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Small Molecules Take a Big Step by Converting Fibroblasts into Neurons

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Direct lineage conversion could provide a rich source of somatic cell types for translational medicine, but concerns over the use of transgenic reprogramming factors have limited its potential. In this issue of *Cell Stem Cell*, Li et al. (2015) and Hu et al. (2015) identify small-molecule cocktails that can convert fibroblasts into functional neurons without exogenous genetic factors.

Not long ago, Yamanaka and colleagues took a giant leap for translational medicine by demonstrating that a simple combination of four genes can reprogram somatic cells (Takahashi and Yamanaka, 2006). Several groups later adapted this approach and showed that it is possible to directly generate cells of many different tissue types using a similar approach, from neurons (Son et al., 2011; Vierbuchen et al., 2010) to cardiomyocytes (Ieda et al., 2010) to hepatocytes (Du et al., 2014). In the context of cell transplantation, direct lineage conversion has substantial appeal because cells do not transit through the tumorigenic pluripotent state. However, the need for using transgenic reprogramming factors in this approach still presents major technical and safety concerns (Xu et al., 2015).

In this issue of *Cell Stem Cell*, both Li et al. (2015) and Hu et al. (2015) take a big step in alleviating these concerns by demonstrating that small molecules alone are capable of converting fibroblasts into neurons.

To identify a neuron-inducing cocktail, Hu and colleagues began with a cocktail of VPA, CHIR99021, and Repsox that they had previously shown to be capable of converting fibroblasts into neural progenitor cells (Cheng et al., 2015), and they supplemented this with chemicals known to promote the differentiation of neural progenitors into neurons (Forskolin, SP600125, GO6983, and Y-27632) (Figure 1). Notably, they showed that the final cocktail converted multiple adult human fibroblast lines into functional neurons at a decent efficiency (one to three neurons for every ten starting fibroblasts).

Li and colleagues took a different approach and leveraged knowledge of a defined set of transcription factors that can convert fibroblasts into neurons (Vierbuchen et al., 2010). They initially screened for chemicals that promote reprogramming in the presence of *Ascl1*. Excitingly, they found that simultaneous administration of four of the chemicals identified as individual promoters of Ascl1-based reprogramming converted mouse embryonic fibroblasts (MEFs) into immature neurons without *Ascl1* overexpression. After further screening, an optimal combination of Forskolin, ISX9, CHIR99021, and I-BET151 (FICB) was determined to generate functional chemically induced neurons (CiNs) from fibroblasts at an extremely high efficiency (nine neurons for every ten starting fibroblasts).

CiNs produced by both cocktails possess several functional hallmarks of neurons, including extensive neurite outgrowth and branching, voltage-dependent ion channels, the ability to fire action potentials and respond to glutamate and GABA, and the capacity to form afferent synapses with primary neurons. Hu et al. used calcium imaging to infer that a large portion (similar to stem cell-derived neuron cultures) of their CiNs were active. RNA-seq analysis by both groups reinforced these results, showing that CiNs are indeed quite similar to stem cell-derived neurons and transcription-factor-induced neurons, while diverging significantly from fibroblasts. In addition, single-cell transcriptional analysis by both groups showed that the vast majority of CiNs express a neuronal transcriptional profile and have silenced the fibroblast network. Interestingly, both groups inferred that most of their CiNs are excitatory, glutamatergic neurons due to strong vGlut expression, while a small fraction of cells express GABAergic neuron markers. No substantial populations of cholinergic or dopaminergic neurons appeared to form.

MEF cultures contain a mixture of embryo-derived cell types, so there was a possibility that the chemical cocktail identified by Li et al. was acting predominantly on neural progenitor cells that lingered in the cultures as opposed to fibroblasts. To distinguish between these possibilities, they performed a lineage-tracing experiment using the fibroblast reporter *Fsp1*-cre MEFs. This experiment confirmed that their small-molecule mixture is capable of efficiently converting fibroblasts into neurons. In addition, the study by Hu et al. provides compelling evidence that mature, adult fibroblasts are also amenable to chemical neuronal conversion.

How do these chemical combinations induce neurogenesis? Both Li et al. and Hu et al. found little evidence for a transition through a neural progenitor state. Cell division stopped within 3 days of either chemical treatment and Hu et al. were unable to detect the induction of canonical neural progenitor genes, suggesting that conversion occurs directly.

