Cell Division Modes and Cleavage Planes of Neural Progenitors during Mammalian Cortical Development

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SUMMARY

During mammalian brain development, neural progenitor cells undergo symmetric proliferative divisions followed by asymmetric neurogenic divisions. The division mode of these self-renewing progenitors, together with the cell fate of their progeny, plays critical roles in determining the number of neurons and, ultimately, the size of the adult brain. In the past decade, remarkable progress has been made toward identifying various types of neuronal progenitors. Recent technological advances in live imaging and genetic manipulation have enabled us to link dynamic cell biological events to the molecular mechanisms that control the asymmetric divisions of self-renewing progenitors and have provided a fresh perspective on the modes of division of these progenitors. In addition, comparison of progenitor repertoires between species has provided insight into the expansion and the development of the complexity of the brain during mammalian evolution.

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1 INTRODUCTION

The vertebrate brain develops from a single epithelial structure called the neural tube. The neuroepithelial (NE) cells that constitute the neural tube initially proliferate and thereby increase their numbers. These cells subsequently enter a neurogenic mode as self-renewing progenitors, and undergo asymmetric divisions to generate cell populations that are committed to differentiate into excitatory neurons (Fig. 1; Box 1). This transition plays a critical role in the development of a stem/progenitor pool in the developing brain and thus determines the final brain size. Thus, a fundamental question is how the division mode of the self-renewing progenitors is controlled during brain development. A classical model of neurogenesis (Rakic 1988; Breunig et al. 2011) has been proposed for neocortical development, in which radial glia (RG) extending from the ventricular surface to the pial surface guide the neurons in their migration into the cortical layer. In this way, the RG cells form a columnar unit of neurogenesis and brain organization. This model, known as the radial unit hypothesis, has gone through several modifications to date. One major change to the radial unit hypothesis became necessary when RGs themselves were found to be neurogenic self-renewing progenitors (Frederiksen and McKay 1988;

Hartfuss et al. 2001; Miyata et al. 2001; Noctor et al. 2001). RG cells undergo successive asymmetric divisions, generating a chain of neurons that migrate along the radial processes of RG cells and into the cortical layer. Here, we summarize the current understanding of mammalian neurogenesis, focusing on the mechanisms by which RG cells generate neurons during embryonic development. We extend our discussion to the transition modes of RG cell division. Cortical neurogenesis is the primary focus of discussion here because of the large number of studies on cortical development. Neurogenesis in zebrafish, chicken, and nonrodent mammals will also be compared to neurogenesis in rodents.

2 NEUROGENIC PROGENITOR CELLS ARE DIVERSE AND DYNAMIC IN CORTICAL DEVELOPMENT

2.1 Self-Renewing Neural Progenitors

The shift in the division mode of self-renewing progenitors from the proliferative to the neurogenic does not occur as a single event but instead as a gradual process that proceeds from the caudal region to the rostral region of the brain. This shift mainly takes place between embryonic days 10 and 12 in the mouse telencephalon (see Gotz and Huttner

Figure 1. Self-renewing progenitors and intermediate progenitors (IPs) in the development of the mammalian cerebral cortex. Both neuroepithelial cells (NE) and radial glia (RG) undergo interkinetic nuclear migration (IKNM) during each cell cycle (see online Movie 1 at cshperspectives.cshlp.org). RG cells repeat asymmetric divisions that generate another RG and a differentiating daughter; neurons or IPs. The second class of self-renewing progenitors are outer (basal) RGs, which maintain the basal process but not apical process, and undergo asymmetric divisions outside of the ventricular zone (VZ). Outer basal (o/b) RG cells and IPs do not undergo IKNM. Among IPs, basal progenitors (BPs) migrate out of the VZ and divide once to produce two neurons. Short neural precursors (SNPs) transiently divide in the VZ to produce a few neurons (see online Movie 1 for time-lapse movie of an RG cell). The RG cell was visualized in a culture slice from an E14.5 wild-type brain sparsely labeled by EGFP and the membrane-bound monomeric Kusabira Orange (mKO2). The nucleus of one daughter migrates faster away from the ventricular surface to undergo IKNM, whereas the other migrates relatively slower to become a neuron.

BOX 1. CLASSIFICATION OF NEURAL PROGENITORS IN THE DEVELOPING CEREBRAL CORTEX

There are several types of neuronal progenitors (Fig. 1) and some of them have more than two different nomenclatures. Here, we describe the classification of neuronal progenitors and their synonyms (see text for references).

Self-renewing neural progenitors—Neural progenitors that replicate themselves at cell division. At the early neural development (proliferative stages), all neural progenitors self-replicate by symmetric divisions. These cells are also called neuroepithelial cells. Self-renewing progenitors that produce neurons (neurogenic), such as RG cells, undergo asymmetric divisions into one daughter equivalent to the parental cell and another daughter committed to differentiation such that progenitors can repetitively self-replicate and produce neurons at divisions.

Radial glia (RG)—A major type of self-renewing neural progenitor. When neurogenesis begins by asymmetric divisions of self-renewing progenitors, their major population maintain the epithelial cell structure and become a highly elongated radial form spanning from the apical end (the ventricular surface) to the basal end (the pial surface) as neurogenesis proceeds. Those progenitors lose the typical tight junction structure that has occupied the most apical domain of the lateral membrane during the proliferative stages, but some tight junction components such as ZO1 remain at adherens junctions of RG cells. As described in the Introduction, the terminology of “radial glia” is used owing to a historical reason, but they are not glial cells. A population of RG cells lose the apical end foot and translocate out of the VZ to become outer (basal) radial glia.

Apical progenitors—A major type of RG cells undergo mitosis at the apical surface after IKNM, and are termed “apical progenitors” as contrasted with “basal progenitors,” a type of transit-amplifying progenitors that divide in the SVZ (see below).

Outer radial glia—Self-renewing progenitors residing outside of the VZ without IKNM, which retain the basal process but not the apical process. See text for details. Progenitors of this type are also called basal radial glia, OSVZ progenitors, intermediate radial glia, or outer VZ progenitors.

