine the number of infected progenitor cells that were capable of dividing in response to EGF to form a neurosphere, we counted the number of infected cells per well 4-6 hours after cells were plated. The number of neurospheres per well in sister cultures was counted 10 days later, and the number of neurospheres derived from infected progenitor cells was determined by staining neurospheres with anti-β-gal antiserum. As noted previously, neurospheres are clonal and nearly all cells in neurospheres that are derived from an infected progenitor cell express virally transduced

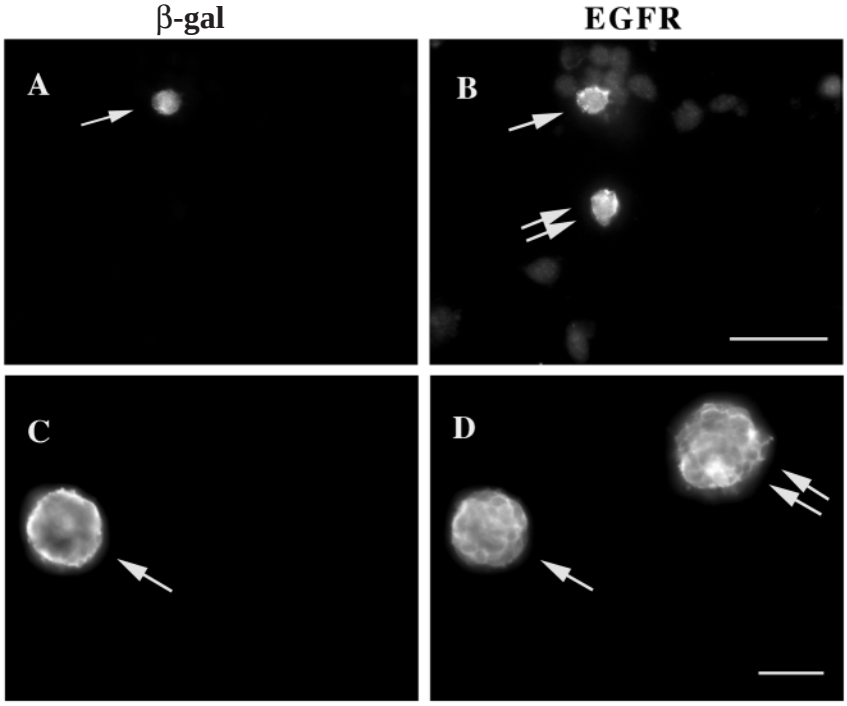

Fig. 1. High levels of EGFR are expressed by some progenitors and by most cells in neurospheres. Explants of E15 cortex were infected with control virus to mark progenitor cells and follow their fate. They were cultured for 4 days, dissociated and stained with β-gal and EGFR antibodies. The virally marked cell in A (β-gal, arrow) expresses high levels of EGFR (B, arrow). Cells that were not infected also express high levels of EGFR (B, double arrows). The majority of cells in this field (B) express low levels of EGFR. The average fluorescence intensity of 20 cells expressing a low level of EGFRs was compared with that of 11 cells expressing a high level of EGFRs. When normalized to background, the fluorescence intensity of cells expressing low levels of EGFR was 1.34±0.02-fold over background (range: 1.18-1.41). In contrast, the average fluorescence intensity of cells expressing high levels of EGFR was 2.74±0.22-fold over background (range: 1.84-3.97). If cells from E15 explants were dissociated after 4 days in culture then grown in EGF for 10 days, some of the marked cells divided to form neurospheres (C, arrow). Note that most cells in the neurosphere in C express the virally transduced marker β-gal, confirming that the sphere is a clone. Most of the cells in this neurosphere also express high levels of EGFR (D, arrow), consistent with their derivation from a progenitor that expressed high levels of EGFR. Neurospheres derived from uninfected progenitor cells also express high levels of EGFR (D, double arrows). Scale bar: 25 μm.

