Developmental Biology 351 (2011) 25–34

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Radial glia regulate Cajal–Retzius cell positioning in the early embryonic cerebral cortex

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ARTICLE INFO

Article history:
Received for publication 1 September 2010
Revised 12 December 2010
Accepted 13 December 2010
Available online 23 December 2010

Keywords:
Cajal–Retzius cell
Radial glia
Neuroepithelial stem cell
Cerebral cortex
β1 integrin
Cell ablation

ABSTRACT

The organization of neocortex, along its radial axis, into a six-layered structure is one of the most exquisite features of the brain. Because of their strategic localization in the marginal zone, and their expression of reelin, a signal that controls spatial ordering of cortical layers, Cajal–Retzius (C-R) cells play a crucial role in cortical patterning along this axis. Yet, it remains less well understood how C-R cell targeting itself is regulated. At the onset of corticogenesis when C-R cells first arrive in the cortex via tangential migration, radial glia (RG) are the main cell type present. This suggests that RG may play a role in C-R cell localization. To test this, we used genetic approaches to perturb RG scaffold during early corticogenesis. We found that disrupting RG endfoot adhesion to basal lamina consistently results in C-R cell displacement. These displacements do not appear to result from primary defects in neural progenitor cell proliferation, deficits in the meninges or basement membrane, or cell autonomous defects in C-R cells. Instead, they show close temporal and spatial correlation with RG endfoot retraction. Moreover, ablation of RG via cell cycle blockade similarly results in local displacement of C-R cells. These lines of evidence thus indicate that, during early corticogenesis, RG play a primary role in regulating spatial targeting of C-R cells. Since RG are also neural progenitors as well as neuronal migration scaffolds, these findings suggest that, during nervous system development, neuroepithelial stem cells may not only be responsible for generating a diverse array of neuronal cell types and facilitating their radial migration. They may also, through regulating the placement of guidepost cells, coordinate spatial patterning of the nervous system along its radial axis.

Introduction

The nervous system originates from a single layer of neuroepithelium. During development, stem cells in the neuroepithelium, according to their positions along the anterior–posterior (A-P) and dorsal–ventral (D-V) axes, produce a specific array of neuronal cell types (Alexander et al., 2009; Jessell, 2000). In many parts of the nervous system, this single layer of neuroepithelium also transforms into a multi-layered tissue, where distinct neuronal cell types are arranged in a spatially coordinated manner along its radial axis (Rakic, 1978). Major progress has been made in the past decades in understanding how the neuroepithelium is patterned along the A-P and D-V axes. For example, key signaling inputs and transcriptional mechanisms have been identified that regulate the segmental expression of *Hox* genes along the A-P axis of the hindbrain (Alexander et al., 2009). Critical insights have also been gained into mechanisms by which morphogens regulate neuronal fate determination along the D-V axis of the ventral spinal cord (Jessell, 2000). By contrast, relatively little is known about how nervous system patterning is regulated along the radial axis.

In the mammalian forebrain, neocortical neurons assemble into six distinct layers along the radial axis. The proper arrangement of cortical plate neurons in an inside-out pattern depends on the function of *reelin* (Caviness and Rakic, 1978; Rice and Curran, 2001; Soriano and Del Rio, 2005). *reelin* encodes a large extracellular glycoprotein predominantly expressed by a unique group of cells in the marginal zone (MZ), the C-R cells (Alcantara et al., 1998; Ogawa et al., 1995). Mutations in *reelin* severely disrupt the normal pattern of cortical lamination, resulting in a roughly inverted order of layers II-VI (D'Arcangelo et al., 1995; Hirotsune et al., 1995). These findings suggest that C-R cells play a crucial role in neocortical patterning along the radial axis. Investigation into mechanisms that regulate C-R cell localization may thus not only improve understanding of neuronal migration during corticogenesis, but also provide insights into nervous system patterning along the radial axis.

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doi:10.1016/j.ydbio.2010.12.026

C-R cells are a heterogeneous population of neurons that originate from several sources outside the neocortex (Meyer, 2010), which include the cortical hem, ventral pallium, and septum (Bielle et al., 2005; Takiguchi-Hayashi et al., 2004; Yoshida et al., 2006; Zhao et al., 2006). Previous studies have shown that cortical meninges play a key role in C-R cell localization. Indeed, genetic and pharmacological perturbation of meningeal chemokine Cxcl12 (also known as SDF-1) signaling resulted in defects in C-R cell positioning (Borrell and Marin, 2006; Paredes et al., 2006b; Stumm et al., 2003). However, only subsets of C-R cells appear affected under these conditions, suggesting involvement of other signals. At the onset of corticogenesis when C-R cells first arrive, RG progenitors are the main cell type present in the cortex. This suggests that RG may play a role in C-R cell positioning. In support, previous studies suggest that C-R cells interact not only with cortical basement membrane (BM) but also with RG (Soriano et al., 1997; Super et al., 1998). Although C-R cell displacement associated with BM breakage is usually taken as evidence for meningeal BM involvement, it does not rule out a role by RG, since disruption of RG scaffold and BM breakage are commonly linked to each other during development. Lastly, in the chick retina, studies have shown that it is the endfoot of RG, but not the basal lamina, that provides guidance for the layer-specific outgrowth of retinal ganglion cell axons (Bauch et al., 1998; Stier and Schlosshauer, 1995). Thus, these findings suggest that cortical RG may not only serve as neural progenitors and neuronal migration scaffolds (Anthony et al., 2004; Kriegstein and Alvarez-Buylla, 2009; Malatesta et al., 2000; Noctor et al., 2001; Rakic, 1972, 2007), but may also play a role in C-R cell targeting. In this article, we describe results from genetic approaches that implicate such a primary role for RG during early corticogenesis.

Materials and methods

Mouse strains

The *emx1-cre* or *wnt3a-cre* transgene was introduced into mice homozygous for *β1 integrin* or *orc3* conditional allele for phenotypic analyses. Homozygous animals without *cre* or heterozygotes with *cre* were employed as controls. Stage of embryos at noon on the day of vaginal plugs is designated as 0.5 day old. 5-Bromo-2-deoxyuridine (BrdU), at 100 μg/g body weight, was injected intraperitoneally into pregnant females at desired stages and animals sacrificed 4 h later for tissue collection and analysis. Animal use was in accordance with UW institutional guidelines.

Primary antibodies

The following primary antibodies were used: anti-BrdU monoclonal supernatant (clone G3G4, Developmental Studies Hybridoma Bank (DSHB), University of Iowa, IA; 1:40), rabbit anti-calretinin (Chemicon, Temecula, CA; 1:1000), mouse anti-chondroitin sulfate (clone CS56, Sigma, St. Louis, MO; 1:100), rabbit anti-laminin (Sigma; 1:2000), mouse rat-401 (anti-Nestin) monoclonal supernatant (DSHB; 1:20), anti-RC2 monoclonal supernatant (DSHB; 1:20), mouse anti-reelin monoclonal (Millipore, Temecula, CA; 1:500), rabbit anti-SDF-1β (Torrey Pines Biolabs Inc., East Orange, NJ; 1:100), and rabbit anti-phospho-histone H3 (Ser10) (Millipore, Temecula, CA; 1:400).