Intermediate progenitors—Transit-amplifying progenitors that have committed into neuronal lineages but transiently retain mitotic activity to divide once or twice without undergoing IKNM. This terminology is sometimes used to collectively include all transit-amplifying progenitors that no longer self-renew, and also used specifically to indicate “basal progenitors” described below.

Basal progenitors—A major population of transit-amplifying progenitors in the developing mammalian cerebral cortex is called “basal progenitors,” because they migrate out to the SVZ without IKNM and divide once into neurons at a “basal position” in contrast to “apical progenitors.” “Basal progenitors” are identical to the “intermediate progenitors” in a narrow sense.

Class	Type	Subtype	Position of division	Self-renewal	Notch activation	Neuronal commitment	Morphology
Self-renewing progenitors	Radial glia	Apical progenitors	VZ	+	+	–	Radial
		Outer (basal) radial glia	Outside of VZ	+	+	–	Radial (no apical process)
Transit-amplifying progenitors	Intermediate progenitors	Basal progenitors	SVZ	–	–	+	Multipolar
		Short neural progenitors	VZ	–	–	+	Short radial

VZ, ventricular zone; SVZ, subventricular zone.

2005 for review). The transition can be studied by following the expression of the antiproliferative gene *Tis-21*, which is expressed in most neurogenic progenitors but not in their proliferative progenitors (Iacopetti et al. 1999; Haubensak et al. 2004). Self-renewing progenitors in both the proliferative and the neurogenic states are highly elongated, have pronounced apicobasal polarity (Fig. 1; also see Box 1), and extend their cytoplasmic process further as neurons accumulate in the cortical layer. Some years ago, RG cells were found to be neurogenic self-renewing progenitors (Frederiksen and McKay 1988; Miyata et al. 2001; Noctor et al. 2001). In this article, the term RG is therefore used to refer to neurogenic self-renewing progenitors with radial morphology. Two extracellular signals that promote the transition from the proliferative to the neurogenic mode have been identified: retinoic acid from the meninges (Siegenthaler et al. 2009) and FGF10 from neuroepithelial cells (Sahara and O'Leary 2009), whereas the intrinsic mechanisms that act downstream from these signals remain to be elucidated. Once RG cells become neurogenic, they first produce neurons that form deeper cortical layers and successively those at upper layers (Shen et al. 2006). A recent study discovered that there is a population of RG cells that exclusively generate upper layer neurons. Those RG cells continue symmetric proliferative divisions when deep layer neurons are produced (Franco et al. 2012). RG cells produce astrocytes later and thus behave like multipotent progenitors (Miller and Gauthier 2007; Okano and Temple 2009). In the last section of this article, we will describe outer (or basal) RG cells, another type of self-renewing progenitor cell that was discovered only recently.

Notch signaling is critical for the self-renewal of neural progenitor cells (de la Pompa et al. 1997; Gaiano and Fishell 2002). In the main germinal zone, called the ventricular zone (VZ), the major ligand of the Notch receptor is Delta-like1 (*Dll1*) (Beckers et al. 1999). *Dll1* is most highly expressed in nascent postmitotic cells and in the intermediate progenitors that are destined to become neurons after RG division (Kawaguchi et al. 2008). Yoon et al. tested whether RG cells require *Dll1* from their own daughter cells to maintain their self-renewal and neurogenic capacity in the VZ, by using clonal analysis of the *Mind bomb1* (*Mib1*) conditional mutant (Yoon et al. 2008). *Mib1* is a ubiquitin ligase that is required for the *trans*-endocytosis of the Notch–Delta complex in cells expressing Delta on their surface, a process that is necessary for Notch cleavage and thereby activation (Kopan and Ilgan 2009). Single cell *Mib1*-mutant clones of RG cells, whose progeny cells were thus defective in expressing functional *Dll1*, generated normal numbers of progeny neurons. Therefore, *Dll1* is provided not only by the sibling cells of the RG cells but also by the surrounding differentiating cells that are born

from other RG cells in the VZ. It is not known how neuroepithelial cells acquire *Dll1* before the generation of neurons. It has been shown that the transcription factor *Hes1* and *Dll1*, both of which are downstream of Notch signaling, can oscillate during the cycling of RG cells (Shimojo et al. 2008). This oscillation might play a role in the self-renewal of the progenitor cells by allowing them to activate Notch signaling in their neighbors (see Kageyama et al. 2008, for review).

2.2 Neural Progenitors Are Highly Dynamic

One prominent feature of self-renewing progenitors in both the proliferative and the neurogenic states is the dynamic nuclear movement in the VZ, termed interkinetic nuclear migration (IKNM). IKNM consists of a slow *G*₁ phase migration in the basal direction and a fast *G*₂ phase migration in the apical direction, both of which occur during a single cell cycle (Fig. 2; see online Movie 1 at cshperspectives.cshlp.org). IKNM arises from the restriction of mitosis to the apical surface, where the centrosome attaches during interphase in proliferative epithelial cells. Therefore, nuclear oscillation is a general phenomenon in proliferative epithelial cells and involves actomyosin function (Norden et al. 2009; Schenk et al. 2009). IKNM contributes to the efficient use of the VZ surface such that a large number of self-renewing progenitors are able to divide in a limited area (the ventricular surface). Studies of mutations in genes responsible for human lissencephaly, such as *Lis1*, have revealed that IKNM in the stratified brain structure also involves the microtubule motor system and is dependent on dynein during *G*₂ (Feng et al. 2000; Niethammer et al. 2000; Sasaki et al. 2000; Tsai et al. 2010) and kinesin during *G*₁ (Meyer et al. 2011). In contrast, *G*₁ phase migration is stochastic in nature, suggesting that it includes a collective displacement effect resulting from *G*₂ phase nuclei migrating toward the ventricular surface (Kosodo et al. 2011; Leung et al. 2011). This combination of active (motor-dependent) and passive (motor-independent) processes during the IKNM of self-renewing progenitors may generate a dynamic homeostasis in which the behavior of individual self-renewing progenitors can be coordinated to permit highly dynamic IKNM within the VZ. Recent studies in zebrafish and mouse models indicate a linkage between IKNM and progenitor self-renewal via Notch activation (Xie et al. 2007; Del Bene et al. 2008).