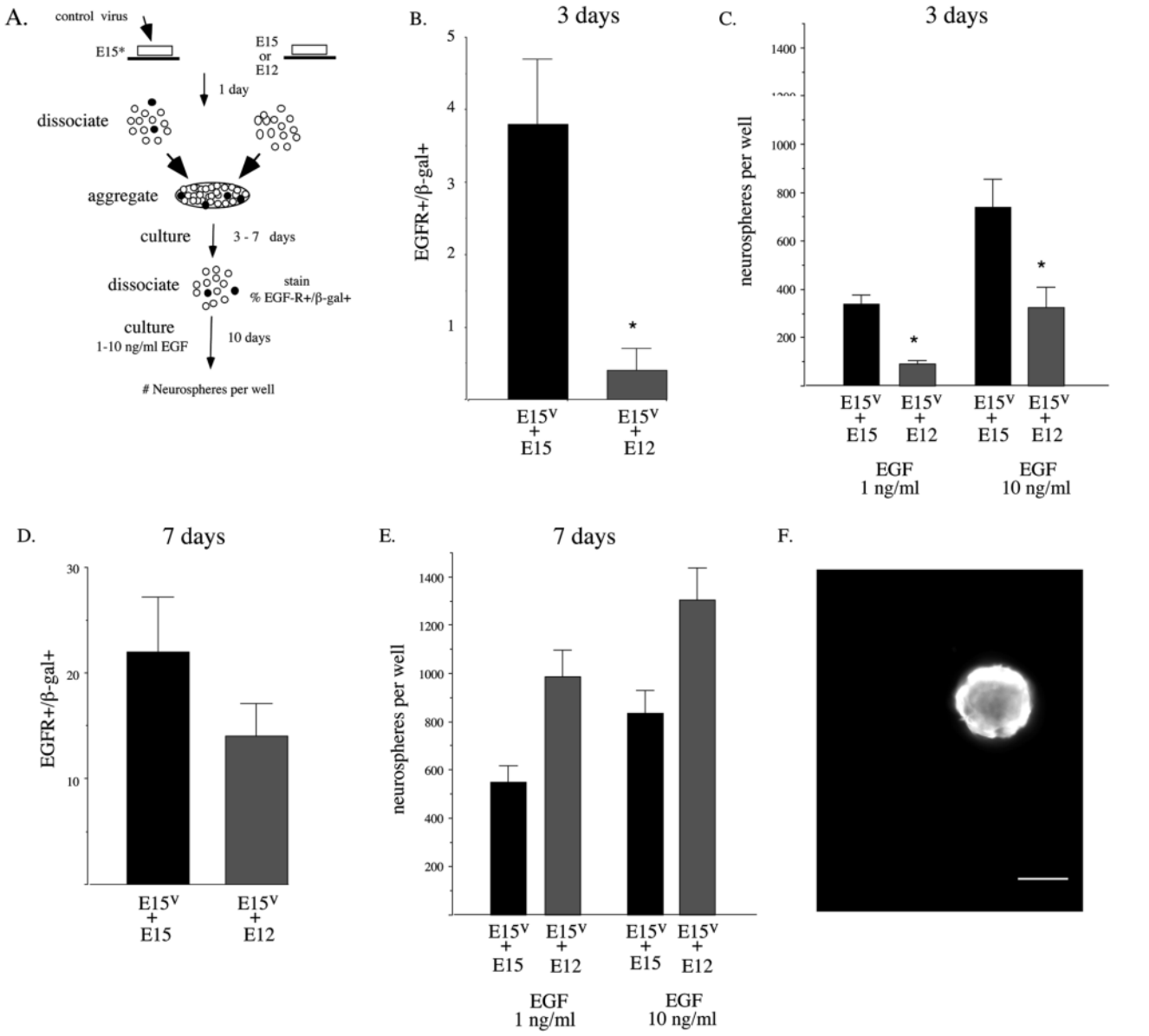

Fig. 2. Early cortical cells delay the development of EGF-responsive stem cells. (A) To determine whether developmentally regulated cell-cell interactions control EGFR expression and mitotic responsiveness, E15 and E12 cells (1:1) were cultured together as aggregates. (B-E) Some of the progenitor cells in E15 explants were marked by infection with control virus (designated by a superscript 'v') and cultured as an aggregate with unmarked cells from E15 or E12 cortex. After 3 days, aggregates were dissociated and stained for expression of EGFR and β-gal (B), or cultured in EGF (1-10 ng/ml) to assess mitotic responsiveness resulting in the formation of neurospheres (C). At the time aggregates were dissociated, we counted the number of β-gal marked cells in the $E15^v$+E15 and $E15^v$+E12 aggregates to determine the representation of E15 cells in the mixed-age aggregates. For example, in one experiment, E15 cells represented 33% of the cells in $E15^v$+E12 aggregates. The number of neurospheres that developed in cultures from $E15^v$+E12 aggregates was therefore corrected to reflect the representation of E15 cells, so that we could compare the number of neurospheres in cultures prepared from E15 and mixed-age aggregates. For example, if a culture prepared from $E15^v$+E12 aggregates contained 20 neurospheres, and included 33% E15 cells when plated, the corrected number of neurospheres was 66. This correction assumed that all neurospheres were derived from the E15 population, based on the observation that none of the neurospheres that developed in cultures of $E12^v$+E15 aggregates came from marked E12 progenitor cells. In D,E, aggregates were cultured for 7 days. They were then dissociated and stained for EGFR expression (D) and assessed for mitotic responsiveness to EGF (E). Exposure to early (E12) cortical cells delayed the rise in EGFR expression among marked E15 progenitor cells (compare B with D). Early cortical cells also delayed the acquisition of mitotic responsiveness to EGF among E15 progenitor cells (compare C with E). (F) After 7 days in culture with E12 cells, some of the virally marked E15 cells became mitotically responsive to EGF and formed neurospheres (stained with β-gal antiserum). Note that the number of neurospheres formed in cultures from 7 day aggregates of E12 and E15 cells appears to be additive between the E15 and E12 populations (see Table 1). Co-culture with early cortical cells appears to impose their developmental timing on later progenitor cells. Significance was determined using paired *t*-tests. $*P<0.05$ (comparing $E15^v$+E12 with $E15^v$+E15 cultures). Scale bar: 25 μm.

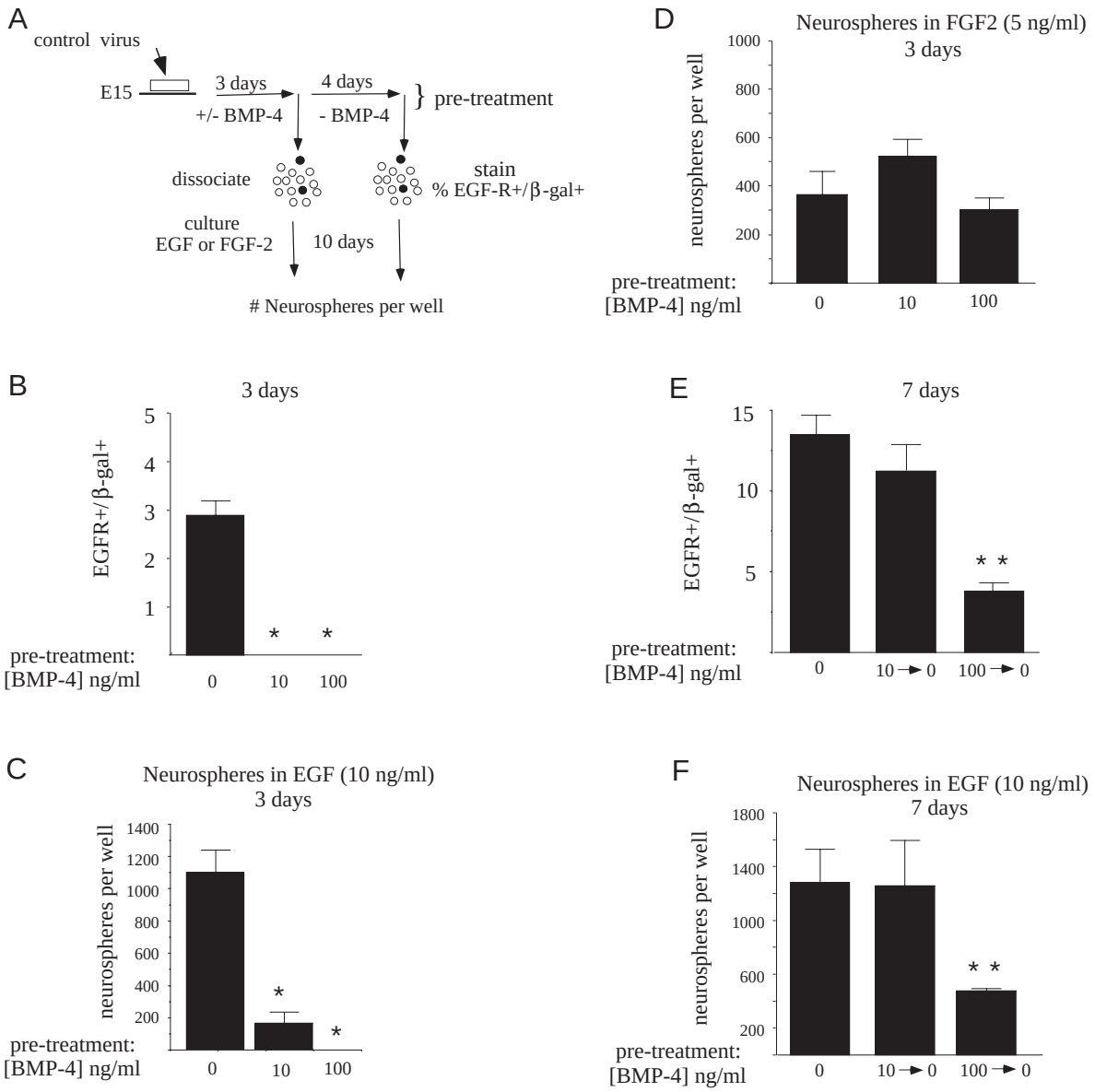

Fig. 3. BMP4 reversibly inhibits the development of EGF-responsive stem cells. (A) Explants of E15 cortex were infected with control virus and cultured in BMP4 for 3 days, then dissociated to assess EGFR expression and mitotic responsiveness. In some cases, explants were rinsed and cultured for 4 more days in the absence of BMP4. (B) BMP4 inhibited the appearance of cells that expressed high levels of EGFR. BMP4 also inhibited the development of mitotic responsiveness to EGF (C), but did not impair mitotic responsiveness to FGF2 (D). If BMP4 was removed for 4 days, high levels of EGFR expression appeared among the marked E15 cells (E) and progenitors acquired mitotic responsiveness to EGF (F). Similar effects on EGFR expression and mitotic responsiveness were observed in virally marked and unmarked E15 cells (data not shown). Note that fewer cells recovered from pre-treatment with higher concentrations of BMP4 (compare 10 and 100 ng/ml) (E,F). Significance was determined using paired *t*-tests. **P*<0.05, ***P*<0.005.

