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Epigenetic regulation of neural stem cell fate during corticogenesis

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ABSTRACT

The cerebral cortex comprises over three quarters of the brain, and serves as structural basis for the sophisticated perceptual and cognitive functions. It develops from common multipotent neural stem cells (NSCs) that line the neural tube. Development of the NSCs encompasses sequential phases of progenitor expansion, neurogenesis, and gliogenesis along with the progression of developmental stages. Interestingly, NSCs steadfastly march through all of these phases and give rise to specific neural cell types in a temporally defined and highly predictable manner. Herein, we delineate the intrinsic and extrinsic factors that dictate the progression and tempo of NSC differentiation during cerebral cortex development, and how epigenetic modifications contribute to the dynamic properties of NSCs.

1. Introduction

The cerebral cortex is composed of two main populations of neurons: projection (or pyramidal) neurons which are glutamatergic and excitatory, and interneurons which are GABAergic and inhibitory. In rodents, the projection neurons originate from neural stem cells (NSCs) in the cortical ventricular zone (VZ), and in contrast, almost all interneurons originate from NSCs located outside the cortex (Gorski et al., 2002; Martin and Rubenstein, 2001). It has been well established that the cerebral cortex is organized in layers that are defined by the densities and morphologies of these neurons.

During mammalian cerebral cortex development, distinct cell types are generated successively in a strictly regulated temporal order. Neurons are first generated followed by glial cells: astrocytes and then oligodendrocytes. Strikingly, sequential shift is also observed in neurogenesis *per se* whereby cortical neurons of different layers are sequentially generated in an 'inside-first outside-last' manner (Molyneaux et al., 2007). The first neurons to exit cell cycle and migrate out of the VZ occupy the preplate, which is subsequently split into two zones by the intercalation of later neurons. The upper zone of the splitted preplate (also known as marginal zone/MZ, and later become layer I) is populated by Cajal-Retzius (CR) neurons which are derived mostly from cortical hem (Zhao et al., 2006), while the lower zone becomes subplate (SP) which mainly functions to mediate axon targeting during development (Kanold and Shatz, 2006). A layer is created between MZ and SP, giving rises to the cortical plate (CP). In the newly formed CP, neurons of the deeper layers (layer V and VI) are generated earlier than neurons of the upper layers (layer II–IV), and these upper-layer neurons migrate past the deeper-layer neurons in the CP. Thus, it appears that fate potential of both neural and neuronal progenitors are finely programmed within NSCs, such that progressive restriction or acquisition of fate potential limits or enables, respectively, their differentiation into specific cell types.

Abbreviations: GABA, gamma-aminobutyric acid; FGF, fibroblast growth factor; BMP, bone morphogenetic protein; WNT, wingless int; SHH, sonic hedgehog; RA, retinoic acid; PRC1/2, polycomb repressive complexes 1/2; EED, embryonic ectoderm development; SUZ12, suppressor of zeste 12; EZH1/2, enhancer of zeste homolog 1/2; RING1A/B, ring finger protein 1a/b; JAK, Janus kinase; STAT3, signal transducer and activator of transcription 3; LIF, leukemia inhibitory factor; CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin-1; gp130, glycoprotein 130; BRN1/2, brain-specific homeobox/POU domain protein 1/2; SATB2, special AT-rich sequence binding protein 2; RELN, reelin; CTIP2, COUP-TF-interacting protein 2; FOXP2, forkhead box P2; SIP1, survival of motor neuron protein interacting protein 1; MTA2, metastasis-associated gene family, member 2; *Emx1/2*, empty spiracle homologues 1/2; *Pax6*, pair box domain 6; *COUP-TF1*, chicken ovalbumin upstream promoter-trascription factor 1; *Sp8*, specific protein 8; *Fezf2*, FEZ family zinc finger 2; *Gfap*, glial fibrillary acidic protein; *Dnmt1*, DNA methyltransferase 1; *S100β*, s100 calsium-binding protein beta; *Nfia*, nuclear factor I/A; *Neurog1/2*, neurogenin 1/2; *Foxg1*, forkhead box G1; *Otx1*, orthodonticle 1; *Svet1*, subventricular-expressed transcript 1; *Cux1/2*, cut-like homeobox 1; *Ntf3*, neurotrophin-3; *Sox5*, sex determining region Y-box5; *Tbr1/2*, T-box transcription factor 1/2; *Ski*, sarcoma viral oncogene homolog.

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Extensive studies in this phenomenon suggest that the regulation of NSC differentiation is orchestrated by an array of intrinsic mechanisms and extrinsic cues. In particular, the involvement of intrinsic mechanism such as epigenetic regulations in fate specification has not only enriched our knowledge, but provide another line of rationale in addition to changes in DNA sequences, in elucidating the complexity of developmental processes. Moreover, alterations of these kinds are retained through meiosis and heritable from generation to generations (Russo et al., 1996). Although this definition is commonly used at present, the dynamic flux of chromatin structure has prompted us to put forward a broader definition which includes chromatin modifications that are not necessarily perpetual, but are still able to cause changes in gene expression. Hence, Bird (2007) has proposed a revised definition of epigenetics as 'adaptations of chromosomal structures so as to register, signal or perpetuate altered activity state'.

There are three major types of epigenetic mechanisms: DNA methylation, histone modifications and non-coding RNA-mediated regulations (Bird, 2002; Goldberg et al., 2007; Wu and Sun, 2006). It is worth to mention that epigenetic modifications display dual mode of actions: exerting direct effects on gene transcription, and/or serve as platforms for the recruitment of chromatin remodeling complexes, resulting in persistent changes in chromatin state. Eventually, these changes activate or repress transcriptional programs either globally or specifically, which ultimately affect cellular phenotypes (Bird, 2002; Robertson, 2005). In this review, we introduce the key mechanisms and machineries, together with epigenetic modifications, that are responsible for the NSCs fate switch during corticogenesis.

