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Mechanisms of radial glia progenitor cell lineage progression

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The mammalian cerebral cortex is responsible for higher cognitive functions such as perception, consciousness, and acquiring and processing information. The neocortex is organized into six distinct laminae, each composed of a rich diversity of cell types which assemble into highly complex cortical circuits. Radial glia progenitors (RGPs) are responsible for producing all neocortical neurons and certain glia lineages. Here, we discuss recent discoveries emerging from clonal lineage analysis at the single RGP cell level that provide us with an inaugural quantitative framework of RGP lineage progression. We further discuss the importance of the relative contribution of intrinsic gene functions and non-cell-autonomous or community effects in regulating RGP proliferation behavior and lineage progression.

Keywords: cerebral cortex; clonal analysis; gliogenesis; mosaic analysis with double markers; neurodevelopment; neurogenesis; neuroscience; radial glia progenitor; single-cell labeling

What makes the human cortex unique and how did the regulatory cell proliferation programs in neural stem cells (NSCs) evolve to accommodate the generation of bigger and more complex brains during evolution? These are key questions that require a clear understanding of the cellular and molecular processes controlling the development of the cortical entity from a relatively simple neuroepithelium. Across most mammals the overall cortical architecture is remarkably well conserved, however, the relative size and neuropil density in human has expanded significantly [1–5]. The cortex is composed of six distinct layers with a diverse array of cell types including excitatory projection neurons, inhibitory interneurons, and astrocyte- and oligodendrocyte glial cells [5–7]. The cellular and molecular mechanisms of generating cell type diversity and regulating NSC lineage progression in the dorsal telencephalon *in vivo* are mostly unknown. Key questions include: What is the quantitative and qualitative output of a single stem cell and how is the output/stem cell potential modulated over time? Which genetic and epigenetic factors regulate the temporal progression of a stem cell along its lineage? What is the relative contribution of cell-intrinsic vs. environmental and/or niche factors? Here, we focus on the above questions and discuss recent progress contributing to our conceptual understanding of cortical radial glia progenitor (RGP) cell lineage progression. For this review we mainly discuss advances that contribute to our quantitative understanding of the production of cortical projection neurons which are generated from dorsal telencephalic RGPs. We refer the reader to excellent recent reviews for the discussion of interneuron and glia genesis, and diversity [8–12].

Abbreviations

GW, gestation week; hESCs, human embryonic stem cells; IKNM, interkinetic nuclear migration; IP, intermediate progenitor; iPSCs, induced pluripotent stem cells; MADM, mosaic analysis with double markers; MST, mitotic somal translocation; NESC, neural epithelial stem cell; NSC, neural stem cell; oRG, outer radial glia cells; oSVZ, outer SVZ; RGP, radial glia progenitor; scRNA-seq, single-cell RNA sequencing; TAPs, transit amplifying progenitors; V-SVZ, ventricular-subventricular zone.

Radial glia progenitors generating cell type diversity in the neocortex

In the neocortex, projection neurons are derived from a common progenitor cell known as the RGP cell [13–16]. RGP themselves are derived from neural epithelial stem cells (NESCs) that compose the early embryonic neuroepithelium. NESCs were first identified by His over 100 years ago and are defined morphologically by a long basal process and an apical process that remains in contact with both the ventricle and the pial surface [17]. Sauer later confirmed that these cells undergo mitosis at the lumen surface, and that they contain apical–basal cell polarity. Furthermore, Sauer introduced the first model for interkinetic nuclear migration (IKNM) [18], where mitosis occurs near the apical side of the neural tube and the two daughter cells migrate away postdivision [19,20]. At approximately embryonic day (E) 9 in mice and around gestation week (GW) 5–6 in humans, NESCs begin to transition into RGPs [3,5,21]. Nascent RGPs initially undergo symmetric proliferative (aka amplification) divisions resulting in the expansion of the progenitor pool [5,22,23]. At around E12, RGPs transition into a neurogenic state and divide asymmetrically thereby producing cortical projection neurons [24,25]. The earliest born neurons (destined to become layer 6 projection neurons) split the preplate into the superficial marginal zone and the deeper subplate [26,27]. Through consecutive waves of neurogenesis, nascent neurons migrate radially along the RGP cell process into the most superficial layer of the developing cortex where they mature and differentiate. This process continues with each new wave of neurons migrating past the previous, resulting in the formation of distinct cortical laminae in an 'inside-out' fashion [28–37]. Early born, deep layer neurons (layers 5–6) are largely composed of corticofugal neurons that innervate brain regions beyond the neocortex including the thalamus, brainstem, and spinal cord [38,39]. Later born superficial neurons (layers 2–4) consist of intracortical neurons that project locally, ipsilaterally, or to the contralateral cortical hemisphere. The neurogenic expansion occurs in a waxing, surging, and waning output pattern of neurons, finishing at E17 in mice and approximately GW20 in humans [40–42]. While the laminar position allows a rough classification of projection neurons it also dictates the ultimate connectivity of cortical projection neurons. Based on physiological connectivity patterns, the concept of a canonical microcircuit has been established [43,44]. In recent years many other criteria have been employed to enable the classification of cortical cell types ranging from morphological, physiological to transcriptomic fingerprints and myelination patterns [45]. In particular, single-cell RNA sequencing (scRNAseq) has ushered in a revolution in our understanding of the dynamic gene expression patterns and states; and their correlation with cellular fate and cortical cell type diversity [46,47]. Many of the technological advances related to the current state of the art scRNAseq methods, and how these advances have expanded our knowledge of cortical projection neuron heterogeneity have been recently reviewed [48–52]. Single-cell transcriptomes and methylomes [53] represent a robust measure to classify distinct cell types and predict lineage trees based on hierarchical clustering algorithms although the mechanistic principles responsible for their generation by RGPs *in vivo* remain to a large extent enigmatic.