2.3 Intermediate Neural Progenitors Increase the Number of Neurons

RG cells can generate either neurons or intermediate progenitors (IPs), the latter of which are transiently pro-

Figure 2. Time-lapse imaging of RG cell division in the brain slice culture. Snapshot series taken from online Movie 1 (at cshperspectives.cshlp.org). An RG cell was visualized in a culture slice over 2 d from an E14.5 wild-type brain sparsely labeled by EGFP and membrane-bound monomeric Kusabira Orange 2 (mKO2). The RG cell divides at the apical surface (2:38). The nucleus of one daughter migrates faster (away from the ventricular surface) to undergo IKNM (2:38–26:44) and divides again at the apical surface (26:44), whereas the other migrates relatively slow to become a neuron (blue arrowhead). The 3D analysis shows the fast-moving basal daughter exclusively inherits the basal process, whereas the apical daughter (blue arrowhead) does not and subsequently loses the apical process. Dividing cells are indicated by asterisks. Time, h:min. Division pattern of this RG cell lineage is schematically shown at the bottom.

liferating neuronal precursors (Fig. 1; Box 1). Notch signaling is essentially inactive in IP cells; therefore, components that are downstream of Notch, such as *Hes1*, are not expressed, whereas a subpopulation of IP cells requires Notch signaling via a noncanonical cascade (Mizutani et al. 2007). IP cells transiently express proneural genes, such as *Neurogenin 2* in the dorsal telencephalon (Miyata et al. 2004; Guillemot 2007) and *Tbr2* (Englund et al. 2005), which acts to convert RG cells into IP cells (Arnold et al. 2008; Sessa et al. 2011). Typical IPs in the developing cerebral cortex undergo a terminal division into two neurons, and IP cells that undergo two rounds of division in the dorsal cortex are found at low frequencies (Haubensak et al. 2004; Miyata et al. 2004; Noctor et al. 2004). Generally, it is not well established whether the daughters of IP cell divisions adopt asymmetric neuronal fates. IP cells typically do not undergo IKNM. Once committed, IP cells retract their apical and basal process (even if they inherit the process), migrate out of the VZ, and become multipolar dividing cells in the subventricular zone (SVZ), the second major germinal zone (Haubensak et al. 2004; Miyata et al. 2004; Noctor et al. 2004, 2008). A minor population of IP cells, termed short neural progenitors (SNPs), possess a short basal connection and undergo terminal divisions into two neurons at the ventricular surface (Gal et al. 2006; Stancik et al. 2010). Thus, the formation of IP cells amplifies the number of neurons generated by an RG cell during the neurogenic period.

3 CLASSICAL MODE OF ASYMMETRIC CELL DIVISIONS: A HISTORICAL PERSPECTIVE

The molecular mechanisms underlying the asymmetric division of progenitor cells have been best studied in invertebrate model organisms, such as *Drosophila melanogaster* and *Caenorhabditis elegans* (Matsuzaki 2000; Knoblich 2008). In these models, cell-fate determinants are asymmetrically distributed in the dividing parent cell either by polar distribution of intrinsic factors or in a noncell-autonomous manner via extracellular signals. In either case, the relationship between the axis of polarity and the orientation of the mitotic spindle (or the orientation of the cleavage plane, which is orthogonal to the spindle orientation) plays a critical role in determining whether a polarized cell will divide symmetrically or asymmetrically (Fig. 3). If the mitotic orientation, defined by the mitotic spindle orientation, is aligned parallel to the polar axis of the cell, the two daughter cells will receive determinants and/or extracellular signals unequally, resulting in asymmetric divisions (Fig. 3A,B). In contrast, division is likely to be symmetric when the polar axis is perpendicular to the spindle orientation (i.e., parallel to the cleavage plane). This general scheme was thought to hold true for neurogenic self-renewing progenitors, such as RG cells during cortical development, because these cells are highly polarized along their radial axis and produce two daughter cells with distinct fates.

Figure 3. Asymmetric divisions in model systems. (A) Typical division modes of polarized cells. The relative orientation of the mitotic spindle and the axis of cell polarity determine the division mode to be either symmetric or asymmetric. (B) *Drosophila* epithelial cells and neuroblasts are typical examples of symmetric and asymmetric divisions, respectively (Knoblich 2008). The apicobasal cell polarity is created by the conserved components of Par3, Par6, and aPKC, which define the apical domain of the cell and localize cell-fate determinants. Mitotic spindle orientations are regulated by another conserved cytoskeletal protein complex consisting of the GoLoco protein Pins, Gai, and the mushroom body defect (Mud) protein in *Drosophila*. Epithelial cells divide symmetrically owing to the orthogonal orientation of the spindles relative to the cell polarity axis. In contrast, neuroblasts, when committed, inherit the apicobasal polarity and express the Inscuteable molecule, which binds to both Par3 and Pins and hence integrates the two complexes, aligning the mitotic spindle parallel to the polarity axis to segregate the cell-fate determinants into one daughter. (C) Typical models for RG asymmetric divisions rely on the separation of the apical surface (green) and basal parts (Chenn and McConnell 1995) into the two daughters. This can be achieved by a slightly oblique cleavage plane relative to the radial axis (the apicobasal orientation) (Kosodo et al. 2004).

