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Cortical neurogenesis from pluripotent stem cells: complexity emerging from simplicity

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Abstract

The cerebral cortex contains dozens of neuronal subtypes grouped in specific layers and areas. Recent studies have revealed how embryonic and induced pluripotent stem cells (PSC) can differentiate into a wide diversity of cortical neurons *in vitro*, while recapitulating many of the temporal and spatial features that characterize corticogenesis. PSC-derived neurons can integrate into the brain following *in vivo* transplantation and display patterns of morphology and connectivity specific of cortical neurons. PSC-corticogenesis thus emerges as a robust model that provides new ways to link cortical development, evolution, and disease.

The cerebral cortex is arguably the most complex structure in our brain, and cortical neuron number and diversity are thought to be at the core of its powerful computational capacities. Most (>85%) cortical neurons are excitatory pyramidal neurons, while the remaining 15% are inhibitory interneurons. Pyramidal neurons and interneurons can be further subdivided into many subtypes, characterized by specific patterns of gene expression, morphology and connectivity [1].

Pluripotent stem cells (PSC), whether embryonic (ESC) [2] or induced (iPSC) [3,4], have emerged as a promising tool to model normal brain development and diseases. Here we will review recent data that demonstrate that a substantial fraction of cortical neuron diversity and complexity can be generated *in vitro* from PSC, while mimicking much of in utero development, revealing that many features of corticogenesis can result from self-organization. We will put special emphasis on studies that used human cells, and the insights that they provide on human brain development, evolution, and disease.

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Starting-up: regional patterning and neuronal specification

The cortical primordium emerges in the telencephalon, the anterior-most part of the forebrain. Interestingly, the telencephalic/forebrain identity first develops largely in the *absence* of any extrinsic morphogenic cues, and is even enhanced through active *inhibition* of morphogen signals such as Wnts or BMPs [5]. The telencephalon then undergoes patterning along the dorso-ventral axis, leading to the parcellation into several neurogenic niches, including the dorsal telencephalon and the ventrally located ganglionic eminences, which will generate cortical pyramidal neurons and most interneurons, respectively [6–8]. These basic features of corticogenesis are essentially recapitulated during directed differentiation of cortical neurons from PSC. Indeed when PSC are cultured without any added (caudalizing) morphogen or in the presence of selected morphogen inhibitors, most of them differentiate into neural precurors displaying a forebrain/telencephalic identity [9–13] [••14–16]. Moreover, if PSC-forebrain differentiation takes place with little or no SHH signalling, it mostly leads to the generation of dorsal telencephalic progenitors and glutamatergic, cortical pyramidal neurons [9, ••14,15, ••17–20]. In contrast, addition of SHH leads to specification of ventral telencephalic progenitors that will differentiate into both GABAergic and cholinergic neurons [19, • 21–23]. Since the majority of cortical GABAergic interneurons in humans, as in rodents, originate in the subcortical telencephalon [• 24,25], ventralized telencephalic differentiation of human PSC also give rise to cortical interneurons [••26–28].

Modeling temporal patterns of corticogenesis

Following early patterning, cortical neurogenesis will start to take place leading to the generation of 6 different neuronal layers, each characterized by specific patterns of gene expression and connectivity [1]. The layer-identity of a cortical neuron is tightly linked to the timing of its generation: this temporal patterning results in the sequential generation of layer-specific types of cortical neurons and is a fundamental process of neuronal diversification [29]. Remarkably, PSC-derived corticogenesis recapitulates this temporal patterning *in vitro*, leading to the sequential generation of a repertoire of neurons displaying specific molecular markers of all six layers [9,15,17,20,30], similarly to what was previously demonstrated using *ex vivo* cultures of early cortical progenitors [31]. Intriguingly, the proportion of each layer-specific neuronal subtype varies considerably depending on differentiation conditions. ESC-derived pyramidal neurons obtained in minimal culture conditions (low cell density without any added morphogens) are strongly skewed towards a deep layer identity [9,15], while a higher proportion of upper layer neurons are obtained when PSC are first differentiated at high density [20] or as cell aggregates [17,32], or when the PSC-derived cortical progenitors are transplanted in the mouse brain [15]. These differences should be explored much further, and it may yield new insights on the mechanism that control the timing and rates of production of specific pyramidal neuron subtypes.