Radial glia progenitors can also produce glia cells [54], including astrocytes and oligodendrocytes, which play important roles in the development, maintenance, and function of neuronal circuits [55,56]. Although gliogenesis has been shown to follow neurogenesis in the developing brain [57–63], the mechanisms of lineage progression from neurogenesis to gliogenesis, especially at the individual RGP cell level remain essentially unexplored [9,11,54,64].

Shortly after birth, the embryonic neuroepithelium transforms into the postnatal NSC niche in the ventricular–subventricular zone (V-SVZ) within the lateral ventricle [54,65,66]. While discrete subpopulations of RGPs give rise to ependymal cells [67], other RGPs transform into V-SVZ type B1 cells [68,69]. Type B1 cells represent the principal stem cell progenitors in adult neurogenesis [70]. Type B1 cells generate type C cells which represent transit amplifying progenitors (TAPs). Type C TAPs significantly expand the lineage and produce neuroblasts destined to populate the olfactory bulb [54,65,66].

The developmental programs regulating the successive generation of postmitotic neurons and glia cells, followed by progressive generation of postnatal progenitor cells by telencephalic RGPs need to be precisely implemented and regulated. Impairments in RGP lineage progression lead to alterations in the cortical cytoarchitecture which is thought to represent the major underlying cause for several neurological disorders including microcephaly or megalencephaly; and more subtle neurodevelopmental diseases including schizophrenia, autism, and epilepsy [3,71–74].

RGP lineage diversity and cortex size

The relative increase in size and complexity of the mammalian cerebral cortex during evolution correlates

with the acquisition of more sophisticated traits. A particular intriguing example is the skilled hand use where the transition from power grip toward precision grip correlates with the physical addition of new cortical fields involved in proprioception [75]. The culmination of brain growth (both in size and complexity) eventually led to the emergence of higher and unique cognitive traits that are characteristic to the human brain. In order to begin to understand how the brain can progressively increase in size, it is essential to obtain the complete RGP lineage trees in different species across evolution. This is a daunting task and currently the RGP-derived lineage of cortical projection neurons is best characterized in mice. However, recent efforts to recapitulate some of the earlier stages of human brain development, including regional patterning *in vitro* using human embryonic stem cells (hESCs) [76] may promise new insight in the future; at least on the general cell biological level by inferring lineage tree branches from cultured cells. Although hESC cultures have great potential, recent studies have employed induced pluripotent stem cells (iPSCs) derived from humans, chimpanzees, and macaques and directed these cell lines to a dorsal telencephalic fate. While these systems do not fully recapitulate the *in vivo* cellular niche (including environmental factors that may have a direct role in regulating cellular fate) the cell-intrinsic potential and putative differences among species-specific iPSC lines is interesting. It has been observed that human iPSCs display more prolonged symmetric proliferative divisions while in macaque they transition to an asymmetric neurogenic division relatively quickly [77]. Once human progenitors switched to asymmetric neurogenic divisions, they continued producing neurons for a longer period of time than macaque. Therefore, it is evident that at the cellular level fundamental differences and species-specific progenitor traits may indicate distinct regulatory mechanisms which may contribute to the evolution of adapted progenitor proliferation potential and thus brain size.

Radial glia progenitors have been shown to not only give rise to neurons directly but also generate a diverse range of distinct transient progenitor cell types with varying degrees of potency. These include intermediate progenitors (IPs), short neural precursors, TAPs and outer SVZ progenitors (oSVZ) or also referred to as outer radial glia cells (oRGs), and have been described [78–81]. The cellular and molecular features of oRGs have been discussed at length in recent reviews [82,83], and here we will only focus on the oRG proliferation potential with regard to the increase in cortex size during evolution. Given the emerging diversity of progenitor cells originating from RGPs it is important to analyze not only the total output of RGPs but also the proliferation potential of every class of IP. What types of neurons and/or glia cells are produced by each type of IP and how does the output change during overall lineage progression? In other words, what is the precise contribution of each progenitor and how do they contribute to quantity and cell type diversity during development and in different species? These are challenging questions for the field and in order to approach them concretely it is critical to observe and quantify neurogenesis at the single progenitor level. At the qualitative level, it will be revealing to characterize and classify the progenitors and their output by using morphological parameters paired with single-cell transcriptomes.

Elegant work from the Kriegstien lab and others has shown that human RGPs transit into distinct morphotypes during development and lineage progression [83–85]. For instance, after GW 17, ventrally located RGPs lose pia-contacting basal processes and transition into a 'truncated' RGP [85] which contribute to the expansion of the human cortical plate. oRGs themselves have been shown to have a huge proliferation and thus output potential since they are capable of producing several hundred neurons and thereby amplify the overall output of individual RGPs along their lineage [86,87]. Based on morphological analyses oRGs have been suggested to divide into several distinct classes which may individually exhibit additional distinctions in their output potential [84]. oRGs exhibit a unique dynamic mode of locomotion called mitotic somal translocation (MST) [79]. Immediately before cytokinesis, the soma rapidly translocates toward the cortical plate, which is independent of mitosis and working through an alternate mechanism to that of IKNM and saltatory nuclear migration [79]. While MST requires the activation of the Rho effector ROCK and nonmuscle myosin II the molecular mechanisms underlying MST are not well understood [88]. It is tempting to speculate that oRG-specific MST may be regulated precisely to optimize and/or tune their proliferation potential along their migration path. oRGs not only amplify the overall RGP output but they also constitute a critical scaffold for radially migrating neurons and their presence and proliferation properties have been implicated in gyrification [78,89–93].