Histology and immunohistochemistry

Histology and immunohistochemistry were carried out essentially as described previously (Huang et al., 2006). Briefly, embryonic and perinatal brains were fixed overnight in 4% paraformaldehyde in PBS at 4 °C and processed by Vibratome sectioning at 50 μm thickness. Sections were blocked with 10% goat serum, 2.5% BSA, 1% glycine, and 0.4% Triton X-100. Primary antibodies were incubated overnight at 4 °C and followed by FITC and/or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) for 4–5 h at room temperature or overnight at 4 °C. Sections were then mounted with ProLong Antifade (Invitrogen Molecular Probes, Carlsbad, CA) and used for image collection under a Nikon *eclipse* Ti microscope. Identical image collection parameters were used for control and mutant specimens.

Results

β1 integrin deletion by emx1-cre results in RG endfoot retraction during early corticogenesis

To evaluate potential roles of RG in C-R cell localization, we took advantage of a conditional knockout allele of *β1 integrin*, to perturb RG scaffold in the developing cortex. Previous studies have shown that deletion of *β1 integrin* using a *nestin-cre* results in RG endfoot detachment, BM breakage, and C-R cell mis-localization at perinatal stages (Graus-Porta et al., 2001). However, since C-R cell displacements at these stages are associated with both RG endfoot detachment and meningeal BM breaches, it is unclear whether RG play a direct role.

To specifically assess the role of RG, we employed *emx1-cre*, a *cre* line expressed in early cortical progenitors starting around embryonic day (E) 9.5 (Gorski et al., 2002), 2–3 days earlier than *nestin-cre* (Graus-Porta et al., 2001; Tronche et al., 1999). We reasoned that early deletion of *β1 integrin* may allow us to temporally separate defects in RG from those in the BM, thereby teasing out potential specific contribution by RG. Indeed, we found that deletion of *β1 integrin* using *emx1-cre* results in dramatic retraction of RG endfeet at an early stage, in the absence of obvious defects in cortical BM. Endfoot retraction first appears around E14.5. At E15.0, it becomes more severe and spreads across the cortex (Fig. 1 and Supplemental Fig. 1). In control cortices at this stage, we observed Nestin-positive RG fibers form a parallel and regularly spaced scaffold, endfeet tightly adhered to the BM (Fig. 1A). By contrast, in many areas of mutant cortex, RG endfeet completely detach from the pial surface and retract within the cortex, leaving significant portions of the upper cortical plate devoid of RG processes (Fig. 1B). Inside the cortical plate, many RG processes also appear wavy, frequently crisscrossing each other. The overall organization of cortical wall, however, appears intact, with well-demarcated cortical plate, intermediate, and ventricular zones (compare Fig. 1B′ to A′, data not shown). Similar observations were also made with another RG marker, RC2 (Supplemental Fig. 1). Of note, in control specimens, we found an expansive RC2 positive web overlying the cortex, corresponding to sites of RG endfeet at the pia. By contrast, in areas of mutant cortex where RG endfeet have retracted, this positive web appears completely absent. The early RG endfoot detachment results in significantly more severe defects in cortical development in *β1 integrin /emx1-cre* than *nestin-cre* mutants (Supplemental Fig. 2). This is consistent with the earlier onset as well as higher levels of *emx1-cre* expression (Gorski et al., 2002; Graus-Porta et al., 2001; Huang et al., 2006). Together, these results indicate that, similar to later stages (Graus-Porta et al., 2001), *β1 integrin* plays a critical role in RG endfoot adhesion to the BM and in cortical lamination during early corticogenesis. This therefore provides a unique opportunity for assessing the involvement of RG in C-R cell targeting.

RG endfoot retraction is closely associated with C-R cell displacement

To determine effects of RG endfoot retraction on C-R cell localization, we used an antibody against calretinin to stain C-R cells. In control cortex at E15.5, we found calretinin positive cells tightly packed and evenly distributed in the MZ, their cell bodies juxtaposed to the pia (Fig. 1A′ and A″; see also Supplemental Fig. 1). By contrast, in many regions of mutant cortex, we found that the vast