2. Forebrain development

The cerebral cortex arises from dorsal telencephalon (pallium) of the prosencephalon (forebrain) during embryonic development. Upon closure of neural tube, the rostral portion of the neural tube (prosencephalon) differentiates into telencephalon and diencephalon. This is followed by regionalization of telencephalon *via* dorso-ventral mechanism, establishing dorsal progenitor and ventral progenitor domains. While the dorsal telencephalon develops into cerebral cortex as mentioned above, the ventral telencephalon (subpallium) becomes the basal ganglia (Götz and Sommer, 2005). Hence, dorso-ventral patterning plays pivotal roles in the early fate determination of telencephalic progenitors, and the initial specification of cortical progenitor identity.

Dorso-ventral patterning is orchestrated by the concerted actions of various morphogens such as FGF, BMP, WNT and SHH. These morphogens are secreted from distinct patterning centers, including the anterior neural ridge, the roof plate, hem, and antihem (Grove and Fukuchi-Shimogori, 2003; Rubenstein et al., 1998). For dorsal patterning, FGF8 regulates the antero-posterior (A-P) axis (Fukuchi-Shimogori and Grove, 2001; Garel et al., 2003), while BMP and WNT molecules induce medio-lateral (M-L) axis (Furuta et al., 1997; Hebert et al., 2002; Rubenstein et al., 1999). SHH and RA instruct the surrounding tissue to acquire ventral and lateral identity, respectively (Shimogori et al., 2004). By spreading in a concentration dependent manner across telencephalic neuroepithelium, such regulatory molecules act in part, in promoting differential yet combinatorial graded expression of selective transcription factors (TFs) which confer positional identities within cortical progenitors that lead to functional arealization (the specification of functional areas) in the adult cerebral cortex. Among the candidate TFs that have been shown to be directly involved in cortical arealization are *Emx1* and *Emx2* (Bishop et al., 2000; Mallamaci et al., 2000), *Pax6* (Bishop et al., 2000), *COUP-TF1* (Zhou et al., 2001), *Sp8* (Zembrzycki et al., 2007), and *Fezf2* (Hashimoto et al., 2000; Jeong et al., 2007).

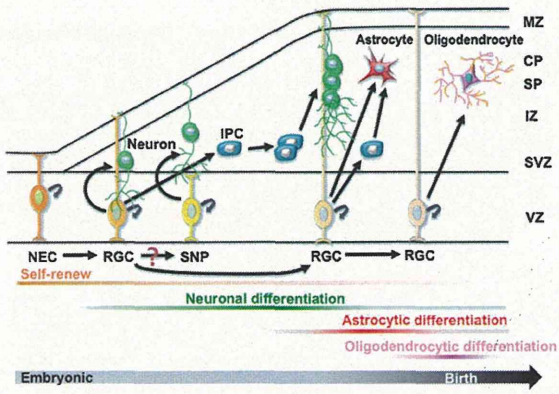

Fig. 1. Schematic representation of the diversity of progenitor populations and sequential developmental stages in the mammalian cortex. Neural development in cortex involves more than one type of progenitors which resided in respective proliferative niche and contribute to distinct cell types in mammalian central nervous system. At early embryonic stage prior to neurogenesis, NECs undergo self-renewal symmetric divisions, resulting in the expansion of neural progenitor pool. Following the onset of neurogenesis, NECs progressively switch into another types of progenitors namely, RGCs and SNPs. Later on, RGCs then give rise to another progenitor cell type, IPCs. All RGCs, SNPs and IPCs contribute to the neurogenic phase during cortical development. At late embryonic stage, gliogenesis is initiated when progenitors progressively differentiate into glial cells, such as astrocytes and then oligodendrocytes.

It is worth mentioning that these morphogens and TFs, do not work independently but cross-regulate each other in conveying defined positional identities to cortical progenitors. For instance, FGF8 of the anterior telencephalic source and WNT and BMP of the cortical hem interact antagonistically (Shimogori et al., 2004). Similar mutually repressive interaction has also been reported between *Pax6* and *Emx2* (Mallamaci and Stoykova, 2006). Intriguingly, numerous studies have indicated that the efficacy of regulatory mechanism is confined within a crucial time window, right before regional cell identity is intrinsically fixed by cell autonomous mechanisms (Backman et al., 2005; Li et al., 2005). Taken together, these facts show that the early stages of forebrain development are fundamentally governed by cross-regulation of morphogens and selective sets of TFs, in a spatially and temporally dependent manner.

3. Diversity and differentiation of NSCs and their progenitors

During embryonic development, the projection neurons arise exclusively from progenitors located within the dorsolateral wall of the telencephalon. Four main types of cortical progenitors have been identified within the developing cortex: neuroepithelial cells (NECs), radial glial cells (RGCs), intermediate progenitor cells (IPCs) and most recently, short neural precursors (SNPs). Each cell type harbors distinct proliferative features, molecular markers and laminar fate of their progeny (Götz and Huttner, 2005).

Prior to neurogenesis, the developing telencephalon is composed of a single pseudostratified layer of NECs lining the lateral ventricles, a region widely known as VZ. NECs contribute to most of the major cell types in the nervous system: neurons, astrocytes and oligodendrocytes. NECs divide symmetrically at the apical surface, producing two daughter cells (Fig. 1). Subsequent continuous self-renewal symmetric divisions then resulted in the expansion of neural progenitor pool and lead to the increased surface area of the VZ. Following the onset of neurogenesis around embryonic day (E)11, NECs progressively switch into another types of apical

progenitors (Fish et al., 2008), namely radial-glial cells (RGCs) and short neural precursors (SNPs) (Fig. 1). Thus, the length of both the progenitor expansion phase and onset of neurogenesis must be tightly regulated to ensure appropriate proportion of progenitors, RG differentiation and ultimately cortical surface area. Notch signaling and Fgf signaling are implicated in determining whether progenitors retain a division mode or acquire the differentiation mode. Fgf2, the only Fgf expressed by progenitors in the cortical VZ has been reported to promote the proliferation of progenitors (Vaccarino et al., 1999). On the other hand, Notch 1 and Fgf10 are involved in driving the timely transition from NECs into RGCs and contribute to the successive generation of basal progenitors and neurons (Gaiano et al., 2000; Sahara and O'Leary, 2009).