Since oRGs are largely absent in the mouse brain, many studies have used variations of cerebral organoid systems which to varying degrees recapitulate some of the cortical structures seen in the human. In order to rigorously study the cellular properties of oRGs, a

number of experimental protocols [90,94–97], including the use of induced LIF/STAT3 signaling [98], have been continuously optimized. Although culture systems present certain caveats when it comes to the study of neurogenesis in human [99], these organoid systems still enable the study of human neural stem, RGP, and oRG cells in a short temporal window of human brain development [100]. Furthermore, they allow the introduction of perturbations into human-derived RGPs and follow the downstream repercussions. This has been particularly beneficial in addressing the role of Zika virus-induced cell death and proliferation deficits in RGPs which results in microcephaly in prenatal infants of infected mothers [98,101–103]. *In vitro* human iPSC model systems have also been surprisingly robust in recapitulating the temporal order of cortical neurogenesis in certain disease contexts including lissencaphaly [95,104].

Outer radial glia cells are also found in other non-primates, including ferrets. However, the mechanisms governing their proliferation and differentiation properties seem to differ from the ones in primates [78,80]. In mouse there is a small number of oSVZ radial glia-like cells (also referred to as basal RG or bRG), however, these differ significantly from oRGs in humans [81,105]. Under normal physiological conditions, mouse bRGs are incapable of symmetric proliferative divisions and express *Tbr2* at midneurogenesis, a marker characteristically expressed by IPs [89]. While expression of specific genes including *TBC1D3* in ventricular cortical progenitors can promote the generation and expansion of bRGs in mice, the underlying mechanisms controlling the generation of these cells is still not entirely clear [106].

Quantitative analysis of RGP lineage progression at population level

In order to decipher the precise path of RGP lineage progression the qualitative and quantitative RGP output has to be assessed [23,51,107]. Traditionally, a key approach to study neuronal output of proliferating RGPs in the developing cortex was to analyze (by pulse-chase labeling analysis) the behavior of a population of cells and infer the patterns of division down to the single RGP level [108,109]. These analyses helped to shape our current model of RGP lineage progression in the neocortex which is based on the concept that multipotent RGPs first undergo symmetric proliferative (amplification) divisions followed by sequential asymmetric divisions generating neurons for distinct laminae in a defined temporal order [5]. Transplantation studies [110,111] showed that cortical RGPs progressively restrict their potential. In other words, 'late' RGPs cannot revert back to produce 'early' lower layer neurons, whereas 'early' RGPs keep their full potential regardless of the age of the host. Indeed, the neurons which were produced by the 'early' progenitors were those expected based on the recipient's stage [110,111]. Interestingly, cortical RGPs retain their ability to sequentially produce distinct cell types in culture conditions [61,112,113].

In order to directly measure the dynamics of RGP proliferation behavior during lineage progression, criteria such as cell cycle length at different developmental time periods and the proportion of cells that exit the cell cycle after each division represent two key measurements. Pioneering experiments from Nowakowski and colleagues predicted that the cardinal RGP undergoes 11 rounds of division beginning at E11 and ending at E17 which marks the end of neurogenesis in the mouse [109]. There are four variables which are commonly used when determining the total RGP output of the neocortex. First, the growth fraction or the proportion of RGP and IP cells that are actively proliferating [109]. Second, the number of RGP cell cycles during the cortical neurogenic time period. Third and fourth, the number of cells exiting the cell cycle and the number of cells remaining in a proliferative state after each round of division, respectively [114]. With these variables, Nowakowski and colleagues were able to very accurately model the growth of the cortex. For example, the absolute number of cells undergoing proliferation will change from one cell cycle to the next. When neurogenesis begins, the number of proliferating (P) RGP would be close to a maximum P value of 1, with 1 implying that every cell in the developing cortex is proliferating. With each cell cycle, a certain fraction of these daughter cells would exit the cell cycle and either become quiescent (Q) or terminally differentiate. The value of Q will gradually increase from 0 until it eventually reaches 1 at the end of neurogenesis. P and Q are inversely correlated and together can be used to calculate the absolute number of cells proliferating during each round of cell division [114]. Correlating these findings with the limited human data at the time was challenging. However, in chimpanzees it appears that similar to mouse the cell cycle length increases, and fewer proliferating cells can be observed, in later time windows [40]. These data led to two major conclusions. First, RGPs gradually become committed, stop proliferating, and eventually differentiate. Second, the key factor determining cortex size across evolution is not only reflected in the added diversity of progenitors such as oRGs (see above) but also in the time period of active RGP

proliferation and the length of the cell cycle at progressive later times [78–80,84,115]. While this model accurately accounts for the growth and absolute number of neocortical neurons, it does not take into consideration more subtle differences such as adjusting for the proportion of different progenitor cell types. It also does not allow for small changes in the ratio of RGP to IP or bRG and how this would affect overall output as illustrated also above in the previous section.