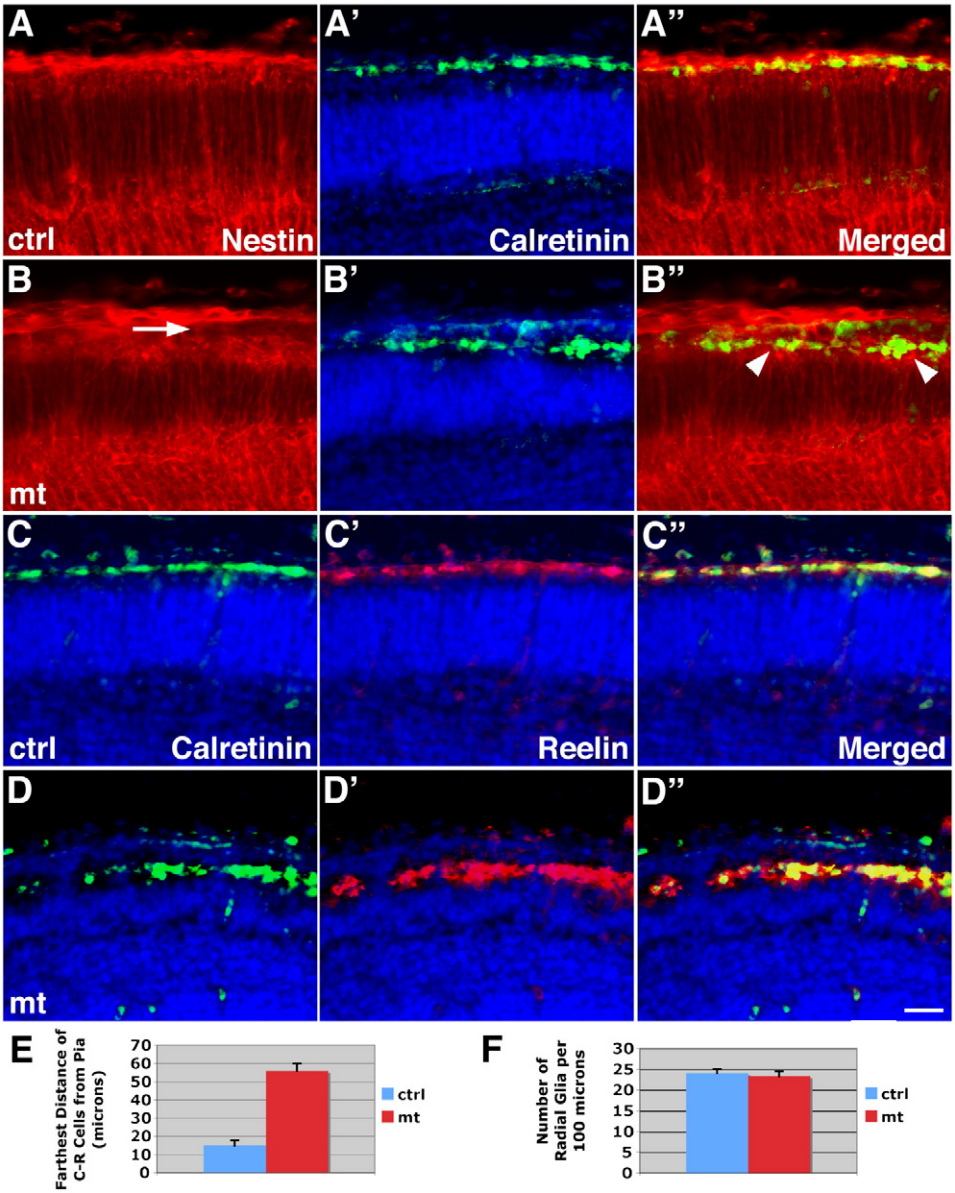

Fig. 1. RG retraction in *β1 integrin/emx1-cre* mutant cortex results in C-R cell displacement. (A–B″) RG morphology and C-R cell localization in *β1 integrin/emx1-cre* control (ctrl) and mutant (mt) cortices at E15.0. In control cortex (A–A″), RG fibers (anti-Nestin, in red) form a parallel and regularly spaced scaffold that reaches the pia, where their endfeet interact with C-R cells (anti-calretinin, in green). Neurons are stained with DAPI (in blue). In mutant cortex (B–B″), RG fibers retract into deeper cortical areas, leaving a gap near the pia (arrow in (B)). Large numbers of C-R cells are concomitantly displaced into deeper cortical areas, where many are still associated with ectopic endfeet (arrowheads in (B′)). (C–D″) C-R cell localization in *β1 integrin/emx1-cre* control (C–C″) and mutant (D–D″) cortices, as determined by anti-calretinin (in green) and anti-reelin (in red) staining. Calretinin and reelin positive cells overlap at normal and ectopic locations in control and mutant cortices, respectively. Scale bar, 50 μm. (E) Quantification of farthest distance of C-R cells from the cortical pia in control and mutant cortices ($n=5$, $P=6.46\times10^{-5}$). (F) Quantification of RG density along the medial–lateral axis in control and mutant cortices ($n=5$, $P=0.62$).

majority of C-R cells are displaced into deeper regions of the cortex (Fig. 1B′ and B″). These displacements appear to correlate closely with sites of RG endfoot retraction, since they are observed only in areas with substantial endfoot detachment but not in areas with normal endfoot adhesion. Quantification shows that in E15.5 control cortex, the maximum distance of C-R cell localization away from the pia is about 15 μms. By contrast, in mutants, C-R cells are frequently displaced from the pia by over 50 μms (Fig. 1E). These results thus suggest that RG scaffold may play a critical role in C-R cell targeting during early stages of corticogenesis.

C-R cells are known for their specific expression of reelin (Alcantara et al., 1998; Ogawa et al., 1995). To corroborate the identity of dislocated cells, we next employed an antibody against reelin (Fig. 1C–D″). In control cortex, we found that, consistent with normal C-R cell localization, reelin and calretinin stainings almost completely overlap near the pia (Fig. 1C–C″). By contrast, in mutants, the vast majority of calretinin and reelin positive cells appear ectopically localized in the middle of cortical plate. At these ectopic locations, however, reelin and calretinin stainings still overlap with each other (Fig. 1D–D″). These findings therefore indicate that the displaced cells are indeed C-R cells. Interestingly, at their ectopic locations, many C-R cells still appear associated with RG endfeet, similar to that observed near the pia in controls (Fig. 1B″, arrowheads; Supplemental Fig. 1). This therefore further supports the interpretation that RG closely interact with C-R cells and may play a role in their localization.

β1 integrin deletion by emx1-cre does not affect RG density or identity

Epithelial cell adhesion to the BM is crucial for cell proliferation. To evaluate whether *β1 integrin* deletion may affect RG proliferation and

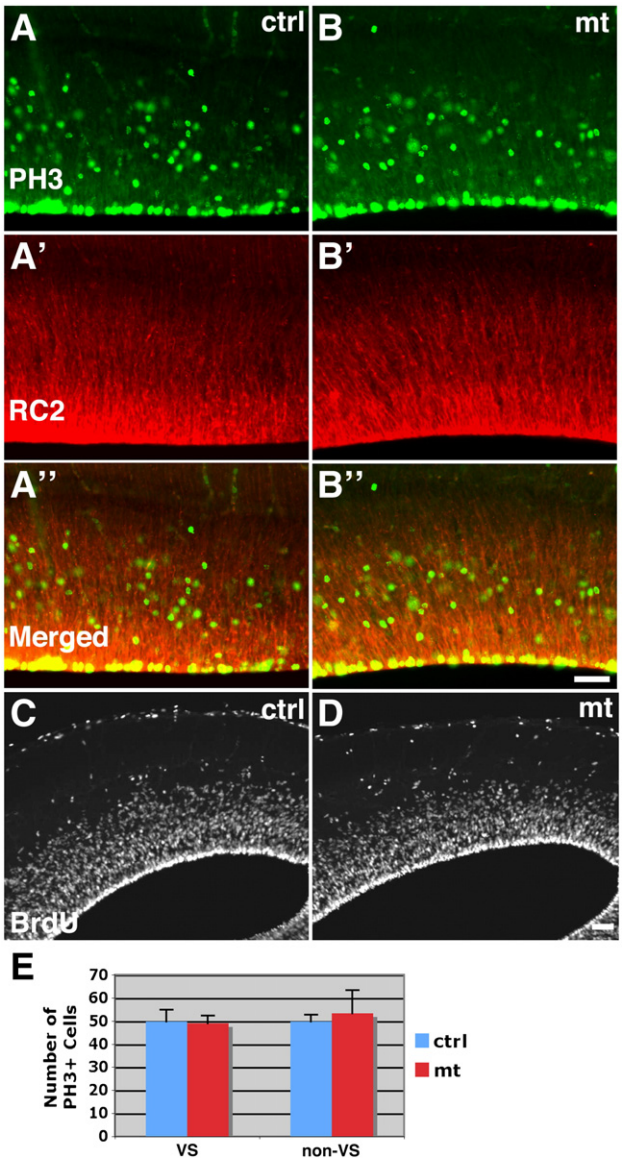

Fig. 2. Normal neural progenitor cell proliferation in *β1 integrin/emx1-cre* mutant cortex. (A–B″) RG morphology and mitotic cell distribution in *β1 integrin/emx1-cre* control (ctrl) and mutant (mt) cortices at E15.5. In control cortex (A–A″), RG fibers (anti-RC2, in red) form a scaffold that spans the ventricular and subventricular zones. Mitotic neural progenitor cells (anti-phospho-histone 3 (PH3), in green) are found both at the apical ventricular surface (radial glial progenitors) and inside the cortical wall. In mutant cortex (B–B″), a similar pattern of RG staining and PH3 positive cell distribution is observed. (C–D) BrdU labeling in *β1 integrin/emx1-cre* control (C–C″) and mutant (D–D″) cortices at E15.5. A similar pattern of S phase cell distribution is observed between control and mutant. Scale bar, 50 μm. (E) Quantification of numbers of ventricular surface (VS) and non-surface (intermediate progenitor, non-VS) cells in control and mutant cortices ($n=4$, $P>0.75$ for both).

density, and consequently cellular organization of the cortical wall, we first quantified RG density by determining the number of RG fibers per 100 μm of cortical width. In control cortex at E15.5, we found RG density to be around 24 fibers per 100 μms. In mutants, despite severe defects in endfoot attachment and fiber organization, we found RG at a similar density (Fig. 1F). Thus, these results indicate that changes in RG density are unlikely to play a significant role in C-R cell displacement.