RGCs, as indicated by its annotation, exhibit morphological characteristics to that of glial cells and characterized by bipolar fibers that extend toward both ventricular and pial surface (Hartfuss et al., 2001; Pinto and Götz, 2007). These long fibers are indispensable in providing migratory guides for newly born neurons (Rakic, 2003). SNPs constitute another subpopulation of progenitors (Gal et al., 2006), and can be distinguished from RGCs by its short processes and the ability to drive the Tα1 α-tubulin promoter (Sawamoto et al., 2001). Albeit the true origin of SNPs remains unknown, it is thought that SNPs are responsible for direct neurogenesis (Stancik et al., 2010). Comparatively, RGCs are often considered to be the most predominant progenitor population owing to their ability to generate neural progenitor cells as well as neurons *via* asymmetric divisions. In the first instance, RGCs can divide into another RGC and a neuron, and later, give rise to another RGC and one basal progenitor (Götz and Huttner, 2005) (Fig. 1). As neurogenesis proceeds, this developmental process then takes place in an additional proliferative platform that lies above the VZ, known as the subventricular zone (SVZ), that comprises exclusively of basal progenitors. Basal progenitors, or more commonly known as IPCs, have their name derived from their division that occurs distant from ventricular surface. The transition from RGC to IPC is associated with the upregulation of *Tbr2* and downregulation of Pax6 (Englund et al., 2005). Although IPCs are known to have limited proliferative potential (Noctor et al., 2004), Kowalczyk et al. (2009) shown that IPCs are responsible for the production of majority (>80%) of pyramidal-projection neurons for all layer, which against the earlier hypothesis that IPCs generated only upper layer neurons (Tarabykin et al., 2001; Zimmer et al., 2004). As a whole, neurogenesis during cortical development appears to involve more than one type of progenitor cells (NECs, RGCs, SNPs and IPCs), which reside in respective proliferative niches (VZ and SVZ) and contribute to the successive phases from expansion of progenitor pool to neurogenic phase, through either symmetric or asymmetric division (Fig. 1).

4. Epigenetic regulations of NSCs development

At late embryonic stage, the termination of neurogenic phase marks the onset of astrogenic phase (Bayer and Altman, 1991). This phenomenon, where neurogenesis precedes gliogenesis occurs only in mammalian neural development. Extensive knowledge advancement has been attained in exploring how such temporal order is maintained in NSC-lineage determination during embryonic development. Apart from the canonical DNA/RNA/protein dogma, epigenetic processes appear to be another level of temporal and spatial regulation in development. Its ability to confer active/repressive marks to DNA/chromatin, and thus modulating the cellular outcome has made such modifications one of the most powerful intrinsic programs that govern developmental processes. Epigenetic regulation is well exemplified in the context of NSC lineage specification, particularly in neuron-to-glia switch (reviewed in Juliandi et al., 2010a,b; Namihira et al., 2008).

Recently, it has been demonstrated that the polycomb group (PcG) proteins are responsible for regulating the balance between self-renewal and differentiation of multipotent progenitor cells in the cerebral cortex (Pereira et al., 2010). PcG modulates chromatin structure by repressive mechanisms (Schwartz and Pirrotta, 2007). It is consisted of two complexes, PRC1 and PRC2. PRC2 contains EED, SUZ12, and methyltransferase EZH1 or EZH2 that catalyzes histone H3 lysine 27 trimethylation (H3K27me3) (Cao and Zhang, 2004; Shen et al., 2008). The resulting histone modification then recruits PRC1, which is composed of ubiquitin ligase RING1A or RING1B for PcG-mediated repression (de Napoles et al., 2004). Among other components, EZH2 is highly expressed in cortical progenitor cells, with slight protein expression in cortical neurons. Pereira et al. (2010) reported that the deletion of *Ezh2* in cortical progenitor cells before neurogenesis resulting in the shift of balance between self-renewal and differentiation toward differentiation, as witnessed by the overproduction of basal progenitors and neurons at early stages but depletion of both populations at later developmental stages. Although the temporal order of NSC differentiation is generally conserved, the loss of *Ezh2* narrows the neurogenic period that in turn reduces the neuronal output and alters the switch to gliogenesis. It is thus postulated that EZH2 is critical in controlling the timing of developmental progression within cortical progenitor cell lineages.

Given that NSCs are multipotent progenitors which can differentiate into either neuron or glial cell, the phenomenon where early NSC development is predominated by neurogenesis clearly indicated that the potency to differentiate into glial cells is repressed within early NSCs. Astrocyte differentiation is promoted following the activation of JAK-STAT3 pathway by extracellular signals such as LIF, CNTF, and CT-1 (Barnabe-Heider et al., 2005; Bonni et al., 1997; He et al., 2005; Johe et al., 1996; Nakashima et al., 1999; Rajan and McKay, 1998). These ligands that are also present during neurogenic phase failed to promote astrocyte differentiation during early cortical development. The irresponsiveness of NSCs in early gestation toward astrocyte-inducing stimulation is found to be attributable to the hypermethylated DNA in the promoter regions of astrocytic genes such as *Gfap* (Takizawa et al., 2001). Moreover, the methylation status of STAT3-binding element (TTC CGA GAA) within the *Gfap* promoter is varied in NSCs at different embryonic stages. STAT3-binding site is hypermethylated in E11.5 NSCs, while it barely methylated in E14.5 NSCs (Takizawa et al., 2001). Given that STAT3 cannot bind to methylated sequences (Takizawa et al., 2001), hence it is conceivable that *Gfap* promoter of early gestational NSCs remains inert toward cytokine-inducible astrocyte differentiation. The role of methylation was then further validated by a conditional knock out model of the *Dnmt1* gene in NSCs whereby the mutation resulted in decreased numbers of neurons, precocious gliogenesis and aberrant upregulation of astrocyte-specific genes such as *Gfap* and *S100β*, and most interestingly, activation of genes involved in gp130-JAK-STAT pathway (Fan et al., 2005).