genes (Burrows et al., 1997 and Fig. 1C). The number of β-gal+ neurospheres per well was divided by the number of β-gal+ cells plated per well to determine the proportion of marked progenitor cells that divided in EGF to form a neurosphere.

Statistical analysis

Each data point represents the mean±s.e.m. of at least three cultures. For immunocytochemical studies, we counted 100 β-gal+ cells per culture. Statistical significance was determined using student's *t*-test. Results were considered to be significant at $P \leq 0.05$.

RESULTS

EGFR signaling develops on schedule in explants and aggregates

We have reported previously that a subpopulation of early embryonic progenitor cells acquires several properties characteristic of late embryonic progenitor cells over 4 days in cortical explant cultures (Burrows et al., 1997). These properties include proliferation as a multipotent stem cell in

response to EGF (Reynolds et al., 1992). These cells form 'neurospheres' after 1-2 weeks in EGF but differentiate into neurons, astrocytes and oligodendrocytes after mitogen withdrawal (Reynolds and Weiss, 1992). EGF-responsive stem cells represent approximately 1-2% of the cells in explants of E15 cortex after 4 days in culture (Table 1). This is similar to their representation in vivo at the equivalent age (E18-E19) (Burrows et al., 1997). In contrast to their behavior in explants, if dissociated and grown as a monolayer culture for 4 days, E15 progenitor cells did not acquire mitotic responsiveness to EGF (Table 1). If cells were dissociated and re-aggregated for 3 days, however, mitotic responsiveness to EGF was acquired as in explants (Table 1). In explants of younger (E12) cortex, mitotic responsiveness to EGF was acquired after 7 days rather than 4 days (Table 1). Changes in responsiveness to EGF therefore developed in cortical explants and aggregates on a schedule that approximates their development in vivo.

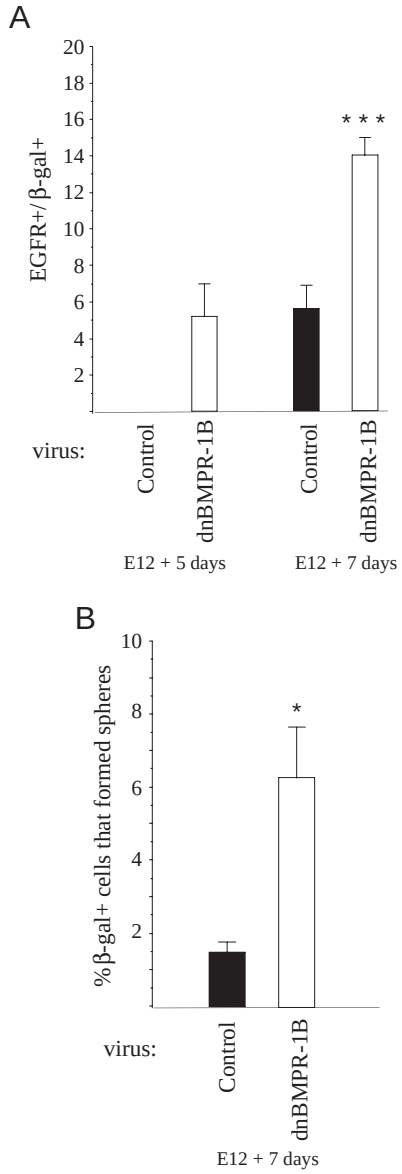

Fig. 4. Blocking BMP receptor signaling promotes developmental changes in EGFR expression (A) and mitotic responsiveness to EGF (B) in progenitor cells infected with dnBMPR1B virus. Explants of E12 cortex were infected with control or dnBMPR1B virus. Their expression of EGFRs was assessed 5 or 7 days later (A). Mitotic responsiveness to EGF (1 ng/ml) was assessed after 7 days (B). Data for mitotic responsiveness after 5 days are shown in Table 2. Blocking BMPR signaling increased the proportion of progenitor cells that expressed high levels of EGFR (A) and divided in response to EGF to form a neurosphere (B). Comparing dnBMPR1B with control virus, $*P<0.05$, $***P<0.0005$ (using paired *t*-tests).

The change in mitotic responsiveness to EGF was associated with the appearance of a population of cells that expressed high levels of the EGFR. These cells developed from progenitor cells that could be infected at E12 or E15 with a retrovirus transducing the histochemical marker β-galactosidase. This virus only infects dividing progenitor cells and can be used to follow their fate and distinguish lineage relationships. At E12 and E15, none of the infected cells expressed high levels of the EGFR, but after 7 or 4 days, respectively, a subpopulation of virally marked cells exhibited high levels of EGFR expression (Table 1 and Fig. 1). Cells expressing high levels of EGFR could be distinguished from cells expressing lower levels of the EGFR by differences in fluorescence intensity observed after staining with EGFR antibody (Fig. 1B). Cells expressing high levels of EGFR developed in aggregate and explant cultures, but not monolayer cultures (Table 1). Approximately half (48±2) of the cells that expressed high levels of EGFR at the time equivalent to E18-E19 also expressed the early astrocyte marker S-100β and 4% express the later astrocyte marker GFAP. Given the recent demonstration that some cells with the antigenic phenotype of astrocytes are EGF-responsive stem cells (Doetsch et al., 1999), it is not clear whether expression of markers associated with astrocytes indicates that a subset of cells expressing high levels of EGFRs are glial-restricted progenitor cells, rather than multipotent stem cells. All neurospheres, however, consisted of cells that expressed high levels of EGFR (Fig. 1D). Together with the findings from our previous study involving viral transduction of EGFRs, these observations suggest that some, if not all, of the cells expressing high endogenous levels of EGFRs at the time equivalent to E18-E19 include EGF-responsive multipotent stem cells that form neurospheres. The appearance of this subpopulation of progenitor cells on schedule in explants and aggregates, but not in monolayers, suggests that cell-cell signaling is required for their development.