Both cocktails clearly required synergy between the small molecules to induce full conversion, and the fact that both sets included CHIR99021 and Forskolin suggest that glycogen synthase kinase-3 inhibition and cyclic AMP stimulation

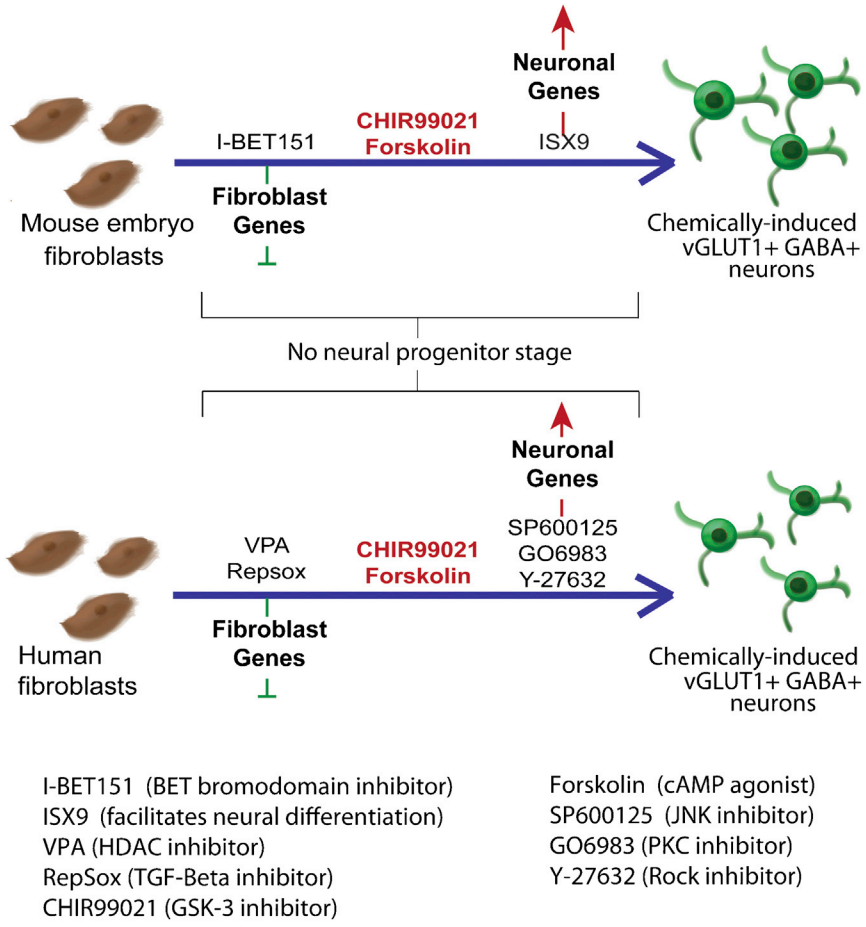

Figure 1. Summary of the Chemical Conversion of Fibroblasts into Neurons
Two different small-molecule cocktails convert fibroblasts into vGlut1+ or GABA+ neurons, neither one transiting cells through a neural progenitor state. Certain chemicals destabilize the fibroblast state while others seem to induce neurogenesis. (Image credit: Kristen Chen.)

play a key role in neuronal induction. Li et al. found that different small molecules acted on different aspects of the conversion process. The bromodomain inhibitor I-BET151 destabilized the starting fibroblast state by reducing the expression of key fibroblast transcription factors. This function is consistent with the known role of bromodomain proteins in coupling histone acetylation to transcription. In contrast, ISX9 treatment activated the expression of neurogenic transcription factors. Importantly, both studies noted a surge in electrophysiological and synaptic maturity when CiNs were co-cultured with primary glia, indicating that glial-derived factors also contribute to the reprogramming process.

The demonstration that small molecules alone can convert fibroblasts into neurons raises tantalizing possibilities and important questions that have direct implications for the translational utility of these cells. Can these cells engraft and function in vivo? Will they form functional, efferent synapses as well as the afferent synapses demonstrated in the papers?

Mammals have an incredible diversity of neuronal subtypes, each having a unique set of molecular properties that combine to enable the complex functions of the brain, sensory, and motor systems. The dysfunction of specific neuronal subtypes also causes the stereotyped symptoms associated with neurological disease. Is it possible to use chemicals to induce a specific neuronal subtype? How closely would these neurons mimic their primary counterparts, and how would this affect their ability to engraft upon transplantation or recapitulate disease in vitro? Hu and colleagues' finding that neurons generated from Alzheimer's disease patient fibroblasts had an increased Aβ42/Aβ40 ratio reminiscent of Alzheimer's disease brains is highly promising, but further experimentation must be done to identify disease processes in the CiN cultures.