In order to move from population analyses to single-cell approaches, many studies used retroviral labeling of individual cells. The lineage of single proliferating RGPs were traced using mainly unicolor marking to then retroactively infer division patterns [116–121]. This method was also combined with live imaging to visualize individual cell divisions in real time, however, due to the technical limitations of the explant culture system, this was limited to shorter time periods of 24–48 h and did not allow for them to follow these cells into their final mature state [16,60]. Nowakowski and colleagues also combined various approaches and compared cell cycle behavior at the population level with the lineage analysis of the single retro-labeled RGP [20,22,40,109]. Their findings were remarkably robust and through mathematical modeling they were able to deduce that multiple populations of progenitor cells must be proliferating in parallel albeit not in synchrony. While *in vivo* lineage analysis provides a snapshot of clone composition at specific timepoints in development, it does not always provide complete information about the birth order of all clonally related cells. By culturing cortical RGPs *in vitro*, the temporal order of neurogenesis could be tracked reliably [61,113,122]. The fate of all progeny was followed through long-term live imaging, allowing for the generation of intricate cell lineage tree models to represent the birth order of each cell [113]. From these data, mathematical modeling was used to generate theoretical predictive models of cortical neurogenesis and identify division patterns of progenitor cells.

In vivo model of RGP lineage progression at single-cell level

While population-based approaches provided a robust frame work of RGP progenitor proliferation patterns and properties, high-resolution single-cell approaches are necessary to establish a definite model of RGP lineage progression *in vivo*. In order to pursue high-resolution single-cell lineage tracing, progenitor stem cells should be marked in a sparse but permanent manner. In the most optimal case, the marker will be transferred to the whole lineal progeny and even after numerous rounds of cell division still robustly mark all daughter cells. To this end, a large number of methods and approaches have been developed in the last decades which afford lineage tracing and clonal studies [51,123–125]. In particular, a variety of combinatorial fluorescent systems have been developed for performing high-resolution *in vivo* lineage analysis. Very prominent is the 'Brainbow' approach which is widely used in a variety of systems and which has recently been used to trace cortical RGP clones [126,127]. Another system, 'CLoNe', utilizes transposition vectors for cortical neuron lineage tracing [128,129] and the astrocyte-specific 'Star Track' specifically labels astrocyte progenitors and progeny [130]. The advantage of all the above systems is that the reporter constructs stably integrate into the genome, and thereby reliably label lineally related daughter cells. Combinatorial labeling with multiple markers also allows for the distinction between closely localized or spatially overlapping clones [127,128].

The monitoring, however, of precise progenitor division patterns *in situ*, and mapping entire lineage trees originating from an individual progenitor still represents a substantial challenge in the field. To this end, we have recently advanced the mosaic analysis with double markers (MADM) technology which provides an unprecedented genetic approach for *in vivo* lineage tracing in the mouse [131–133]. For MADM, two reciprocally chimeric marker genes are targeted separately to identical loci on homologous chromosomes. The chimeric marker genes (*GT* and *TG* alleles) consist of partial coding sequences for green (eGFP[G]) and red (tdT[T], tandem dimer Tomato) fluorescent proteins separated by an intron containing the loxP site. Following Cre recombinase-mediated interchromosomal recombination during mitosis, functional green and red fluorescent proteins are reconstituted resulting in two daughter cells each expressing one of the two fluorescent proteins (upon G2-X events: recombination in G2 of the cell cycle followed by X segregation, for technical details refer to Refs [131–133]). Analysis of MADM-based G2-X events in conjunction with temporally controlled tamoxifen (TM)-inducible CreER can provide exact information on birth dates of RGP (and other stem cell) clones and their cell division patterns (i.e., symmetric vs. asymmetric) [23,132,134]. An added MADM feature is the possible introduction of gene mutations allowing clonal two-color labeling with concomitant genetic manipulation. As such, these MADM applications permit the tracing of stem cell lineage progression in genetic mosaics with wild-type daughter cells labeled with one color (e.g., red) and

homozygous mutant siblings with the other (e.g., green) in an unlabeled heterozygous environment. In summary, MADM can provide an unambiguous quantitative optical readout of the proliferation mode (symmetric vs. asymmetric) of progenitors at the single-cell level and thus permit the determination of the developmental progenitor potential *in situ*.