Younger cortical cells delay development of EGF-responsive stem cells

The change in EGFR expression and responsiveness occurred on schedule in explant and aggregate cultures and appeared to involve cell-cell signaling. These signals could act in a positive or negative manner. For example, the concentration of positive signals that promote EGFR expression could be low at early stages of development, but increase over time. Alternatively, the concentration of negative signals that suppress EGFR expression could be higher at early stages of development. To address the contribution of positive and negative extrinsic regulatory signals, we asked whether younger cortical cells could delay the change in EGFR signaling in older progenitors and/or whether older cortical cells could accelerate the change in younger progenitors. To test these possibilities, we made aggregate cultures containing either E15 cells or E12 cells alone, or a mixture of E12 and E15 cells, and cultured the

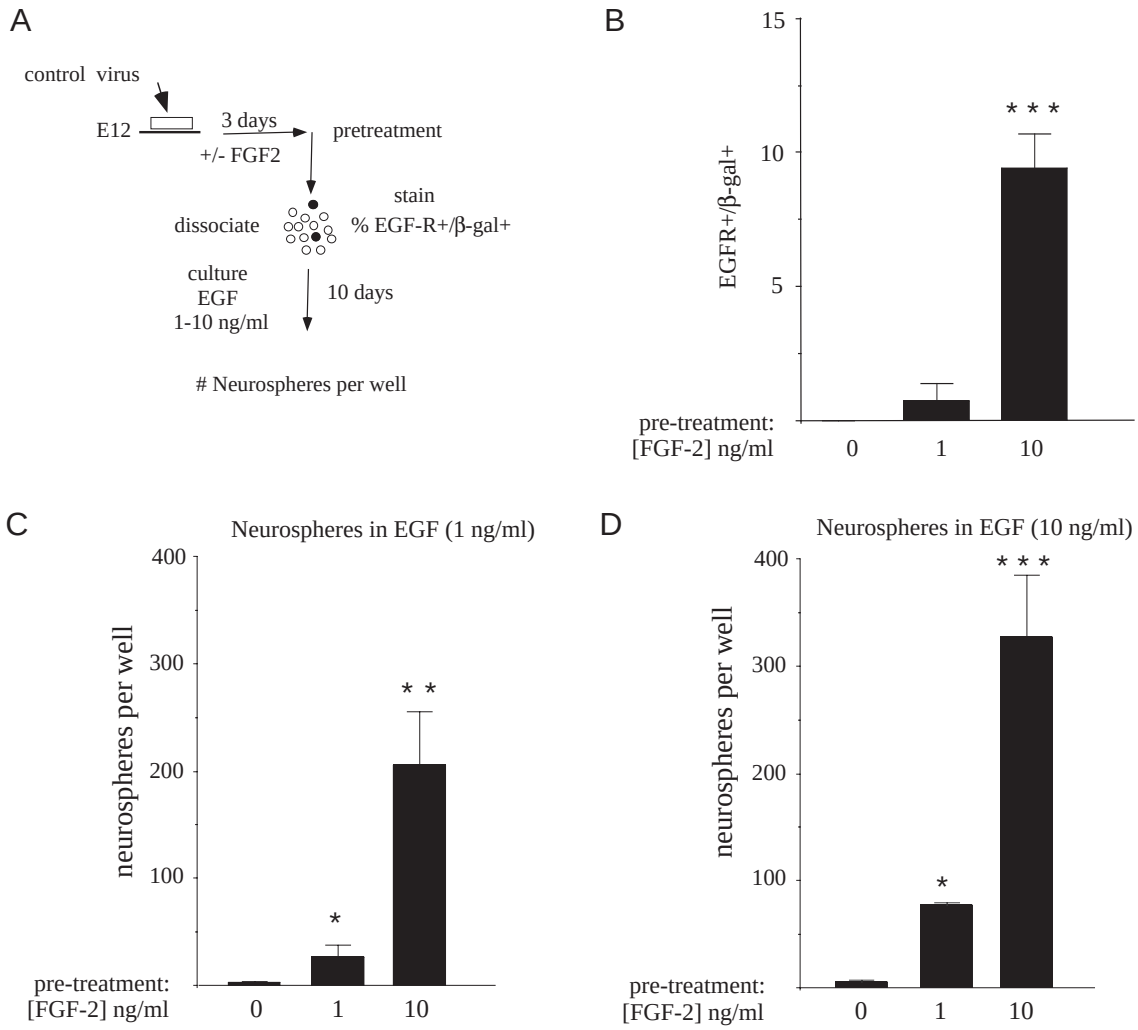

Fig. 5. FGF2 promotes developmental changes in EGFR expression and mitotic responsiveness to EGF. Explants of E12 cortex were infected with control virus. One day later, FGF2 (1-10 ng/ml) was added to the cultures. (A) After 3 days of pre-treatment with FGF2, explants were dissociated and expression of EGFR and mitotic responsiveness to EGF were assessed. FGF2 induced premature changes in EGFR expression (B) and mitotic responsiveness to EGF (C, 1 ng/ml EGF; D, 10 ng/ml EGF). Comparing FGF2-treated cultures to untreated cultures: * $P<0.05$, ** $P<0.005$, *** $P<0.0005$.

aggregates for 3 days (Fig. 2A). A small population of either the E12 or the E15 progenitor cells was marked with a retrovirus transducing β-gal to distinguish them from cells of the other age.

In mixed-age aggregate cultures, younger cells (E12) delayed the development of EGF-responsive stem cells. For example, when E15 progenitors were cultured with E12 cells, fewer E15 progenitor cells expressed high levels of EGFRs after 3 days (Fig. 2B). Moreover, fewer E15 cells divided in response to EGF to form neurospheres if they had been cultured as aggregates with E12 cells (Fig. 2C). The results in Fig. 2 were corrected to reflect the representation of E15 cells in the mixed-age cultures, as described in the legend. When complementary experiments were performed (infected E12 + uninfected E15), none of the spheres that developed were derived from the virally marked population of E12 cells (data not shown).

These observations suggested that early cortical cells inhibited developmental changes in EGFR expression and responsiveness of the E15 population. This could reflect delayed maturation, differentiation into another type of cell, or death. To distinguish between these possibilities, E15+E12 aggregates were cultured for an additional 4 days (7 days total). EGFR expression among virally marked E15 cells increased in the E12 co-cultures over time (compare Fig. 2D with 2B), and neurospheres developed in large numbers (Fig. 2E). These neurospheres could have been generated from E12 or E15 progenitor cells. To confirm that some of the neurospheres were derived from the E15 population, the cultures were stained with anti-β-gal (Fig. 2F). The presence of β-gal+ neurospheres demonstrates that E15 cells marked with β-gal virus become competent to divide in response to EGF to form a neurosphere after co-culture with E12 cells, but acquire this property with a timecourse characteristic of younger cells. After co-culture with younger cells, the likelihood that a marked E15 progenitor behaved like an EGF-responsive stem