Could this approach work for other, more easily obtainable cell sources such as peripheral blood mononuclear cells? For regenerative medicine, would it be possible to generate new neurons in situ by injecting a chemical cocktail into the brain? Do the CiNs retain any properties of the starting cells such as their age or specific chromatin and gene expression signatures?

Together, these groups have taken a major step in showing that small molecules alone can convert fibroblasts into neurons. While there remain many important unanswered questions about subtype specification and translational utility, these studies provide a key starting point for these efforts. They also show that small molecules can drastically alter the plasticity and fate of somatic cells. Here, Li et al. used a step-wise procedure in which they first screened for molecules to enhance transcription-factor-mediated conversion and later determined if a combination of the resulting hits could replace the transcription factors altogether. The same group previously employed this approach to identify a chemical cocktail capable of reprogramming fibroblasts into iPSCs (Hou et al., 2013), suggesting that this strategy could serve as a blueprint for developing chemical combinations to reprogram cells into other lineages.

Interestingly, VPA and Repsox from the Hu et al. protocol were previously identified in iPSC reprogramming screens (Ichida et al., 2009) and are part of the all-chemical iPSC cocktail (Hou et al., 2013), as are CHIR99021 and Forskolin, the two molecules common to both neuron-inducing cocktails. Although the scientific basis for this convergence remains to be determined, it may suggest that fibroblasts enter a highly plastic, but post-mitotic, cell state before transitioning into neurons. Slight modifications of the chemical recipe may yield additional lineages. Understanding, optimizing, and harnessing the small-molecule reprogramming approach will lead

to remarkable advances in disease modeling and regenerative medicine in the future.

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Resistance in the Ribosome: RUNX1, pre-LSCs, and HSPCs

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Therapeutic targeting of pre-leukemic stem cells (pre-LSCs) may be a viable strategy to eradicate residual disease and prevent leukemia relapse. Now in *Cell Stem Cell*, Cai et al. (2015) show that loss-of-function mutations in *RUNX1* reduce ribosome biogenesis and provide pre-LSCs a selective advantage over normal hematopoietic cells through increased stress resistance.

The myelodysplastic syndromes (MDSs) are a heterogeneous group of clonal bone marrow malignancies characterized by ineffective hematopoiesis, the presence of dysplastic cells in the bone marrow, and peripheral blood cytopenias. MDS occurs more frequently in older males and in individuals with prior exposure to cytotoxic therapy (Garcia-Manero, 2012), and individuals with MDS have an increased risk of developing acute myeloid leukemia (AML) (Heaney and Golde, 1999). Recent experimental evidence suggests that MDS arises from a series of transforming events that accumulate to generate pre-leukemic stem cells (pre-LSCs), the precursors of fully transformed LSCs (Pandolfi et al., 2013). Transformational genetic and epigenetic changes are believed to selectively expand pre-LSCs in the bone marrow, which then out-compete normal hematopoietic stem and progenitor cells (HSPCs). Genome-wide studies have recently identified a number of genetic lesions that are implicated in this process and the development and/or progression of MDS. These lesions have so far been found in splicing factor genes (e.g., SF3B1 and SRSF2) as well as genes involved in regulating DNA methylation (e.g., TET2, IDH, and DNMT3A), histone modification (e.g., ASXL1 and EZH2), and several signal transduction and transcription factors (e.g., RUNX1, p53, EVI1, JAK2, and FLT3). In this issue of *Cell Stem Cell*, Cai et al. (2015) show that mutations in the transcription factor RUNX1 reduce ribosomal biogenesis and provide a competitive advantage to pre-LSCs by enhancing stress resistance.

Almost half of MDS patients present with recurring karyotypic abnormalities affecting chromosomes 5, 7, 8, and 20, many of which impact the ribosome. Hemizygous loss of the ribosomal protein gene *Rps14* contributes to the development of anemia in 5q$^-$ syndrome (Ebert et al., 2008). Nucleophosmin, which is located on chromosome 5q35.1, has been implicated in MDS pathogenesis and is also critical for ribosome function (Grisendi et al., 2006; Reschke et al., 2013). Other genetic abnormalities cause impaired ribosome biogenesis (Ribi) and function—a collection of disorders known as ribosomopathies. Researchers have also found an association between ribosomal stress and activation of p53. In their current study, Cai et al. have focused on *Runx1*, a DNA binding transcription