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Asymmetric Distribution of EGFR Receptor during Mitosis Generates Diverse CNS Progenitor Cells

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Summary

It has been debated whether asymmetric distribution of cell surface receptors during mitosis could generate asymmetric cell divisions by yielding daughters with different environmental responsiveness and, thus, different fates. We have found that in mouse embryonic forebrain ventricular and subventricular zones, the EGFR can distribute asymmetrically during mitosis in vivo and in vitro. This occurs during divisions yielding two Nestin$^+$ progenitor cells, via an actin-dependent mechanism. The resulting sibling progenitor cells respond differently to EGFR ligand in terms of migration and proliferation. Moreover, they express different phenotypic markers: the EGFR^{high} daughter usually has radial glial/astrocytic markers, while its EGFR^{low} sister lacks them, indicating fate divergence. Lineage trees of cultured cortical glioblasts reveal repeated EGFR asymmetric distribution, and asymmetric divisions underlie formation of oligodendrocytes and astrocytes in clones. These data suggest that asymmetric EGFR distribution contributes to forebrain development by creating progenitors with different proliferative, migratory, and differentiation responses to ligand.

Introduction

Asymmetric cell division, in which unequal distribution of determinants during mitosis results in two daughter cells with different fates, is a fundamental mechanism underlying the generation of neural cell diversity. In invertebrates and, more recently, in mammals, multipotent neuroblasts and neural stem cells have been observed to undergo repeated asymmetric cell divisions in which one daughter cell is a restricted neuroblast, while the other retains progenitor cell identity (Doe and Skeath, 1996; Miyata et al., 2001; Noctor et al., 2001; Qian et al., 1998).

Significant insight into intracellular mechanisms of asymmetric division has been gained from simple systems (Wodarz and Huttner, 2003). In *Drosophila*, as neuroepithelial cells delaminate and form neuroblasts, a protein complex that includes Bazooka, DmPar-6, atypical protein kinase C (aPKC), and the G protein subunit Gαi forms a crescent on the apical side. When neuroblasts divide, this complex directs Prospero (pros), Numb, and prosRNA, with their adaptors, Miranda, Partner of Numb, and Staufen, respectively, to the basal pole. With the coordination of oriented cleavage planes, these basally localized asymmetric determinants are segregated preferentially into the basal daughter (ganglion mother cell). Functional analyses of these asymmetric cell determinants with genetic approaches have revealed their key roles in binary cell fate choices; for instance, loss of Numb eliminates asymmetric fates (Buescher et al., 1998; Spana et al., 1995).

Much of this intracellular asymmetric machinery is conserved in vertebrate neural development. ASIP, the mouse homolog of Bazooka, with mPAR-6 and aPKC localizes at the apical side of mouse neuroepithelial cells (Manabe et al., 2002). Mouse Numb shows asymmetric distribution at cortical neural progenitor/stem cell divisions during neurogenesis (Shen et al., 2002; Zhong et al., 1997) and has a conserved function in mediating diverse fate determination (Li et al., 2003; Petersen et al., 2002; Petersen et al., 2004; Shen et al., 2002).

These asymmetric determinants act via regulation of specific intracellular signaling pathways. Numb downregulates Notch signaling, blocking nuclear transport of the activated form of Notch. Thus, the daughter cell that inherits Numb has a lower level of Notch signaling (Berdnik et al., 2002), which in turn directs the cell fate choice. In this way, two sibling cells can have equal Notch expression on the cell surface and be exposed to equivalent ligand, but show unequal Notch signaling.

It has been debated whether asymmetric cell division can also occur at the cell surface receptor level, directly leading to unequal signaling between sibling cells. Numb can stimulate Notch endocytosis in *Drosophila*, and it has been suggested that this could clear receptors from one sibling cell (Berdnik et al., 2002). In vertebrates, unequal Notch has been described on ferret cortical neuroepithelial cells (Chenn and McConnell, 1995), but this result was not confirmed in mouse (Zhong et al., 1997); unequal Notch distribution remains an intriguing possibility for asymmetric neural cell division.

While in *Drosophila* asymmetric neural cell divisions are largely dependent on Numb, and all known Numb functions act via the Notch pathway, in vertebrates this may not be the only receptor system that can generate asymmetric cell divisions. Many environmental signals are indispensable for aspects of neural cell fate determination, such as progenitor/stem cell maintenance and neuronal and glial differentiation. Murine neural stem cell proliferation can be stimulated by fibroblast growth factor (FGF) and epidermal growth factor (EGF) signaling. Moreover, EGF receptor (EGFR), a member of the tyrosine kinase receptor family, can influence cortical progenitor fate choice, as its overexpression at midgestation pushes cells into the astrocyte lineage at the expense of neuron formation (Burrows et al., 1997; Caric et al., 2001). Hence, we decided to examine how EGFRs might be distributed during mitoses of cortical progenitor cells.

EGFR expression is upregulated at midgestation, and

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EGFR activation in late progenitor cells stimulates cell proliferation, migration, and astrocyte differentiation (Burrows et al., 1997). We examined EGFR distribution during and after progenitor cell divisions in vivo and in clonal cultures, allowing us to follow the progeny of individual cells in order to determine their fate. Interestingly, we found that a subpopulation of forebrain progenitor cells shows asymmetric EGFR distribution at late stages of mitosis, resulting in different levels of EGFR on daughter cells and subsequent differences in their behavior. Our study provides evidence that asymmetric EGFR distribution acts as a mechanism for generating asymmetric neural cell divisions.

Results

Asymmetric Localization of EGFR in Mitotic Mouse Forebrain Progenitor Cells In Vivo

As shown previously (Caric et al., 2001), EGFR expression in cortex varies temporally, being low prior to embryonic day 13 (E13), and regionally, being higher in lateral than in medial cortex. It also varies within laminae: from E13–15, EGFR is exclusively localized in ventricular (VZ) and subventricular zones (SVZ), and, starting from E16, its expression is most intense in subplate and marginal layers, with more diffuse staining in the intermediate zone and cortical plate. EGFR staining is stronger in basal than in dorsal embryonic forebrain.

We found that EGFR appears concentrated at the luminal surface of the VZ (Figures 1A–1C)—at low levels from E13–14—but more marked at E15–17, when cells also began to show clear membrane localization of EGFR. We focused on dividing cells, identifying mitotic cells by their condensed chromosome/chromatid morphology revealed by chromosome staining. Mitoses were found mostly in the VZ; fewer were found in the SVZ and intermediate zone, and a few were dispersed in the cortical plate. Most dividing cortical cells have EGFR distributed evenly over the entire cell membrane or show no expression at all (Figures 1M–1O). However, some dividing cortical progenitor cells show EGFR asymmetrically localized to one side (Figures 1D–1L and Movies S1 and S2 in the Supplemental Data available with this article online). In 21 E17 brain sections, a total of 217 dividing cortical progenitor cells, from metaphase to telophase, were examined; 111 were $EGFR^+$ and 24 (21.6%) of these showed asymmetric distribution. Of these 24 mitotic progenitor cells, 16 were in the VZ and 7 were in the SVZ (1 was in the lateral migratory stream); 21 cells were at anaphase/telophase and 3 were at metaphase.

Cortical cleavage planes can vary from perpendicular or parallel with respect to the ventricular surface (Chenn and McConnell, 1995; Haydar et al., 2003). For VZ cells with asymmetric EGFR, there was a change during development from predominantly parallel cleavage planes at E14–E15 (6/9 [67%]) to predominantly perpendicular at E17 (14/16 [87%]), mirroring a similar change in plane orientation described for the general dividing cell population (Haydar et al., 2003). In the SVZ, dividing cells usually had cleavage planes perpendicular with respect to the nearby ventricular surface.

Asymmetric Distribution of EGFR in Mitotic Cells Results in Daughter Cells with Different EGFR Levels

To determine if asymmetric EGFR distribution during mitosis can indeed lead to daughter cells with different EGFR levels, we used a pair assay (Shen et al., 2002) in which the progeny of each division can be followed. Single E13–E17 cortical cells were plated into culture wells at low density in serum-free medium containing 10 ng/ml FGF2 (this medium was used throughout these studies, unless stated otherwise) and mapped to determine their location. For studies of E15–E17 cortex, we enriched the minor progenitor population, using the surface marker LeX (Capela and Temple, 2002), via magnetic bead sorting (MACS). After 24 hr, pairs of daughter cells were identified, then fixed, and stained for EGFR. In some pairs, one daughter cell had EGFR expression ($EGFR^{high}$), but the other ($EGFR^{low}$) did not (Figure 2A). The frequency of these pairs with asymmetric EGFR staining decreased with developmental stage: 26.7% ± 5.4% at E13 and 17.8% ± 1.7% at E17 (Student's t test, $p < 0.001$; Figure 2B). Some cortical progenitor cells that were observed in mitosis had asymmetric EGFR distribution, usually at telophase and cytokinesis, as we saw in vivo (Figure 2C and Table 1). We did not observe any asymmetric EGFR during metaphase in culture, unlike in vivo; this discrepancy may be explained by loss of contact with neighboring cells. The frequency of EGFR asymmetric distribution at telophase and cytokinesis in E15–16 dividing cells was 38.8%, while 23.1% of the resulting pairs had asymmetric EGFR. Thus, the initial asymmetric EGFR distribution is lost in some pairs; for example, the $EGFR^{low}$ daughter may reexpress EGFR after mitosis, as occurs with Numb expression in *Drosophila* neuroblasts. EGFR asymmetry was also observed when cells were grown in serum-free medium containing EGF rather than FGF2 (17.3% of dividing E17 cortical pairs in EGF were asymmetric for EGFR, compared to 17.9% in FGF2).