Another developmental signal, Notch, has also been shown to contribute in neurogenic to gliogenic switch of NSCs. Namihira et al. (2009) indicated that committed neuronal progenitors and their derived young neurons confer astrocytic differentiation potential to the residual neural progenitors through Notch signaling-induced demethylation of astrocyte-specific gene promoters. Following the activation of Notch signaling, the expression of *Nfia* is upregulated in residual neural precursors, leading to the dissociation of DNMT1 and subsequently demethylation of astrocyte-specific gene promoters (Fig. 2). However, it is also suggested that Notch signaling only potentiates gliogenesis, by preparing neural precursors in a 'poised' state, astrocyte-inducing cytokines are still required to induce astrocyte differentiation (Namihira et al., 2009). In addition, Hirabayashi et al. (2009) showed that PcG-mediated epigenetic

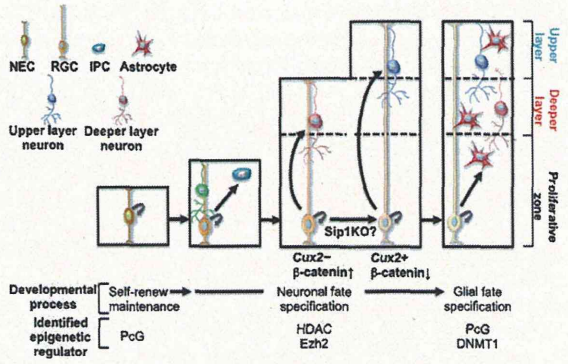

Fig. 2. Regulation of neural progenitors development in mammalian cortex. Intrinsic and extrinsic mechanisms together with epigenetic modifications are responsible for progenitor expansion, neuronal fate specification, and neurogenic to gliogenic switch. PcG proteins are responsible for regulating the balance between self-renewal and differentiation of multiple progenitor cells in the cerebral cortex *via* repressive mechanisms on differentiation genes. HDACs and *Ezh2* plausibly regulate the temporal progression of neuronal differentiation, as HDAC-inhibition leads to a fate switch from deep layer-producing progenitors into upper layer-producing progenitors, and deletion of *Ezh2*, encoding a histone methyltransferase component of PcG, leads to a reduction of upper layer neurons production. NSCs gradually acquire the potency to differentiate into glial cells, following a Notch-activated dissociation of DNMT1 from astrocytes-specific genes, and PcG proteins-mediated suppression of neuronal lineage genes.

mechanism promotes the transition from neurogenic to gliogenic phase in neural progenitors during cortical development (Fig. 2). Accordingly, in late-stage neural progenitors, PcG proteins contribute to the suppression of *Neurog1* locus and thus allowing STAT proteins to activate astrocyte-specific genes. Furthermore, it was shown that the repressive histone methylation H3K27me3 was gradually increasing at the *Neurog1* and *Neurog2* promoters. Collectively, these studies underpin that fate switch in neural progenitors is critically dependent on whether or not cells are intrinsically prepared, whereby epigenetic events in part control the responsiveness of progenitor cells to external cues and dictate cell fate decisions.

5. Sequential order in the generation of cortical projection neurons

In mammals, the neocortical structure and organization are best characterized by the presence of six layers that are different in their cytoarchitecture and functional properties (Peters and Jones, 1984). This fact raises a fundamental question: how these layers are formed?

To enable the sequential development of cortical layers, neurons destined to populate different cortical layers must be produced in a precise temporal order. In other words, the birthdate of cortical progenitors is directly correlated with their laminar identity (Angevine and Sidman, 1961; Rakic, 1971; Takahashi et al., 1997) and to a lesser extent, their projection identity. Besides the reported correlation between the birthdate of progenitors and laminar fate of their progeny, *in vivo* transplantation experiments (Desai and McConnell, 2000; Frantz and McConnell, 1996) and *in vitro* lineage analysis (Gaspard et al., 2008; Shen et al., 2006) further demonstrated that progenitor birthdate also defines the developmental potential of an individual progenitor. It was shown that early born progenitors acquire multipotency and are competent to generate both deep and upper layer neurons, while late born progenitors experience relatively increasing restricted options by producing only neurons of upper layers (Desai and McConnell, 2000; Frantz and McConnell, 1996). Given that the birthdate of progenitor specify only a time frame, extensive studies are conducted toward the identification of *de facto* factors that determine laminar fate and developmental potential especially during the advance of birthdate. To date, the precise molecular mechanism has yet to be revealed, owing to the multifaceted and complexity of the system. However, all studies converged toward a common understanding that, both intrinsic properties of progenitors and environmental cues concomitantly determine the fate of cortical progenitors.

5.1. Intrinsic programs and fate specification of cortical projection neurons

The ability of isolated cortical progenitor cells to produce an array of cortical neurons when cultured *in vitro*, which recapitulates the sequential corticogenesis *in vivo*, indicates the presence of an intrinsic genetic program (Gaspard et al., 2008; Shen et al., 2006). The regulation of cell cycle machinery has been suggested to impact progenitor fate because the laminar position is associated with the day precursor exits the cell cycle (Polleux et al., 1997). It has also been postulated that fate determination is governed by an internal clock mechanism that count the number of cell divisions progenitors have undergone before they are released for differentiation (Seuntjens et al., 2009). Recently, two cyclin-dependent kinase inhibitors, $p57^{KIP2}$ and $p27^{KIP2}$, were shown to affect the generation of deep and upper layers neurons respectively. The loss of $p57^{KIP2}$ in mice led to increased neurons in layer VI while the loss of $p27^{KIP2}$ exhibited overproduction of upper layer neurons (Mairet-Coello et al., 2012).