Two types of neural progenitors have been identified in the developing cortex (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004): RG progenitors that adhere to and undergo mitosis at the ventricular surface, and basal (intermediate) progenitors that have no direct contact with the ventricular surface and divide in the interior of cortical wall. To determine whether *β1 integrin* deletion may affect the relative proportion of these two cell populations, we stained brain sections with an antibody against phospho-histone 3 (PH3), a mitosis specific marker (Fig. 2A–B″). We found that, in control cortex at E15.5, the vast majority of cells at the ventricular surface appear to be undergoing mitosis, as is the case for a similar number of cells in the interior (Fig. 2A). Interestingly, in *β1 integrin* mutants, we found a similar pattern in the distribution of PH3 positive cells (Fig. 2B). Quantitative analysis confirmed that, between control and mutant, there are no significant differences in the number of mitotic cells either at the ventricular surface or in the interior. In addition, BrdU labeling also showed a similar distribution of S phase cells (Fig. 2C and D). Thus, these results indicate that *β1 integrin* deletion does not substantially affect the distribution of cortical RG or basal progenitors. These observations are in contrast to those made, following acute blockade of *β1 integrin* function using monoclonal antibodies (Loulier et al., 2009), where substantial displacements of RG from the ventricular surface was observed. As pointed out by Loulier et al., these differences may reflect compensation for the long-term genetic loss of one integrin by other heterodimers. Further, we found that expression of several RG specific markers, such as Nestin, RC2, and Pax6, is also unaffected in *β1 integrin* mutants (Figs. 1A and B, and 2A′ and B′, and data not shown). Thus, these results altogether indicate that *β1 integrin* deletion does not substantially affect the density or identity of cortical RG and basal progenitors. They suggest that, among potential defects, it is defects in RG scaffold organization, but not defects in neural progenitor density or identity, that likely play a key role in C-R cell displacement.

C-R cell displacement is not due to defects in cortical meninges or the BM

Recent genetic and pharmacological experiments showed that cortical meninges, especially meningeal mediated Cxcl12 signaling, play an important role in C-R cell localization (Borrell and Marin, 2006; Paredes et al., 2006b). To determine whether meningeal defects may contribute to C-R cell displacement in *β1 integrin* mutants, we used an antibody against laminin to examine cortical BM organization (Fig. 3). In control cortex at E15.5, we observed a thin layer of laminin-positive staining overlying the cortex (Fig. 3A–A″). Interestingly, in many areas of the mutant cortex, despite widespread displacement of C-R cells, we observed a similar pattern of laminin staining and no obvious breaches in the BM (Fig. 3B–B″). Similar observations were also made at E15.0 (data not shown). These findings thus argue that major defects in BM structural integrity are unlikely to play a primary role in C-R cell displacement at these early stages. Since meningeal cells are the main source of cortical BM components (Chen et al., 2009; Yin et al., 2003), these findings also suggest that meningeal expression of BM components is also likely unaffected in *β1 integrin* mutants.

To further assess other potential defects in cortical meninges and BM, we also examined chondroitin sulfate proteoglycan (CSPG) expression (Supplemental Fig. 3). CSPG expression is known to be mainly associated with preplate neurons in the developing cortex, but meningeal cells also appear to express moderate levels (Paredes et al., 2006b; Sheppard et al., 1991). We found that CSPG expression in the meninges is largely normal in mutants at E15.5. The main changes appear to be reduced expression in the MZ, as well as enhanced expression in the subplate. This altered pattern is likely a result of preplate splitting defects, since preplate is known to give rise to the MZ and subplate. In addition, we found that meningeal cell division, as revealed by BrdU incorporation at E15.5, also appears to be largely normal (Supplemental Fig. 3), which suggests unperturbed renewal of meningeal fibroblasts. Thus, together with our earlier results on laminin levels, these findings argue against a major contribution to C-R cell displacement by perturbed meningeal gene expression or cell proliferation.

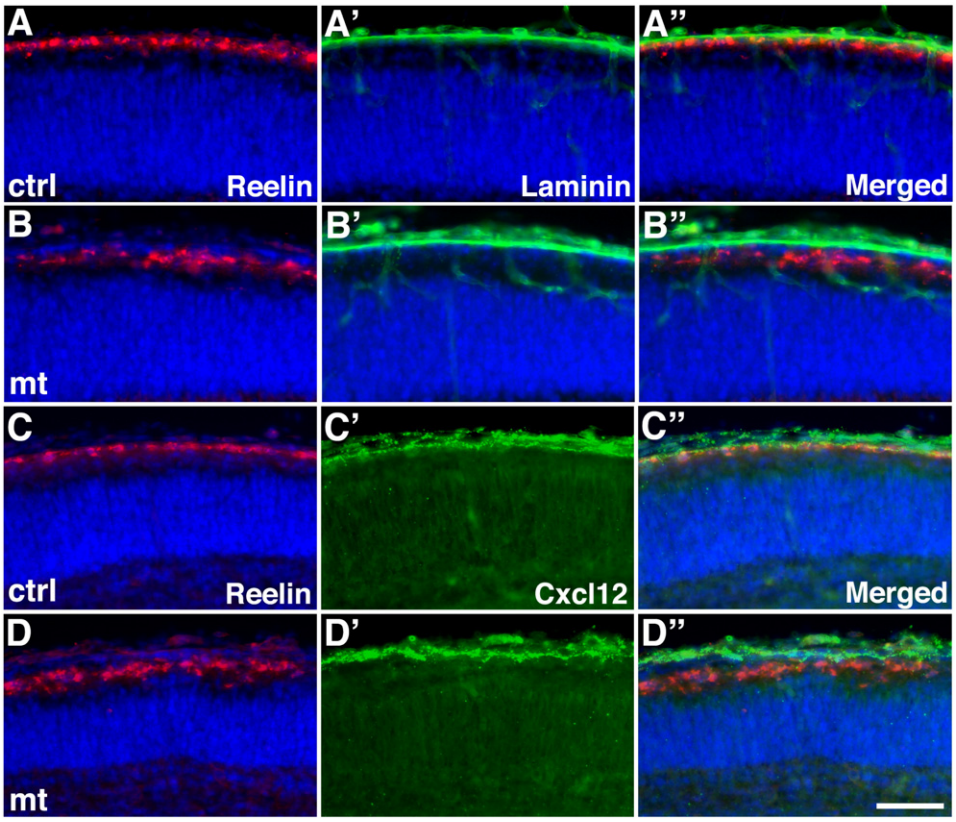

Fig. 3. Normal pial basement membrane organization and Cxcl12 expression at sites of C-R cell displacement in *β1 integrin/emx1-cre* mutant cortex. (A–B″) BM morphology and C-R cell localization in *β1 integrin/emx1-cre* control (A–A″) and mutant (B–B″) cortices at E15.5. Similar stainings for laminin (in green) in the pial BM were observed between control (A′) and mutant (B′), despite C-R cell (anti-reelin, in red) displacement in mutants. (C–D″) Cxcl12 expression and C-R cell localization in *β1 integrin* control (C–C″) and mutant (D–D″) cortices at E15.5. Similar stainings for Cxcl12 (in green) were also observed at the pial meninges between control (C′) and mutant (D′), despite C-R cell (anti-reelin, in red) displacement in mutants. Scale bar, 50 μm.