To confirm the asymmetric distribution of EGFR with an independent method, we used a plasmid construct containing EGFR fused with enhanced GFP (eGFP) at the C-terminal region (p-eGFP-N1-hEGFR; Carter and Sorkin, 1998) to transfect freshly isolated E12–E13 cortical progenitor cells. Approximately 24 to 48 hr post-transfection, 2%–3% of cells expressed detectable hEGFR-eGFP protein, mostly at low levels and in the cell membrane, similar to endogenous EGFR (Figure 2D). Of 20 dividing cortical progenitor cells that were followed, 2 (10%) showed asymmetric eGFP distribution during cell division (Figure 2D).

We also confirmed this result using a ligand-labeling strategy. Fluorescently conjugated EGF binds to EGFRs, and the resulting internalized complex can be visualized (Carter and Sorkin, 1998). After cortical progenitor cells had generated pairs of daughter cells, Alexa488-conjugated EGF was added to the culture medium for 5–10 min. The pairs were then fixed and stained for EGFR. There was a high correlation between asymmetry in internalized Alexa488-conjugated EGF and EGFR staining (Figures 2E and 2F; Student's t test and χ^2 analysis: $p < 0.01$). In summary, both the hEGFR-eGFP fusion construct and Alexa488-conjugated EGF ligand-labeling experiments confirmed that EGFR is asymmet-

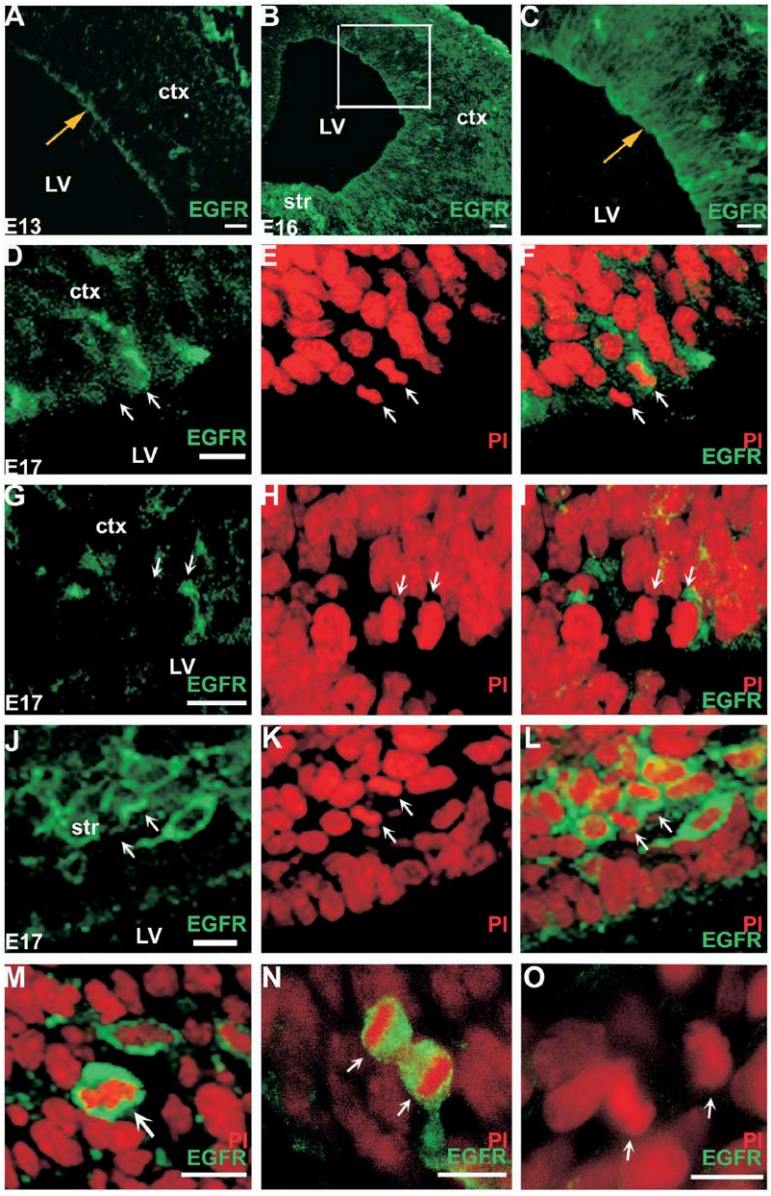

Figure 1. Asymmetric EGFR Distribution in Dividing Cortical Progenitor Cells In Vivo

(A–C) Immunostaining of EGFR in cortex at E13 (A) and E16 (B); boxed region is shown at higher power in (C). EGFR is concentrated on the luminal edge of cortex indicated by arrows. (D–L) Confocal images of asymmetric EGFR distribution in dividing forebrain progenitor cells ([D–F] and [G–I], Movies S1 and S2, respectively, examples from cortical VZ). (J–L) example from striatal SVZ; the EGFR level is higher in striatum compared to cortex. (M–O) Many cortical progenitor cell divisions show symmetric EGFR distribution (M and N) or no detectable EGFR (O). White arrows indicate dividing cortical progenitor cells. PI, propidium iodide dye (to reveal chromosomes); LV, lateral ventricle; str, striatum; ctx, cortex. Scale bar, 20 μm.

rically distributed during mouse cortical progenitor cell divisions.

Sibling Progenitor Cells with Different Levels of EGFR Are Functionally Different

We then assessed whether differential distribution of EGFR resulted in a functional difference between daughter cells. EGFR signaling can stimulate proliferation and migration of neural cells (Burrows et al., 1997; Caric et al., 2001). Hence, we assessed EGF-driven BrdU incorporation and migration in these cell pairs.

Single E15–16 cells were plated in medium containing 20 ng/ml EGF with no FGF2 (to exclude interference with FGFR signaling) for 24 hr. The resulting cell pairs were mapped, 10 μg/ml BrdU were added, and 8 hr later, the pairs were fixed and stained for EGFR. In pairs with asymmetric EGFR, the $EGFR^{high}$ daughter cell had a significantly higher frequency of BrdU incorporation than its $EGFR^{low}$ sibling (Figures 3A and 3B; χ^2 analysis, Student's t test, $p < 0.01$). However, when these cortical progenitor pairs were grown in medium containing both FGF2 and EGF, $EGFR^{high}$ and $EGFR^{low}$ daughter cells incorporated BrdU at a similar frequency (data not shown), showing that the correlation between EGFR expression and BrdU incorporation in these asymmetric pairs is ligand dependent. Thus, progenitor siblings can have asymmetric EGFR while being symmetrically responsive to FGF2, and, consistent with this, double-staining reveals pairs asymmetric for EGFR, while both cells of the pair have FGFR2 (Figure 3F).

To measure the migration of each daughter cell, we generated pairs from E17 LeX-enriched cortical progenitor cells, as described above, photographed them to map their initial position, and then rephotographed

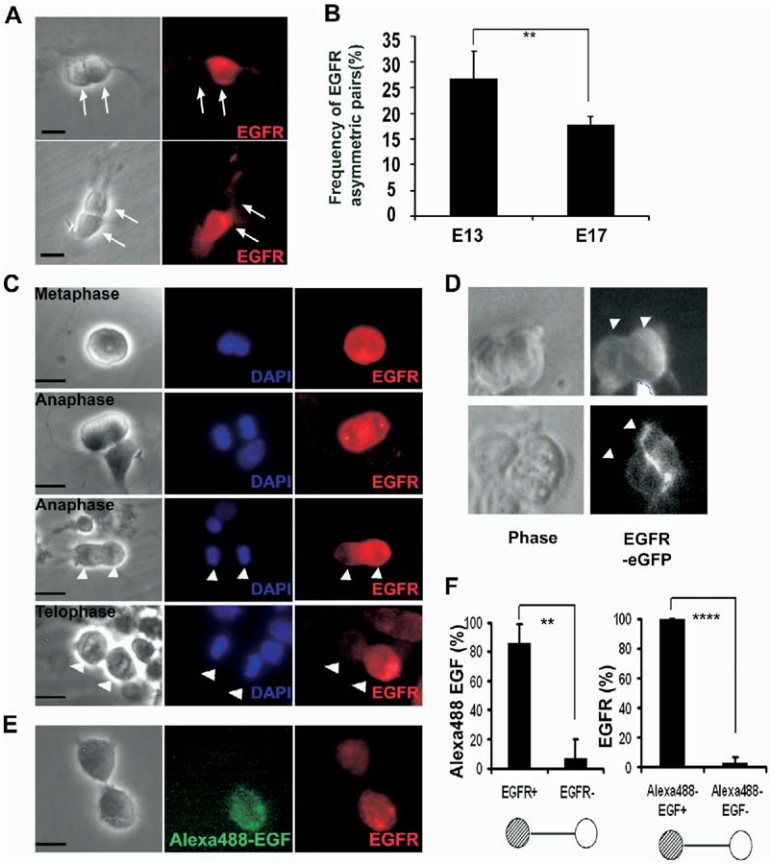

Figure 2. Asymmetric EGFR Distribution in Dividing Cortical Progenitor Cells In Vitro