Notably, early and late cortical progenitors have been also shown to harbor molecular differences, in which TFs are expressed differentially in distinct progenitors, at distinct times (Alcamo et al., 2008; Bedogni et al., 2010; Britanova et al., 2008; Han et al., 2011; Hevner et al., 2001; Joshi et al., 2008; Kwan et al., 2008; Lai et al., 2008; McKenna et al., 2011). It is thus conceivable that early and late molecular programs lie intrinsically within progenitors, regulated by sequentially activated or repressed TFs, are one of the determining factors on how cortical progenitors choose between deep and upper layer fate. Consistent with this notion, the constitutive expression of *Foxg1* in cortical precursors was demonstrated to be critical in suppressing CR neuron fate in later born neurons (Hanashima et al., 2004). Besides, the congruency of *Fezf2* (Arlotta et al., 2005; Inoue et al., 2004) and homeodomain gene *Otx1* expression (Frantz et al., 1994), first in apical progenitors and later in their deep layer progeny neurons, made these TFs as an attractive candidates in controlling the fate specification toward deep layer neurons. Remarkably, *Otx1* mRNA is also relatively less abundant in the ventricular zone during late embryogenesis. In case of late progenitors, available evidence shows that *Svet1*, *Cux1* and *Cux2* are expressed in a subset of precursors in the SVZ as well as postmitotic neurons of layers II-IV during the generation of upper layer neurons (Nieto et al., 2004; Tarabykin et al., 2001; Zimmer et al., 2004). This is in accordance with the latest finding where Franco et al. (2012) demonstrated the presence of fate restricted radial glial cells sublineages in murine cerebral cortex. By using *in vivo* genetic fate mapping and *in vitro* clonal analysis, they have identified a distinct CUX2-positive progenitor population, in which intrinsically committed to generate only upper layer neurons, regardless of their niche and birthdate. Conversely, CUX2-negative progenitors' lineage was shown to primarily gives rise to deep layer neurons (Fig. 2). Notably, the findings of this model challenge the prevailing view that birth date dictates the laminar fate. Instead, they suggested that molecular fate specification facilitates proper birth order of cortical neurons (Franco et al., 2012).

5.2. External cues and fate specification of cortical projection neurons

Experimental evidence on the ability of early progenitors to give rise to upper layer instead of deep layer neurons when transplanted into brain of older embryonic stage suggests that apart from being intrinsically programmed, progenitor cell commitment is subjected to influence by external cues. β-catenin and SIP1 are among the probable candidates that have been shown to participate in progenitor and laminar fate determination during corticogenesis (Fig. 2). These factors are proposed to exert instructive external cues by establishing a gradient environment that mediates the subsequent cascade activation and repression of other downstream factors.

In accordance with this model, during the progression of cortical development, β-catenin signaling in progenitor cells is characterized by a temporal gradient. The expression of β-catenin is higher in progenitor which gives rise to deep layer neurons while lower when upper layer neurons are being produced (Mutch et al., 2009) (Fig. 2). Hence, overactivation of β-catenin led to increased proportion of deep layer neurons, which expressed the deep layer markers such as CTIP2 and FOXP2. Conversely, increased fraction of upper layer neurons, as represented by upper layer markers SATB2 and BRN1, was observed following inhibition of β-catenin signaling. Despite these facts, β-catenin overactivation seemed to affect only a fraction of late progenitors with the majority of them remained irresponsive to β-catenin signaling (Mutch et al., 2009). Additional mechanisms might be required to reset the fate of late progenitors to early cell fate which include intrinsic programs or another extrinsic factors, or synergistic action of both.

Seuntjens et al. (2009) provided the first evidence of postmitotic neuron-to-progenitor negative feedback signaling mechanism in regulating the fate of uncommitted progenitors. This signaling mechanism is reported to mediate by *Sip1*, a transcriptional repressor whose expression in postmitotic neuron is upregulated in embryonic cortex. Specific ablation of SIP1 in the neocortex not only induced precocious cell fate change from deep to upper layer neurons at E12.5–13.5 but also coincides with earlier neuron-glial fate switch at E16.5–17.5, owing to the premature onset of *Ntf3* and *Fgf9* expression (Fig. 2). This study provides an example of extrinsic signaling controlled by transcriptional regulator in dictating the fate of respective progenitors and subsequently leading to a shift of development potential. Taken together, these studies showed that an array of genes, TFs, and signaling molecules collectively play an important role in progenitor fate switch. Nonetheless, none of them is able to completely reset the switch, indicating that the *de facto* mechanism is unrevealed.

6. Cortical neuron subtype specification

Following the acquisition of laminar- and neuronal-specific properties, postmitotic neurons undergo subsequent specification programs that confer their projection subtype identity. In line with the progressive generation of neurons from different layers, the specification of projection neurons also follows a coordinated temporal order. From E10.5 to E16.5, neuronal specification is characterized by waves of transition that flow from SP neuron (layer I), corticothalamic neuron (layer VI), subcerebral projection neuron (layer V), and finally to callosal projection neuron (layer II–VI) (Molyneaux et al., 2007) (Fig. 3). In general, subcerebral projection neuron of layer V and corticothalamic neuron of layer VI are classified as corticofugal neurons and project subcortically to different targets. On the other hand, callosal projection neuron falls into the class of commissural neurons and extends their axons only within the cortex (Molyneaux et al., 2007). As yet, the only insight into subtype specification is *via* the direct transcriptional repression of each