Although previous studies have mainly used electron microscopy to examine the mitotic orientation of neural progenitor cell divisions in the cerebral cortex (Smart 1973), Chenn and McConnell published a groundbreaking work in 1995 in which divisions of self-renewing progenitors were first analyzed in slice cultures using time-lapse imaging of fluorescent dye-labeled chromosomes. Using this technique, these investigators identified a change in

the division axis that occurs during the neocortical development of the fetal ferret brain (Chenn and McConnell 1995) and proposed that divisions in which the cleavage plane orientation is perpendicular to the ventricular (apical) surface are symmetric and proliferative, whereas divisions in which the cleavage plane is parallel or oblique to the ventricular surface are asymmetric and neurogenic. However, subsequent studies using these same techniques

did not generate results that support the above model (Adams 1996; Das et al. 2003; Kosodo et al. 2004; Konno et al. 2008; Noctor et al. 2008). Huttner and colleagues have proposed a mechanism that may possibly reconcile these disparate observations (Huttner and Brand 1997; Kosodo et al. 2004); they suggested that a slight tilt of the cleavage plane orientation from the radial axis is sufficient to ensure asymmetric partition of the apical domain facing the ventricle, given its limited surface area (Fig. 3C). This mechanism suggests that the asymmetric inheritance of the apical membrane, in addition to the cleavage plane orientation, is a key feature of daughter cell-fate determination. Both models are based on the concept that the differential inheritance of the apical or basal epithelial structure is critical for daughter cells to adopt different fates. Recent progress in live imaging techniques has enabled us to precisely capture the dynamic behaviors of RG cells in slice cultures. Combined with molecular perturbations via gene knockout and knockdown techniques, these imaging studies have allowed us to verify the prevailing models. Below, the current understanding of the mechanisms underlying the neurogenesis and self-renewal of RG cells will be reviewed.

4 MITOTIC SPINDLE ORIENTATION IS CONTROLLED IN RG CELL DIVISION

4.1 Basic Mechanisms Regulating Spindle Orientation

The regulation of mitotic spindle orientation involves an evolutionarily conserved cytocortical protein complex (Schaefer et al. 2000; Yu et al. 2000; Gotta and Ahringer 2001) that comprises three essential components (Fig. 3B): the α subunit of the heterotrimeric G protein $G\alpha_i$, a GoLoco domain protein, and another component that binds to the GoLoco domain protein (Du et al. 2001). This cytocortical protein complex is directly anchored to the cytoplasmic membrane through $G\alpha_i$, which, in turn, binds to GoLoco domains in the GoLoco protein (e.g., LGN and AGS3 in mammals [Mochizuki et al. 1996; Takesono et al. 1999; Du et al. 2001; Yu et al. 2003], Partner of In-scuteable/Pins in *Drosophila* [Schaefer et al. 2000; Yu et al. 2000; Bellaiche et al. 2001], and GPR1/2 in *C. elegans* [Colombo et al. 2003]). The GoLoco protein, in turn, binds to a nuclear-mitotic apparatus protein (NuMA) or a functional homolog of this protein (e.g., *Drosophila* mushroom body defect), which then associates with the dynein–dynactin motor complex. The motor complex hauls the plus end of astral microtubules (Du et al. 2001; Srinivasan et al. 2003; Bowman et al. 2006; Izumi et al. 2006; Siller et al. 2006). This cytocortical protein complex thus provides the

membrane-anchoring footing for the dynein–dynactin complex to generate the force that pulls the astral microtubules and, hence, orients the mitotic spindle (see Morin and Bellaiche 2011 for review).

The loss of two GoLoco domain proteins, AGS3 and LGN, creates different phenotypes during development of the mouse cortex. The depletion of LGN randomizes cleavage plane orientation in the neurogenic chicken spinal cord (Morin et al. 2007; Peyre et al. 2011) and in the mouse cerebral cortex during both the proliferative and neurogenic stages (Konno et al. 2008). Thus, LGN plays a central role in orienting the mitotic spindle in parallel to the ventricular plane during RG cell division. In addition, the loss of LGN converts the RG cells in neurogenic stages into ectopic progenitors that are translocated out of the VZ (Fig. 4; online Movie 2 at cshperspectives.cshlp.org). In the LGN-mutant brain, 40% of the self-renewing progenitor cells migrate out of the VZ (Konno et al. 2008), although RG cells in the proliferative stage are not perturbed by randomized spindle orientations. These ectopic progenitors are generated by oblique cleavage planes that bypass the apical membrane (see below); in slice cultures of LGN-mutant brains, approximately half of the daughters on the basal side become self-renewing progenitors that continue asymmetric divisions and neuronal production, whereas the apical daughter commits to differentiation (into neurons or IP cells). This finding is inconsistent with the traditional model, which suggests that the apical daughter cell acquires self-renewability. Surprisingly, there are no significant changes in either net neurogenesis or brain size in the LGN mutant, suggesting that these ectopic progenitors mostly yield correctly fated neurons. Together, these observations imply that cleavage planes in the wild type are not as oblique as those in the LGN-mutant RG cells, although a certain degree of fluctuation is present in their cleavage plane orientations.

How does the mitotic spindle orient parallel to the ventricular plane in RG cell division? Mitotic spindles in wild-type RG cells in slice culture show highly dynamic rotations and orientations in metaphase but become static and orientated parallel to the ventricular surface (with the cleavage plane perpendicular to this surface) from anaphase onward (Konno et al. 2008; Peyre et al. 2011). However, the depletion of any component of the LGN- $G\alpha_i$ -NuMA complex impedes this controlled dynamism of the mitotic spindle (Peyre et al. 2011) owing to the lack of cortical attachments to the astral microtubules in the mitotic spindle. The LGN- $G\alpha_i$ -NuMA complex is distributed as a lateral belt, and this distribution of the complex likely allows mitotic spindle orientation to be parallel to the ventricular plane in the dividing RG cells. In this way, one daughter cell from each neurogenic division inherits the complete epithelial

Figure 4. Time-lapse imaging of the generation of the o/bRG glia in wild-type brain slice. RG cells occasionally undergo oblique divisions that split into the apical and basal daughters in a culture slice from the E14.5 wild-type brain (0:00). Both daughters migrate together out of the VZ. The apical daughter (blue arrowhead) keeps a more apical position relative to the basal daughter. Both daughters are often in contact with each other via a thin process. The apical daughter of this RG division becomes an IP that divides once to produce a pair of neurons (12:07–18:11). The daughter that inherits the basal process without the apical process acquires the ability to repeat asymmetric divisions that generate postmitotic daughters (white and green arrowheads); this daughter cell is thus o/bRG cells. The apical surface is down. Time, h:min. (See online Movie 2 at cshperspectives.cshlp.org, which is a time-lapse movie of an RG cell in a culture slice from the E14.5 wild-type brain undergoing an oblique division, dividing into apical and basal daughters.)

structure of the mouse developing neocortex as described below (Konno et al. 2008; Peyre et al. 2011).