(A) Asymmetric EGFR distribution in sibling cell pairs derived from E13 cortical progenitor cells. (B) The incidence of pairs asymmetric for EGFR decreases with age of cortical development (n = 2728 pairs from 16 independent experiments at E13; n = 875 pairs from 10 independent experiments at E17). (C) Asymmetric distribution of EGFR is not detected at metaphase (upper panels), is occasionally seen during anaphase (middle panels), and is most prominent at telophase and cytokinesis (bottom panels). (D) Time-lapse recording revealed EGFR asymmetric distribution during division in live cells with human EGFR-eGFP fusion construct. The blue line in the upper panel outlines fluorescence from a neighboring cell; arrowheads mark the two daughter cells. (E) Labeling with Alexa488-conjugated EGF ligand shows asymmetric distribution consistent with asymmetric EGFR staining in sibling cells. (F) Correlation between asymmetry in internalized Alexa488-EGF complex and asymmetry in EGFR: the daughter cell with the most internalized Alexa488 EGF shows higher EGFR expression (n = 42 pairs for left panel; n = 35 pairs for right panel). Student's t test, **$p < 0.01$; ****$p < 0.0001$. Scale bar, 10 μm. Two ovals joined by a bar, pairs of daughter cells. Error bars represent the mean ± SD.

them after an additional 8 or 16 hr of culture. The distance between the initial and final position seen in the overlaid images (Figure 3C) was then determined. (Pairs that divided again during the observation period were excluded, because cells usually stop migrating during mitosis.) We noticed that the two daughter cells usually migrated together initially and later separated. Migration ability varies from pair to pair, but $EGFR^{high}$ daughter cells consistently migrated farther in this assay than did their sibling $EGFR^{low}$ cells in EGF-containing medium (Figure 3D; paired t test, $p < 0.05$).

Asymmetric EGFR Distribution Correlates Closely with Numb Asymmetry in a Stage-Dependent Manner

We examined the relationship between EGFR distribution and that of a well-established asymmetric determinant, Numb. Numb has a PTB binding domain that would allow it to associate with EGFR; however, no direct association between EGFR and Numb has been found (Dho et al., 1998). Nevertheless, it was important to determine whether there was a correlation between Numb and EGFR distribution in these cortical cells. When we double-stained $EGFR^{high}/EGFR^{low}$ E13 cortical pairs with Numb antibody, we found that most, 76%, were also asymmetric for Numb. Moreover, Numb colocalized with EGFR, being found in the $EGFR^{high}$ daughter, while the $EGFR^{low}$ daughter was usually negative for Numb (Figures 4A and 4B). However, this correlation decreased at E16–17, at which stage pairs asymmetric for EGFR are mostly (74%) symmetric for Numb (Figures 4A and 4B).

To find out whether the EGFR distribution might be dependent on Numb asymmetry, we compared the frequency of EGFR asymmetric pairs from dorsal forebrain in wild-type and $Emx1^{IREScre}$ Numb and Numblike double mutant mice (Li et al., 2003) and found no significant difference (Figure 4C). Thus, EGFR asymmetry is not dependent on the presence of Numb.

Table 1. E15–E16 Cortical Dividing Cells with Asymmetric EGFR In Vitro

Mitosis Stage	Incidence of EGFR Asymmetric Distribution	Total Dividing Cells
Metaphase	0	19
Anaphase	9.1%	22
Telophase & cytokinesis	38.8%	67

EGFR asymmetric distribution is most prominent at late stages of mitosis.

Asymmetric EGFR Distribution Is Actin Dependent

In *Drosophila*, the asymmetric distribution of Numb and Prospero are actin-dependent processes. We tested the effect of disrupting the actin cytoskeleton on EGFR asymmetric distribution using Latrunculin A, a potent actin polymerization inhibitor (Knoblich et al., 1997;

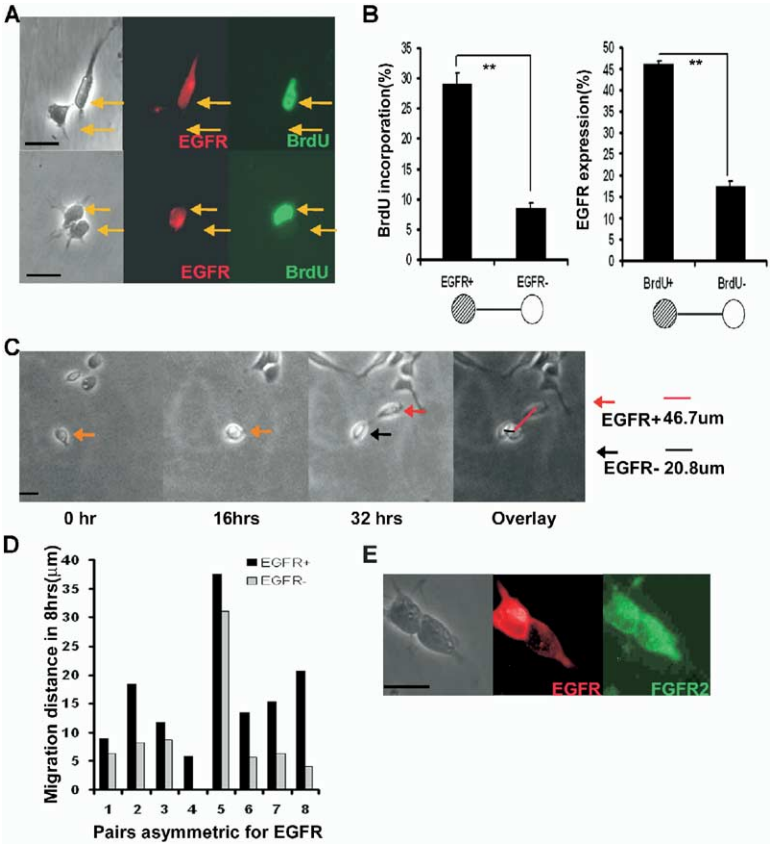

Figure 3. Cell Pairs with Different Levels of EGFR Differ in Proliferation and Migration

(A and B) Colocalization of EGFR and BrdU in E15 cortical progenitor cell pairs exposed to EGF and then given BrdU for 8 hr. (A) Examples of stained pairs. (B) The $EGFR^{high}$ daughter cells show higher BrdU incorporation. Student's t test, ** $p < 0.01$; n = 76 pairs, left panel; n = 53 pairs, right panel. (C) Migration assay example. A single cortical progenitor cell was imaged immediately after plating (0 hr; orange arrow). After overnight culture, this progenitor cell had divided and generated a cell pair (16 hr; orange arrow). 16 hr later, the pair was recorded again (32 hr) and then fixed and immunostained for EGFR (red arrow, $EGFR^+$; black arrow, $EGFR^-$). The 16 and 32 hr images have been overlaid, and changes in position of the daughter cells from 16 hr to 32 hr are indicated by a straight line (overlay, red and black line for the $EGFR^+$ and $EGFR^-$ daughter, respectively). (D) The $EGFR^{high}$ daughter shows significantly more EGF-dependent migration than the $EGFR^{low}$ daughter, paired t test, $p < 0.01$. (E) A representative pair asymmetric for EGFR is symmetric for FGFR2. All scale bars, 15 μm. Error bars represent the mean ± SD.

Zhao et al., 2002). When E15–E16 cortical progenitor cells were cultured for 16 hr and then treated with 0.5 or 1 μM Latrunculin A for 1.5 hr, most mitotic cells exhibited defects in cell cleavage (Figure 4D). In these treated cells, EGFR accumulated in the center of the dividing cells and did not distribute asymmetrically, in contrast to control (Figures 4D and 4E; Student's t test, $p < 0.01$). TUNEL staining showed no significant difference in cell apoptosis between control and Latrunculin A groups (Figure 4E; Student's t test, $p > 0.05$), excluding the possibility that dividing cells with asymmetric EGFR distribution are selectively dying. This shows that EGFR distribution during mitosis is actin dependent.

