Brief Communication

Identification of a *Pax6*-Dependent Epidermal Growth Factor Family Signaling Source at the Lateral Edge of the Embryonic Cerebral Cortex

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In an emerging model, area patterning of the mammalian cerebral cortex is regulated in part by embryonic signaling centers. Two have been identified: an anterior telencephalic source of fibroblast growth factors and the cortical hem, a medial structure expressing wingless-int (WNT) and bone morphogenetic proteins. We describe a third signaling source, positioned as a mirror image of the cortical hem, along the lateral margin of the cortical primordium. The cortical antihem is identified by gene expression for three epidermal growth factor (EGF) family members, Tgfα, Neuregulin 1, and Neuregulin 3, as well as two other signaling molecules, Fgf7 and the secreted WNT antagonist Sfrp2. We find that the antihem is lost in mice homozygous for the *Small eye* (*Pax6*) mutation and suggest the loss of EGF signaling at least partially explains defects in cortical patterning and cell migration in *Small eye* mice.

Key words: **cortical patterning; Tgfα; Neuregulin; Sfrp2; Fgf7; *Small eye*; Pax6; Emx2**

Introduction

Recent studies indicate that regional specification and growth control of the cerebral cortex is initiated by signaling centers operating on an originally homogeneous embryonic field (Fukuchi-Shimogori and Grove, 2001; Ragsdale and Grove, 2001; Hebert et al., 2002; Garel et al., 2003). Anteroposterior position is conferred by an anterior fibroblast growth factor (FGF) source (Fukuchi-Shimogori and Grove, 2001), and mediolateral pattern is regulated by the cortical hem, a medial signaling center enriched in bone morphogenetic proteins (BMPs) and wingless-int (WNT) proteins (Grove et al., 1998; Lee et al., 2000; Ragsdale and Grove, 2001; Hebert et al., 2002). Given the size and complexity of the final cortical area map, however, additional cortical signaling sources are almost certain to exist.

We hypothesized that the lateral edge of the cortical primordium, where the dorsal and ventral telencephalon meet, might be the site of an additional signaling center. The lateral cortical margin is readily identified by gene expression for a WNT antagonist, the secreted frizzled-related protein Sfrp2 (see Fig. 1) (Ragsdale et al., 2000; Kim et al., 2001). *Sfrp2* expression is detected in this territory as early as embryonic day 10.5 (E10.5) (data not shown) and, by E12.5, describes a curve that laterally mirrors the medial cortical hem, itself identifiable by the expression of multiple *Wnt* genes (see Fig. 1*A*). Together, these territories mark the lateral and medial limits of the cortical ventricular zone (VZ) (see Fig. 1*C*,*D*). Two-color *in situ* hybridization experiments suggest a pincer arrangement between the hem and the *Sfrp2*-rich antihem; the two territories are well separated in rostral telencephalon but approach one another to meet in the caudal telencephalon (see Fig. 1*C*).

If the antihem is an important embryonic signaling center, it is expected from studies of other cortical signaling centers to express multiple members of at least one secreted signaling molecule family. Two lines of research prompted a survey of epidermal growth factor (EGF) family members. First, a classic series of *in vitro* experiments implicates EGF family members in the development of cerebral cortical areas linked to the limbic system (Ferri and Levitt, 1993; Levitt et al., 1997). The limbic system-associated membrane protein LAMP is expressed in limbic cortical areas (Levitt et al., 1997) and is upregulated in cells from non-limbic cortical domains in response to EGF family ligands (Ferri and Levitt, 1995). Second, EGF receptor-mediated signaling controls dorsoventral neuronal specification in the development of the *Drosophila* ventral nerve cord (Skeath, 1998; von Ohlen and Doe, 2000). Two EGF ligands are involved in this dorsoventral signaling: Spitz, a *Tgfα*-like molecule, and Vein, which is similar in structure to the Neuregulin proteins, a subfamily of vertebrate EGF ligands (Golembo et al., 1999).