Meningeal fibroblasts express high levels of Cxcl12, while C-R cells express Cxcl12 receptor Cxcr4 (Borrell and Marin, 2006; Paredes et al., 2006b; Stumm et al., 2003), which suggests regulation of C-R cells by meningeal Cxcl12 signaling. Indeed, perturbations in Cxcl12 signaling by genetic and pharmacological approaches have been found to result in C-R cell displacement (Borrell and Marin, 2006; Paredes et al., 2006b). However, defects under these conditions appear significantly milder than those in *β1 integrin* mutants, affecting only subsets of C-R cells. Thus, it appears unlikely that defects in Cxcl12 signaling, if any, in *β1 integrin* mutants are solely responsible for C-R cell mis-localization. To directly assess involvement of Cxcl12 signaling, we used an antibody against Cxcl12 to evaluate its expression (Fig. 3C–D″). We found that, in control cortex at E15.5, Cxcl12 puncta were prominently associated with cortical meninges (Fig. 3C′–C″), consistent with its reported sites of expression (Borrell and Marin, 2006; Paredes et al., 2006b; Stumm et al., 2003). Interestingly, in mutants, despite severe mis-localization of C-R cells, we observed a similar pattern of Cxcl12 staining (Fig. 3D′–D″). Thus, these results argue against a major contribution by perturbed Cxcl12 signaling. Together, our results in this section have thus ruled out substantial involvement by known meningeal pathways in C-R cell displacement following *β1 integrin* deletion.

C-R cell displacement is not due to cell autonomous requirement for β1 integrin

Besides cortical plate neurons and astrocytes, *emx1-cre* also targets the vast majority of C-R cells, including those derived from the cortical hem and the septum (Bielle et al., 2005; Gorski et al., 2002; Yoshida et al., 2006). Thus, one possibility is that *β1 integrin* may be required in C-R cells for normal localization near the cortical BM. To address this possibility, we employed *wnt3a-cre*, a *cre* line specifically expressed in hem-derived C-R cells, a subpopulation that constitutes their vast majority (Yoshida et al., 2006). We found that *β1 integrin* deletion by *wnt3a-cre* has no obvious effects on either C-R cell localization or cortical lamination (Fig. 4). Thus, these observations indicate that *β1 integrin* is not required in C-R cells for their proper localization. This interpretation is further supported by the fact that *wnt3a-cre* has been shown to efficiently induce loxP site recombination in C-R cells. For example, *wnt3a-cre*-mediated induction of diphtheria toxin expression completely eliminates targeted C-R cells by E12.5 (Yoshida et al., 2006), several days before the stage of our analysis. Thus, we conclude that it is not cell autonomous requirements for *β1 integrin* but changes in cortical environment that are likely responsible for C-R cell displacement in *β1 integrin* mutants.

RG endfoot retraction and scaffold disruption temporally precede C-R cell displacement

Our results so far point to RG scaffold, but not cortical meninges or BM, as a prime candidate in regulating C-R cell targeting during early corticogenesis. If so, one would predict that defects in RG scaffold organization should precede those in C-R cell localization. To test this, we examined RG morphology and C-R cell localization in *β1 integrin* mutant cortex around E14.5, a stage when C-R cell displacements first appear (Fig. 5). We found that, although there are occasional areas with C-R cell displacement, most cortical areas show normal C-R cell localization at this stage (Fig. 5A and B). By contrast, consistent disruptions in RG scaffold were observed across the cortex (Fig. 5A and B′). Unlike the parallel scaffolds observed in controls (Fig. 5A′), RG fibers in many areas of the mutant cortex appear wavy and crisscross each other, especially in the cortical plate (Fig. 5B′). Their loose morphology suggests a lack of proper endfoot attachment. Indeed, we

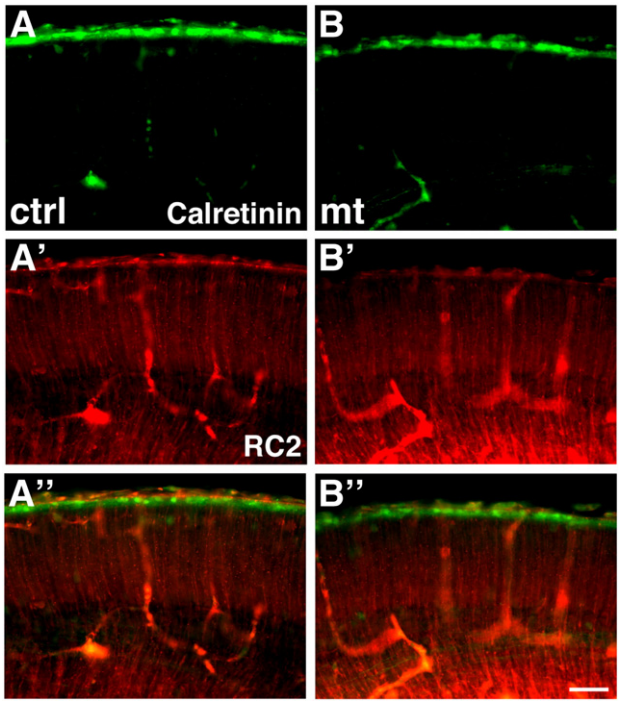

Fig. 4. Normal C-R cell localization and RG scaffold in E15.5 *β1 integrin /wnt3a-cre* mutant cortex. (A–A″) In control cortex, C-R cells (anti-calretinin staining, in green) occupy a thin layer near the pia (A and A″). RG (anti-RC2 staining, in red) form a parallel scaffold spanning the cortical wall (A″ and A″). (B–B″) In *β1 integrin/wnt3a-cre* mutant cortex, where *β1 integrin* is deleted from hem-derived C-R cells, a similar pattern of C-R cell localization and RG organization is observed as in control. Scale bar, 50 μm.

found that the web of RC2 staining normally associated with RG endfeet is substantially reduced in many regions of the mutant cortex (compare Fig. 5B′ to A′, arrows). Thus, these results indicate that RG endfoot retraction precedes C-R cell displacement.

To further assess this interpretation, we next examined RG morphology and C-R cell localization at E15.0, a stage between first appearance of C-R cell displacement (at E14.5) and near full phenotypic manifestation (at E15.5). We found that, in the lateral cortex at this stage, despite substantial disruption in the RG scaffold, C-R cells are localized normally (data not shown). By contrast, in the medial cortex, significant displacement of C-R cells was observed, in association with RG scaffold disruption. This medial-to-lateral gradient of phenotypic progression is consistent with observations made in several other mutants (Beggs et al., 2003; Graus-Porta et al., 2001; Niewmierzycka et al., 2005), where the medial cortex appears more susceptible to perturbations in RG-BM interaction. These observations therefore provide further evidence supporting the interpretation that RG endfoot retraction temporally precedes C-R cell displacement. Thus, together, our results in this section provide additional evidence supporting the conclusion that RG play a primary role in C-R cell localization during the early stages of corticogenesis.

Deletion of orc3 by emx1-cre results in ablation of RG

In *β1 integrin* mutants, many C-R cells are associated with RG endfeet even at ectopic locations, suggesting intimate interactions between these cell types. To assess whether C-R cells may be simply dragged down by retracting endfeet, as well as to further evaluate the role of RG, we sought to perturb RG scaffold, in an independent approach. To this end, we took advantage of a conditional knockout allele of *orc3* (unpublished data), a gene that encodes a core subunit of origin recognition complex (ORC), a protein complex essential for DNA replication from yeast to human cells (Bell, 2002). We chose *orc3* because as neural progenitors, RG are the main neural cell type undergoing division in the developing cortex. Thus, interference with cortical neural cell division may allow RG ablation in a relatively selective manner.