Sibling Daughters with Different Levels of EGFR Have Different Fates

If EGFR asymmetry is important for asymmetric divisions, then we should find that sibling cells in asymmetric pairs would acquire different fates. Hence, we double-stained asymmetric EGFR pairs grown in FGF2-containing medium with different neural cell markers.

In previous studies we showed that, at E13–14, some divisions generate two progenitor cells (P/P divisions), some generate a progenitor and a neuron (P/N divisions), and others generate two neurons (N/N divisions) and also that Numb can be asymmetrically distributed at all of these types of divisions (Shen et al., 2002). Here we find that asymmetric EGFR is seen largely at P/P divisions and rarely at P/N divisions or N/N divisions. At all ages examined, from E13–E17, the two daughter cells with different levels of EGFR are usually both negative for β-tubulin III and positive for Nestin, a neural progenitor cell marker. EGFR asymmetric pairs at E13–14 consist of 72% P/P divisions, 9% P/N divisions, and 13% N/N divisions, while at E16–17 very few are P/N or N/N divisions (Figures 5A and 5B and Table 2). Therefore, EGFR asymmetric distribution generates two progenitor cells that differ in EGF responsiveness ($P^{EGFRhigh}/P^{EGFRlow}$).

The developing cortex contains a heterogeneous mix of progenitor cells; for example, some are restricted progenitors and others, stem cells, while some may have a cortical, and others, a basal forebrain origin. When these are plated in culture and allowed to form pairs, most are born in the 4 hr before fixation, but some are born earlier in the 24 hr culture period, so that pairs have different levels of maturity. With this in mind, heterogeneity in phenotype among the sister cell pairs is expected. Nevertheless, we found significant trends in phenotypic differences between sibling cells.

We double-stained E16–17 cortical cell pairs asymmetric for EGFR with known progenitor markers, including LeX, RC2, and GLAST. LeX (also known as Lewis X, SSEA-1, or CD15) is expressed in CNS germinal zones, including subpopulations of radial glia (Mai et al., 1998) and is found on neural stem cells from embryonic and adult stages (Capela and Temple, 2002, unpublished observations). RC2 and GLAST are also expressed by radial glia, and GLAST, by the astrocyte lineage (Misson et al., 1988; Shibata et al., 1997).

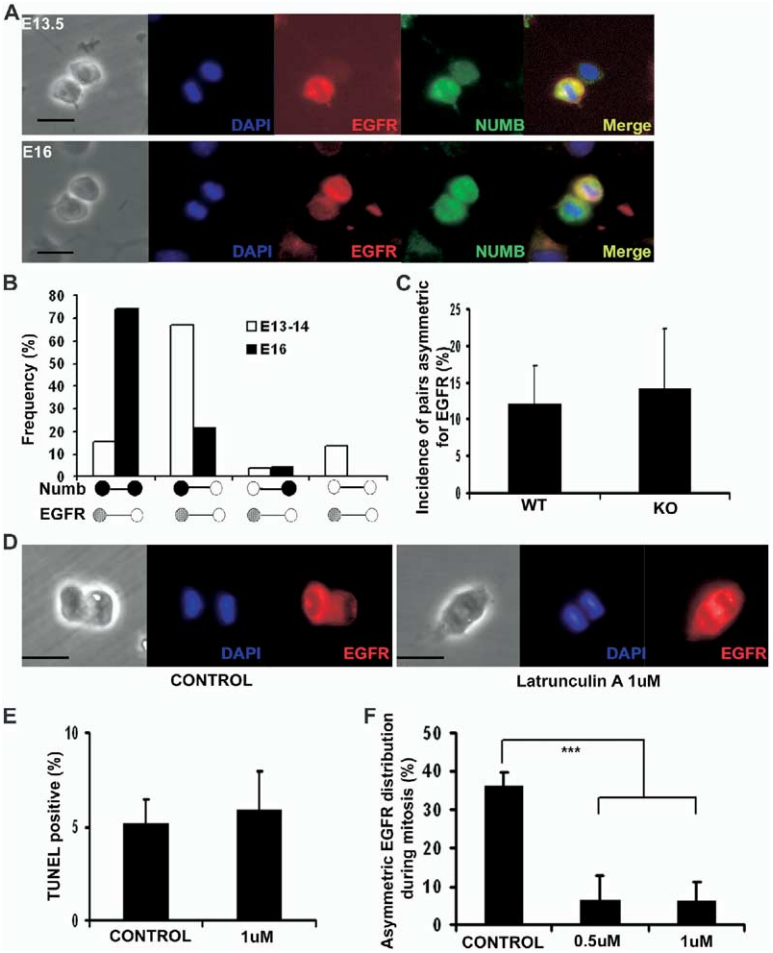

Figure 4. EGFR Asymmetry Is Correlated with Numb Asymmetry in an Age-Dependent Manner and Shows F-Actin Dependence

(A and B) At E13, EGFR usually colocalizes with Numb in asymmetric pairs ([A], upper panels), while at E15–E16, daughter cells asymmetric for EGFR usually show symmetric Numb distribution ([A], bottom panels); the frequencies are shown in (B). n = 72 E13 pairs; n = 69 E15–16 pairs. (C) Asymmetric pairs for EGFR in Emx1^{IREScre}-mediated Numb and Numblike double knockout (KO) mice occur at similar frequency to wild-type (WT) (Student's t test, p = 0.71; n = 195 pairs for WT and n = 300 for KO). (D) E15–16 cortical progenitor cells grown overnight in FGF2-containing medium and then treated with the actin disrupter Latrunculin A at 1 μM or 0.5 μM for 1.5 hr do not show EGFR asymmetric distribution. (E) No significant difference in apoptosis of progenitor cells after treatment with Latrunculin A versus control vehicle (Student's t test, p = 0.54; n = 1012 cells in control and n = 1116 cells in Latrunculin A). (F) The frequency of EGFR asymmetric distribution in dividing E16 cortical progenitor cells is significantly decreased after Latrunculin A treatment compared to DMSO (vehicle control) (MANOVA, ***p < 0.001; n = 114 mitotic cells analyzed in control, and n = 67 and 45 cells with 0.5 μM and 1 μM Latrunculin A treatment, respectively). Error bars represent the mean ± SD.

The expression of these markers was highly correlated with EGFR^{high} distribution in asymmetric pairs (Figure 5C and Table 2). GLAST expression in the EGFR^{high} daughter cell occurs less frequently than RC2 and LeX expression at this stage, but a pair-study of cortical progenitor cells 1 day older revealed that EGFR^{high} RC2^{+} daughter cells have a higher probability of coexpressing GLAST than their sibling EGFR^{low} RC2^{+} cells (data not shown). These observations suggest that the EGFR^{high} daughter cell has a radial glial character, which later acquires GLAST as it enters the astrocyte lineage. Consistent with this, in E17.5 forebrain sections, EGFR is colocalized with RC2 in cortical progenitor cells with radial morphology (Figure 5D). The EGFR^{low} daughter was found to stain with few markers other than Nestin and an antibody recognizing olig1 and olig2, early transcription factors involved in neuronal and oligodendrocyte cell differentiation (Zhou et al., 2000) that can be expressed in early astrocytes (Liu and Rao, 2004); usually, both daughters in the EGFR asymmetric pair were olig1/2^{+} (Table 2; χ^2 analysis, p > 0.05). Early oligodendrocyte precursor markers, including NG2, PDGFRα, and O4, were not expressed in these pairs that have recently finished division. Hence, the EGFR^{low} daughter is characterized by being RC2^{-}, GLAST^{-}, β-tubulin III^{-}, and Oligo1/2^{+}. Given that these late cortical clones only generate two cell types in culture—astrocytes and oligodendrocytes—and that oligodendrocytes are known to have low levels of EGFR (Kalyani et al., 1999; Maric et al., 2003) and to lack RC2 and GLAST (Diers-Fenger et al., 2001; Schools et al., 2003), these data are consistent with the EGFR^{low} daughter cells being in the early oligodendrocyte lineage. Consistent with this, when we examined EGFR expression of NG2^{+} cells derived from E16 cortical progenitor cells cultured for 6 days, 85% were negative for EGFR. Moreover, we found that EGFR^{+} cells in E17 forebrain sections were generally NG2^{-} (see Figure S1).

Oligodendrocytes and Astrocytes Are Generated from Asymmetric Divisions of Cortical Progenitor Cells In Vitro

To understand how late cortical progenitor cells generate oligodendrocytes and astrocytes over time in culture and how EGFR asymmetry might contribute to this, we time-lapse recorded single E15.5 cortical progenitor cells growing in either EGF- or FGF2-containing medium for 4 days. Clones were then stained for β-tubulin III, NG2, and RC2. Clone lineages were reconstructed and correlated with the immunostaining to provide information about the origin of each progeny.