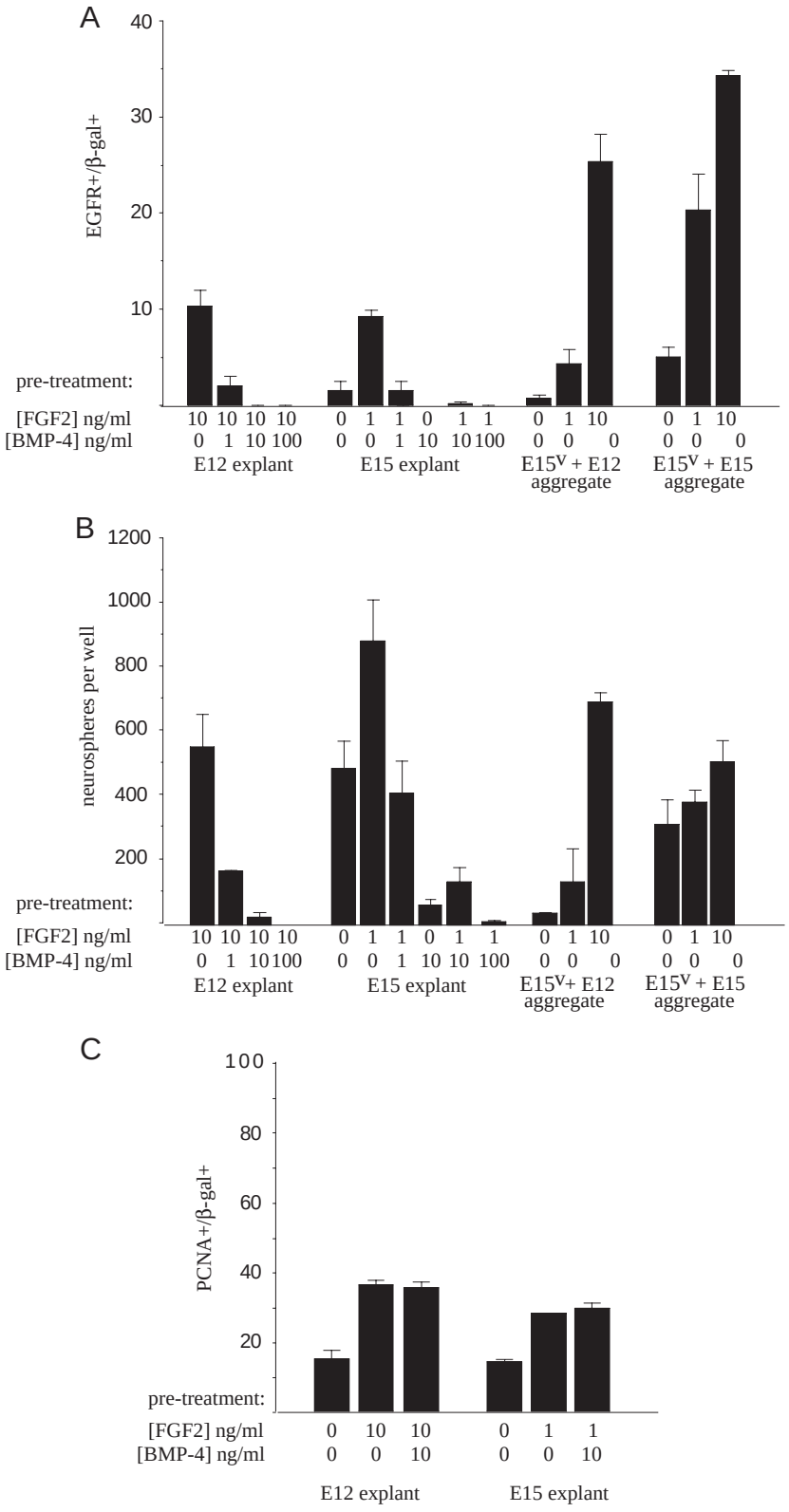

Fig. 6. FGF2 antagonizes effects of BMP4 and early (E12) cortical cells. Explants of E12 and E15 cortex were pre-treated for 3 days with the indicated concentrations of FGF2 and BMP4. Alternatively, aggregates of E15 cells alone or E15 cells+E12 cells were cultured in the absence or presence of FGF2 at the indicated concentrations for 3 days. Explants and aggregates were dissociated and their expression of EGFRs (A) and mitotic responsiveness to EGF (1 ng/ml) (B) were assessed. The data in B for aggregates was normalized for the representation of E15 cells as described in Fig. 2. (C) PCNA cyclin expression was determined as an indicator of proliferation in explants of E12 and E15 cortex after treatment with FGF2 alone or in combination with BMP4. Concentrations of BMP4 (10 ng/ml) that antagonize the FGF2-induced increase in EGFR expression and responsiveness do not inhibit the stimulation of proliferation by FGF2 (1-10 ng/ml).

cell was also three- to sixfold greater compared with cultures of E15 cells alone (18-36% with E12 cells versus 5-6% without E12 cells). These observations suggest that younger cortical cells produce a factor(s) that delays the development of EGF-responsive stem cells.

BMP4 mimics inhibitory effect of younger cortical cells

To identify candidates for the inhibitory factor(s) produced by younger cortical cells, we screened several peptide growth factors that are expressed in the early embryonic cortex. One factor, BMP4, mimicked the inhibitory effect of E12 cortical cells (Fig. 3). When explants of E15 cortex were cultured in BMP4 (10-100 ng/ml) for 3 days, the normal increase in EGFR expression was inhibited (Fig. 3B). Pre-treatment of explants with BMP4 also inhibited the acquisition of mitotic responsiveness to EGF (Fig. 3C,D). The inhibitory effect on mitotic responsiveness was specific for EGF family members, as BMP4-treated progenitors remained mitotically responsive to FGF2 (Fig. 3D).

BMPs elicit several responses in cortical progenitor cells, including cell cycle withdrawal and premature differentiation into neurons (Li et al., 1998) or astrocytes (Gross et al., 1996), and cell death (Furuta et al., 1997; Mabie et al., 1999). To confirm that BMP4 delayed the change in EGFR signaling in progenitor cells, rather than preventing it by promoting differentiation or death, E15 explants were exposed to BMP4 (10-100 ng/ml) for 3 days, then allowed to recover for 4 days in the absence of exogenous BMP4. After withdrawal of BMP4, increased expression of EGFRs (Fig. 3F) and mitotic responsiveness to EGF (Fig. 3G,H) were observed, though recovery from pre-treatment with very high concentrations of BMP4 (100 ng/ml, Fig. 3F-H) was reduced. The reversible inhibition seen with lower concentrations of BMPs (10 ng/ml), however, indicates that BMP4 does not cause premature differentiation or death of the progenitor cells that give rise to the EGF-responsive stem-cell population, but instead delays their development. Other TGFβ superfamily