Materials and Methods

Gene expression patterns were studied in CD-1 mice, *Small eye* (*Sey*, or $Pax6^{Sey\text{-}Neu}$) mice maintained on a C3H/He background, and *Emx2*-targeted mice maintained on a C57BL/6 background. The day of plug discovery was designated E0.5. *In situ* hybridization of E9.5–E18.5 forebrains and genotyping of the *Sey* and *Emx2* mice were performed as described previously (Pellegrini et al., 1996; Xu et al., 1997; Grove et al., 1998). Antihem marker genes were studied with mouse cDNAs for *Areg*/

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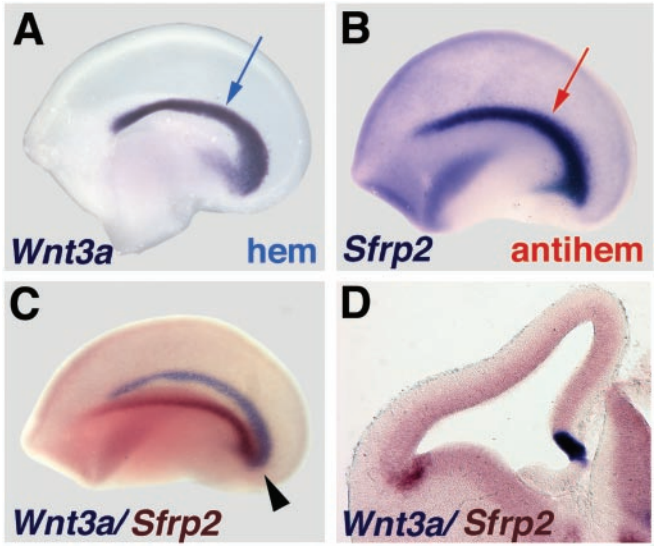

Figure 1. Location of the cortical hem and antihem. *A–C*, E12.5 cerebral hemispheres, processed with one- or two-color *in situ* hybridization and viewed from the medial (*A*) or lateral (*B*, *C*) faces. Anterior to the left. *D*, Coronal section through E12.5 cerebral hemisphere processed with two-color *in situ* hybridization. *A*, *B*, The "swoosh" of the medial cortical hem (*A*, blue arrow) is mirrored by the lateral antihem (*B*, red arrow). Together, they form a pincer arrangement around the cortical primordium (*C*). The cortical hem is marked by strong *Wnt3a* expression (purple in *A*, *C*,*D*), and the antihem is identified by expression of *Sfrp2* (purple in *B*; reddish-brown in *C*, *D*). Arrowhead (*C*) indicates meeting of the hem and antihem in the caudal cerebral hemisphere.