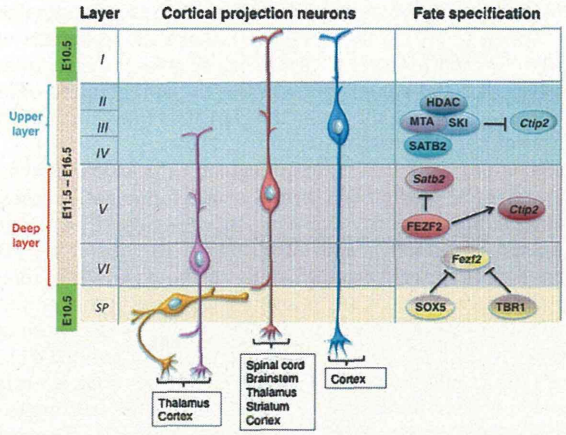

Fig. 3. Schematic representation of cortical neuronal subtype specification. After the acquisition of laminar- and neuronal-properties, postmitotic cortical neurons undergo subsequent specification programs that confer their projection subtype identity in a coordinated temporal order. Each neuronal subtype projects axon to different targets and their subtype identity is governed by distinct transcriptional factors. Transcriptional repression appears to be the central mechanism in controlling subtype identity.

subtype fate, such that the key TFs are mutually exclusive. It is also worth noting that genetic variations in these key TFs (SOX5, FEZF2 and SATB2) have been found within patients with developmental and language delays, intellectual disability, schizophrenia and autism spectrum disorders (Cooper et al., 2011; Lamb et al., 2012; Potkin et al., 2009; Rosenfeld et al., 2009, 2010). These are likely due to defective arrangement and formation of functional cortical circuitry since these TFs have been implicated in neuronal migration and positioning. Hence, alterations in these TFs not only change the neuronal identity of cortical neuron, but also contribute to a spectrum of cognitive and motor neurodevelopmental disorders.

Among the TFs that co-regulate multifaceted developmental aspects of early-born neurons are *Sox5* and *Tbr1* (Fig. 3). Throughout embryonic development, SOX5 is absent from cortical progenitors resided in VZ and SVZ, and newly born migrating neurons of the intermediate zone. *Sox5* is expressed only after cells exit the cell cycle, indicating that SOX5 mediates postmitotic refinement in shaping the molecular identities of early born neurons (Fishell and Hanashima, 2008; Kwan et al., 2008). The expression of *Sox5* is enriched in SP neurons, layer VI corticothalamic neurons and a subset of layer V subcerebral projection neurons (Fig. 3), and the loss of SOX5 differentially affects each of the three aforementioned corticofugal neuron subclasses. Collectively, analyses of *Sox5*-null mice demonstrated that SOX5 postmitotically regulates the migration, postmigratory differentiation and axonal projections of SP and deeper layer neurons (Kwan et al., 2008; Lai et al., 2008). Concomitant with its role in neuronal migration, the *Sox5*-null neocortex exhibits failure in preplate splitting, with SP neurons ectopically positioned in the upper edge of layer II instead of beneath layer VI (Kwan et al., 2008; Lai et al., 2008). Furthermore, it has also been shown that SOX5 is required for the migration of newborn deeper layer but not upper layer neurons. The lack of this ability led to laminar inversion of deeper layer neurons in the *Sox5*-null mice (Kwan et al., 2008). In addition to neuronal migration, *Sox5*-deficient mouse model interpreted a post migratory differentiation role for SOX5, whereby the loss of *Sox5* gene function led to reduced number of SP neurons and apparent defects in their proper differentiation as evidenced by the lack of normally distinctive SP neuron morphology (Fishell and Hanashima, 2008). SOX5 plays crucial roles in the development of layer-dependent corticofugal projections, including both corticothalamic neurons and subcerebral projection neurons. In *Sox5*-null mice, SP and layer VI

corticothalamic axons aberrantly projected to the hypothalamus (Kwan et al., 2008; Lai et al., 2008). Similar striking defects were also observed in subcerebral projections of layer V which failed to reach the pons and spinal cord, despite the upregulation of layer V-subtype specific TFs, following the abolished function of SOX5 as a transcriptional repressor (Kwan et al., 2008).

TBR1, is another transcriptional regulator that actively involved in a number of early-born neurons developmental processes, including laminar identity and position, molecular differentiation and axonal pathfinding (Han et al., 2011; Hevner et al., 2001; McKenna et al., 2011) (Fig. 3). Similar to the postmitotic roles of SOX5, the expression of TBR1 is also not detected in progenitors at VZ and SVZ, but confined to corticothalamic neurons in both layer VI and SP (Fig. 3), and CR neurons of the MZ (Hevner et al., 2001). The role of TBR1 in neuronal migration is evidenced by complex migration defects upon the loss of *Tbr1* gene function. Interestingly, this complicated migration phenotype is characterized by ectopic clustering of both deeper layer and upper layer neurons, subjected to area-dependent differences attributed to the rostral-caudal gradient expression of *Tbr1*. Indeed, the migration phenotype of *Tbr1*−/− mice might be partially due to the decrease of *Reln* mRNA levels, since RELN signaling has been shown to be critical for cortical layer formation. In addition, the expression of *Tbr1* is vital for correct corticothalamic and callosal projections, since the loss of TBR1 resulted in corticothalamic neurons reaching only the internal capsule and callosal projections terminated in the Probst bundle without reaching the midbrain (Leone et al., 2008). It is thus, seemingly clear that both *Sox5* and *Tbr1* contribute to the molecular identities and projections of SP and layer VI corticothalamic neurons.