To generate a conditional *orc3* allele, we flanked three coding exons (exons 5–7) in the 5′ end of *orc3* gene with a pair of loxP sites. This design is predicted to generate an early stop codon following *cre*-mediated excision. Indeed, we found that *emx1-cre*-mediated deletion results in a severe loss of Orc3 protein from mutant cortices (Supplemental Fig. 4). On the other hand, animals homozygous for the *orc3* allele are viable and fertile, without obvious phenotypes, indicating that *orc3* locus has been properly targeted.

We found that, consistent with the role of ORC in regulating DNA replication (Bell, 2002), *orc3* deletion by *emx1-cre* resulted in significant reductions in neural progenitor division at E13.5, especially near the ventricular surface (data not shown). However, we observed no obvious changes in RG scaffold at this stage (Fig. 6A and B). Despite occasional appearance of condensed nuclei, Nestin staining revealed RG fibers spanning the entire cortical thickness similarly in mutants as in controls (Fig. 6A′–B′). By E14.5, however, we found severe blockade of neural progenitor division (data not shown). This in turn results in severe loss of RG and consequently frequent gaps in the RG scaffold (Fig. 6C and D). On the other hand, *orc3* deletion by *emx1-cre*, a *cre* line that also targets C-R cells, appears to have no effects on C-R cell production, as substantial numbers of C-R cells were found in the mutant cortex (see Fig. 8). This lack of effects may be due to the early birth of C-R cells and/or the perdurance of Orc3 protein. In addition, *orc3* deletion from C-R cells alone, using *wnt3a-cre*, also has no obvious effects on either C-R cell number or their final localization (data not shown), which further argues against requirements for *orc3* in C-R cells for their migration or targeting. Thus, these results indicate that *orc3* deletion by *emx1-cre* results in a relatively selective RG ablation at E14.5, providing an independent opportunity for assessing their role in C-R cell localization.

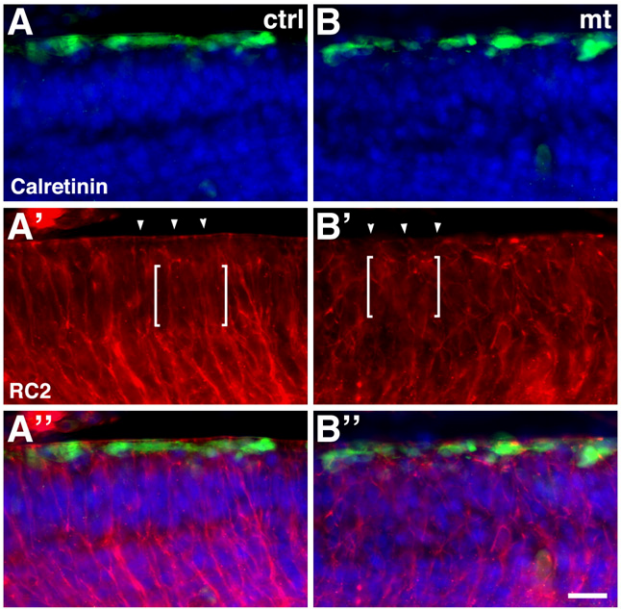

Fig. 5. RG endfoot retraction precedes C-R cell displacement in *β1 integrin/emx1-cre* mutant cortex. (A–A″) At E14.5, a regular array of RG fibers (anti-RC2 staining, in red) is observed in the control cortex (A′), concomitant with a tight layer of C-R cells (anti-calretinin staining, in green) near the pial surface (A). (B–B″) In mutant cortex, RG fibers appear disrupted, do not form a parallel scaffold, and frequently crisscross each other (brackets in (B′), compared to (A′)). RC2 staining from RG endfeet at the pia is also largely absent (arrowheads in (B′), compared to (A′)). By contrast, C-R cell localization at this stage appears normal, suggesting that C-R cell displacement at later stages take place following RG endfoot retraction. Scale bar, 50 μm.

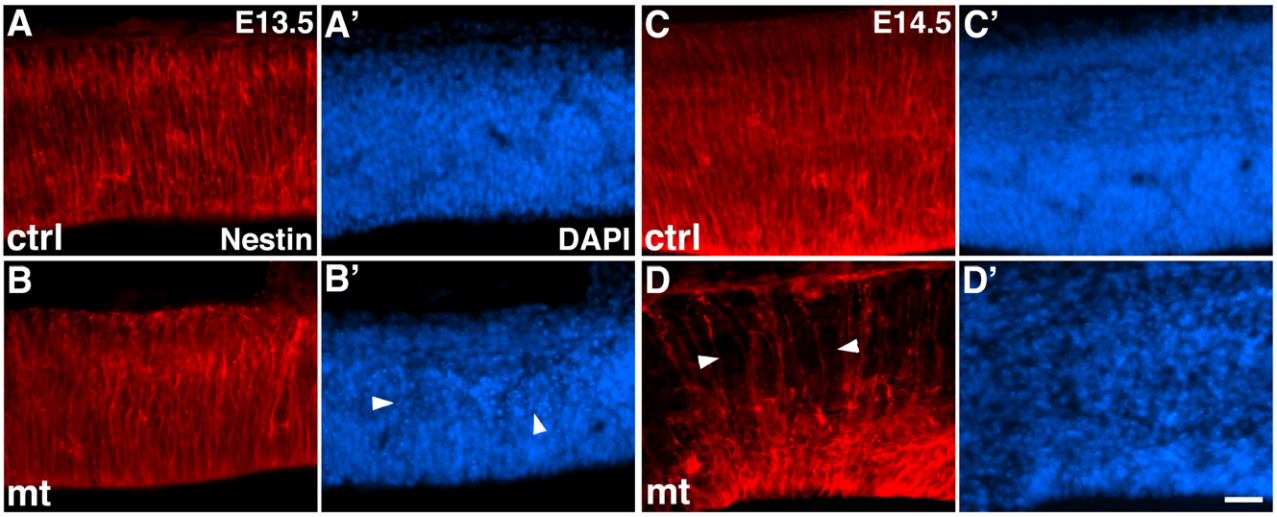

Fig. 6. Ablation of RG by *emx1-cre*-mediated *orc3* gene deletion. (A–B′) RG organization in *orc3*/*emx1-cre* control and mutant cortices at E13.5. RG (anti-Nestin staining, in red) form similarly organized scaffolds in the mutant (B) cortex as in control (A), despite occasional appearance (arrowheads in (B′)) of chromatin condensation (DAPI staining, in blue) in mutants. This indicates limited effects of *orc3* deletion on RG scaffold at this stage. (C–D′) Severe loss of RG in *orc3*/*emx1-cre* mutant cortex at E14.5. Unlike the regularly spaced RG scaffold in control (C), large and frequent gaps (arrowheads in (D)) were observed in the RG scaffold of mutant cortex (D) at E14.5, especially in the upper cortical wall. Cell density also appears lower in mutants (D′), likely resulting from blockade of neural progenitor cell division. Scale bar, 50 μm.