In order to gain insight into the precise patterns of RGP division patterns and proliferation behavior, during neuron and glia production we have recently performed MADM-based quantitative clonal analysis [135]. Our systematic clonal analysis suggests that the behavior of individual RGPs is remarkably coherent and predictable across all developmental stages. RGPs initially undergo symmetric division with a predictable proliferation potential before transiting to asymmetric neurogenic division. Importantly, the explicit identification of asymmetric neurogenic MADM clones enabled a quantitative assessment of the neurogenic potential of individual RGPs as they switch from symmetric proliferative division to asymmetric neurogenic division. We found that RGPs in the neurogenic phase do not undergo terminal differentiation in a stochastic manner but rather follow a defined nonrandom program of cell cycle exit resulting in a unitary output of about eight to nine neurons per individual RGP. Perhaps interestingly, the size of asymmetric neurogenic clones was similar across neocortical areas with distinct functions, suggesting that the unitary neuronal output is a general property of cortical RGPs. Upon completion of neurogenesis, a defined fraction of individual RGPs proceed to gliogenesis, whereby about one in six neurogenic RGPs proceed to produce glia—astrocytes and/or oligodendrocytes—indicating a coupling between gliogenesis and neurogenesis at a predictable rate. Altogether, these MADM-based clonal analyses revealed definitive ontogeny of neocortical excitatory neurons and glia [135] (Fig. 1). While the MADM analysis detailed above provides a quantitative framework of lineage progression at the individual RGP cell level, the quality of distinct clones with unitary output remains to be determined. In other words, while the canonical RGP output is approximately eight to nine neurons, their distribution in the cortical plate may be fixed or display heterogeneity to various degrees. Furthermore, the clonal distribution pattern could differ in distinct functional areas. In order to address these questions it will be important in the future to monitor potential clonal heterogeneity at single-cell resolution and correlate the neuronal distribution with the functional areas in the neocortex. While we focus here mainly on neuronal output from RGPs, the predictable rate of glia production based on MADM analysis suggests a specific inherent gliogenic

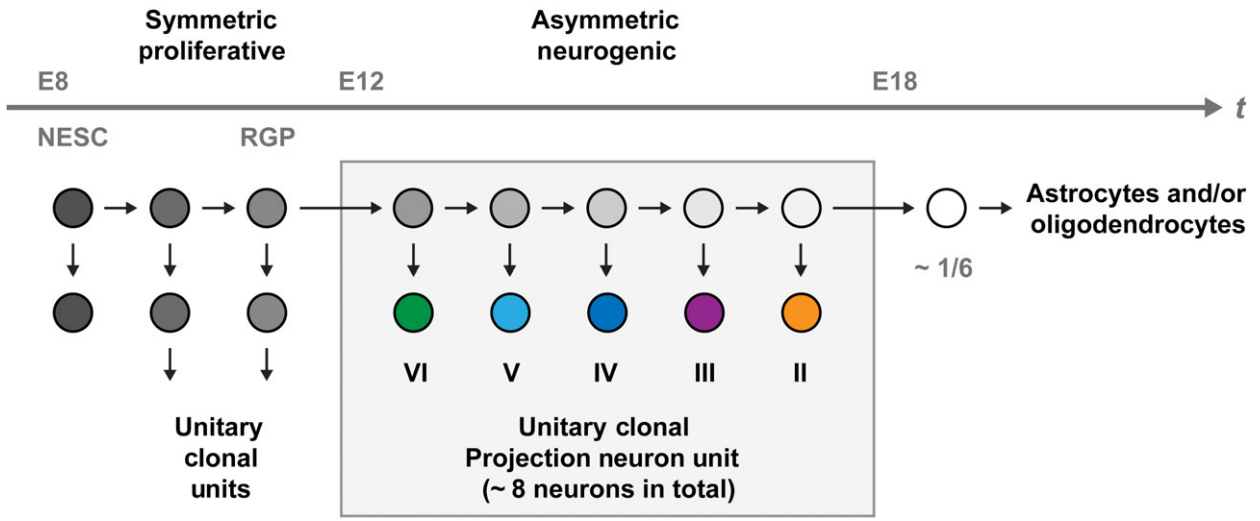

Fig. 1. Deterministic RGP behavior and unitary production of projection neurons in the neocortex. Systematic clonal analysis suggests that the behavior of RGPs is coherent and predictable across all developmental stages. RGPs initially undergo symmetric division with a predictable proliferation potential before transitioning to asymmetric neurogenic divisions. RGPs in the neurogenic phase do not undergo terminal differentiation in a stochastic manner but rather follow a defined nonrandom program of cell cycle exit resulting in a unitary output of about eight to nine neurons per individual RGP. Roman numerals VI to II refer to the serial production of neurons destined to cortical layers which are numbered accordingly. Upon completion of neurogenesis, a defined fraction of individual RGPs proceed to gliogenesis whereby about one in six neurogenic RGPs proceed to produce glia—astrocytes and/or oligodendrocytes—indicating a coupling between gliogenesis and neurogenesis at a predictable rate (Adapted from Ref. [135]).

potential which requires further analysis. Future studies should also integrate the quantitative concept of unitary neuron production at the cortical neuronal circuit level and evaluate the functional implications with respect to the canonical cortical wiring diagram.