Of the clones followed, none contained both neurons

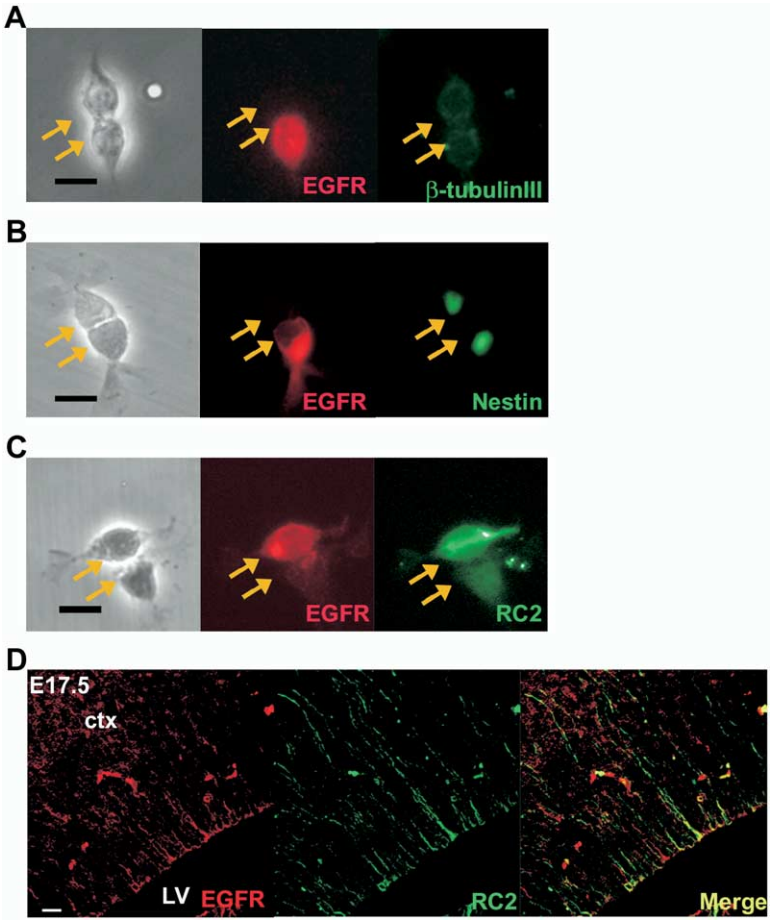

Figure 5. Cells Showing Asymmetry in EGFR Are Usually Progenitor Cells, and the EGFR^{high} Daughter Cell Is Frequently RC2$^+$

(A–C) In most cases, both daughters in cell pairs asymmetric for EGFR are β-tubulin III$^-$ (A) and Nestin$^+$ (B). The EGFR$^+$ daughter cell is usually RC2$^+$ (C). (D) Confocal images of E17.5 cortex show colocalization of EGFR and RC2. Scale bars, 15 μm (A–C) and 30 μm (D). LV, lateral ventricle; ctx, cortex.

and glia, consistent with the rarity of this phenotype at late stages (Qian et al., 2000; Walsh and Reid, 1995). Small neuronal clones developing from restricted neuroblasts showed no difference in their lineage trees when grown in EGF versus FGF2 medium (data not shown). However, glial lineages were different in EGF versus FGF2 (Figure 6). When cultured in EGF, clone size was smaller than in FGF2 and, surprisingly, some E15.5 cortical gliogenic progenitors showed asymmetric lineage trees, a pattern typical of neurogenic progenitors (Qian et al., 1998). In contrast, all gliogenic progenitors grown in FGF2 had symmetric, proliferative lineage patterns, as reported previously (Qian et al., 1998; Qian et al., 2000). We know that 93% of E15.5 EGFR$^+$ cortical cells cultured for 24 hr have one of the FGFR subtypes, FGFR2, which allows them to respond to FGF2, and that the majority of EGFR$^+$ progenitor cells can divide in FGF2, so that differences in lineage patterns are less likely to be due to different sets of progenitor cells dividing in EGF versus FGF2. Rather, this result, combined with our previous observations, suggests that asymmetric distribution of EGFR occurs

Table 2. Neural Markers in Asymmetric EGFR Pairs from E16–E17 LeX Enriched Cortical Cells

Cell Marker	Marker Description	% in EGFR$^+$ Sibling Cell	% in EGFR$^-$ Sibling Cell
β-tubulin III	Neuronal progenitor cell and postmitotic neuron	0 (n = 2)	5.8 ± 5.8% (n = 2)
Nestin	Neural progenitor cell	91.7 ± 3.4% (n = 2)	86.2 ± 2.1% (n = 2)
RC2**	Radial glial cell	68.5 ± 11.1% (n = 4)	37.2 ± 11.9% (n = 4)
MMA*	Neural stem cell/neural progenitor cell	74. 1 ± 8.3% (n = 2)	48.4 ± 16.4% (n = 2)
GLAST*	Astrocyte progenitor and astrocyte	47.2 ± 29.2% (n = 3)	21.5 ± 18.7% (n = 3)
Oligo1/2	Oligodendrocyte progenitor cell/neural progenitor cell	55.8 ± 19.9% (n = 2)	47.1 ± 26.0% (n = 2)
NG2	Oligodendrocyte progenitor cell/neural stem cell	0 (n = 3)	0 (n = 3)
PDGFRα	Oligodendrocyte progenitor cell	0 (n = 3)	0 (n = 3)
O4	Preoligodendrocyte cell	0 (n = 2)	0 (n = 2)

EGFR$^+$ and EGFR$^-$ daughters show different profiles of neural markers.
χ^2 analysis: *$p < 0.05$, **$p < 0.01$; n, number of experiments (>15 EGFR asymmetric pairs for each experiment).

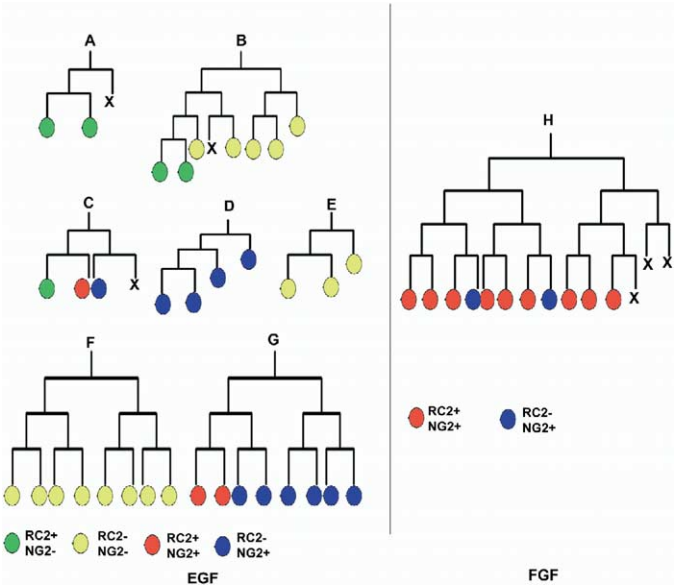

Figure 6. Time-Lapse Lineage Trees Showing Clones Developing from E15.5 Cortical Progenitor Cells in EGF or in FGF2

Isolated cortical progenitor cells were cultured in 20 ng/ml EGF (A–G) or in 10 ng/ml FGF2 (H). Clones were recorded using time-lapse video microscopy for up to 4 days, then stained for β-tubulin III, NG2, and RC2. Lineage trees were reconstructed from the recorded images and immunostaining. All terminal progeny shown are β-tubulin III$^-$. X = dead cell. Note that lineages A, B, D, and E show asymmetric patterns in EGF similar to cortical neuroblast lineages; all lineages in FGF2 were proliferative as shown in lineage H, but had asymmetric patterns revealed by phenotypic differences in progeny.

repeatedly during growth of these clones, while FGFR distribution does not, so that the asymmetry in the proliferation pattern is only revealed when they are grown in EGF without FGF2.

We found that the glial progeny of E15.5 cortical progenitors grown in FGF2 fell into two categories: RC2$^+$NG2$^+$ and RC2$^-$NG2$^+$; the latter are likely to be early oligodendrocytes. Clones growing in EGF contained a different phenotype: RC2$^-$NG2$^-$. These cells had a similar morphology to the RC2$^-$NG2$^+$ oligodendrocytic cells seen in FGF-containing medium, with small cell bodies and bipolar or multiple processes. Given that most cortical progenitors in vivo and in vitro are initially RC2$^+$ (Gotz et al., 2002; Hartfuss et al., 2001; our unpublished data), this suggests that while down-regulation of RC2 occurs effectively without added FGF2, acquisition of NG2 is FGF2 dependent, consistent with previous data showing that FGF2 stimulates oligodendrocyte differentiation (Kessaris et al., 2004).

When we reconstructed lineage trees from clones grown in FGF2, we found that, despite the overall symmetry of the tree, there was an underlying asymmetric pattern to production of these different RC2, NG2-expressing cells (Figure 6). Thus, even in the presence of FGF2, the cells were undergoing asymmetric divisions to generate astrocyte and oligodendrocyte progeny.