While the sequential generation of pyramidal neurons from PSC is a robust feature, observed from ESC and iPSC of mouse and human origin, direct comparison between mouse and human PSC-corticogenesis revealed that it is greatly extended in time with

human PSC [15,17,19,20,32], even when using identical culture conditions [15]. Consistent with the protracted period of cortical neurogenesis in humans, human ESC-cortical progenitors start to generate postmitotic neurons after about 4 weeks instead of 6–8 days in the mouse, which is correlated with appearance of radial glia (RG)-like progenitors, the main neurogenic cortical progenitor [33]. Thereafter, mouse ESC-corticogenesis takes 2–3 weeks to be completed, while it takes 10–15 weeks starting from human ESC (Figures 1 and 2) [9]. These temporal specificities are strikingly similar to in vivo corticogenesis [34–38], and they may be directly relevant to the links between development and evolution of the cortex. Indeed, many of the species-specific features of the primate/human cortex, including a larger number and diversity of neurons, are thought to be linked to differences in the mechanisms underlying the generation of cortical neurons [36,38,39]. The mechanisms by which the primate embryonic brain can generate more neurons for prolonged periods might be linked to species-specific properties intrinsic to cortical progenitors, such as differential cell cycle control or tuning of self-renewal *vs*. terminal differentiation [40]. The emergence of other types of progenitors may also contribute to evolutionary changes in cortical neurogenesis. These progenitors include the recently described “outer” radial glial (oRG) cells [35,37,41–44], which share many features with RG cells, including the potential for self-renewal, but they lack any apical projection. Most strikingly, while human oRG cells can generate neurons directly, their progeny undergoes multiple rounds of divisions before final differentiation, thus providing a mechanism for increased neuronal output and cortical expansion. Importantly, the detection of oRG-like cells was reported following *in vitro* differentiation from human PSC [••20,45,46] but not from mouse PSC [46], providing further evidence of species-specific features of PSC-corticogenesis directly relevant to evolution.

A third aspect of corticogenesis that appears to be species-specific is neuronal maturation: once generated human cortical neurons display much prolonged patterns of morphological and functional maturation, such as dendrite patterning and synaptogenesis [47,48]. Similarly, PSC-derived human cortical neurons mature very slowly at the molecular and functional levels [15,20,32]. Even more strikingly, comparison of human vs. mouse PSC-derived cortical neurons transplanted into the mouse neonatal cortex revealed that the human pyramidal neurons follow a species-specific program of delayed neuronal maturation and synaptogenesis [15,49]. For instance, while ESC-derived mouse pyramidal neurons develop full blown and specific axonal and dendritic projections after 4 weeks [9], similarly differentiated and transplanted human neurons take more than 6 months to develop subcortical projections and at least 9 months to develop complex dendrite arborization pattern, dendritic spines, and functional synaptic activity [15]. Similar results are found with GABAergic cortical interneurons derived from human PSCs. While synaptogenesis and reasonably mature-appearing action potentials can be detected within one month of co-culturing with mouse cortical pyramidal neurons [27], transplantation studies into the neocortex of neonatal mice show limited terminal differentiation of these cells even 6 months later [28].

Overall, these data point to cortex-intrinsic mechanisms that control the clock of several key aspects of corticogenesis, for which PSC-based models may provide attractive experimental

set-ups to dissect the underlying mechanisms [30], with potentially important relevance to basic mechanisms linking cortical development and evolution.

Importantly, the very slow rate of maturation of cortical cells from human PSC presents a major challenge to the widespread application of this system for the study or treatment of human disease.

Modeling spatial patterns of corticogenesis

The cytoarchitecture of the cortex is crucial to its function, and despite its apparent complexity, key aspects of the patterned, three dimensional (3D) organization of the developing cortex can also be recreated *in vitro* (Figure 1B). When PSC are cultured as bowls of cells and differentiated into cortical-like progenitors, this leads to robust polarized neurons accumulating at their periphery, following an organization highly reminiscent of a nascent cortical primordium, including a ventricular-like zone and a cortical plate-like mantle region. Using long-term culture systems of 3D differentiation from PSC, the emergence of specific domains of progenitors and even cortical layer-like structures have been reported [45,46]. Most remarkably, using a long-term cortical 3D model, the final position of the neurons within a cortical plate-like structure was found to depend on its neuronal birthdate, where neurons born earlier were found in deeper positions than later ones, thus recapitulating the inside-out pattern characteristic cortical neurogenesis [45]. These data constitute striking demonstration that a cortical-like cytoarchitecture can self-organize *in vitro*, and it will be fascinating to test how far one can go to model further the complexity of cortical architecture, and perhaps function, in a dish. For example, it may be possible to use focal application of Shh signalling agonists in this system to create an interneuron-generating domain, then use the system to study the migration and integration of cortical interneurons within the developing human cortex.