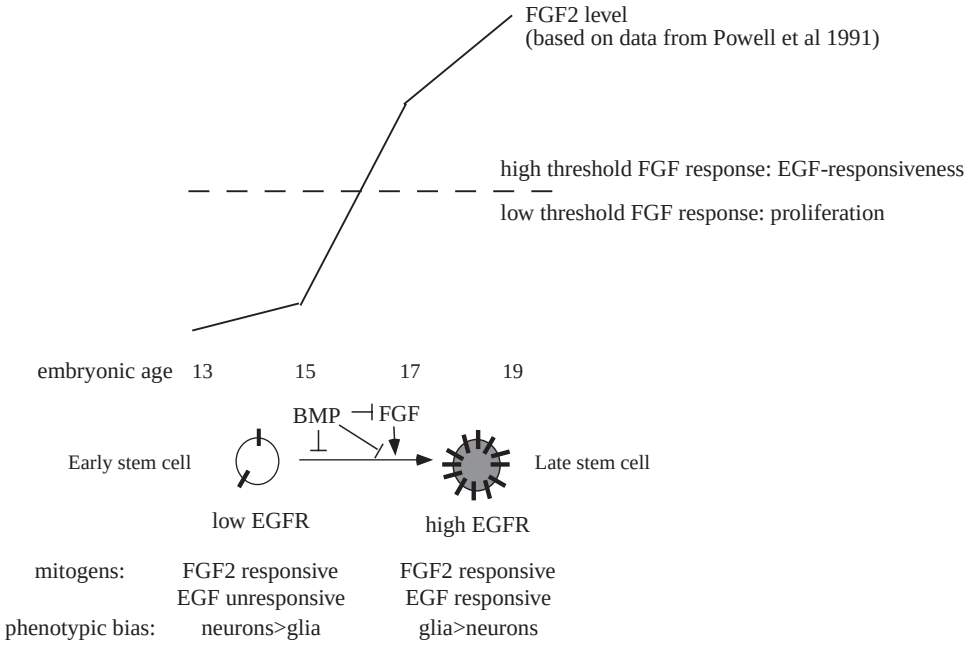

Fig. 7. Mechanisms that could regulate the development of EGF-responsive ('late') stem cells. BMPs could act at several levels to antagonize the development of EGF-responsive stem cells: directly blocking EGFR expression, selectively antagonizing responsiveness to a positive signal such as FGFs, and/or inhibiting the expression of a positive signal such as FGF2. Our finding that dnBMPR1B acts at least in part by a non-autonomous mechanism suggests that negative regulation of a positive signal such as FGF is an important component of the regulation of stem cell development by BMPs. A previous study has shown that FGF2 levels increase in the embryonic brain between E13 and E19 (Powell et al., 1991). This increase in FGF2 could serve as the trigger for the onset of changes in EGF-responsiveness among stem cells. Other work suggests that the expression of BMPRs in the brain declines during mid-embryonic development (Zhang et al., 1998). This could reduce the effectiveness of a given concentration of BMP, relieving negative regulation of FGF expression by BMPs.

members, including activin A and TGFβ 1-3, did not mimic this effect of BMP4, though BMP2 and BMP6 had effects comparable with BMP4 (data not shown).

dnBMPR1B promotes changes in EGFR signaling

Our observations suggested that BMP4, or a related family member, was a good candidate for the inhibitory signal made by E12 cortical cells. To determine whether endogenous BMPs in the early embryonic cortex normally suppress the developmental changes in EGFR expression and responsiveness, we reduced endogenous BMPR signaling by infecting progenitor cells with a retrovirus transducing dnBMPR1B (Zou and Niswander, 1996). A previous study using a virus that expressed dnBMPR1A in cortical progenitors reported an inhibition of migration among infected cells in explants (Li et al., 1998). To confirm the biological activity of our retrovirus, explants of E15 rat cortex were infected with dnBMPR1B virus and the migration of infected cells compared with explants infected with the control virus IZAP, which expresses the histochemical marker β-galactosidase (Burrows et al., 1997). Cells infected with pLIG-dnBMPR1B failed to leave the proliferative region (not shown), as reported for virus transducing dnBMPR1A (Li et al., 1998), indicating that pLIG-dnBMPR1B blocks responses to BMPs in cortical progenitor cells. Expression of dnBMPR1B also enhanced proliferation. In explants of E12 cortex, 28.3±1.8% of progenitor cells infected with dnBMPR1B virus expressed PCNA cyclin (a marker of dividing cells) 4 days post-infection compared with 15±2.5% of cells infected with control virus (P=0.01).

Expression of EGFRs and mitotic responsiveness to EGF were assessed 4-7 days after infecting explants of E12 cortex. We found that the proportion of cells expressing high levels of the EGFR was greater in the cells infected with dnBMPR1B virus compared with control virus (Fig. 4A). Comparable effects on EGFR expression were seen after infection of E15 progenitors (data not shown). The probability of dividing in response to EGF to generate a neurosphere was also three- to sixfold greater in progenitors infected with dnBMPR1B virus than with control virus (Fig. 4B). Neurospheres derived from dnBMPR1B-infected progenitor cells generated neurons and glia after withdrawal of EGF (data not shown), confirming the multipotent nature of the infected progenitor cell that generated the neurosphere.

We expected most of the neurospheres that developed prematurely to be derived from progenitor cells infected with dnBMPR1B virus. Surprisingly, most of the neurospheres that developed prematurely from explants infected with dnBMPR1B virus were derived from uninfected cells (Table 2). This suggested that blocking BMP receptor signaling increased an extrinsic signal that promotes the development of EGF-responsive stem cells. Blocking BMPR signaling could therefore regulate EGFR signaling at least in part by a non-cell-autonomous mechanism.

FGF2 accelerates the development of EGF-responsive stem cells

An antagonistic relationship between BMP and FGF family members has been described in the developing limb (Niswander and Martin, 1993), tooth (Neubuser et al., 1997), feather bud (Noramly and Morgan, 1998) and CNS (Li et al., 1998; Mabie et al., 1999). Moreover, BMP has been shown to negatively regulate the expression of several FGF family members in the limb (Pizette and Niswander, 1999; Zuniga et al., 1999). This raises the possibility that BMPs in the cortex

Table 2. Mitotic responsiveness to EGF

Virus	Time in explant culture	Neurospheres per well	b-gal+ neurospheres per well
Control	4 days	2.3±1.6	0
dnBMPR1B	4 days	21.7±10.4	0
Control	5 days	145±41	1.6±0.1
dnBMPR1B	5 days	610±147*	10.1±4.1

Explants of E12 rat cortex were infected with virus transducing β-gal ('control virus') or virus transducing β-gal and a dominant negative form of BMPR1B ('dnBMPR1B'). After 4 or 5 days, explants were dissociated and mitotic responsiveness to EGF (10 ng/ml) resulting in the formation of a neurosphere was determined 10 days later. In cultures prepared 4 days after infection, all of the neurospheres were generated from uninfected cells. In cultures prepared 5 days after infection, many more neurospheres developed from explants infected with dnBMPR1B virus than control virus. Some of these neurospheres were derived from infected cells, but most were derived from uninfected progenitor cells. These observations suggest that blocking signaling via BMPRs promotes the development of EGF-responsive stem cells by a non-cell-autonomous mechanism. Significance was determined using a paired *t*-test.

*$P<0.05$.

suppress the expression of FGF, and that an FGF family member might promote EGFR expression and mitotic responsiveness to EGF. Consistent with such a model, FGF2 and its receptors are expressed in the embryonic cortex (Powell et al., 1991; Weise et al., 1993; Qian et al., 1997) and FGF2-responsive stem cells have been described in the early embryonic telencephalon (Gage et al., 1995; Johe et al., 1996; Qian et al., 1997), prior to the appearance of an EGF-responsive population.