In contrast to LGN, the knockdown of AGS3 by short hairpin RNA (shRNA) produces cleavage planes that are more oblique to the ventricular surface than those of the wild-type brain and premature neurogenesis at the expense of the RG cells (Sanada and Tsai 2005). This finding suggests that AGS3 destabilizes the default cleavage plane orientation, allowing oblique cleavage and asymmetric division. Knockout of the AGS3 gene, however, causes no significant changes in either gross brain morphology (Blumer et al. 2008) or cleavage plane orientation (our unpublished results). Understanding of the role of AGS3 in cortical neurogenesis requires further study.

4.2 The Role of Fluctuations in Spindle Orientation

One interesting question has been whether cleavage plane fluctuations from the radial axis play a role in the fate of the

RG cell progeny. Mouse Inscuteable (mInsc) is able to reorient the mitotic spindle to be perpendicular to the ventricular surface when overexpressed as observed in *Drosophila* (Zigman et al. 2005; Konno et al. 2008). Because the endogenous mInsc expression is very low in the telencephalon, it does not seem to be able to rotate mitotic spindle orientations by 90° during RG cell divisions, but may be able to alter cleavage angles. A recent study (Postiglione et al. 2011) indicated that the conditional knockout of mInsc causes cleavage planes to become more perpendicular to the ventricular surface, whereas RG cell divisions in the wild-type brain show variable cleavage angles compared with those in the mInsc KO mice. This mutant phenotype suggests that the low level of mInsc leads to fluctuating cleavage planes in RG cells (Fig. 5). In the mInsc mutant, IP cell production is significantly decreased, which reduces the number of upper layer neurons and thus overall cortical thickness. In contrast, mInsc overexpression increases variability in spindle orientation and causes an increase in the number of IP cells and a correspondingly thicker brain with

Figure 5. The division mode of RG cells and its transitions. (A) Proliferative neuroepithelial cells undergo symmetric divisions. The Par complex is necessary for the epithelial polarization of RG cells, whereas the mammalian Pins homolog LGN orients the mitotic spindle. (B) The transition from neuroepithelial cells to RG cells requires the retinoic acid signal from meninges and FGF10. At the neurogenic stage in the cerebral cortex, most RG cells undergo asymmetric divisions in terms of subcellular inheritance, to generate one daughter that inherits the entire epithelial structure and another daughter with only the apical membrane; the daughter inheriting the epithelial structure is the origin of self-renewing RG cells, whereas the other daughter differentiates into a neuron or an IP cell. Fluctuations of spindle orientation contribute to the formation of IP cells. (C) Oblique cleavage planes that bypass the apical membrane generate a basal daughter that possesses only the basal process without the apical process, which migrates out of the VZ. This migrating daughter acquires self-renewability and becomes an outer (basal) RG that continues neurogenic asymmetric divisions. On the other hand, the apical daughter that has lost the basal process differentiates. This type of oblique division frequently occurs in the absence of LGN or by mInsc overexpression (see the text), whereas it is infrequent in the wild-type neurogenic stage.

an unchanged RG cell population. Fluctuations of the cleavage plane therefore appear to yield a greater number of IP cells, although the direct production of neurons from the RG cells may not require an Insc-dependent fluctuation of spindle orientations. Comparisons between LGN, AGS3, and mInsc are currently difficult owing to discrepancies in the wild-type cleavage plane orientations among individual studies (Sanada and Tsai 2005; Konno et al. 2008; Postiglione et al. 2011). Epistatic analyses of these three genes under the same experimental conditions may increase our understanding of how they are functionally related to one another. It had been thought that Insc could bind simultaneously to both the LGN complex and the Par complex, as described above. Recent structural studies, however, have indicated that LGN interacts with NuMA and mInsc in a mutually exclusive manner and has a higher affinity for mInsc (Culurgioni et al. 2011; Yuzawa et al. 2011; Zhu et al. 2011). Therefore, the mechanisms by which mInsc recruits the LGN and Par complexes for the determination of the cleavage plane may not be as simplistic as was previously thought and remain to be solved.

4.3 Spindle Orientation Defects May Lead to Diseases

A link between cleavage plane orientation and RG daughter cell fate has been previously discussed in studies of the molecular regulation of mitotic spindles and centrosomes. Mutations in dynein subunit genes, such as Lis1 and Nde1, are involved in spindle assembly and may also be involved in cortical–astral interactions. Mutations in these genes are known to cause human lissencephaly (Tai et al. 2002; Feng and Walsh 2004; Vallee and Tsai 2006; Yingling et al. 2008). Fetal mouse brains defective in these genes display aberrant cleavage plane orientation in addition to abnormalities in IKNM. There are more than seven genes responsible for autosomal recessive primary microcephaly (MCPH) (Megraw et al. 2011) including ASPM (abnormal spindlelike microcephaly-associated); all of these genes are now known to encode centrosomal proteins (Bond et al. 2002; Kaindle et al. 2010). These genes are also required for the normal formation of centrioles, centrosomes, and/or mitotic spindles. The disruption of these genes by knockout or knock-down induces abnormal cleavage plane orientations and

affects progenitor populations and neurogenesis (Feng and Walsh 2004; Fish et al. 2006; Lizarraga et al. 2010). ASPM knockdown affects cleavage orientation, whereas mutations in ASPM do not (Pulvers et al. 2010), suggesting that the acute effects of ASPM knockdown on cleavage plane orientation may be rescued by other gene functions in the permanent absence of ASPM. The depletion of huntingtin, a centrosomal protein that is mutated in Huntington's disease, produces a more oblique cleavage plane orientation and defects in progenitor maintenance (Godin et al. 2010). These observations suggest that proper spindle orientation is important for the cell-fate choice between self-renewal and differentiation. It should be noted that these factors also influence other microtubule processes, such as cilia formation, intracellular transport, and DNA damage responses. The abnormal neurogenesis phenotypes of these mutants may result from defects in multiple processes involving these genes. Careful phenotypic evaluation is needed to determine the extent to which such abnormal phenotypes are caused by impaired cleavage plane orientations.