Taken together, these data suggest that asymmetric distribution of EGFR in late cortical progenitor cells in vitro is a point of divergence of astrocyte and oligodendrocyte lineages, so that the EGFR^{high} daughter retains expression of RC2 and later becomes an astrocyte, while the EGFR^{low} daughter cell has the capacity to generate oligodendrocytes.

Overexpression of EGFR Can Promote Astrocyte Generation and Differentiation at the Expense of Oligodendrocytes in the Presence of EGF

To find out whether differences in EGFR levels might determine the astrocyte/oligodendrocyte lineage choice, we overexpressed EGFR via retroviral infection (Figure 7A). In vivo, overexpression of EGFR stimulates astrocyte generation, but an influence on oligodendrocyte lineage cells has not been described (Burrows et al., 1997). E14–E15 cortical cells were plated at clonal density, and on the second day in vitro, one group was infected with control virus and another group, with EGFR virus; the groups were grown in medium containing either 10 ng/ml FGF2 or 10 ng/ml FGF2 plus 20 ng/ml EGF. Using 0.5–1.0 × 10^6 cfu/ml retrovirus concentration, 50%–70% cortical progenitor cells were infected. After 6 to 7 days in culture, clones were fixed and stained with RC2, GFAP, NG2, or O4.

The EGFR construct is tagged with eGFP, which allows it to be visualized but does not appear to alter its ability to be ligand activated (Carter and Sorkin, 1998). We found that in retroviral infected cells, EGFR^{eGFP} is distributed on the membrane, as is the endogenous receptor seen in control cells. E14 cortical progenitor cells infected with EGFR retrovirus and grown in FGF2+EGF gave rise to smaller clones, usually containing two to six cells, compared to FGF2 alone (Figure 7B). However, there was no difference in clone size between those infected with control virus grown in either growth medium and the EGFR^{eGFP} virus group grown in FGF2 alone. This finding is consistent with previous studies showing that increased EGFR signaling in late progenitor cells can actually reduce proliferation (Burrows et al., 1997).

To examine whether there was a difference in final fate in EGFR overexpressing cells, infected clones were immunostained for astrocyte and oligodendrocyte markers (Figures 7C–7G). In E15.5 cortical cells cultured for 7 days, the frequency of O4$^+$ cells was similar in the control virus group grown with FGF2 (80.3% ± 20.5%) and the EGFR^{eGFP} virus group grown with FGF2 (72.7% ± 23.3%), showing that oligodendrocyte generation was not altered by overexpression of EGFR in the absence of ligand. However, the frequency of O4 cells was sig-

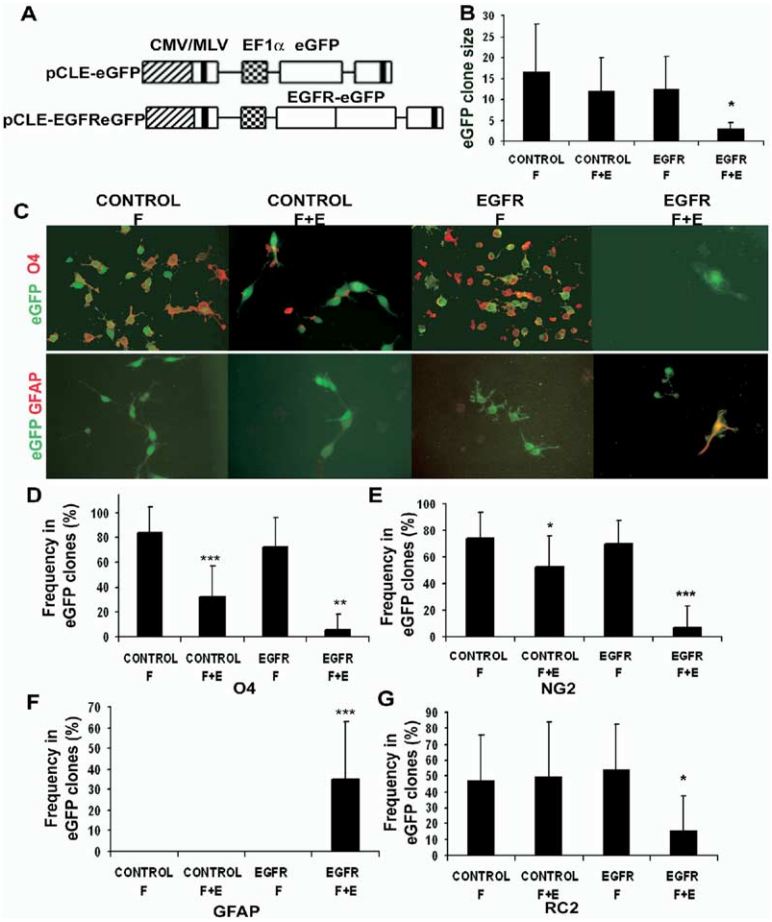

Figure 7. Retroviral Overexpression of EGFR in E14–E15 Cortical Progenitor Cells

(A) Schematic of retroviral constructs for control and EGFR. (B) The size of E14 cortical eGFP$^+$ clones (number of cells) after 7 days of culture: comparison of control versus EGFR retroviral infections in different media. (C) O4 (oligodendrocyte lineage cells) and GFAP (astrocytes) in eGFP$^+$ cell clones with control or EGFR virus. O4 expression is suppressed, and GFAP expression is enhanced in EGFR overexpressing cell clones growing in EGF. (D–F) Comparison of the frequency of O4, NG2, GFAP, and RC2$^+$ cells in eGFP$^+$ clones from 7 day cultures of E14 cortical cells. CONTROL = control virus; pCLE = original retrovirus; EGFR = EGFR virus; F = FGF2; E = EGF; F+E= FGF2 + EGF. MANOVA, Newman-Keuls test, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. For each condition, $\geq$3 experiments; for each experiment, $\geq$20 clones analyzed. Error bars represent the mean ± SD.

nificantly decreased in the control virus group grown in FGF2+EGF (32.0% ± 25.5%; MANOVA, Newman-Keuls test: $p < 0.001$) and further decreased in the EGFR virus group grown in FGF2+EGF (5.9% ± 12.2%; MANOVA, Newman-Keuls test: $p < 0.01$) (Figures 7C and 7D). Consistent with this, the NG2$^+$ population showed a similar change among the four groups (Figure 7E), demonstrating that with increased EGFR signaling, even in control cells, oligodendrocyte generation decreases and that overexpression of EGFR magnifies this decrease. In contrast, we found the opposite effect on astrocyte differentiation with increased EGFR signaling. Very few GFAP$^+$ cells appeared in control groups and in the EGFR^{eGFP} virus group grown in FGF2 medium at the 7 day time point. However, when EGFR^{eGFP} overexpressing cells were grown in FGF2+EGF, they showed significantly increased GFAP expression (35.0% ± 28.0%; MANOVA, $p < 0.001$; Figures 7C and 7F) and reduced RC2 expression (Figure 7G; MANOVA, $p < 0.05$). The control group showed no significant change in the frequency of RC2$^+$ or GFAP$^+$ cells in each clone grown in FGF2+EGF compared to FGF2. Thus, increased EGFR signaling via EGFR overexpression induces premature astrocyte generation from progenitors, corroborating findings in vivo (Caric et al., 2001). It is interesting that in control cells, addition of EGF is unable to stimulate early astrocyte production. This is consistent with a dose-dependent effect of EGFR described previously (Burrows et al., 1997): increasing EGFR signaling by raising ligand concentration stimulates progenitor cells to divide and slowly differentiate into astrocytes, but higher levels of signaling from overexpression of EGFR results in cessation of proliferation and premature astrocyte differentiation.

These data show that increased EGFR expression can induce astrocyte generation at the expense of oligodendrocytes in late cortical progenitor cells in a ligand-dependent manner. We speculate that overexpression of EGFR prevents progenitor cells from effectively segregating EGFR during mitoses. The presence of high EGFR on a normally EGFR^{low} daughter may prevent this cell from generating alternative lineages and push it into the astrocyte lineage.

Discussion

Extracellular factors play essential roles in neural cell fate determination. Some of these, such as FGF2 and sonic hedgehog, act as morphogens, which are distributed in a gradient and evoke different cell responses at high versus low concentrations. This ligand-based patterning mechanism is important for regionalization of large areas of the embryo. At the same time, diverse progeny have to be generated within the same local

environment from an individual progenitor. In this case, asymmetric cell division plays a key role. One mechanism for generating such divisions is by unequal segregation of cell-intrinsic determinants. This does not exclude the role of environmental factors, but rather regulates the ability of cells to respond to them. Here, we describe another mechanism for generating asymmetric cell divisions: unequal distribution of surface receptors during mitosis. We show that EGFR can be asymmetrically distributed during cortical progenitor cell divisions, and we provide evidence that this asymmetry, in the presence of EGFR ligand, can be a point of progenitor cell divergence.

