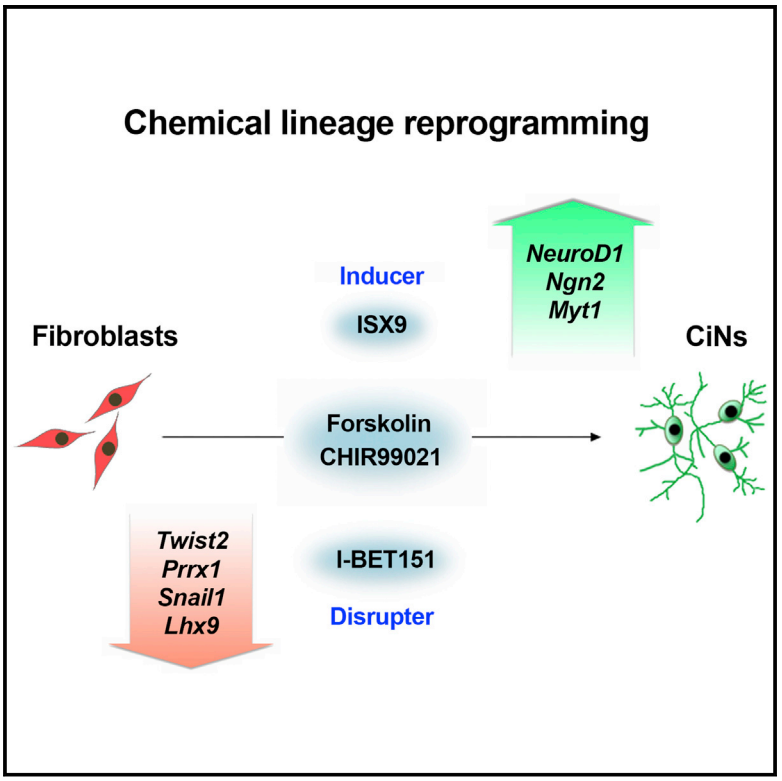
Short Article

Cell Stem Cell

Small-Molecule-Driven Direct Reprogramming of Mouse Fibroblasts into Functional Neurons

Graphical Abstract



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In Brief

In this article, Deng and colleagues show that a cocktail of small molecules can drive direct lineage reprogramming of mouse fibroblasts into functional neurons, via chemical disruption of the original cell program and induction of an alternate cell fate.

Highlights

- Chemical screening identifies a small molecule cocktail for reprogramming
- Functional mature neurons can be induced from fibroblasts with chemicals alone
- BET family protein inhibition suppresses the fibroblast-specific program
- The neurogenesis inducer ISX9 is required for induction of neuronal genes

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Small-Molecule-Driven Direct Reprogramming of Mouse Fibroblasts into Functional Neurons

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SUMMARY

Recently, direct reprogramming between divergent lineages has been achieved by the introduction of regulatory transcription factors. This approach may provide alternative cell resources for drug discovery and regenerative medicine, but applications could be limited by the genetic manipulation involved. Here, we show that mouse fibroblasts can be directly converted into neuronal cells using only a cocktail of small molecules, with a yield of up to >90% being TUJ1-positive after 16 days of induction. After a further maturation stage, these chemically induced neurons (CiNs) possessed neuron-specific expression patterns, generated action potentials, and formed functional synapses. Mechanistically, we found that a BET family bromodomain inhibitor, I-BET151, disrupted the fibroblast-specific program, while the neurogenesis inducer ISX9 was necessary to activate neuron-specific genes. Overall, our findings provide a "proof of principle" for chemically induced direct reprogramming of somatic cell fates across germ layers without genetic manipulation, through disruption of cell-specific programs and induction of an alternative fate.

INTRODUCTION

A major question in regenerative medicine is how best to obtain customized functional cell types. Recently, direct lineage reprogramming has emerged as a promising, fast, and direct approach for manipulating cell fate that avoids the teratoma risks associated with pluripotent stem cells. Fibroblasts can be directly converted into diverse functional cell types by the viral introduction of known cell-fate-determining transcription factors or microRNAs (Davis et al., 1987; Vierbuchen et al., 2010; Xu et al., 2015; Yoo et al., 2011). The approach of direct reprogramming has been used in disease modeling, suggesting promising applications in regenerative medicine (Xu et al., 2015). Moreover, direct reprogramming of cell fates in vivo could potentially provide a route to in situ regeneration for therapeutic purposes (Xu et al., 2015).

However, the low efficiency of induction, the technical challenges involved, and the genomic integration of the viral vectors used for direct reprogramming have raised concerns about the potential for future applications of this approach. An alternative strategy based on small molecules to induce cell-lineage reprogramming would be advantageous because such a strategy would be non-immunogenic, cost effective, and easy to manipulate and standardize. In addition, the application of small molecules is reversible and does not require cell permeabilization. Because of these advantages, such a strategy could potentially be translated into therapeutic applications (Yu et al., 2014).