5 WHAT DETERMINES ASYMMETRY IN CELL FATE IN RG CELL DIVISION?

5.1 Asymmetric Segregation of Epithelial Substructures during RG Cell Division

Classical models predict that the asymmetric inheritance of the apical domain leads to unequal daughter cell fates during RG cell division. It has been technically difficult to accurately detect the segregation of the apical membrane and its basal process during RG cell division because both processes become very thin during mitosis (Miyata et al. 2001; Noctor et al. 2001). Live imaging of brain slices can overcome this technical difficulty. The expression of ZO-1- or Par3-EGFP fusion proteins (which localize to the zonula adherens) marks the cell–cell boundary at the apical junctional level (Konno et al. 2008; Shitamukai et al. 2011). Imaging these fluorescent markers from the apical side of brain slices allows the direct observation of the partitioning of the apical junction into daughter cells and has revealed that the segregation of the apical membrane exclusively to a single daughter cell occurs in only 10%–20% of RG divisions during the neurogenic stage of the developing murine brain (Konno et al. 2008; Asami et al. 2011; Shitamukai et al. 2011); indeed the apical junction is partitioned between both daughter cells in the majority of RG cell divisions. The reextension of the lost apical end is rarely observed in divisions during the midneurogenic stage (E14–E16). If asymmetric inheritance of the apical junctions is only one mechanism that confers distinct fates to

the daughter cells, the observed rate of asymmetric apical membrane partition cannot account for the neuronal production rate at this neurogenic stage (approximately half of the daughter cells adopt the neuronal fate, whereas the other half self-renews). Thus, the majority of RG cell divisions that partition the apical junction into both daughter cells must be asymmetric in their daughter cell fate. Moreover, no correlation between the inherited size of the apical domain and the RG cell fate has been detected in brain slice cultures (Shitamukai et al. 2011). It is noteworthy that randomized spindle orientations, which are caused by the loss of LGN, do not affect the elongated shape of neural progenitors at proliferative stages, suggesting that the missing processes owing to oblique cleavage can be regenerated during early developmental stages in the rodent cerebral cortex. Indeed, a high level of active Notch expression induces regeneration of the missing radial process even in neurogenic stages (Shitamukai et al. 2011).

The basal process of self-renewing progenitors, which reaches the basal lamina facing the meninges, splits into two daughter cells during the proliferative stage (Figs. 3 and 4) and becomes very thin during division in the neurogenic stage (Noctor et al. 2001; Miyata et al. 2004). Kosodo et al. reported that the basal processes of RG cells in the early neurogenic stages split before cytokinesis begins (Kosodo et al. 2008). These split processes are occasionally inherited by one or both daughter cells. In contrast, during the midneurogenic stage (~E14), the basal processes appear to segregate into a single daughter cell in RG cell divisions (Miyata et al. 2001; Noctor et al. 2001; Weissman et al. 2003; Shitamukai et al. 2011).

In summary, time-lapse observations of slice cultures indicate that the majority of asymmetric RG cell divisions during the murine neurogenic stage segregate the apical membrane to both daughters, whereas the basal process is segregated to only one daughter (Figs. 3 and 4; see online Movie 1 at cshperspectives.cshlp.org). Consequently, this mode of RG division generates one daughter cell with the complete parental epithelial structure and another daughter with the apical membrane and without the basal processes, as shown in Figure 3 (Miyata et al. 2001; Noctor et al. 2001, 2002; Konno et al. 2008). These observations are inconsistent with the hypothesis that the asymmetric partitioning of the apical membrane determines the daughter fates of RG cell divisions during murine cortical development (Chenn and McConnell 1995; Huttner and Kosodo 2005). On the other hand, the importance of the epithelial inheritance for progenitor self-renewal has been shown by a recent study in zebrafish hindbrain. This study revealed that the Crumb protein complex, which is located at the subapical domain, is critical for Notch activation in self-renewing progenitors, and Notch activation, in turn, promoted

apicobasal polarity in a noncanonical manner (Ohata et al. 2011). Thus, Notch functions in a positive feedback loop between the self-renewal and the epithelial polarity of neural progenitor cells.

In both zebrafish hindbrain (Alexandre et al. 2010) and chicken spinal cord (Das and Storey 2012), the asymmetric inheritance of the apical surface has been observed to give rise to daughter cells with different fates; the apical daughter that inherits a larger area of the apical membrane adopts a differentiated fate, whereas the basal daughter first loses and then regrows its apical process. As described above (Konno et al. 2008), when the mitotic spindle is artificially tilted in the mammalian cerebral cortex, the basal daughter acquires the ability to self-renew, and the apical daughter is differentiated. Together, these findings suggest that this pattern of differential fate acquisition between the basal and apical daughters is a universal feature of cell-fate decisions in the development of the vertebrate brain, when RG cell divisions are sufficiently oblique for RG epithelial structure to split into two daughter cells. Interestingly, the behavior of the basal daughter is different in the mouse cerebral cortex and the zebrafish hindbrain (or the chicken spinal cord); in mice, the restoration of epithelial apicobasal polarity does not appear to occur frequently during RG cell divisions in the neurogenic stage (Fig. 5). This low capacity for regrowth of apical processes appears to be influenced by developmental stages, which may be characteristic of neurogenic RG cells in mammalian cortical neurogenesis.

