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RESEARCH HIGHLIGHT

A chemical logic for reprogramming to pluripotency

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Pluripotency can be experimentally induced from somatic cells by nuclear transfer, fusion with embryonic stem cells, or ectopic transcription factor induction, but attempts to recapitulate this process by chemical means alone have previously failed. In a recent paper published in *Science*, Hou *et al*. pursue a rational, albeit laborious approach to identify cocktails of small molecules whose treatment restores pluripotency in adult somatic cells.

The defining characteristics of pluripotent stem cells (PSCs) — self-renewal and the potential to differentiate into any cell type — herald promise for use in regenerative medicine. By leveraging the knowledge that embryonic stem cells (ESCs) have reprogramming capacity, Takahashi and Yamanaka [1] identified the minimal set of transcription factors — the now-famous quartet of Oct4, Sox2, Klf4 and c-Myc — required to confer the developmental potential of an ES cell onto a terminally differentiated somatic cell to generate induced PSCs (iPSCs). The development of non-integrative methods to generate human iPSCs has reduced risks emanating from residual expression of transgenes [2]. However, efficient iPSC generation still relies on the transient delivery of transgenes that in other contexts are oncogenic. Therefore, it is theoretically appealing to envision that complete replacement of gene transfer with small-molecule compounds might improve the safety of human iPSCs.

Efforts to replace the canonical four factors using cell-fate modulators such as microRNAs and small molecules found a recurrent theme: among the four factors, Oct4 was the most difficult to be reproducibly replaced by any means. Several groups have reported genetic replacement of Oct4 by Nr5a2, Nr5a1, *etc* [3]. However, chemical replacement of Oct4 was believed to be challenging, if not impossible, to achieve in reprogramming adult cells. A single report described the use of a G9a methyltransferase inhibitor BIX-02194 to replace exogenous Oct4 during reprogramming of mouse fetal neural progenitor cells, but reprogramming of adult cells has not been described [4]. A new landmark study in *Science* [5] reports the generation of mouse iPSCs using small molecules alone. In a powerful demonstration of experimental perseverance, the authors combined small-molecule combinations from three different screens to create a reprogramming method that does not require the introduction of exogenous transgenes (Figure 1). The resulting chemical iPSCs (CiPSCs) possess the developmental potential of authentic ESCs and iPSCs, including the ability to colonize the pre-implantation blastocyst and germline transmission.

The *Science* study is an extension of an earlier study, in which a first screen searched for chemical cocktails that would efficiently replace Sox2, Klf4 and c-Myc. This yielded a small-molecule combination containing histone deacetylase inhibitor valproic acid (VPA), GSK3-β inhibitor CHIR99021, TGF-β inhibitor E-616542, and monoamine oxidase inhibitor Tranylcypromine (VC6T) [6]. A second screen identified small molecules that would drive reprogramming in the absence of ectopic Oct4 but in the presence of Sox2, Klf4 and c-Myc, leading to identification of cAMP agonist Forskolin. The chemical "substitutes" for Oct4, Sox2, Klf4 and c-Myc were then combined, but reprogramming failed to progress beyond an early stage.

The stalled reprogramming prompted a third screen to identify compounds that drive progression from the mid to final phases of iPSC generation. Using a doxycycline (DOX)-inducible Oct4 expression screening system, ectopic Oct4 expression was induced during the first 4-8 days, followed by starvation of ectopic Oct4 and candidate compound treatment. Epigenetic modulators, particularly 3-deazaneplanocin A (DZNep), an S-adenosylhomocysteine hydrolase inhibitor, were identified. The additions of DZNep and application of MEK and GSK3-β inhibitors, also known as "2i", to finalize chemical reprogramming [7], completed the chemical reprogramming protocol. The final cocktail consisted of VPA, CHIR99021, E-616542, Tranylcypromine, Forskolin and DZNep (VC6TFZ) followed by 2i treatment.

Hou *et al*. next proceeded to develop a plausible model of how exogenous Oct4 was replaced. Analysis of the early response to chemical induction revealed that Sall4, Sox2 and several extraembryonic endoderm-associated master regulators Gata4, Gata6 and Sox17 were up-regulated. Sall4 is a core regulator of both pluripotent stem cells and XEN cells, the *in vitro* counterpart to the primitive endoderm [8]. Sall4, Gata3, Gata4 and Gata6 replace exogenous Oct4 [9, 10]. Chemical reprogramming was inhibited when Sall4 and Gata family members were knocked down. These observations led the authors to postulate that chemical reprogramming proceeds through a nascent XEN-like state before consolidation of ground state pluripo-