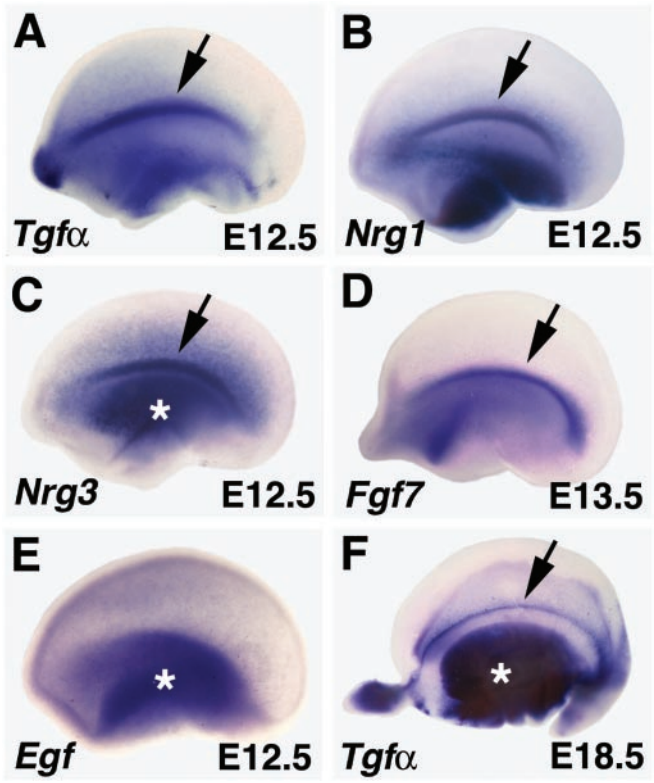

Figure 2. EGF family members are expressed in the cortical antihem. *A–E*, Embryonic cerebral hemispheres viewed from the lateral face; anterior to the left (*A–C*, *E*, E12.5; *D*, E13.5). *F*, E18.5 hemisphere viewed from the inside looking laterally. *A–C*, Peaks of expression of *Tgfα*, *Nrg1*, and *Nrg3* mark the curving longitudinal lateral band of the antihem (arrows in *A–C*). *Tgfα* expression is maintained for several days in this position (arrow, *F*). *D*, *Fgf7* is also expressed in the antihem at E13.5. *E*, The founding member of the EGF family, *Egf* itself, is expressed in the ventral telencephalon and in the cortical primordium without a peak of expression at the antihem. Asterisks mark expression in ventral telencephalon (*C*, *E*, *F*).

Amphiregulin (Das et al., 1995), *Egf* (Pascall and Brown, 1988), *Hegfl*/*Heparin-binding EGF-like growth* factor/*Diphtheria toxin receptor* (IMAGE Consortium, GenBank accession number W80035), *Fgf7*/*Keratinocyte growth factor* (IMAGE Consortium, GenBank accession number BF159111), *Nrg1* (IMAGE Consortium, GenBank accession number AI197081), *Nrg2* (IMAGE Consortium, GenBank accession number AW476657), *Nrg3* (Zhang et al., 1997), *Nrg4* (IMAGE Consortium, GenBank accession number AA238077), *Sfrp2* (Rattner et al., 1997), *Tgfa*/*Tgfα* (Vaughan et al., 1992; Kornblum et al., 1997), *Tmeff1*/*Transmembrane protein with EGF-like and two follistatin-like domains 1* (IMAGE Consortium, GenBank accession number BF147745) and *Tmeff2*/*Tomoregulin* (IMAGE Consortium, GenBank accession number AI098476), and a rat cDNA for *Ereg*/*Epiregulin* (Taylor et al., 1999).

Results

We examined mRNA expression of 11 EGF family members: Egf itself, Tgfα, Neuregulins 1–4, Amphiregulin, Epiregulin, Heparin-binding EGF-like growth factor, Tmeff1, and Tmeff2. Our screen disclosed that the lateral margin of the cortical primordium is enriched in expression of three Spitz and Vein homologs, *Tgfα*, *Neuregulin1* (*Nrg1*), and *Nrg3*. At E12.5, *Tgfα* expression marks the antihem, which appears as a curve of strong *Tgfα* expression when viewed from the lateral face of the cortical primordium (Figs. 1,2*A*). Coronal sections show that *Tgfα* expression is in the VZ of proliferating cells (Fig. 3). *Nrg1* and *Nrg3* show overlapping high expression in the same curving band (Fig. 2*B*,*C*). All three genes display some graded expression in the cortical VZ, which is most pronounced in *Nrg3* preparations. For both *Nrg* genes, expression in the cortical primordium increased as development proceeded (last age examined, E18.5). For all three genes, however, the cortical antihem is the peak of expression in the cortical primordium.

Other EGF ligands, including *Egf* itself, were at least weakly detected in the embryonic telencephalon (Figs. 2*E*, 3*K*,*L*) (Kornblum et al., 1997) but not concentrated in the antihem. At E10.5, E12.5, and E14.5, *Amphiregulin*, *Egf*, *Epiregulin*, *Hegf1*, *Nrg2*, *Nrg4*, *Tmeff1*, and *Tmeff2* are expressed in the ventral telencephalon, usually increasing in intensity with age. Most EGF family members are expressed in the dorsal telencephalon from E12.5 onward, with the exception of *Nrg2*, which is detectable at E10.5. No striking patterns of expression were detected except for *Epiregulin* and *Tmeff1*, which display expression gradients in far lateral and medial embryonic cortex.

