

# Stem cells take the stairs

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Human embryonic stem cells progress through multiple stages in their path to neural differentiation, but the steps taken along the way are difficult to distinguish, limiting our understanding of this important process. Jing and colleagues (2) now report comprehensive analyses of transcriptome dynamics during this process that reveal five discrete stages, defined in part by highly connected transcription factor networks that link progressive stages. Surprisingly, the third stage, which appears to be critical for neural fate commitment, depends almost entirely on intracellular signaling.

One of the major challenges in developmental biology is to explicitly define unique stages and dynamic transitions within the stem cell differentiation process. This is largely because, as Conrad Waddington famously depicted in his epigenetic landscape, gene expression changes and cellular identity appear to roll down a hill of differentiation instead of taking easily defined steps (1). However, just as a ramp may become a pixelated staircase when more details are defined, the field has begun to identify small, definable substages of differentiation by in-depth analysis of molecular landscapes (1). These landscapes are regulated by both intrinsic signals (*i.e.* molecular events originating within cells) and extrinsic signals (cues from the external niche that feed information to the cell). Defined substages therefore depend on a delicate balance of extrinsic and intrinsic signaling. In this issue, Jing and colleagues (2) explore this balance using RNA sequencing (RNA-seq)<sup>3</sup> to create a detailed blueprint of transcriptome dynamics during differentiation of human embryonic stem cells (hESCs) into the neural fate, and they identified five distinct stages throughout the process (2). These data provide compelling evidence that differentiation consists of multiple unique steps defined by both extrinsic and intrinsic signals.

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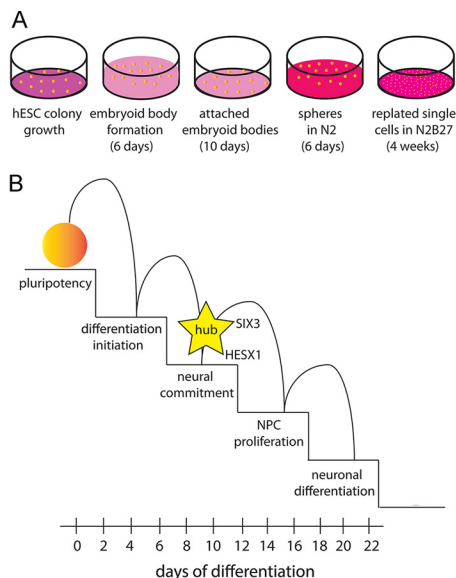
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<sup>3</sup> The abbreviations used are: RNA-seq, RNA sequencing; hESC, human embryonic stem cell; TF, transcription factor.

Among developmental processes, neurogenesis is particularly complicated due to its dynamic spatiotemporal progression, and errors in this intricate process could lead to developmental disorders such as autism or schizophrenia (3). Historically, rodents and amphibians were used to study brain development, but gaining a full picture of unique features of human brain development requires a more accurate human model. In particular, hESCs and induced pluripotent stem cells have provided a very useful platform to understand basic processes of human brain development and to manipulate specific genes to examine their functional roles. Previous work, including data from transcriptome analysis by RNA-seq, has created snapshots of the molecular state of the cell during hESC neural differentiation but only at a low resolution (4, 5).

To obtain a systematic view of neural development with a high temporal resolution, Jing and colleagues (2) modified a published protocol for hESC neural differentiation (6), so they could analyze cells across several developmental stages en route to cortical projection neurons (Fig. 1A) (2). They then performed RNA-seq analyses of samples collected every other day during the first 22 days of hESC neural differentiation. Hierarchical clustering, principal component analyses, and gene ontology analysis defined five major stages of hESC progression into neural fate: (i) pluripotency (day 0), (ii) differentiation initiation (days 2–6), (iii) neural commitment (days 8–10), (iv) neural progenitor cell proliferation (days 12–16), and (v) neuronal differentiation (days 18–22) (Fig. 1B). In addition to unique gene expression patterns, each stage involved distinct extrinsic signaling pathways, with the exception of stage 3 when almost all extrinsic signaling pathways were silenced. This is interesting because stage 3, or days 8–10 of hESC differentiation, represents the neural commitment stage. To correlate this finding with *in vivo* brain development, they compared gene expression of hESC differentiation with previously published transcriptomes of mouse embryonic day 7 epiblasts *in vivo*, which is a critical time point for cells to commit to the neural fate. Modules found in the stage 3 transcriptome were specifically enriched in the part of the embryo that will later form the brain. This result suggests that stage 3 gene expression profiles from cultured hESCs successfully represent cellular profiles of neural commitment *in vivo* during gastrulation.