Molecular and cellular mechanisms of RGP lineage progression

The MADM-based clonal analysis provides an inaugural quantitative framework of RGP lineage progression (Fig. 1), but the cellular and molecular mechanisms are not well understood. Key questions include: How is the switch from symmetric proliferative RGP divisions to asymmetric neurogenic RGP divisions controlled? Which mechanisms determine the neurogenic and gliogenic RGP potential? How is the deterministic mode of cortical neuron production regulated? In order to address the above questions the MADM system provides a platform for directed candidate gene approaches [132,136–141]. To this end we recently commenced to functionally analyze the molecular requirements controlling the first critical step in RGP lineage progression: the switch from symmetric (expanding) to asymmetric (neurogenic) RGP progenitor division. One key regulator of the mode of cell division is the signaling protein LGL1 [aka Llgl1, lethal giant larvae homolog 1 (*Drosophila*)], which regulates intracellular polarity in a variety of cellular contexts, and likely plays an important role in RGPs in mouse *in vivo* [142–146]. Albeit being predicted to contribute to embryonic RGP lineage progression, how *Lgl1* controls this process is not entirely clear. Furthermore, the relationship between LGL1-mediated cell polarity, ventricular zone architecture, and cortical RGP behavior has not been extensively studied *in vivo*. The functional requirement of *Lgl1* at later stages during NSC lineage progression, and including gliogenesis, is essentially unknown due to lethality of *Lgl1* knockout mice at birth. The analysis of RGP lineage progression in *Lgl1* mutant mice is somewhat compromised due to the severe and progressive disruption of the VZ resulting in disorganization and tumor-like growth of RGPs in the form of rosettes [143]. This, however, also raises the possibility that substantial aspects of the phenotype in whole tissue *Lgl1* knockout could be the result of a combination of cell-autonomous and non-autonomous and/or community effects. In order to address this issue, and to determine the relative contribution of cell-autonomous *Lgl1* signaling and non-cell-autonomous mechanisms in RGP lineage progression, we capitalized on the MADM system. We developed the following genetic strategy: subtractive phenotypic RGP analysis in genetic *Lgl1* mosaics (heterozygous, normal background; *Lgl1*-MADM) and conditional *Lgl1* knockouts (mutant background; cKO-*Lgl1*-MADM). In other words, *Lgl1* mutant RGP cells are either surrounded by an environment with 'normal' heterozygous and wild-type cells (*Lgl1*-MADM), or by mutant cells (cKO-*Lgl1*-MADM) [147]. The above genetic strategy represents a unique experimental paradigm (Fig. 2) which can be applied in principle to any candidate gene of interest to determine the relative contribution of intrinsic gene function and the effect of non-cell-autonomous effect on the overall phenotype *in vivo*. Interestingly, in cKO-*Lgl1*-MADM (but not *Lgl1*-MADM) the formation of heterotopic masses or subcortical band heterotopias was a predominant phenotype correlating with the downregulation of basolateral adherens junctional components similar like in the full knockout of *Lgl1* [143]. The cKO-*Lgl1*-MADM appeared to phenocopy *Numb*/*Numbl* double mutants [148–150]. NUMB localizes to the basolateral cadherin–catenin adhesion complex and is thought to control the trafficking of components such as N-cadherin (CDH2) [150]. Intriguingly, the loss of CDH2 [151] or αE-catenin [152], both resulted in the formation of heterotopias. A recent study observed that nonphosphorylated LGL1 strongly bound CDH2, whereas LGL1 with amino acid substitutions that mimicked phosphorylation did not interact with CDH2 [153]. These data suggest that LGL1 plays a critical role in adherens junction formation by regulating junctional CDH2 integrity presumably by regulating its internalization and/or intracellular trafficking [153]. Since *Lgl1* has also been suggested to play a role in polarized secretion and exocytosis regulation [154] it will be interesting to determine any putative functional relationship of *Lgl1* and *Numb* in regulating adherens junctional integrity and/or in controlling RGP proliferation dynamics. In mosaic *Lgl1*-MADM mice, where *Lgl1* was deleted only sparsely and/or removed from single RGPs, the mutant progenitors did proliferate normally with a unitary neuron output. These data indicate that wild-type progenitors surrounding mutant ones, maintain their integrity in the VZ in a non-cell-autonomous manner and that the exuberant RGP proliferation (and thus disturbed RGP lineage progression) in cKO-*Lgl1*-MADM and heterotopia formation is the result of community effects rather than the consequence of cell-autonomous *Lgl1* deficit.

One striking observation in individual *Lgl1*-MADM clones was that loss of *Lgl1* did not change the unitary neuron output but led to a massive increase in clonally related parenchymal astrocytes. However, it is currently not clear whether the increased proliferation of