The patterning of cortical areas is another complex process [6,50] that can be surprisingly modelled using PSC differentiation, combined with intracortical transplantation. Indeed, when mouse or human ESC-derived cortical neurons obtained in minimal culture conditions are grafted in the mouse neonatal cortex, they send most of their axonal projections to targets of the visual and limbic occipital cortex [9,15], with a pattern that is similar to grafted embryonic visual cortical tissue [9,51]. This was observed despite the fact that the cells were transplanted in the frontal cortex, suggesting that the highly selective ‘occipital-like’ pattern of projections was not likely due to respecification of the grafted neurons by the host. In line with this hypothesis, examination of the molecular identity of ESC-derived cortical progenitors and neurons before grafting revealed that most of them expressed markers of the occipital cortex [9]. On the other hand, the areal fate of ESC-derived cortical progenitors can be modified *in vitro* by the addition of extrinsic cues known to induce frontal cortical fates *in vivo* [17,45]. Interestingly, the human cortical cells, but not the mouse, tended to lose markers of occipital identity following longer periods post-transplantation, and their axonal projections corresponded to a wider range of areal identities with time [15]. These observations suggest that specific patterns of areal identity may be acquired *in vitro*, but that following grafting some of the cells can be specified to other areal identities over time, possibly in relation with their relatively early stage of maturation at the

time of grafting, and therefore higher susceptibility to extrinsic cues from the host cortex [52]. In any case, these data indicate that areal specification can occur in a specific way from PSC, which has interesting implications for our understanding of arealization mechanisms, and also in the long-run prospect of area-specific cortical repair strategies.

Modeling pathological corticogenesis

The advent of iPSC technology [4] offers in principle many novel opportunities to model brain diseases, including those that strike the developing cortex [53,54]. So far few studies have relied on iPSC-derived cortical cells to model neurodevelopmental diseases. Among these, one striking example is Timothy syndrome (TS), caused by a mutation in a L-type voltage-gated calcium channel, and leading to developmental delay and autism. Examination of iPSC-derived cortical cells from TS patients revealed several interesting phenotypes. These included, as expected, defects in calcium signaling and neuronal activity, but also more surprising defects such as the generation of specific types of neurons [55]. Together with other studies [56] [57] [46] [58] [59] these findings illustrate that PSC-derived corticogenesis can be used to model some aspects of complex human cortical diseases, particularly in the case of synaptic or electrophysiological abnormalities that are not restricted to a specific neuronal subtype.

Conclusion and perspectives

In sum, recent years have shown tremendous progress in the generation of cortex-like neurons from mouse and human pluripotent cells. Human PSC can generate cortically-patterned tissue both in 2D and 3D cultures, where they replicate key aspects of temporal and spatial patterning. Xenographic transplantation studies with both cortical pyramidal neurons and GABAergic interneurons derived from human PSC corroborate the in vitro studies, suggesting that bona fide cortical neurons are being produced. The protracted differentiation process, and the difficulty in generating and identifying more specific cortical neuronal subtypes, present important technical and conceptual challenges for the use of this technology to study human cortex development and disease. That said, with the generation of transgenic reporter stem cell lines allowing the identification of increasingly specific cell types, and the steady improvement of differentiation protocols, PSC-corticogenesis models may help to transform how we understand and treat diseases of the cerebral cortex.

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Highlights

- Pluripotent stem cell differentiation models spatial and temporal patterns of generation of cortical pyramidal and interneurons.
- Pluripotent stem cell derived corticogenesis displays species-specific features relevant to brain evolution.
- Pluripotent stem cell modelling is a promizing tool to reveal insights on neurodevelopmental diseases.