5.2 Localized Cell-Fate Determinants

Asymmetric divisions typically occur via the asymmetric segregation of cell-fate determinants. As in the *Drosophila* model (Fig. 3B), Numb (and its homolog Numb-like) is a strong candidate for a cell-fate determinant (Zhong et al. 1996). Numb is a regulator of the membrane trafficking that is involved in the internalization of Notch (Couturier et al. 2012) and negatively regulates Notch activation, thereby promoting neural differentiation. However, the role of Numb (and Numb-like) has not been fully delineated in cortical development owing to the phenotype of the double KO mouse (Numb and Numb-like), which is indistinguishable from that of the Notch1 mutant (i.e., they both produce premature differentiation of neural progenitor cells) (Petersen et al. 2002, 2004). Rasin et al. (2007) indicated that the double KO of Numb and Numb-like compromises the transport of cadherin to the cytoplasmic membrane and abolishes epithelial integrity. This ultimately results in the failure to maintain the RG cells, and could explain why the loss of Numb causes a phenotype similar to the Notch mutant. Zhou et al. (2007) proposed that the

Golgi component ACBD3 is involved in Numb asymmetry. ACBD3 is released into the cytoplasm only in dividing RG cells, where it functions together with Numb, in Notch inhibition; in other situations, ACBD3 is sequestered by the Golgi apparatus and is therefore not functional. On the other hand, a recent study showed that Par3 is released from the cell membrane and asymmetrically distributes in the cytoplasm in dividing RG cells resulting in the unequal segregation of Par3 into daughter cells (Bultje et al. 2009). This study suggests that greater amounts of Par3 result in reduction in functional Numb, leading to self-renewal.

In the zebrafish forebrain, Mindbomb (Mb), which is associated with endosomes, was recently identified as a localized determinant that specifies the differentiated daughter cell fate during RG cell division (Dong et al. 2012). Clonal analysis revealed that more Mb was distributed into the daughter that is migrating at the more apical position than into the other daughter during basally oriented IKNM. Cells inheriting more Mb are generally associated with higher Delta activity, and hence should adopt more a differentiated fate according to the lateral inhibition mechanism of Notch. Indeed, this daughter cell migrating at the more apical position is differentiated, whereas the other daughter at a more basal position is self-renewing. This finding is consistent with observations in the mammalian cerebral cortex showing that the daughter cell inheriting the basal process migrates faster than the other daughter during the G₁ phase of IKNM right after RG cell division and that the more basally positioned daughter cell acquires self-renewability. Dong et al. also showed that the asymmetric segregation of Mb requires Par3 and that the daughter with more Par3 also inherits more Mb and is more differentiated (Dong et al. 2012). Thus, the requirement of Par3 for asymmetric daughter cell fate holds true in both the zebrafish and mouse, although the interpretation of the role of Par3 is different from that which has been proposed for the mouse brain in which Par3 promotes self-renewal (Costa et al. 2008; Bultje et al. 2009). Thus, previous studies raise the possibility that there might be multiple localized components, mainly in Notch pathways, that determine the asymmetric cell-fate decisions in RG division. The contributions of these components may depend on the mode of divisions and be species-specific. A critical issue to resolve is which aspect of the structural asymmetry is associated with the inheritance of localized components.

5.3 Roles of Organelles in Cell-Fate Decision

Although the asymmetric daughter cell fates of RG cell division are correlated with the polar epithelial structure of RG cells as detailed above, the localization of the

candidate molecules may also depend on the asymmetry of intracellular structures or organelles as discussed above. The centrosomes are one such asymmetric intracellular structure and were first associated with the self-renewability of stem cells in *Drosophila* germ line stem cells (Yamashita et al. 2003). The two centrosomes that are segregated at mitosis are asymmetric in terms of centriole age and the degree of centrosome maturation and are replicated in a semiconservative way. Centrosome maturation (which involves the accumulation of pericentriolar materials) is normally coupled to centriole age, although there are exceptions (Conduit and Raff 2010; Januschke et al. 2011). Wang et al. suggested that the self-renewing daughters of RG divisions inherit the more mature centrosome with the mother centriole, whereas the other daughter receives the immature centrosome (Wang et al. 2009). The perturbation of centriole maturation by ninein knockdown promotes premature differentiation of RG cells. These findings suggest that centrosomes may play critical roles in the asymmetric division of neural progenitors. Given that membrane trafficking components such as endosomes often become associated with the mitotic spindle poles, one intriguing possibility is that components involved in the intracellular and/or extracellular components are asymmetrically divided between the two centrosomes and thereby segregate asymmetrically into the resulting daughter cells. Microcephaly genes that encode centrosomal and/or centriolar proteins (Kaindl et al. 2010) may contribute to the cell-fate choices of self-renewal versus differentiation through centrosome asymmetry.

The primary cilia, which function as a signaling center in vertebrate cells, can affect the asymmetric divisions of RG cells. RG cells grow a short cilium during the G₁ phase. The G₁-S transition and the G₁-G₀ transition have been found to be affected by cilia formation in vertebrate cells (Kim et al. 2011; Li et al. 2011; Inoko et al. 2012). Because the centriole becomes the basal body of the cilia, centriole components, including some microcephaly genes, may contribute equally to the formation of cilia. Therefore, one question to be addressed is whether the centrosome components impact the centriole, the basal body, or both, in the decision of RG cell fate.

6 THE SECOND TRANSITION IN THE DIVISION MODE OF SELF-RENEWING NEURAL PROGENITORS

It is known that primates develop an additional germinal zone (the outer SVZ) outside the SVZ in the middle of neurogenesis and that this zone is a major region of progenitor production during cortical development (Dehay and Kennedy 2007). Recently, certain progenitors in the outer

SVZ were identified as a new subtype of self-renewing progenitors. This new subtype was designated outer RG (oRG), or alternatively, basal RG (bRG) cells (Fietz et al. 2010; Hansen et al. 2010; Reillo et al. 2011), and is indicated as o/bRG cells in this article. These cells share some properties with RG cells, such as the ability to self-renew, the expression of RG markers, and the presence of basal processes. Like RG cells, o/bRG cells divide asymmetrically to self-renew and produce more committed cells (Hansen et al. 2010). However, they do not possess an apical process or apicobasal polarity and they do not undergo IKNM (Fietz et al. 2010; Hansen et al. 2010). Consistent with these observations, o/bRG cells do not appear to express apical structural components for adherens junctions or apical and subapical polarity complexes such as the Par complex (Fietz et al. 2010). These properties are almost identical to those of the self-renewing progenitors that are generated by the loss of apical membranes owing to oblique divisions in the LGN-deficient mice (Konno et al. 2008; Shitamukai et al. 2011).