Consistent with the notion that distinct transcriptional program governs specific subsets of projection neurons, two zinc finger transcription factors, namely *Fezf2* and *Ctip2* are the key determinants of subcerebral projection specification in layer V (Leone et al., 2008) (Fig. 3). This long-range projection constitutes the corticospinal tract (CST), descends through the brain stem into the spinal cord, and is crucial in providing a high degree of control over accurate motor functions (Eyre, 2007; ten Donkelaar et al., 2004). FEZF2 is highly expressed in early cortical progenitors starting at E8.5, and retained in their postmitotic deeper layer progenies while the onset of *Ctip2* expression is recorded only during postmitotic differentiation. Notably, the loss of *Fezf2* expression in mice cortices is accompanied by the lost of *Ctip2* expression (Chen et al., 2005a; Molyneaux et al., 2005), whereas the opposite is not the case. Hence, *Fezf2* appears to act upstream of *Ctip2* in regulating the fate of layer V subcerebral projections. When ectopically expressed in neurons of other subtypes, either *Fezf2* or *Ctip2* is sufficient to redirect their virtual axonal targets to subcortical targets. Furthermore, FEZF2 is able to alter molecular profile and promote subcortical projection neuron fate despite the absence of CTIP2 (Chen et al., 2008). It was also demonstrated that the forced expression of *Fezf2* in progenitors of GABAergic medium spiny neurons successfully induced fate switch and generation of subcortical projection neurons (Rouaux and Arlotta, 2010). However, knockouts of either *Fezf2* or *Ctip2*, both led to the abnormal development of subcortically projecting axons, in which the corticospinal motor neurons (CSMN) failed to extend into the CST (Chen et al., 2005a,b; Molyneaux et al., 2005). Given that FEZF2 harbors a putative repressor domain similar to that of engrailed protein, and CTIP2 is known to repress transcription of $p57^{KIP2}$ (Brayer and Segal, 2008; Brayer et al., 2008; Topark-Ngarm et al., 2006), it is conceivable that FEZF2 and CTIP2 regulate the fate specification of subcerebral projections *via* direct transcriptional repression.

In order to maintain the co-existence of multiple subtypes of projections within the developing cortex, the spatio-temporal dynamic activity of cortical FEZF2 is inhibited by SOX5 and TBR1 in SP and layer VI corticothalamic neurons (Shim et al., 2012) (Fig. 3). The earlier activation of *Fezf2* in cortical progenitors and its continued expression in deeper layer neurons is repressed in SP and layer VI neurons by SOX5 and TBR1, leading to a postnatal layer V with FEZF2-enriched pattern. It was recently reported that SOX5 is a functional competitor of SOX4 and SOX11, which exert cis-regulation on *Fezf2*. SOX4 and SOX11 functionally compete with the repressor SOX5 in binding to a conserved non-exonic element (E4) located adjacent to the *Fezf2* gene and acts as a cortical *Fezf2* specific enhancer, resulting in the transactivation of E4 and *Fezf2* expression (Shim et al., 2012). Therefore, the earlier expression of *Sox4* and *Sox11* as compared to *Sox5*, facilitates their role in the activation of *Fezf2* before upregulation of *Sox5* in layer VI. Besides, SOX5 also appears to delay the onset of *Ctip2* expression in layer V corticospinal neurons, allowing them to first migrate to their correct laminar position before initiating their projection to the spinal cord. Similar to SOX5, TBR1 abolishes FEZF2 activity in layer VI corticothalamic neurons *via* direct transcriptional repression (Han et al., 2011) (Fig. 3).

Interestingly, the subtype identities of upper layer-neurons might be controlled by a distinct mechanism that is independent from transient downregulation of *Fezf2* and *Ctip2* (Fig. 3). Upper layer-neurons do not express *Fezf2* and *Ctip2* at any time during their development (Alcamo et al., 2008; Britanova et al., 2008). Comparatively, much less is known about the mechanisms that control the fate specification of intracortical projection neurons. Recent studies have revealed that *Satb2*, is essential for the formation of callosal projection neurons and the loss of *Satb2* results in axon extension toward subcortical targets. SATB2 is expressed by a subset of postmitotic neurons in layer II–V (Fig. 3), but its expression is predominantly enriched in callosal projection neurons (Alcamo et al., 2008; Britanova et al., 2008). In layer V, *Satb2*-expressing callosal projection neurons appear to be intermingled with *Ctip2*-expressing subcortical projection neurons (Koester and O'Leary, 1993). The re-specification of axonal targets of callosal neuron to that of subcerebral projections in the absence of SATB2 is largely attributed to the constitutive activation of *Ctip2*, particularly within the population of upper layer neurons (Alcamo et al., 2008; Britanova et al., 2008). These data imply that SATB2 actively represses *Ctip2* expression in the specification of callosal neurons (Fig. 3). Conversely, the expression of FEZF2 was not significantly affected, indicating that *Satb2*-deficient neurons acquire only some but not all of the characteristics typical of CSMN (Alcamo et al., 2008). Collectively, these studies suggested that remarkable fidelity in the regulation of timing, location, and level of gene expression is crucial in controlling the phenotypic specification and evolution of neural circuits during cortical development.

7. Epigenetic modifications and cortical neuron specification

The involvement of epigenetic regulation in fate commitment of cortical progenitors has been largely unexplored, with only a handful of discrete literatures available to date. Despite the quantity, these literatures have shed some light on epigenetic modifications as another critical regulator that governs the fate specification and differentiation of progenitor cells. In particular, histone methylation has been shown to alter the proportionate production of deep layer and upper layer neurons. The deletion of *Ezh2*, encoding histone methyltransferase led to a 2-fold reduction of BRN2-expressing and SATB2-expressing upper layer neurons (Fig. 2). Meanwhile, the number of deep layer neurons in both layer V and VI remains equal. Furthermore, the expression of CUX1, normally detected in layer II–IV is almost undetectable in cortical plate at perinatal stage (Pereira et al., 2010). However, it seemed unlikely that histone methylation governed the fate specification of

deep or upper layer neuron since only upper layer neuronal population is affected. Therefore, the underlying rational between histone methylation and the production of upper layer neuron remains to be elucidated.