To test the idea that FGF2 promotes changes in EGFR signaling, explants of E12 cortex were exposed to FGF2 (1-10 ng/ml) for 3 days, then dissociated to assess their expression of EGFRs and mitotic responsiveness to EGF (Fig. 5). FGF2 induced the premature appearance of a subpopulation of cells that expressed high levels of EGFRs (Fig. 5B) and divided in response to EGF to form neurospheres (Fig. 5C,D). A premature change in EGFR signaling was also observed if monolayer cultures of E12 cells were exposed to FGF2 (data not shown). In E12 explants, a very small effect was observed with 1 ng/ml FGF2, but a much greater premature induction of EGFR expression and responsiveness was elicited by 10 ng/ml FGF2 (Fig. 5). The neurospheres produced in these cultures generated neurons and glia after removal of EGF (data not shown), indicating their derivation from a multipotent progenitor. The premature change in EGFR signaling induced by FGF2 was not mimicked by FGF6, FGF8, EGF family ligands (EGF, TGFα, HB-EGF), LIF, NT3, or SHH (data not shown).

FGF2 antagonizes inhibitory effects of BMP4 and younger cortical cells

The observations described above suggest that temporal changes in the responsiveness of cortical progenitor cells to EGF family ligands could be regulated by a combination of negative and positive signals. BMPs are a good candidate for the negative signal, FGFs for the positive signal. To determine whether BMPs and FGFs act antagonistically to regulate EGFR expression and responsiveness in cortical progenitors, explants of E12 or E15 cortex were exposed to combinations of BMP4 and FGF2.

An antagonistic relationship was observed between exogenous FGF2 and BMP4; however, this antagonism was selective for effects on EGFR expression. In explants of E12 or E15 cortex, BMP4 (10 ng/ml) inhibited the induction of EGFRs by FGF2 (1-10 ng/ml), but did not inhibit proliferation induced by FGF2 (Fig. 6C). BMP4 can therefore inhibit changes in EGFR signaling by a mechanism that does not inhibit proliferation, and FGF2 can stimulate proliferation without inducing EGFR expression prematurely. Exogenous FGF2 (10 ng/ml) also antagonized the endogenous inhibitory signal(s) made by E12 cells in aggregates of $E15^{v}$+E12 cells (Fig. 6A,B). At this concentration of FGF2, the proportion of virally marked E15 cells that generated neurospheres was comparable in cultures derived from $E15^{v}$+E15 and $E15^{v}$+E12 aggregates (5.1% versus 5.5%).

DISCUSSION

Temporal and spatial patterns of proliferation and differentiation in the nervous system reflect complex interactions between extrinsic and intrinsic regulatory mechanisms. Our previous studies demonstrated that one mechanism for achieving temporal changes in progenitor cell responsiveness to extrinsic signals involved altering their expression of EGFRs (Burrows et al., 1997; Lillien and Wancio, 1998). We found that progenitor cells that express high levels of EGFRs and divide in response to EGF in the late embryonic cortex are lineally related to early cortical cells that express lower levels of EGFRs and do not divide in response to EGF (Burrows et al., 1997). This change in cortical progenitor cells normally begins during mid-embryonic stages of development. As a consequence of the increase in EGFR expression, progenitor cells acquire the competence to respond to EGF family ligands in several ways, including proliferation as a multipotent progenitor/stem cell and astrocyte differentiation (Burrows et al., 1997). The choice between dividing as a multipotent stem cell and differentiating into an astrocyte depends on ligand concentration once a threshold level of EGFR expression is achieved (Burrows et al., 1997). Regulation of the change in expression of EGFRs by progenitor cells therefore has profound consequences for their fate. In the present study, we have provided evidence that the change in this intrinsic property of progenitor cells is regulated by antagonistic extrinsic signals.

BMP reversibly inhibits development of EGF-responsive stem cells

We found that early embryonic cortical cells delayed developmental changes in EGFR signaling among later progenitor cells. This suggested that early cells produce a signal that inhibits the increase in EGFR expression and the acquisition of mitotic responsiveness to EGF. Several lines of evidence support a role for BMPs in the negative regulation of

developmental changes in EGFR signaling. First, exogenous BMP4 mimicked the inhibitory effects of E12 cortical cells, reversibly blocking developmental changes in EGFR signaling. The negative effects of BMP4 were specific for mitotic responsiveness to EGF family ligands because BMP4 treated progenitor cells remained mitotically responsive to FGF2. Second, reducing endogenous BMP receptor signaling with a virus transducing a dominant-negative form of the receptor enhanced developmental changes in EGFR expression and increased the probability that cells divided in response to EGF to form neurospheres. Third, several BMPs are expressed in the embryonic cortex (Furuta et al., 1997; Li et al., 1998), and cortical progenitors express BMPR1B, BMPB1A and BMPRII during the embryonic stages under investigation (Zhang et al., 1998). Although the highest levels of BMP 2, 4, 5, 6 and 7 mRNA have been observed in the dorsal (medial) region of the telencephalon, BMP4 mRNA is also expressed more laterally (Furuta et al., 1997), in the dorsolateral regions used in the present study. Cellular sources of BMPs include radial glia (Schluesener and Meyermann, 1994) and choroid plexus (Furuta et al., 1997).

Previous studies have described a variety of responses to BMPs among embryonic cortical progenitor cells. For example, exogenous BMP2 and BMP4 have been shown to promote cell cycle withdrawal and differentiation into neurons (Li et al., 1998) or astrocytes (Gross et al., 1996), and to promote cell death (Furuta et al., 1997). More recently, the BMP antagonist noggin (Zimmerman et al., 1996) was reported to promote the development of oligodendrocytes (Mabie et al., 1999). Although these reports appear to conflict with our observations, they can be reconciled with our findings if one considers the possibility that specific responses to BMPs depend on cellular context, i.e., the type of progenitor cell. The inhibitory effect of BMP4 on EGFR signaling in stem cells was reversible, in contrast to the irreversible inhibition of proliferation and premature differentiation reported for the majority of cortical progenitor cells. Although it appeared that fewer cells recovered from exposure to very high concentrations of BMP4 (Fig. 3F-H), consistent with death of some cells (Mabie et al., 1999), this could also reflect failure to wash out the BMP4 during the recovery phase of the explant cultures. BMPs have been reported to promote the development of *Drosophila* germline stem cells (Xie and Spradling, 1998). This observation and our findings raise the possibility that BMPs also promote a stem cell state among neural stem cells, rather than inducing cell cycle withdrawal and differentiation as observed for most cortical progenitor cells.

Mechanism of BMP action

BMPs could regulate EGFR signaling at several levels. For example, BMPs could alter EGFR expression directly, by transcriptional and/or post-translational mechanisms (Shou et al., 1999). The antagonism between exogenous FGF2 and BMP4 suggests that BMPs also inhibit responses to FGF2, albeit selectively (see Fig. 6). The non-cell-autonomous change in EGF-responsiveness observed after infection with dnBMPR1B virus, however, suggests that a component of the inhibitory effect of BMP4 is indirect, and involves negative regulation of signals such as FGF2 that promote the development of EGF-responsive stem cells (Fig. 7).