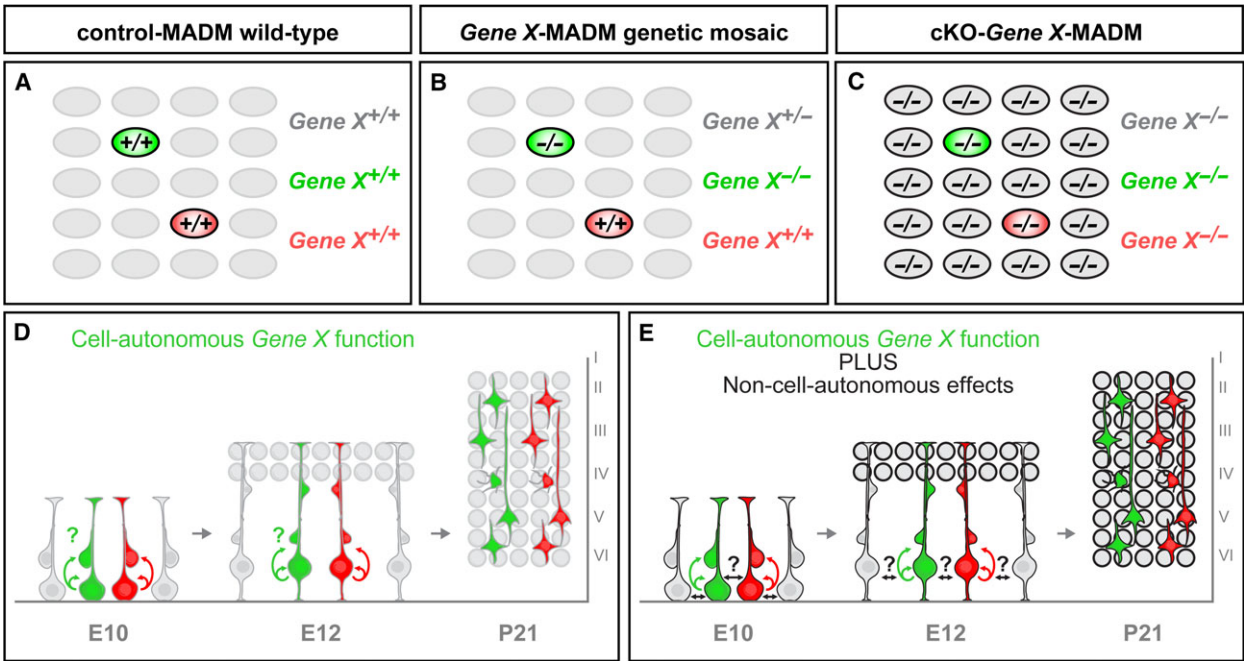

Fig. 2. Mosaic analysis with double markers-based genetic dissection of cell-autonomous gene function and non-cell-autonomous effects regulating RGP lineage progression. The genetic assay relies on comparative analysis of multiple MADM paradigms to distinguish cell-autonomous genetic functions from non-cell-autonomous effects. MADM-based genetic dissection of a gene of interest (*Gene X*) requires mutant alleles to be introduced distal to the MADM cassettes via meiotic recombination (for details how to introduce mutant alleles into the MADM system see also Ref. [132]). (A–C) Schematic illustration of experimental paradigm in control-MADM (A, wild-type), *Gene X*-MADM (B, genetic mosaic), and cKO-*Gene X*-MADM (C, conditional/full knockout). In control-MADM, GFP$^+$ (green), tdT$^+$ (red), and unlabeled (vast majority) cells are all WT. In *Gene X*-MADM, GFP$^+$ (green) cells are *Gene* $X^{-/-}$, tdT$^+$ (red) cells are *Gene* $X^{+/+}$, and unlabeled cells are *Gene* $X^{+/-}$. In cKO-*Gene X*-MADM, GFP$^+$ (green), tdT$^+$ (red), and the vast majority of unlabeled cortical projection neurons are all *Gene* $X^{-/-}$. By phenotypically comparing the GFP$^+$ *Gene* $X^{-/-}$ cells in *Gene X*-MADM (B, D) to the genotypically identical GFP$^+$ *Gene* $X^{-/-}$ cells in cKO-*Gene X*-MADM (C, E) the cell-autonomous gene functions and relative contribution of non-cell-autonomous effects can be identified and quantified at single-cell resolution (Adapted from Ref. [147]).

astrocyte intermediate progenitor cells [54] solely is responsible for the increased number of *Lgl1*$^{-/-}$ cortical astrocytes. The loss of *Lgl1* could in addition also lead to changes of the inherent gliogenic potential in RGPs. It will thus be informative to determine whether the fraction of gliogenic RGPs (1/6 in wild-type) is increased in *Lgl1*-MADM. Furthermore, the increased astrocyte production in RGPs lacking *Lgl1* was dependent on *Egfr* suggesting a functional relationship. In this regard, it is tempting to speculate that astrocyte overproduction could reflect the loss of a specific *Lgl1*-dependent function in polarized secretion and/or exocytosis, in order to regulate cell surface abundance of astrocyte production-stimulating (such as for instance EGFR) and/or -inhibiting factors. It is intriguing to speculate that the control of polarized secretion, exocytosis [154,155], and possibly further intracellular trafficking events, could actually represent one unifying function of *Lgl1* in the control of proliferating RGPs during several sequential stages in their lineage progression (Fig. 3). Indeed, the loss of *Lgl1* also compromises postnatal neurogenesis in a cell-autonomous manner although the underlying mechanisms remain to be clarified [147].

Outlook and perspectives

A rough framework of RGP lineage progression has been defined based on clonal analyses but a number of outstanding questions need to be addressed in the future. First, what is the degree of progenitor cell type diversity in the developing neuroepithelium and later in the VZ and SVZ? Do all RGPs harbor the same quantitative and qualitative potential for neuron/glia output? Recent lineage tracing and fate-mapping experiments employing distinct Cre/CreER-based approaches in combination with defined promoter elements suggest a significant level of progenitor cell type diversity [156–160]. While many promoters driving Cre/CreER recombinases lead to clones spanning all