In a screen of *Fgf* gene expression in and near the cortical primordium, we found that *Fgf7* gene expression marks the antihem by E13.5 (Fig. 2*D*). Transient embryonic expression of *Fgf7* was noted previously in a lateral embryonic cortical region (Mason et al., 1994). Thus, together with *Sfrp2*, five secreted signaling molecules are expressed along the lateral edge of the cortical primordium.

The cortical hem is part of the true cortical primordium as characterized by progenitor cell behavior and gene expression (Grove et al., 1998). *Tgfα* expression at the antihem also lies within cortical primordium, defined by expression of *Neurogenin2* (*Ngn2*) (Fig. 3*A*,*B*; two-color *in situ* data not shown). *Sfrp2* expression is within the *Tgfα* domain and marks the extreme margin of the cortical primordium (Fig. 3*G*; *Ngn2*/*Sfrp2* two-color *in situ* data not shown). *Fgf7* expression, which is strongest in the posterior antihem, overlaps that of *Sfrp2* (Fig. 3*J*). In contrast, expression of *Nrg1* and *Nrg3* is not restricted to the cortical VZ but stretches into the VZ of the basal forebrain (Fig. 3*C*,*H*,*I*).

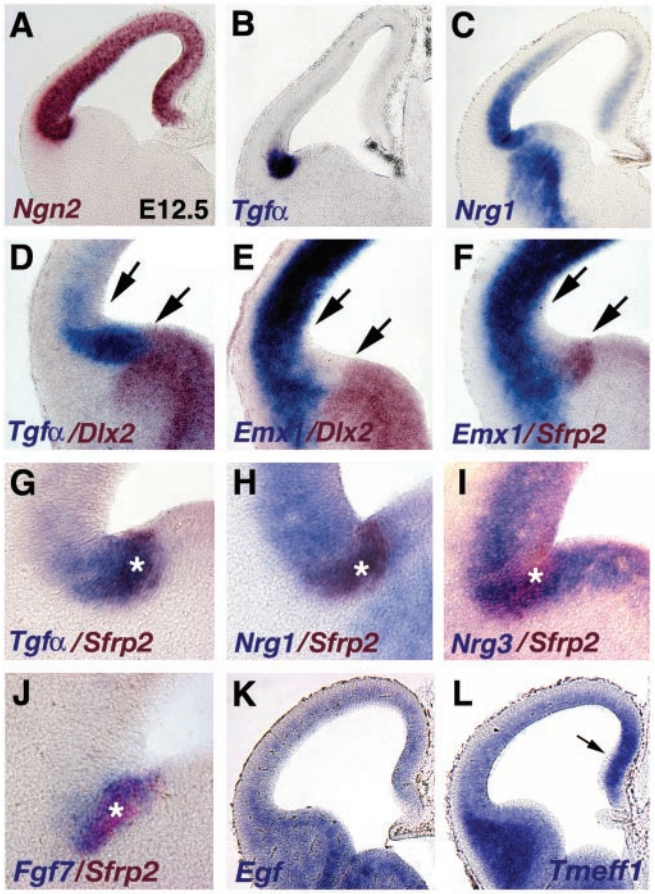

Figure 3. Location of the antihem relative to the transition between dorsal and ventral telencephalon. *A–L*, Coronal sections through E12.5 and E13.5 (*J*) cerebral hemispheres processed for one- or two-color *in situ* hybridization. *A*, *Ngn2* expression marks the boundaries of cortical neuroepithelium. *B*, The *Tgfα*-expressing domain lies within the cortical primordium (compare *A*, *B*). *C*, *Nrg1* expression peaks in the *Tgfα*-rich domain but extends as a decreasing gradient into dorsolateral cortical neuroepithelium, with an additional zone of expression in the medial cortical primordium. *D–F*, At E12.5, a wedge-shaped territory lacks expression of *Emx1* or *Dlx2* (between arrows). This territory is filled by *Tgfα* expression (*D*), with *Sfrp2* and *Fgf7* expression nested in the *Tgfα* domain (*F*, *G*, *J*). *Tgfα*, *Fgf7*, and *Sfrp2* expression remain within cortical neuroepithelium at this site (*D*, *F*, *J*), but *Nrg1* and *Nrg3* extend into the VZ of the basal telencephalon (*H*, *I*). Asterisks (*G–J*) mark the *Sfrp2*-expressing zone, the most lateral margin of the cortical neuroepithelium. *K*, *L*, At E12.5, *Egf* (*K*) and *Tmeff1* (*L*), like other EGF family members, do not show gene expression peaks in the cortical antihem. Arrow (*L*) notes increased *Tmeff1* expression in medial embryonic cortex, as seen in *Nrg1*-labeled sections (*C*).