Indeed, o/bRG-like cells have been identified in other species, including ferrets and mice (see online Movie 2 at cshperspectives.cshlp.org). These cells are generated during the middle to late neurogenic stages during normal brain development (Fietz et al. 2010; Reillo et al. 2011; Shitamukai et al. 2011; Wang et al. 2011), although they remain a minor population in mice, comprising ~10% of the self-renewing progenitors. This finding is consistent with the notion that the progenitor population is generated by infrequent oblique divisions in mice. Based on these data, we proposed a model in which the o/bRG cells are produced from the basal daughter derived from the oblique RG cell divisions that split the apical and basal regions into two daughters as observed in LGN mutants (Shitamukai et al. 2011). Together, these findings suggest that the formation of o/bRG cells from RG cells is a common feature in mammalian brain development.

The presence of the basal process in both o/bRG cells and RG cells suggests that these processes have an important role in the maintenance of self-renewing progenitors during cortical development. Indeed, Fietz et al. suggested the importance of integrin-mediated signaling from the basal lamina (Fietz et al. 2010) in o/bRG and RG cells. Furthermore, Tsunekawa et al. showed that cyclin D2, whose mRNA and proteins are localized to the basal end of the radial fiber in RG cells during the neurogenic stage (Glickstein et al. 2007, 2009), is segregated into the daughter cell that inherits the basal process during RG cell division; hence, cyclin D2 affects the daughter cell fates of RG cell divisions (Tsunekawa et al. 2012). Finally, other intrinsic and/or extrinsic signals such as Notch may also be used by the basal processes. We note that Notch activation in o/bRG cells depends on a supply of Delta from their

own daughter cells in contrast to RG cells in the VZ in mice (Shitamukai et al. 2011).

In summary, self-renewing progenitors undergo two successive transitions in their division mode during cortical development; they first transition from a symmetric to an asymmetric division mode, and they then transition from apical RG cells to o/bRG cells. One intriguing model that has been proposed (Konno et al. 2008; Fietz et al. 2010; Shitamukai et al. 2011; Wang et al. 2011) suggests that this transition occurs with the loss of apical processes during oblique divisions. The second transition allows the RGs in the VZ to adapt to a different niche, the outer SVZ. Indeed, the number of o/bRG cells is correlated with the degree of gyration (e.g., humans, monkeys, and ferrets), suggesting (Lui et al. 2011; Reillo et al. 2011; White et al. 2011) that the new niche, where the self-renewing progenitors reside after the second transition, may contribute to the increased proliferative capacity of o/bRG cells in mammalian species. Thus, the second transition of the division mode from RG cells to o/bRG cells may be key to the evolution of mammalian brain size. The outer SVZ is formed at the middle stages of neurogenesis, and progenitors in the zone contribute to the generation of the upper layer neurons in primates. Taking into account this fact and the recent discovery of RG cells specific for the upper layer neurons (Franco et al. 2012), these RG cells may be capable of undergoing the second transition to generate o/bRG cells, contributing to the expansion of brain size during mammalian evolution.

7 CONCLUDING REMARKS

Recent live imaging studies in rodents as well as those in other vertebrate models have revised our knowledge of the mechanisms underlying the asymmetric divisions of neurogenic RG cells from classical models, and revealed a common feature in RG divisions among vertebrates; when the epithelial structure is split unequally into daughter cells, the apical daughter differentiates and the basal daughter acquires the potential to self-renew via Notch activation. Although oblique divisions likely occur frequently in the neurogenesis of zebrafish and chicken, with the basal daughter regrowing the apical end foot to become an RG cell, the typical division mode of neurogenic RG cells in the mammalian cerebral cortex appears to be different from those cases. One daughter cell of RG divisions mostly inherits the entire epithelial structure owing to spindle orientations nearly parallel to the epithelial surface, and then self-renews. In the mammalian cerebral cortex, fluctuations in spindle orientations rather promote the generation of IP cells, and generate o/bRG cells, as the extreme case, by the loss of the apical end foot. It should be clarified whether this difference between the rodent neurogenic cerebral cortex and the

zebrafish brain comes from distinct developmental stages or species. Spindle orientation fluctuations are promoted by Inscuteable (and possibly AGS3) and suppressed by the LGN-NuMA-G α i complex, but detailed mechanisms also remain to be elucidated. One issue that needs to be addressed is differences in measuring spindle orientations among laboratories. Advances in live imaging techniques will enable us to accurately correlate spindle orientations, apical membrane partitions, and daughter cell fate in RG cell divisions. Even if the apical area, inherited by daughters, does not influence the daughter cell fate of mammalian RG cell divisions as observed in brain slice cultures, multiple asymmetric features originate from a slight tilt of the cleavage plane, including the cell volume, the basal process, and intracellular organelles such as centrosomes (Fig. 5). Which is most critical among them to create distinct daughter cell fates? Is asymmetric partition of such structural elements coupled with each other? These questions remain unanswered. Structural asymmetry in RG divisions is highly likely associated with the asymmetric activation of self-renewing signals including Notch. It will therefore be important to clarify the spatiotemporal manners, in which (1) Notch signaling components are transmitted into daughters from the parental cell, and (2) Notch signaling is activated in the daughters during the cell cycle. Thus, in vivo live imaging of signaling processes upstream of and downstream from Notch (as well as other extra- and intracellular signals) will greatly enhance our understanding of the mechanisms underlying the asymmetry in RG cell divisions.

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