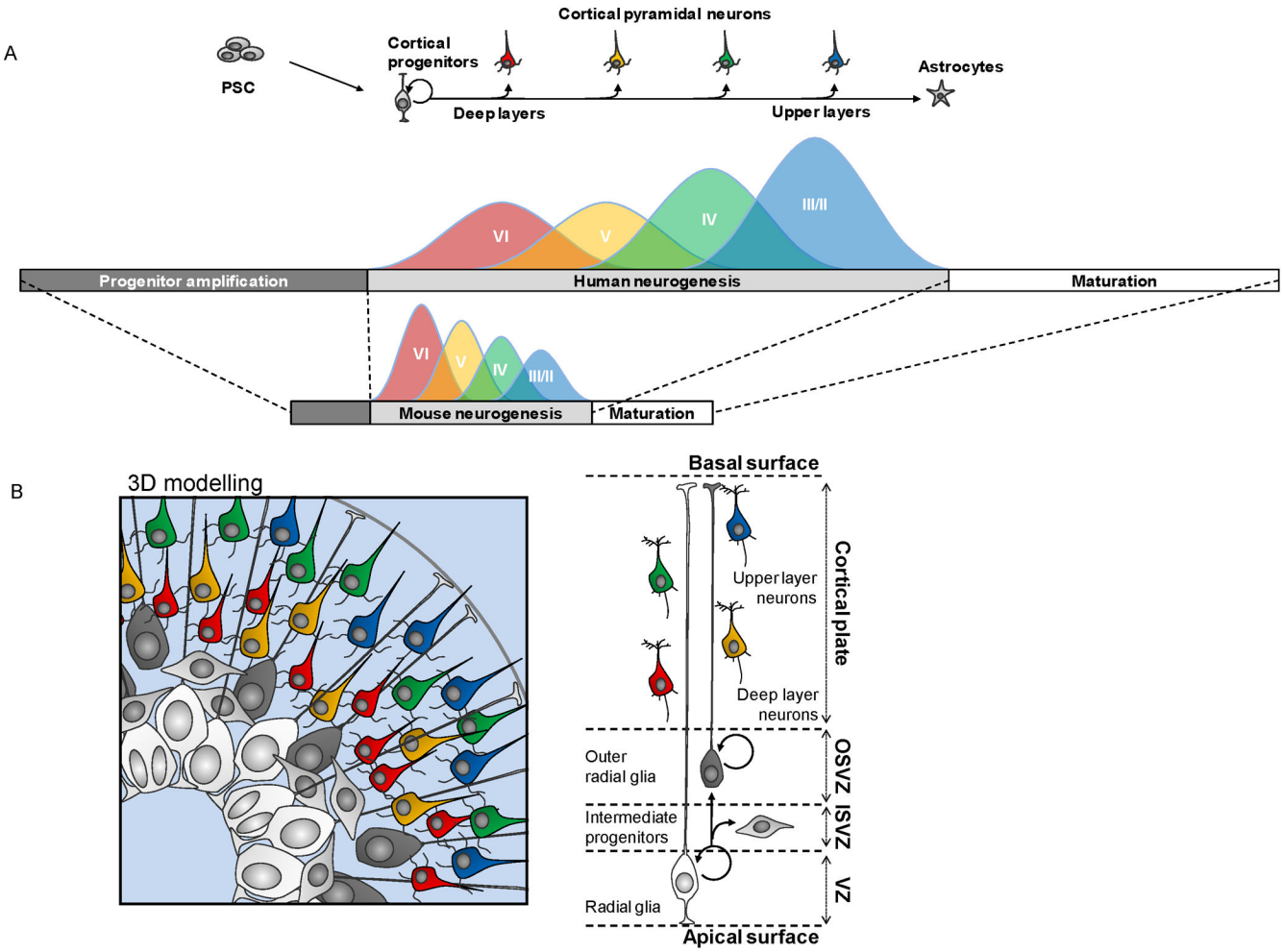

Figure 1. Modelling temporal and spatial patterning of cortical neuron neurogenesis

(A) PSC cultured under minimal conditions or in the presence of Wnt/Tgfβ/BMP morphogen inhibitors, undergo differentiation towards forebrain/telencephalic identity. In absence or low levels of Shh-signalling, PSC will mostly differentiate into a collection of progenitors of dorsal telencephalon/cortical identity. Subsequent generation of cortical pyramidal neurons follows a temporal patterning, with deep layer neurons being generated earlier than upper layer neurons, eventually followed by a switch to astrocyte production, like *in vivo*. Human PSC-derived corticogenesis follows a much more protracted time-course than the mouse counterpart, highly reminiscent of the *in vivo* situation. **(B)** Schematics of the relationships between the various cellular players of corticogenesis found in vivo. OSVZ/ISVZ, outer/inner subventricular zone. 3D models can recapitulate in a strikingly faithful way the *in vivo* organization of cortical progenitors and neurons, thereby providing unique tools to study spatial patterning and cytoarchitecture formation.