Histone acetylation is plausibly important for the specification of upper-layer neurons during late cortical development (Fig. 2). Transient histone deacetylases (HDACs) inhibition by valproic acid (VPA) in mouse embryonic stem cell (mESC)-derived neural progenitors not only induced neuronal differentiation, but also selectively enriched the neuronal population of upper layers (Juliandi et al., 2012). As yet, although the mESC-based system is able to recapitulate *in vitro* corticogenesis, particularly in the generation of repertoire of cortical neurons, the upper layer neurons are relatively under-represented (Au and Fishell, 2008; Gaspard and Vanderhaeghen, 2010). This bias implicates that *in vivo* cues are necessary for the full generation of upper layer neurons. Nevertheless, VPA treatment could increase the proportion of CUX1-expressing neurons accompanied by reduced population of RELN-positive early born or CTIP2-positive deep layer neurons (Juliandi et al., 2012). It was proposed that HDAC-inhibition promotes the temporal progression of neuronal differentiation during cortical development, leading to a fate switch from deep layer-producing progenitors into upper layer-producing progenitors. However, the underlying mechanism resulting in the temporal progression after HDAC-inhibition still remains elusive. Furthermore, the apparent heterogeneity in neural precursors (Stancik et al., 2010) and the presence of CUX2-expressing fate restricted radial glial cells sublineage et early developmental stage (Franco et al., 2012) are not in agreement with the temporal progression and fate switch in a seemingly single progenitor population. Therefore, whether the epigenetic regulation amplifies the number of upper-layer-fate restricted progenitors, resulting in an increase in the total of upper layer neurons produced or promotes the transition of these initially proliferative progenitors to precociously differentiate into upper layer neurons should be addressed. Meanwhile, the role of epigenetic modifications in promoting the temporal progression of neuronal differentiation cannot be ruled out.

As compared to the accumulating knowledge on how multifaceted epigenetic modifications determine the differentiation potential of NSCs and committed cortical progenitors, little is known about epigenetic-mediated restriction of subtype fate in cortical neurons. The first insight was provided by a MYST family histone acetyltransferase, *Querkopf*, which was proposed to regulate cell differentiation in the cortex through chromatin modifications. This is supported by the finding whereby *Querkopf* mutant mice displayed severe defects in the central nervous system, particularly a smaller cortical plate with significantly fewer cells as compared to that of wild type. Most importantly, accompanied with the prominent reduction of cells numbers in CP, the number of large pyramidal cells expressing *Otx1* in layer V was found largely affected among others. Furthermore, this reduction seemed to be layer-specific since the number of *Otx1*-expressing cells in layer VI were unaffected (Thomas et al., 2000).

Another example of epigenetic-mediated molecular program of subtype specification is provided by the specification of callosal projection neurons against subcerebral projection neurons. While both projection subtypes are generated from the same progenitor origin, their specification required distinct molecular programs such that FEZF2 and CTIP2 are essential for the specification of the latter (Arlotta et al., 2005; Chen et al., 2005a,b; Molyneaux et al., 2005), and SATB2 is the regulatory determinant of callosal projection neurons (Britanova et al., 2005). Various studies have substantially shown that SATB2 promotes callosal projection fate by antagonizing the expression of *Ctip2* in an epigenetic associated manner (Alcamo et al., 2008; Britanova et al., 2008), mediated by proto-oncogene *Ski* (Baranek et al., 2011) (Fig. 3). *Satb* family of TFs regulates transcription by binding to AT-rich DNA sequences, also known as matrix attachment regions (MARs). SATB2 is a chromatin remodeling protein that recognizes and binds to MARs, leading to the change of chromatin structure and subsequently affecting the accessibility of transcriptional machinery to its candidate targets (Britanova et al., 2005; Cai et al., 2003; Dobreva et al., 2003; Jenuwein et al., 1997). *In silico* analyses identified the presence of multiple MARs in both upstream and downstream of the *Ctip2* open reading frame (Britanova et al., 2008). Experimental evidence from interactive studies further substantiate the physical interaction of these two elements, whereby SATB2-binding was indeed detected within the 3.5 kb upstream region of the *Ctip2* transcription start site (Britanova et al., 2008). Following the lost of *Satb2* expression in mice, *Ctip2* expression was expanded across upper layers. Conversely, overexpression of SATB2 in NSCs *in vitro* markedly decreased the amount of differentiated CTIP2-positive neurons (Alcamo et al., 2008). Interestingly, Britanova et al. (2008) reported that SATB2 does not work alone but as a component of a protein complex that can interact with different MAR regions in the cortex *in vitro*. SATB2 represses the expression of *Ctip2* by interacting with histone deacetylases, HDAC1 and MTA2, both are members of the nucleosome remodeling and deacetylase (NuRD) complex (Fig. 3). In line with this finding, another transcription regulator SKI, appears to co-regulate the specification of callosal neurons since the loss of SKI also leads to ectopic expression of *Ctip2* and thus suppression of callosal identity. Accordingly, in upper layer but not deeper layer neurons, SKI and SATB2 are largely co-expressed and constitute a repressor complex *in situ*. The formation of repressor complex consisting of SATB2, SKI, HDAC1 and MTA2, is essential for transcriptional repression of *Ctip2* in callosal neurons (Fig. 3). While SATB2 binds to MAR-sequences in the *Ctip2 cis*-regulatory region and recruits MTA2 to the same site, SKI is required for recruiting HDAC1, and subsequently allowing the formation of a functional NuRD complex (Baranek et al., 2011). Furthermore, consistent with the notion that SATB2 induces changes in chromatin state, Alcamo et al. (2008) revealed that in the absence of SATB2-containing NuRD complex binding to MAR regions, H3K4me2 and histone H3 acetylation in the *Ctip2* locus are markedly increased, both of which correlate with an 'open' chromatin status. Hence, it is conceivable that these transcriptionally active marks permit the ectopic activation of *Ctip2* in *Satb2*-deficient callosal neurons.

8. Conclusion

Several lines of evidence described in this review support the notion that TFs and epigenetic modifications are among the important regulatory determinants of NSCs fate specification. The key mechanism to cell-type specification during corticogenesis is as yet debatable, with progressive restriction of fate *versus* lineage restriction as the most prevailing but contradicting views. Given that epigenetic modifications are highly cell-type specific, reversible, and occur only within a crucial time window, epigenetic mechanisms could be the key to impose such restrictions and thus managing the diversity of progenitors and ultimately the subsequent specification events. Taking into consideration that transcriptional regulation in neocortical development is highly pleiotropic, the elucidation of molecular determinants governing fate specification of cortical progenitors is an important challenge in the future.

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