RG ablation also results in spatially specific C-R cell displacement

To assess effects of RG ablation on cortical BM, we again employed antibodies against laminin (Fig. 7). As expected, in controls at E14.5, we found a thin layer of continuous laminin staining overlying the cortex (Fig. 7A and A″). By contrast, in mutants, we found severe disruption in the RG scaffold, with frequent large gaps between a reduced number of glial fibers (Fig. 7B and B″). Despite these defects, however, laminin staining in the BM remains as a continuous layer (Fig. 7B′ and B″). These results thus indicate that the overall organization of cortical BM is unaffected by RG ablation.

To determine effects of RG ablation on C-R cell localization, we next employed antibodies against calretinin. We found that disruption of RG scaffold by *orc3* deletion also results in severe C-R cell displacement (Fig. 8). In contrast to their juxtaposition to the pia in controls (Fig. 8A), in *orc3* mutants, large numbers of C-R cells enter deeper regions of the cortical wall (Fig. 8B). Although there appear to be a relative increase in the number of C-R cells, due to a reduced cortical width, C-R cell displacements are localized and correlate closely with sites of radial glial loss. In areas devoid of RG, C-R cells appear dramatically displaced, entering regions as far as half way through the cortical wall (Fig. 8B, inset b). By contrast, in areas with significant remnants of RG, C-R cells retain their positioning near the pia (Fig. 8B, inset a). This spatial correlation argues against displacement due to a general over-accumulation of C-R cells. Since *orc3* deletion results in complete loss of RG instead of retraction of glial endfeet, these findings also argue against passive displacement of C-R cells due to retracting RG endfeet. Thus, together, our results in this section provide an independent line of evidence supporting a primary role of RG in C-R cell positioning during the early stages of corticogenesis.

Discussion

C-R cells occupy a strategic location in the MZ during cortical development, providing a localized source of reelin for cortical layer

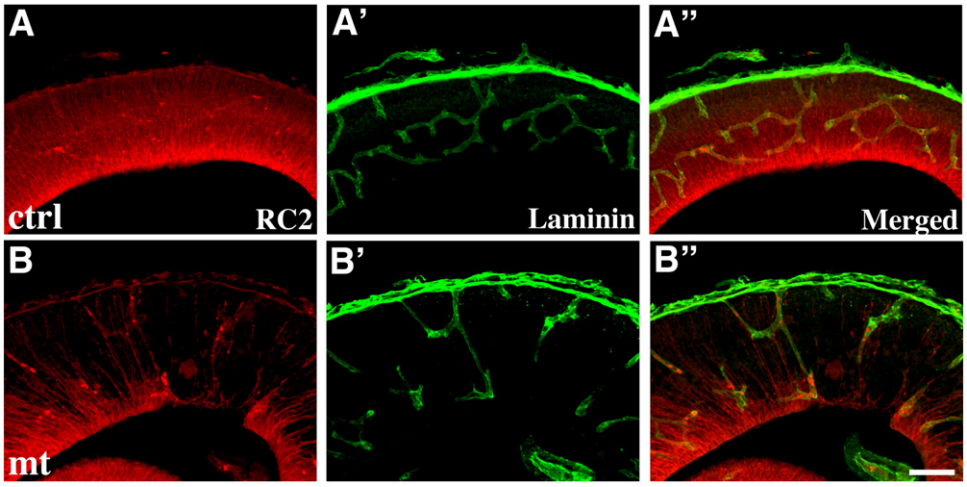

Fig. 7. Basement membrane organization is largely normal in *orc3*/*emx1-cre* mutant cortex at E14.5. (A–A″) Dense and evenly spaced RG fibers (anti-RC2 staining, in red) are observed in control cortex at E14.5, which interact with cortical BM (anti-laminin staining, in green) at the pia. (B–B″) Despite severe loss of RG (B), cortical BM appears continuous and intact E14.5 (B′). The laminin staining above the cortical BM (A′, A″, B′ and B″) is from pial blood vessels, which sometimes detach from the brain surface during processing. Scale bar, 100 μm.

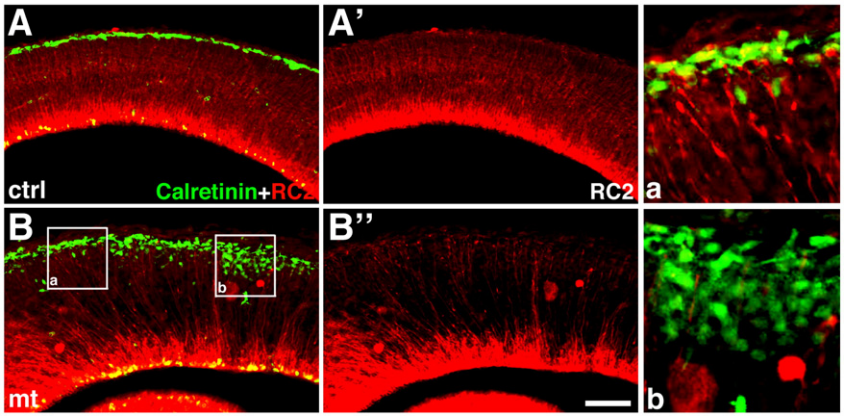

Fig. 8. RG ablation in *orc3/emx1-cre* mutant cortex results in local C-R cell dislocation. C-R cell localization and RG morphology in *orc3/emx1-cre* control (ctrl) and mutant (mt) cortex at E14.5. In control cortex (A–A″), RG fibers (anti-RC2, in red) form an evenly spaced scaffold spanning the cortical wall (A′), with C-R cells (anti-calretinin, in green) localized at the pia (A). In mutant cortex (B–B″), frequent gaps appear in the RG scaffold (B′), in association with local displacement of C-R cells (B). Boxed areas in (B) are shown at higher magnification in the two right panels. In these regions, moderate loss of RG has limited effects on C-R cell positioning (a), while severe loss of RG results in dramatic displacement of C-R cells (b). Scale bar, 100 μm.

ordering along the radial axis (Caviness and Rakic, 1978; Rakic, 1978; Rice and Curran, 2001; Soriano and Del Rio, 2005). To determine how C-R cells are themselves spatially targeted, we have tested the role of RG through two independent genetic approaches. In the first, we have employed *emx1-cre*-mediated deletion of *β1 integrin* to disrupt RG endfoot adhesion to cortical BM. We have found that this results in severe C-R cell displacement (Fig. 1), in close spatial and temporal correlations with disruption of RG scaffold near the pia (Figs. 1 and 5). Importantly, RG scaffold disruption at these stages does not affect the overall structural integrity of the cortical wall (Fig. 1). Nor does it also affect the density of neural progenitors (Fig. 2). Further, no obvious defects were found in the organization of cortical BM or in meningeal pathways known to regulate C-R cell localization (Fig. 3). Lastly, *β1 integrin* is not required cell autonomously in C-R cells (Fig. 4). These results, taken together, therefore implicate a novel and primary role of RG in regulating C-R cell localization during early corticogenesis.