EGFR Asymmetry during Mitosis Is Revealed by a Number of Independent Methods

Immunostaining of E13–E17 forebrain sections reveals that EGFR is asymmetrically distributed in dividing cortical progenitor cells in both the VZ and SVZ. Of dividing cells expressing EGFR, approximately 20% show asymmetry. We might expect the frequency of asymmetric divisions to be low, given that at this stage restricted precursors that undergo symmetric divisions predominate. Nevertheless, since the frequency is low, it was important to confirm this finding by a number of independent methods. There are several lines of supporting evidence: (1) EGFR asymmetric distribution occurs in forebrain in vivo at different stages; (2) it can be seen in vitro; (3) introduced human EGFR-eGFP fusion construct shows asymmetric distribution; (4) Alexa488-conjugated EGF ligand reveals asymmetric binding on sibling cells that correlates with EGFR levels seen with antibody staining; (5) BrdU incorporation, cell migration analyses, and progenitor marker expression support functional differences that correlate with EGFR asymmetry in cell pairs just a few hours after mitosis; and (6) there is evidence for repeated asymmetric segregation of EGFR in late-stage progenitors, seen by the generation of asymmetric lineage trees in EGF. Taken together, these findings strongly support this mechanism for generating differences in neural progenitor sibling cells.

The Asymmetric Localization of EGFR during Cortical Cell Divisions Is an Actin-Dependent Process

Numb and Prospero asymmetry occurs at metaphase, and Cornetto asymmetry, at anaphase (Bulgheresi et al., 2001). EGFR asymmetric distribution occurs at late stages of mitosis, usually anaphase or telophase, although rarely, asymmetry at metaphase is observed in vivo. In *Drosophila*, the asymmetric segregation of Numb, Prospero, and Cornetto is actin dependent (Bulgheresi et al., 2001; Knoblich et al., 1997). Treatment of *Drosophila* embryos with Latrunculin A prevents asymmetric localization of Numb and Prospero. In contrast, other asymmetric determinants, e.g., Oskar mRNA, Bicoid mRNA, and Staufen, are distributed by a microtubule-dependent mechanism (Brendza et al., 2000). We found that treatment with Latrunculin A completely inhibits the asymmetric localization of EGFR in cultured cortical cells, resulting in an accumulation of EGFR in the center of the cell. This shows that like Numb, Prospero, and Cornetto asymmetry, EGFR asymmetry depends on F-actin.

Although EGFRs are primarily localized at the cell surface, they constantly undergo intracellular vesicular trafficking, including internalization and recycling through the cell, even in the absence of ligand (Wiley, 2003). The distribution of asymmetric determinants during mitosis depends on the creation and maintenance of distinct subcellular domains, and this involves polarized vesicle trafficking and retention mechanisms. Hence, it is possible that asymmetric distribution of EGFR depends on the polarized trafficking of EGFR-containing vesicles, a process requiring the normal F-actin network.

Interactions between EGFR and Numb

Given that Numb is asymmetrically localized at an earlier stage of mitosis than EGFR and that Numb has a possible binding site for EGFR, it was possible that there was a dependence on Numb for EGFR localization, at least at midgestation when their asymmetry was highly correlated. However, our observations that Numb and EGFR asymmetry do not always coincide and that EGFR asymmetry is the same in Emx1^{IREScre} Numb and Numblike knockout mice (Li et al., 2003) versus wild-type mice, show that this is not the case.

Nevertheless, there is likely to be a functional implication of sibling cells exhibiting asymmetry in EGFR and/or Numb. It is possible to create, at a single mitosis, cells with diverse levels of ability to respond to Notch and EGFR ligands. Numb could also interact with EGFRs via its endocytic role: Numb can be seen in vesicles containing EGFR, and Numb overexpression inhibits EGFR endocytosis in CV1 fibroblasts (Santolini et al., 2000). It is difficult to predict how EGFR endocytosis might influence EGF signaling, given that it can reduce or increase it depending on context (Wiley, 2003). However, it is clear that the ability to generate cells with different levels of EGFR could add to the diversity of progenitor cell response by generating further combinations of receptor signaling.

Model for the Role of EGFR Asymmetry during Cortical Development

In the cortex, EGFR expression increases at E13, around peak neurogenesis and before gliogenesis (here, as in most CNS regions, neurons arise before glia). Around this time, EGF-reponsive stem cells arise (Kornblum et al., 1997; Tropepe et al., 1999). Ligand distribution also alters during development: from E12 in the rat, TGFα is produced largely from basal forebrain and the choroid plexus (Kornblum et al., 1997), while from E17, heparin binding EGF is expressed in the SVZ (Nakagawa et al., 1998) and by developing neurons in the cortical plate (Kornblum et al., 1999).

At E13–15, cortical divisions that are asymmetric for EGFR usually occur in the VZ, are mostly parallel to the ventricular surface, and result in Numb asymmetry, with Numb moving into the EGFR^{high} daughter (Figure 8). While Numb can sustain the progenitor phenotype (Petersen et al., 2002; Petersen et al., 2004; Verdi et al., 1999), it can also stimulate neuroblast differentiation in vertebrates, analogous to its role in *Drosophila* neural development (Li et al., 2003; Verdi et al., 1999; Waka-

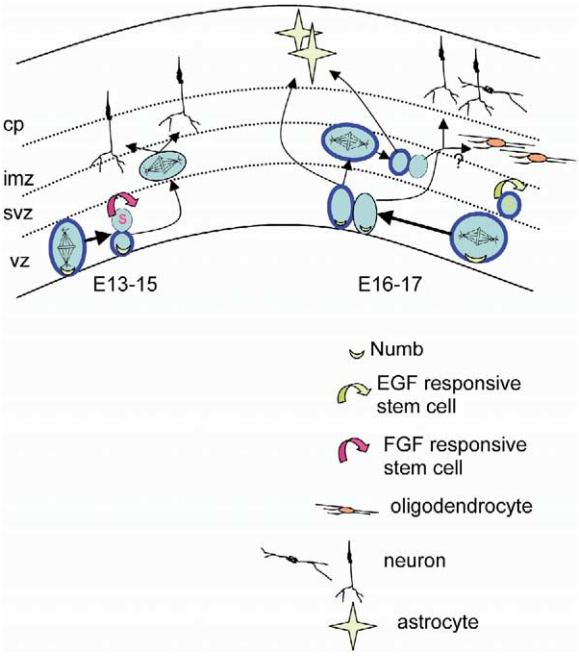

Figure 8. Model Describing the Role of Asymmetric EGFR Distribution during Cortical Progenitor Mitoses

Blue indicates EGFR; vz, ventricular zone; svz, subventricular zone; imz, intermediate zone; cp, cortical plate.

matsu et al., 1999), and we found that in cultured cortical progenitors at midgestation, Numb preferentially moves into the differentiating neuronal daughter at P/N divisions (Shen et al., 2002). Overexpression of EGFR in cortical progenitor cells in vivo causes them to migrate to the SVZ, toward localized ligand, indicating that translocation of progenitors from the VZ to the SVZ compartments may be EGFR dependent (Burrows et al., 1997; Caric et al., 2001). The SVZ is now appreciated as a major location for terminal mitoses of restricted neuroblasts (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). We saw that the EGFR^{high} daughter exhibits further EGF-dependent migration than its EGFR^{low} sister. Hence, one possibility is that, at around E13, asymmetric segregation of EGFR into the VZ daughter that receives Numb will stimulate its translocation to the SVZ to undergo its final divisions and then differentiate. Because we rarely saw asymmetric EGFR divisions at terminal divisions produce a progenitor and a neuron, and EGFR was weak in the SVZ at E13–14, we believe that asymmetric distribution of EGFR is infrequent in SVZ neuroblasts. If this scenario does occur in vivo, the EGFR^{high} cell must rapidly lose EGFR expression once it reaches the SVZ.

The cell that remains in the VZ has FGFR, but lacks Numb, so it has active Notch and is therefore likely to continue as a progenitor; it might be either a multipotent neuroblast or a multipotent FGF-dependent stem cell. At all stages examined, we also observed a low frequency of EGFR^{high} Numb^{-} daughters. These may retain progenitor cell status, becoming EGF-responsive stem cells.

We found that at later stages, E16–17, most VZ cell divisions that are EGFR-asymmetric occur perpendicular to the cell surface and have symmetric Numb (Figure 8). Again, EGFR acquisition might help one daughter to translocate to the SVZ. High levels of EGFR stimulation, especially in late-stage cells, slow their proliferation, probably as part of the astrocyte differentiation step (Burrows et al., 1997; our unpublished data). Hence, asymmetric EGFR segregation at this stage, along with Numb acquisition, may help ensure that the EGFR^{high} daughter begins to differentiate into an astrocyte and translocates toward the cortical plate and higher ligand concentration. At E16–17, asymmetric EGFR cell divisions also occur in the SVZ, usually parallel to the nearby ventricle surface. We speculate that the EGFR^{low} cells generated in the VZ or SVZ may take on a nonastrocytic fate, either neuronal, or, possibly, oligodendroglial.