The transcription factor genes *Emx1* and *Dlx2* are differentially expressed in dorsal and ventral telencephalon. At E12.5, the boundaries of expression of *Emx1* and *Dlx2* leave an intermediate wedge clear of expression of either gene in the VZ of the lateral margin of the cortical primordium (Fig. 3*E*) (Fernandez et al., 1998). Two-color *in situ* hybridization shows that this wedge-shaped zone is antihem territory: it is filled by dense expression of *Tgfα*, and *Sfrp2* expression marks its ventral limit (Fig. 3*D–G*). Although the cells that compose the antihem do not express *Emx1* at E12.5, they appear to do so at a later stage of development (Fernandez et al., 1998). Genetic fate mapping of *Emx1*-expressing cells suggests that cells in this region give rise to portions of the amygdala and lateral cortex (Gorski et al., 2002).

The antihem shows both similarities and differences with other cortical signaling sources. For example, expression of EGF family genes are not detectable in the antihem at E10.5, an age at which *Fgf*, *Wnt*, and *Bmp* expression is prominent at other cortical signaling sources. *Nrg1* and *Tgfα* are only barely detectable in the lateral cortical primordium at E11.5. Moreover, EGF gene expression does not overlap as neatly as the expression of multiple *Wnt* genes in the cortical hem or even of *Fgf* genes at the anterior pole. Particularly for the *Nrg* genes, mRNA expression extends as a gradient from the antihem into the more dorsolateral cortical VZ. Nonetheless, the antihem is similar to other signaling sources in representing a peak of expression of several members of a single signaling molecule family.

The *Small eye* (*Sey*) homozygote mutant mouse lacks function of the transcription factor Pax6 and displays impaired cortical neurogenesis, cell migration, and patterning (Chapouton et al., 1999; Bishop et al., 2000; Stoykova et al., 2000), as well as localized defects in the antihem region, losing *Sfrp2* expression (Wawersik et al., 1999; Ragsdale et al., 2000; Kim et al., 2001). *Tgfα* and *Nrg1* expression is also lost in the antihem (Fig. 4*A*,*B*,*E–H*), suggesting a complete absence of this signaling center in the homozygote mutant. Because of a possible general developmental delay in the mutant, these mice and littermate controls were analyzed for gene expression from E12.5 to E16.5 for *Sfrp2* ($n = 9$ homozygotes; $n = 18$ controls), *Nrg1* ($n = 6$ homozygotes; $n = 10$ controls), and *Tgfα* ($n = 12$ homozygotes; $n = 21$ controls). At E14.5, *Tgfα* expression marks the antihem in controls but is eliminated in homozygote mutants (Fig. 4*A*,*B*,*E*,*F*). Expression of *Nrg1* in the lateral cortical VZ is almost undetectable in the homozygote *Sey* mouse, with the result that the gradient of expression is reversed, medial to lateral, with highest *Nrg1* expression in the hippocampal primordium (Fig. 4*H*, asterisk). Dense expression of *Nrg1* appears at the antihem region in littermate mice at E14.5 (Fig. 4*E*), with a stronger gradient in the rest of the cortical primordium than in CD-1 mice (compare with Fig. 2*B*).