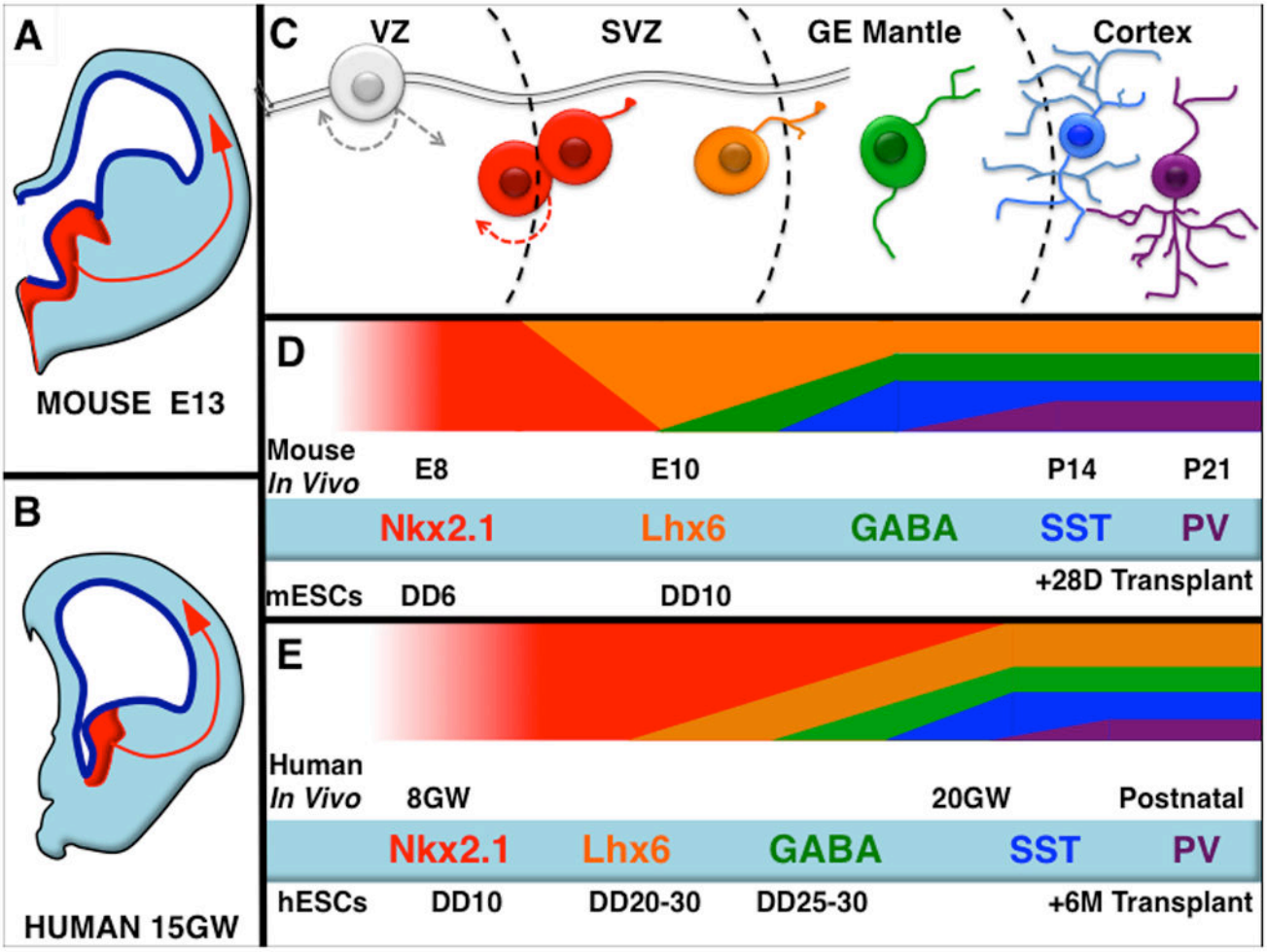

Figure 2. Comparison of Mouse and Human *In Vivo* and ESC-based *In Vitro* PV and SST Interneuron Development

Schematic of a mouse (A) and a human (B) half coronal section at comparable ages during neurodevelopment, embryonic day 13.5 and 15 gestational weeks (not to scale) showing in red the Nkx2.1-expressing medial ganglionic eminence (MGE). The MGE is the progenitor domain for both PV and SST expressing cortical interneurons and is relatively well conserved across species (A, B), as is the progression (C) from Nkx2.1-expressing progenitors, to Lhx6 and then GABA-expressing migratory precursors, then finally to terminally differentiated interneurons. Mouse ESC derived PV and SST expressing cells mature at analogous rates, with both makers detectable by approximately 4 weeks post transplantation into mouse neonatal cortex (D). Conversely human ESC-derived PV and SST expressing cells mature very slowly- also analogously to their *in vivo* counterparts (E). E=Embryonic Day, DD=Differentiation Day, GW=Gestational Weeks, P=Days After Birth.