In a second approach, we have employed lineage specific cell ablation to assess the role of RG. We have found that *emx1-cre*-mediated *orc3* deletion results in severe blockade of RG renewal in the early cortex, and consequently frequent gaps in RG scaffold (Fig. 6). These perturbations do not appear to significantly compromise cortical BM (Fig. 7). However, similar to *β1 integrin* deletion, they also result in displacement of C-R cells, at sites that correlate with RG loss (Fig. 8). These findings thus provide an independent line of evidence supporting a primary role of RG in C-R cell positioning during early corticogenesis. Together, our results therefore reveal an unexpected and primary role by RG in the spatial targeting of C-R cells. Because of the prominent role played by C-R cell-mediated reelin signaling in cortical layer ordering, as well as the unique nature of RG as neural progenitors, these findings shed new lights, not only on cellular mechanisms of cortical layer formation, but also on potential functions of neuroepithelial stem cells in nervous system patterning along the radial axis.

Regulation of C-R cell positioning in the developing cortex

C-R cells are one of the earliest born cortical neuronal types (Meyer, 2010). They originate from several sources outside the neocortex (Bielle et al., 2005; Takiguchi-Hayashi et al., 2004; Yoshida et al., 2006; Zhao et al., 2006). One of their most prominent features is their spatially specific localization to the MZ. Previous experiments have implicated cortical meninges and in particular meningeal Cxcl12 signaling in C-R cell migration and localization (Borrell and Marin, 2006; Paredes et al., 2006b; Stumm et al., 2003). However, Cxcl12 signaling appears to regulate only subsets of C-R cells, suggesting involvement of other as yet unidentified cues.

C-R cells populate the MZ between E10.5 and E12.5, before the onset of cortical plate neuron production. At this stage, the main cell types present in the cortex are the RG, which suggests a role of RG in C-R cell targeting. Indeed, previous studies have found that perturbation of cell division using the teratogen methylazoxymethanol (MAM) results in severe C-R cell displacement (Noctor et al., 1999). However, since MAM treatment affect not only neural cell proliferation, but also meningeal cell division and consequently meningeal Cxcl12 signaling (Paredes et al., 2006a, 2006b), the specific role played by RG remained undetermined. In this article, we have employed genetic approaches to specifically perturb RG scaffold, without affecting cortical meningeal function and BM integrity. Our results have revealed a specific and primary role of RG in C-R cell localization in the early cortex.

RG are cortical neural progenitors responsible for generating the vast majority of cortical neurons and glia (Anthony et al., 2004; Malatesta et al., 2000; Noctor et al., 2001), either directly or indirectly (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). Our perturbation of corticogenesis by *β1 integrin* or *orc3* gene deletion affects not only RG, but also cortical neurons, as well as intermediate progenitors. This raises the question of whether the observed effects may be indirect, resulting from abnormalities in neurons and/or intermediate progenitors. Several lines of evidence argue against this interpretation. In *reeler* mutants, for example, *reelin* loss of function results in an inverted lamination pattern, preventing normal interactions between cortical plate neurons and C-R cells. Yet, C-R cell localization is normal (Derer, 1985; Ogawa et al., 1995). Similarly, mutations in *Tbr2*, a gene required for intermediate progenitor specification, result in complete elimination of progenitor cells in the subventricular zone (Sessa et al., 2008). Yet, normal neuron migration and laminar order is observed. Thus, these findings, together with our results, suggest that RG most likely play a direct role in regulating C-R cell positioning during early corticogenesis.

Our finding of RG regulation of C-R cell localization is also compatible with regulation by meningeal signaling (Borrell and Marin, 2006; Paredes et al., 2006b; Stumm et al., 2003). These two mechanisms may cooperate to regulate C-R cell positioning or they may affect distinct subpopulations. Our results show that RG perturbation appears to affect a substantially larger number of C-R cells, suggesting a major role in this process. The close interactions that we observed between RG endfeet and C-R cells also suggest a potentially specific role by RG endfeet. Interestingly, a similar role for

RG endfeet has been observed in the developing retina, where they guide the spatially specific outgrowth of retinal ganglion cell axons (Bauch et al., 1998; Stier and Schlosshauer, 1995). This suggests that such roles of RG endfeet may be conserved throughout the developing nervous system. Our results may facilitate identification of underlying molecular cues.

Intrinsic polarity of RG and nervous system patterning along the radial axis

As neuroepithelial stem cells, RG possess an intrinsic apical–basal polarity, a feature that is crucial to RG functions as neural progenitors (Anthony et al., 2004; Malatesta et al., 2000; Noctor et al., 2001). On the one hand, the apical domain of RG is key to the maintenance of their progenitor fate. For example, perturbations in the apical complex Par3/Par6/aPKC, or in cadherin mediated cell adhesion, both result in transformation of RG into basal progenitors (Bultje et al., 2009; Cappello et al., 2006; Costa et al., 2008; Imai et al., 2006; Rasin et al., 2007).

On the other hand, the basal process of RG is long known for its role in supporting radial neuron migration (Elias et al., 2007; Rakic, 1972, 2007). Our results suggest that the basal process, especially the basal endfeet, may have one additional function, in guiding the localization of C-R cells that arrive through tangential migration. Our findings, together with earlier ones, therefore highlight a distinct function of the basal process of RG. Consistent with this notion, specific molecules have been found localized to the basal process. These include a surface antigen with homology to the SPARC family of proteins (Gongidi et al., 2004), the mRNA transcript of Transitin, a Nestin-like intermediate filament (Lee and Cole, 2000), and adenomatous polyposis coli, a protein required for mRNA anchoring at the plus ends of microtubules (Mili et al., 2008; Yokota et al., 2009). These findings thus suggest that the basal process of RG may indeed possess unique properties. Further investigations may therefore reveal new insights into the function of RG basal process during corticogenesis, including in C-R cell localization.

C-R cells are unique in that they are strategically localized in the MZ and are the near-exclusive source of reelin during corticogenesis. Because they regulate reelin signaling and, as a consequence, the order of cortical layering, C-R cells are key to cortical patterning along the radial axis. Our results show that RG play a primary role in C-R cell localization in the early cortex. This suggests that RG may, through the placement of guidepost cells, regulate nervous system patterning along the radial axis. As such, our findings point to a potential and so far underappreciated feature of neuroepithelial cells as progenitors, in that their intrinsic apical–basal polarity may play a role in nervous system patterning along the radial axis. If so, this may open up a new venue for better understanding nervous system patterning in all dimensions.

RG/C-R cell interaction and cortical malformation

Defective cortical neuron migration and positioning can result in several neurological diseases, including epilepsy and mental retardation. Significant progress has been made in understanding the genetic basis of neuronal migration disorders, especially in identifying genes that affect the cytoskeletal dynamics of migrating neurons or the integrity of cortical BM (Olson and Walsh, 2002). Our findings here uncover a novel interaction between RG and C-R cells in the early cortex, an interaction that is critical for spatial targeting of C-R cells. Since C-R cell-mediated reelin signaling is crucial to the guidance of radial neuron migration, our findings suggest that defects in RG/C-R cell interaction might also play a role in certain types of cortical malformation. As such, further understanding of the molecular basis that underlies RG/C-R cell interaction may also facilitate better insights into cortical malformation.

Acknowledgments

We thank Dr. Louis F. Reichardt (UCSF) for support of this project at its initial stage, Dr. Elizabeth A. Grove (University of Chicago) for providing the *wnt3a-cre* strain, Dr. Kevin Jones (University of Colorado, Boulder) for providing the *emx1-cre* strain, and members of the Reichardt and Huang labs for discussions and assistance. This work was supported by startup funds from UW-Madison.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ydbio.2010.12.026.

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