Mice lacking the transcription factor Emx2 also show widespread defects in neurogenesis and patterning (Bishop et al., 2000; Mallamaci et al., 2000) with concomitant defects in signaling sources (Muzio et al., 2002). WNT signaling in the cortical hem region is affected, as is anteroposterior gene expression of the FGF receptor *Fgfr3* (Muzio et al., 2002), suggesting that signaling along both mediolateral and anteroposterior axes is abnormal. We found that the antihem, marked by *Tgfα* expression, is retained in the *Emx2* homozygote mutant but appears dorsally displaced (Fig. 4*C*,*D*; data not shown).

Discussion

Several roles can be hypothesized for the cortical antihem. A likely possibility is that the antihem serves as a barrier between the dorsal and ventral telencephalon, in both pattern formation and cell migration. Localized Sfrp2 may limit the spread of WNT signaling between dorsal and ventral telencephalon (Ragsdale et al., 2000; Kim et al., 2001). More broadly, both at the antihem and within the cortical primordium, EGFs may antagonize BMP and WNT signaling. Genetic studies of *Drosophila* development show that EGF receptor-mediated signaling attenuates both Decapentaplegic (BMP) and Wingless (WNT) signals (O'Keefe et al., 1997; Szuts et al., 1997; Kubota et al., 2000). Thus, the lateral EGF source may assist in maintaining a distinction between the dorsal and ventral telencephalon and in regional patterning of the cerebral cortex. The latter role is supported by the ability of *Tgfα* to convert nonlimbic to limbic cortex *in vitro* (Ferri and Levitt, 1995).

EGFs may also regulate cortical cell migration. In rodents, a large proportion of cortical interneurons derived from the ventral telencephalon migrate past the region of the antihem to populate the cortical primordium (Anderson et al., 2001). During fly