The Role of EGFR Asymmetry in the Oligodendrocyte/Astrocyte Fate Choice

In cultured asymmetric EGFR pairs from E16–E17 cortex, the EGFR^{high} daughter usually had progenitor/astrocyte lineage markers, while the EGFR^{low} daughter usually lacked them. It is possible that the EGFR^{low} daughter could later reexpress astrocyte markers. However, the simplest explanation is that the sibling cells have become different, and that after losing progenitor/astrocyte markers, the newly generated EGFR^{low} daughter would go on to acquire a different fate. Our studies suggest that in vitro it likely acquires oligodendrocyte characteristics, stimulated by FGF2 signaling. We know that after longer periods of culture, cells derived from these progenitors are RC2^{-}NG2^{+} and EGFR^{-}. The fact that EGFR overexpression pushes the cells into the astrocyte pathway at the expense of oligodendrocytes strengthens the idea that creating cells lacking EGFR is important for this fate choice in cultured cells.

While progenitors in vitro can robustly produce both oligodendrocytes and astrocytes, these bipotent cells are found rarely in vivo, comprising only 10%–17% of the progenitor population (Luskin and McDermott, 1994; Parnavelas, 1999; Zerlin et al., 2004). It is, of course, possible that cells exhibiting glial bipotency are rare, because once the lineages diverge, they may be amplified by symmetric divisions of restricted progenitors. Moreover, EGFR asymmetry is a relatively rare event. We found that NG2 overlaps rarely with EGFR in vitro and in embryonic forebrain sections, indicating that from early stages, oligodendrocyte lineage cells don't express this receptor, which is consistent with the recent finding that PDGFRα-expressing progenitors in basal forebrain lack EGFR (Chojnacki and Weiss, 2004). Overexpression of EGFR in vivo pushes cortical progenitor cells toward the astrocyte lineage (Burrows et al., 1997; Caric et al., 2001), but whether this happens at the expense of oligodendrocyte generation has not been examined, and knockouts of EGFR die too early to determine an effect on oligodendrogenesis (Sibilia et al., 1998; Threadgill et al., 1995). Hence, it will be important in the future to determine whether glial divergence in the developing forebrain in vivo has a dependence on EGFR asymmetric distribution.

In conclusion, these data show that the EGFR can be asymmetrically segregated in dividing neural progenitor cells in vivo and in vitro and can generate functionally different sister cells in the presence of ligand. EGFR ligands are present in germinal zones and are secreted from the choroid plexus into the cerebrospinal fluid; hence, an effective way to reduce EGFR signaling in the presence of abundant ligand is simply to remove the surface receptor. Asymmetric distribution of surface receptors during mitosis is another means of generating receptor complexity on CNS progenitor cells, allowing divergent proliferative, migratory, and differentiation responses that contribute to the emerging cellular complexity.

Experimental Procedures

Tissue Dissociation and Cell Culture

Cerebral cortices from timed pregnant Swiss Webster mouse embryos (Taconic) at E13–E17 (plug date designated as day 0) were dissociated to single cells using papain (Worthington, 10–12 Units/ml) with 32 mg/ml DNase (Qian et al., 1998). For E15–17 stage cells, progenitors were enriched by LeX labeling and magnetic bead sorting (MACS; Miltenyi Biotech).

Single cells were plated into poly-L-lysine coated Terasaki wells in serum-free medium: DMEM (Gibco) with L-glutamine, sodium pyruvate, B-27, N-2 (Gibco), 1 mM N-acetyl-cysteine (Sigma). 10 ng/ml FGF2 (invitrogen) and/or 20 ng/ml EGF (invitrogen) was added as a mitogen, unless otherwise indicated. Plated cells were incubated at 35°C with 6% CO2 and 100% humidity.

Immunohistochemistry

For cryostat sections (6–10 μm thick), E13–E17.5 mouse embryo brains were fixed for 3–4 hr in 4% paraformaldehyde (PFA) in PHEM buffer (Rieder and Bowser, 1985). For immunostaining of EGFR, sections were blocked in 5% fetal bovine serum (FBS) in PBS with 0.1% Triton X-100 (PBT) and incubated with sheep anti-EGFR antibody (Caric et al., 2001) (Upstate Biotechnology, 1:50) (a specific antibody that recognizes a single band corresponding to EGFR by Western blot) at 4°C overnight. Immunoreactivity was visualized using cy3 donkey anti-sheep antibody (Jackson ImmunoResearch, 1:500) for 45 min or biotinylated donkey anti-sheep IgG secondary antibody (Jackson ImmunoResearch, 1:200) in 0.1% PBT for 1.5 hr and then Alexa488-conjugated streptavidin (Molecular Probes; 1:200) in PBT for 45 min. Counterstaining was done with propidium iodide (Molecular Probes; 1 mg/ml in PBS for 5 min).

For immunostaining of cultured cells, cells were fixed in 4% PFA in PHEM buffer for 15 min and rinsed in PBS for 5 min three times. The remaining staining procedure was the same as above, but using PBS instead of 0.1% PBT.

The following primary antibodies were used: FGFR2 (Bek, rabbit, Santa Cruz Biotech, 1:200), RC2 (mouse, Developmental Studies Hybridoma Bank [DSHB], 1:2), β-tubulin III (mouse, Sigma, 1:400), Nestin (mouse, DSHB, 1:4), BrdU FITC (mouse, Becton Dickinson, 1:10), NG2 (rabbit, Chemicon, 1:400), Numb (rabbit, W. Zhong, 1:400), O4 (mouse, DSHB, 1:2), PDGFRα (rabbit, A. Nishiyama, 1:20,000), Oligo1/2 (guinea pig, D.J. Anderson, 1:20,000), CD15/LeX (mouse, Becton Dickinson, 1:10), GFAP (rabbit, DAKO, 1:400), and GLAST (guinea pig, Chemicon, 1:4000).

Pair Analyses: EGFR Labeling, BrdU Labeling, and Cell Migration Assays

Daughter cell pair analysis was performed as previously described (Shen et al., 2002). Briefly, single cortical cells were plated at 30–40 cells per Terasaki well for 3–4 hr, and then the position of each cell was mapped. After overnight culture (for E13 to E14) or 30 hr culture (E15 to E17—older cells divided more slowly), pairs of daughter cells were identified on the basis of their original mapped positions, then fixed and stained.

EGFR Ligand-Labeling Assay

10 μg/ml Alexa488 EGF complex (Molecular Probes, recommended concentration) was added to cell pair cultures. After incubating at 35°C for 5–10 min, cells were fixed and stained for EGFR.

BrdU Incorporation

Cells were cultured with 20 ng/ml EGF (Invitrogen). After cortical pairs were identified, 10 μg/ml BrdU were added to the wells for 8–10 hr. Cells were washed, fixed in 4% PFA, stained for EGFR, and then stained with anti-BrdU antibody.

Migration Assay

E17 LeX MACS-enriched cortical progenitor cells were plated in EGF medium. Images were taken of each well after 3–4 hr, 18 hr, and 24 or 32 hr. Cells were then fixed and stained with EGFR antibody. After asymmetric pairs for EGFR were identified, for each, the initial pair image and the final prefixation image were overlaid using Photoshop 5.0. The midpoint between the initial pair was assigned as the start position for each daughter cell. A line was drawn from the start position to the final position and measured as an indicator of migration.

Visualization of EGFR Distribution with eGFP Fusion Construct

Dissociated E11–E13 cortical cells were transfected with p-eGFP-N1-EGFR (gift from A. Sorkin) 2–3 hr after plating with Fugene 6 (Roche) (1 μg cDNA:6 μl Fugene 6). The medium was changed to culture medium 24 hr later, and cells with eGFP expression were observed under the fluorescence microscope. The transformed cells were identified, and dividing cells were recorded by taking images during mitosis.

EGFR-eGFP Retroviral Construct and Infection

For EGFR retroviral construction, the IRES and hPLAP in the pCLE retroviral vector (gift from N. Gaiano) was replaced with EGFR-eGFP from p-eGFP-N1-EGFR or with eGFP for control virus. The EcoPack2-293 (Clontech) packaging cell line produced retrovirus with Lipofectamine Plus (Invitrogen). Viral titers were determined using NIH3T3 cells. For infection, E14–E15 cortical cells were plated at 50–60 cells per Terasaki well. Virus was added 24 hr later at 0.5–1.0 × 10^6 cfu/ml. After 6–7 days of culture, cells were fixed and stained.

Supplemental Data

Supplemental data include Supplemental Experimental Procedures, one figure, two movies, and Supplemental References and can be found with this article online at http://www.neuron.org/cgi/content/full/45/6/873/DC1/.

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