BMPs and FGFs have been reported to act antagonistically in several systems, including the feather bud (Noramly and Morgan 1998), tooth (Neubuser et al., 1997) and limb (for example, Niswander and Martin, 1993). The mechanisms underlying their antagonism are complex (Vogt and Duboule, 1999). In the limb, FGFs antagonize BMP signaling through SHH and the BMP antagonist gremlin (Zuniga et al., 1999), and by inhibiting the expression of BMPR1B (Merino et al., 1998). BMPs antagonize FGF signaling at several levels as well. BMP2 and BMP4 alter expression of FGFRs in the feather bud (Noramly and Morgan, 1998). Moreover, BMPs inhibit the transcription of FGF4 and FGF6 in the developing limb (Pizette and Niswander, 1999). This kind of non-cell-autonomous mechanism could explain the effect of dnBMPR1B virus we observed in cortical cultures. It raises the possibility that BMPs in the cortex negatively regulate FGF expression, in addition to the possibility that they directly antagonize expression of EGFRs and/or responsiveness to FGF (Fig. 7).

Several FGF family members, including FGF2, are expressed in the embryonic cortex (Powell et al., 1991; Weise et al., 1993; Ozawa et al., 1996), as are at least three FGFRs (Qian et al., 1997). FGF2 has been reported to elicit multiple responses from cortical progenitor cells (for example, Qian et al., 1997). FGF-responsive stem cells appear before EGF-responsive stem cells, making the FGF pathway a reasonable candidate for a positive regulator of EGFR signaling. Our finding that exogenous FGF2 accelerates the timing of this change in progenitor cells provides further support for this idea. A recent report also demonstrated that FGF2 could induce premature EGF-responsiveness among a subpopulation of striatal progenitor cells (Ciccolini and Svendsen, 1998). Although responsiveness was assayed as CREB phosphorylation, as in the present study, only 10-20% of progenitor cells responded to EGF after "priming" with FGF2. Most early progenitor cells can divide in response to FGF2, but only a subpopulation have the self-renewal property characteristic of stem cells (Qian et al., 1997). These observations raise the possibility that FGF2 may be able to alter responses to EGF only among the subpopulation of progenitor cells that are stem cells.

Although we found that exogenous FGF2 could promote changes in EGFR signaling and antagonize the inhibitory effects of an endogenous signal(s), it is still not clear whether FGF signaling normally promotes these changes in progenitor cells in vivo, or whether FGF2 is the relevant family member. The effects of FGF2 on EGFR signaling were not mimicked by FGF6 and FGF8, two other family members expressed in the embryonic cortex (Ozawa et al., 1996). The pattern of SHH expression (Echelard et al., 1993) argues against it as a candidate for regulating cortical progenitor cells; however, the BMP antagonist noggin is expressed in a temporal and spatial pattern (Mabie et al., 1999), suggesting it could also play a role in modulating BMP signaling and thereby influence EGFR signaling.

Regulation of developmental timing

BMPs and FGFs are pleiotropic and could control the appearance of EGF-responsive stem cells during development by regulating one or more cellular processes. For example, FGFs could regulate the onset of the appearance of EGF-

responsive stem cells by stimulating the proliferation of their precursors, presumably FGF-responsive, EGF-unresponsive stem cells. Conversely, BMPs could delay the onset of their appearance by inhibiting the proliferation of their precursors. If the development of EGF-responsive stem cells was controlled by such a proliferation-dependent mechanism, however, one could argue that the timing mechanism was intrinsically regulated, and needed a mitogen merely to drive it, as proposed for O-2A progenitor cells (Temple and Raff, 1986). The results shown in Fig. 6C, however, argue against such a mechanism. BMP4 selectively inhibited responses to FGF2, allowing proliferation in the absence of an induction of EGF-responsiveness. This could reflect the threshold mechanism suggested to specify responses to FGF2, with lower thresholds of FGFR stimulation supporting proliferation and higher thresholds promoting glial cell development (Qian et al., 1997). Appropriate concentrations of BMP4 may therefore block high-threshold responses, such as EGFR expression, but not low-threshold responses such as proliferation. Our results provide support for the idea that BMPs and FGFs act instructively to regulate timing by an extrinsic mechanism, rather than modulating a proliferation-dependent intrinsic timing mechanism.

What then triggers the onset of the appearance of EGF-responsive stem cells? It is appealing to consider a feedback mechanism, whereby cells that produce BMPs or FGF2 achieve appropriate numbers or states of maturation at mid-embryonic development, resulting in the generation of a net positive signal. BMPs are produced by radial glial cells (Schluesener and Meyermann, 1994) and by the choroid plexus (Furuta et al., 1997). FGF2 is made by progenitor cells and choroid plexus (Raballo et al., 2000). The numbers of these cells do not change in an appropriate manner at mid-embryonic development to provide a trigger, suggesting that the cellular event(s) that initiates the change in EGFR expression may be more complex and involve a change in the level of expression of FGF, BMP and/or their receptors. It has been reported that the level of FGF2 increases during mid-late stages of embryonic development (Powell et al., 1991; Weise et al., 1993) (Fig. 7). Thus, an increase in FGF2 expression could be the trigger. The level of expression of BMPRs in the brain appears to decline during embryonic development (Zhang et al., 1998), suggesting that BMP signaling might decline. Given the observation that BMPs in the limb negatively regulate the expression of FGFs (Pizette and Niswander, 1999; Zuniga et al., 1999), together with our finding that dnBMPRs have a non-cell-autonomous positive effect, it is possible that a reduction in BMP signaling triggers the increase in FGF2 expression in the CNS (Fig. 7). In our explant cultures, we observed that a lower concentration of FGF2 (1 ng/ml) had a greater effect on E15 explants than on E12 explants (compare Fig, 5 with Fig. 6). This observation is consistent with an increase in the level of an endogenous positive signal such as FGF2 with age, or with a decline in the level or effectiveness of endogenous BMPs.

In addition to the change in EGFR expression and responsiveness, cortical progenitor cells and stem cells change is several other ways during mid-embryonic development. Early progenitor cells and stem cells tend to generate more neuronal progeny, while later progenitor cells and stem cells tend to generate more glial progeny (Zhu et al., 1999). Early and late progenitor cells also differ in their competence to generate deep layer neurons (McConnell, 1988). The developmental change in the latter property also appears to be controlled by cell-cell signaling (Bohner et al., 1997). Our preliminary studies using viral transduction of EGFRs suggest that mitotic stimulation of progenitor cells with EGF does not control developmental changes in the ratio of neuronal and glial progeny (L. L., unpublished). It will be interesting to determine whether the signals we have now identified as regulators of EGFR expression and responsiveness also control other properties of progenitor cells that change during embryonic development, or whether these properties are controlled by distinct mechanisms.

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