Jing and colleagues (2) then go further to identify specific markers and key transcription factor (TF) networks for each stage. The TFs expressed at each stage correlated strongly with the gene ontology of the stage-specific transcriptomes, rein-



**Figure 1. Defined stages of neural differentiation from hESCs.** *A*, the differentiation protocol, from hESCs, to nonadherent embryoid bodies (three-dimensional aggregates of stem cells), attached embryoid bodies, neurospheres in N2 medium, and then single cells in N2B27 medium. *B*, hESCs progress through distinct stages of differentiation, drawn here as a stairway over the timeline of analysis. Each step is interconnected and controlled by unique TF hubs. The third step, neural commitment, has multiple hub TFs including *SIX3* and *HESX1*, which are required for progression of differentiation but not for maintenance of pluripotency.

forcing the notion that TFs drive cellular identity. In addition, they identified networks of interstage-connected TFs, with highly connected “hub” genes being critical for defining each state and mediating the transition between stages. *PAX6*, *SIX3*, *SIX6*, *HESX1*, and *ID3* were the top hub TFs identified in stage 3. These TFs were highly connected to stages 4 and 5, while they were mostly independent from earlier stages. *PAX6* is already known to be important for neural differentiation, so they sought to validate the function of the other hub genes by applying CRISPR/Cas9 gene editing technology to obtain deletion hESC lines. Whereas stages 1 and 2 (pluripotency and differentiation initiation) were largely unaffected by *SIX3* and *HESX1* deletions, these mutant hESCs could not progress through neural fate commitment and showed severe deficits in forming neural epithelium and neurons. By analyzing the expression levels of TFs within the *SIX3* and *HESX1* networks, they concluded that *SIX3* and *HESX1* regulate downstream TF networks to promote neural differentiation. Stable hESC lines with deletion of either *SIX6* or *ID3* could not be generated, likely due to their role in hESC maintenance. Future studies using conditional knock-out strategies may provide additional insight.

The study by Jing and colleagues (2) not only provides a very useful database of transcriptomes and TF networks for the field, but also identifies a key transition stage of neural commitment that surprisingly is associated with mostly intrinsic signaling. This foundational study also sets the stage for a number of future investigations. First, the dataset provides an entry point for validating the molecular transitions observed here in an *in vivo* context such as human fetal brain tissues. Similarly, it will be exciting to see additional confirmation that the neural fate commitment stage mostly involves intrinsic signaling by models

that better resemble *in vivo* human brain development, such as 3D brain organoids (7). Second, the current study established several hallmark stages, but future studies can apply single-cell RNA-seq with a higher density of temporal sampling in combination with bioinformatics tools, such as Monocle (8) and Waterfall (9) to obtain a more holistic picture of molecular cascades underlying the continuous process of human neural differentiation. Third, the emergence of a new field of “epitranscriptomics” and the recent finding of the widespread and critical role of m<sup>6</sup>A mRNA methylation for cortical neurogenesis (10) begs the question of how transcriptome composition, consisting of both unmodified transcripts and transcripts with different modifications from the same gene, changes during the course of human neural differentiation.

In summary, by identifying the specific point of neural fate commitment and molecular landscapes during the human neural differentiation process, Jing and colleagues (2) provide a significant step forward on the staircase of understanding molecular dynamics in differentiation, which will help to isolate the disruptions most likely to cause neurodevelopmental disorders.

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