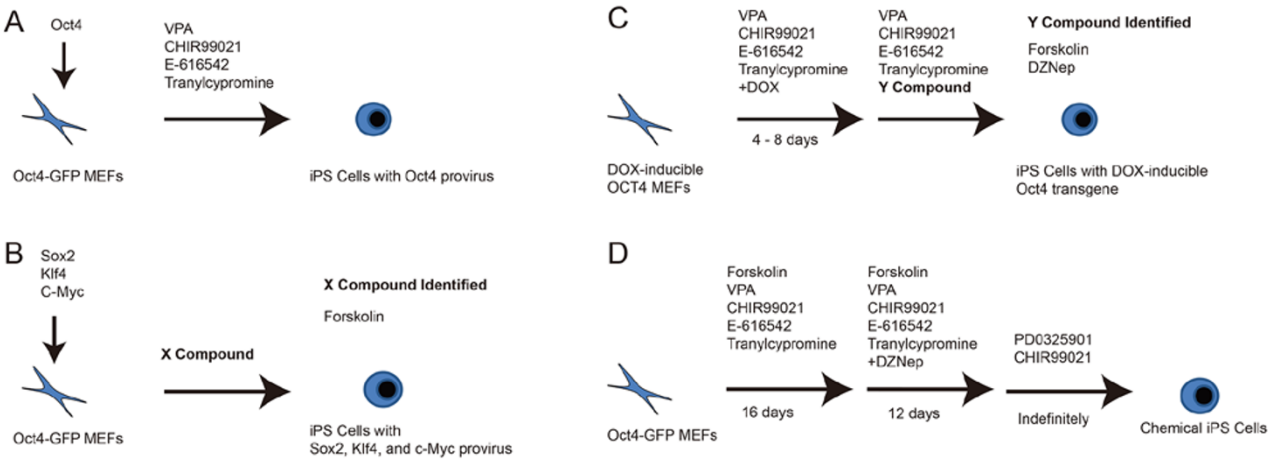

Figure 1 Achieving chemical reprogramming by a "Screen-And-Combine" approach. Chemical combinations sufficient to induce pluripotency were identified from three screens. **(A)** Screen 1 searched for compounds that replaced Sox2, Klf4 and c-Myc, yielding VPA, CHIR99021, E-616542 and Tranylcypromine (VC6T). **(B)** Screen 2 searched for chemicals (compound X) that replaced Oct4 when reprogramming with exogenous Sox2, Klf4 and c-Myc, yielding several compounds including Forskolin. Combining chemical "substitutes" from the two screens was not sufficient for reprogramming. **(C)** Screen 3 searched for chemicals (compound Y) that complete reprogramming by using MEFs containing a DOX-inducible *Oct4* transgene. The authors pulsed reprogramming cultures with DOX for 4-8 days followed by DOX withdrawal. Compounds identified included Forskolin and DZNep. **(D)** Final chemical reprogramming protocol. Forskolin and DZNep were added to cocktail VC6T. Reprogramming was finalized by 2i, consisting of MEK inhibitor PD0325901 and GSK3-β inhibitor CHIR99021.

tency by 2i. Thus, induction of Sall4, Sox2 and Gata family members in the setting of small molecule-mediated epigenetic derestriction and late treatment with 2i cooperatively replaced exogenous Oct4. This model sheds insight on how replacement of Oct4 was achieved, and most importantly, provides the first hints into principles for generating human CiPSCs.

The work of Hou *et al*. suggests that rather than merely serving as modulators of a transcription factor-driven reprogramming process, combinatorial perturbations of signaling pathways and epigenetic modulations by small molecules may allow any desirable cell fate to be programmed chemically. However, full appreciation of chemical reprogramming can only occur once human CiPSCs are generated. Compared to mouse iPSCs, conventional human iPSCs possess starkly distinct extrinsic requirements for their establishment and maintenance, a distinction that is ascribed to the notion that mouse and human iPSCs correspond to different stages of embryonic development [11]. For example, human iPSCs do not self-renew in response to 2i but instead reside in a MEK/GSK3-driven self-renewing state. Therefore, it remains unclear whether application of VC6TFZ to human somatic cells would drive the generation of human CiPSCs. Nonetheless, it should be emphasized that all of the small molecules used for chemical reprogramming have been reported to function in human reprogramming. A recent report shows that GATA3 can efficiently replace OCT4 in humans [12]. Finally, small molecule replacement of all other factors except OCT4 during human reprogramming has been described [13], meaning that the primary obstacle to human chemical reprogramming — chemical replacement of exogenous OCT4 — may not be insurmountable. Human CiPSCs may be on the horizon.

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