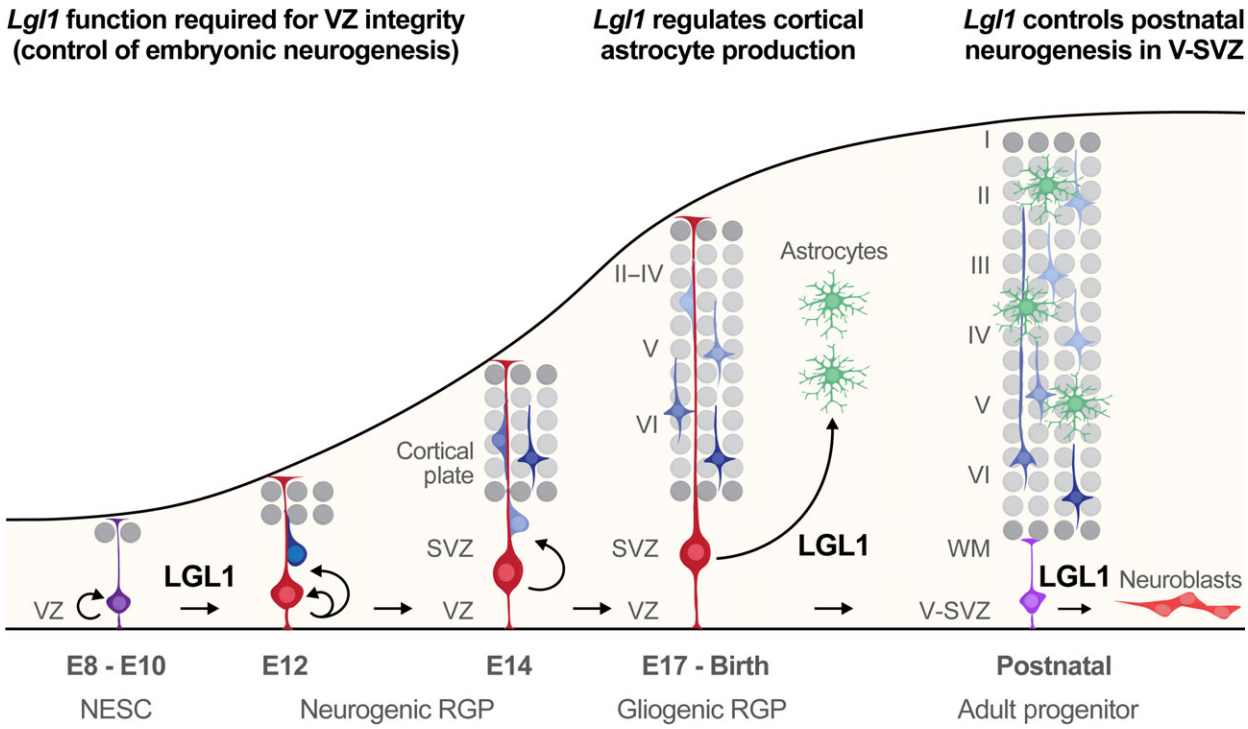

Fig. 3. Discrete sequential functions of *Lgl1* in regulating RGP behavior in the developing neocortex. Schematic model of RGP lineage progression and *Lgl1* functions at distinct stages of cortex development. See text for details (Adapted from Ref. [147]).

cortical layers, others appear to mark clones with more restricted laminar distribution. In most of the above studies, however, the analysis was not carried out at the single progenitor cell level. It thus remains an open question how diverse the RGP population really is with regard to the neurogenic and gliogenic inherent potential. The intrinsic RGP output potential could also be adjusted along the path of lineage progression and distinct RGP populations could respond differently to such regulation. It is interesting to note in this regard that once RGPs switch from symmetric to asymmetric neurogenic division about five of six RGPs lose the capacity to produce glia [135]. The advancement of scRNA-seq technologies holds great promise that in the near future the transcriptional profiles of large fractions of RGPs can be mapped in more detail, and the level of RGP cell diversity determined, at least at the level of gene expression. Building upon such data it will be important to correlate the gene expression profile with neuro- and gliogenic potential at the individual RGP level to evaluate the full spectrum of RGP cell type diversity.

What are the cellular and molecular mechanisms in cortical RGPs regulating the fine balance between proliferation and differentiation into neurons and/or glia cells, to specify the cerebral cortex of the correct size and cellular composition? While previous efforts greatly contributed to our current framework of neocortical genesis, experimental paradigms addressing the function of specific genes were mostly based upon whole population approaches (e.g., full and/or conditional knockout studies). However, the lack of true single-cell resolution of progeny fate vital for dissecting progenitor division patterns has previously often precluded a definitive understanding. MADM offers a promising solution and permits quantitative clonal analysis, concurrent with genetic manipulation, of precise division patterns and lineage progression at unprecedented individual progenitor cell resolution. With MADM it is also possible to define and quantify the relative contributions of molecular genetic cell-autonomous and non-cell-autonomous mechanisms controlling lineage progression in RGPs at single-cell resolution [147]. Future MADM analyses hold the potential to systematically analyze lineage progression in any stem cell and tissue, and probe the relative contributions of the intrinsic and extrinsic components of any gene function to the overall phenotype.

While lineage analysis in higher order mammals remains technically challenging, recent studies have cleverly utilized naturally occurring endogenous retroelements, to create lineage maps in the human brain [51,161]. However, it is still unclear whether the deterministic and/or unitary mode of neuron production

in RGPs represents a general principle. Thus, it will be important to establish models or RGP lineage progression at high single-cell resolution in other species than the mouse and especially in human. Recent studies have begun using cerebral organoids to approach the above issue [90,162–164] but complementary approaches beyond culture systems will be needed to obtain a more realistic model reflecting the *in vivo* condition. Ultimately, the combination of multidisciplinary approaches in cell culture and *in vivo*, and involving distinct species including human may promise a deeper understanding of the molecular mechanisms controlling (a) RGP lineage progression; (b) regulation of brain size in general; and (c) why human brain development is so sensitive to disruption of particular signaling pathways in pathological neurodevelopmental microcephaly, megalencephaly, or psychiatric disorders. In a broader context, the anticipated results will likely contribute to our knowledge of cortical neuron and/or glia specification and may potentially reveal a logic that can generate neuronal/glia diversity, thus providing a possible foundation for prospective future embryonic stem cell-based approaches in the context of directed brain repair [165–168].

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