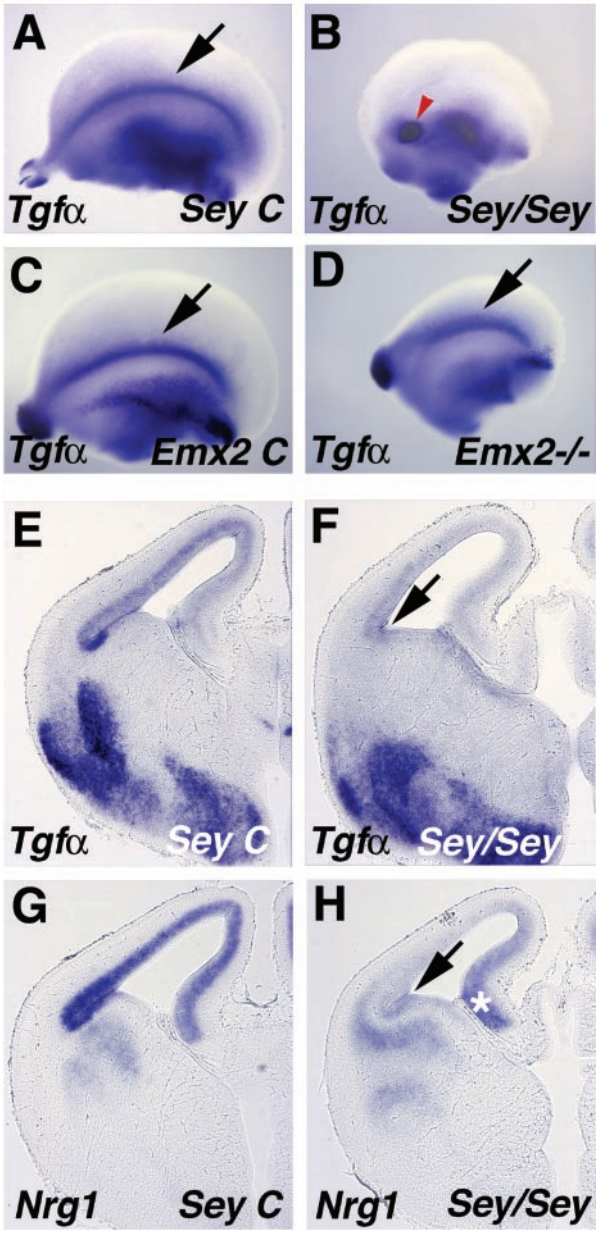

Figure 4. The antihem is lost in the *Small eye* mouse. *A–D*, Cerebral hemispheres viewed from the lateral face; anterior to the left. *E–H*, Coronal sections through E14.5 cerebral hemispheres. *A*, *B*, *E*, *F*, The antihem marked by *Tgfα* expression is missing in an E14.5 mouse homozygous for the *Small eye* mutation (*B*, arrow in *F*) but is present in a littermate control (arrow in *A*, *E*). The spot of *Tgfα* labeling in *Sey* homozygote mice (red arrowhead in *B*) is likely the olfactory bulb remnant (Jimenez et al., 2000). *C*, *D*, A *Tgfα*-expressing antihem is present in an E13.5 *Emx2* homozygote mutant mouse (*D*, arrow) and littermate control (*C*, arrow) but appears dorsally displaced in the mutant (*D*). *G*, *H*, *Nrg1* expression is missing from the antihem region (arrow in *H*) and dorsolateral cortical neuroepithelium in an *Sey/Sey* mouse at E14.5, but expression in the hippocampal primordium remains (asterisk).

oogenesis, the *Tgfα*-like ligand Gurken guides dorsal migration of border cells (Duchek and Rorth, 2001). Both overexpression of EGF family ligands and misexpression of a constitutively activated form of the EGF receptor inhibit border cell migration (Duchek and Rorth, 2001). Therefore, by analogy with fly developmental mechanisms, the antihem region in mouse, rich in EGF family members, could promote migration of the correct ventral telencephalic cells or inhibit the migration of incorrect cells. In retroviral studies of rodent telencephalic development, Caric et al. (2001) have found that increasing the EGF receptor levels in VZ cells promotes their radial migration away from the VZ. Thus, neurons born in the antihem region, which are thought to migrate toward ventrolateral cortical areas (Bayer et al., 1991), could be guided in part by the EGF ligands of the antihem.

Each of the above hypotheses receives support from analysis of the *Sey/Sey* mutant mouse, which lacks functional Pax6 and also appears to lack an antihem. First, patterning defects in the presumptive area map are seen in *Sey/Sey* cerebral cortex just before birth, with shrinkage of lateral and rostral cortical domains (Bishop et al., 2000). Second, gene expression patterns normally confined to the ventral or dorsal telencephalon transgress their usual boundaries in the *Sey/Sey* mutant; thus, the dorsoventral identity of the region around the antihem becomes ambiguous (Stoykova et al., 2000; Toresson et al., 2000; Yun et al., 2001). Third, ventral to dorsal cell migration is enhanced in the mutant, suggesting that incorrect cells are crossing the ventrodorsal telencephalic boundary and that Pax6 is essential to allow the correct cells to immigrate (Chapouton et al., 1999). We propose that Pax6 regulates development of the antihem and associated EGF ligand gradients and that the patterning and migration defects in the *Sey/Sey* mutant are at least in part mediated by the loss of the antihem. Finally, mice that lack Emx2 show a subtler, but consistent, abnormality in this region. The antihem is present but shifted dorsally (this report), and LAMP expression, marking limbic cortex, is dorsally displaced in parallel (Mallamaci et al., 2000).

The antihem differs from other signaling centers by expressing signaling molecules later than other centers and in a more graded manner. However, these features may be significant to its potential cortical patterning function. As the cortical primordium grows larger, it becomes more difficult to explain how patterning could occur according to the classic model of a morphogen diffusing over an embryonic field with a width of 0.5 mm or less (Wolpert, 1969; Gurdon et al., 1994). Yet, cortical pattern remains labile relatively late in corticogenesis, when the embryonic cortex is larger than a typical embryonic field (Ragsdale and Grove, 2001). One way to prolong cortical patterning would be to introduce secondary signaling sources that generate a gradient of signaling protein directly. Thus, a gradient of EGF mRNA expression with a peak at the antihem may directly set up a patterning gradient of EGF proteins in the older and larger cortical primordium without a need for long-distance protein diffusion.

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