Recently, we developed a small-molecule approach to induce pluripotency from mouse somatic cells without the use of exogenous factors (Hou et al., 2013), demonstrating the feasibility of chemically reprogramming mouse somatic cell fates toward pluripotency. However, the possibility of direct cell fate conversion between two differentiated cell types by pure chemicals has not been examined. In the past, several studies have employed small molecules to enhance the efficiency of direct reprogramming or replace part of the reprogramming transcription factor cocktail required (Ladewig et al., 2012; Liu et al., 2013; Xu et al., 2015). However, a pure small-molecule approach for direct cell fate reprogramming without genetic manipulation has not been described.

In this study, we identified a cocktail of small molecules capable of establishing neuronal features in mouse fibroblasts efficiently and directly. The small molecules disrupt the fibroblast-specific program, activate the endogenous expression of neuronal-specific genes, and convert fibroblasts to functional neurons.

RESULTS

Small-Molecule Screening for Compounds Inducing Neuronal Fate

To identify neuronal-fate-inducing small molecules, we started out with a primary chemical screen. In a previous study, a set

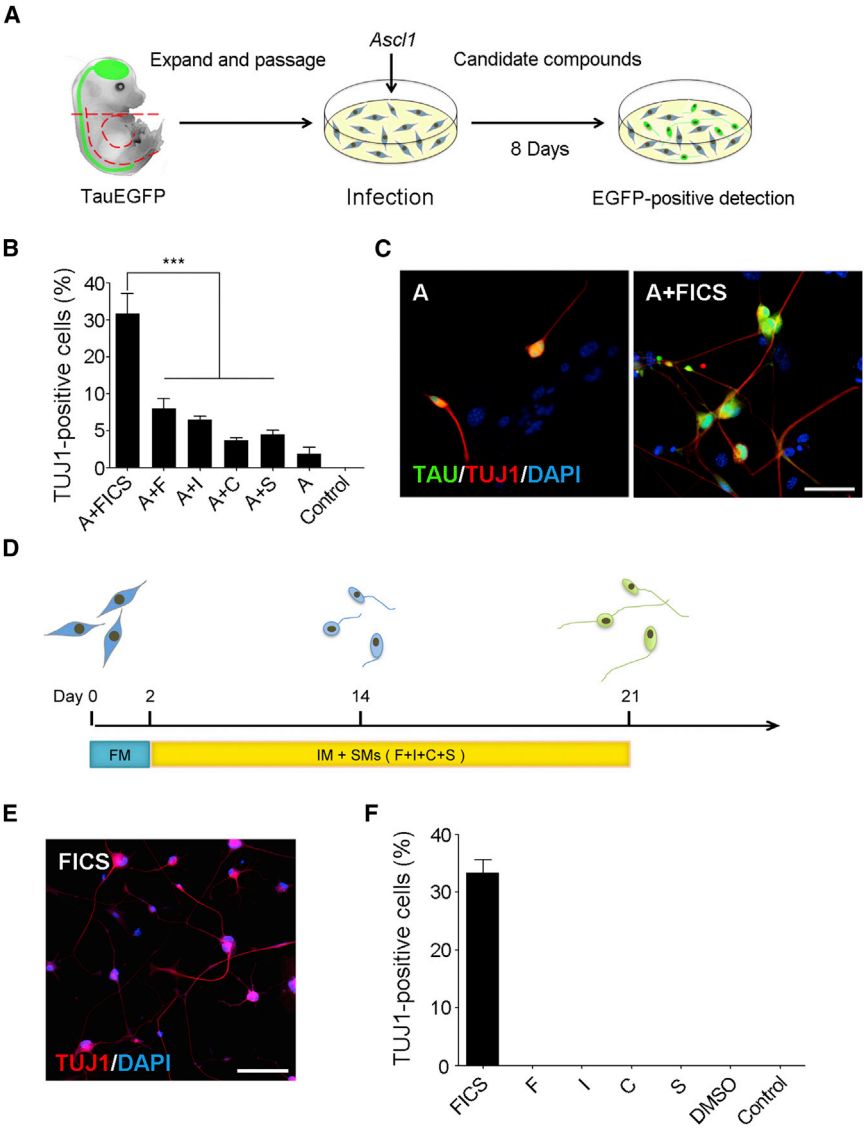


Figure 1. Identification of Neuronal Fate-Inducing Small Molecules

(A) Diagram of the small-molecule screening for candidate compounds.

(B and C) Four small molecules were found to substantially potentiate fibroblast-to-neuron conversion with *Ascl1*. (B) Quantification of TUJ1-positive cells with circular cell bodies and neurite outgrowth that is at least 3-fold longer than the cell body. Ten randomly selected visual fields (20×) were used to determine the cell number, and the graph represents the percentage of the qualified TUJ1-positive cells relative to DAPI-stained cells. (C) Representative TUJ1-positive cells induced by A only and A+SMs.

(D) Diagram of the SM induction process. FM, fibroblast medium; IM, neuronal induction medium.

(E) Representative TUJ1-positive cells induced by SMs.

(F) Quantification of TUJ1-positive cells induced by SMs (ten 20× visual fields were randomly selected to determine the number relative to DAPI-stained cells).

A, *Ascl1*; Control, not induced (NI); S, SB431542; C, CHIR99021; I, ISX9; F, Forskolin; DMSO, vehicle control. The data are presented as the mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (Student's t test). The scale bars represent 100 μm.

of three transcription factors (*Ascl1*, *Brn2*, and *Myt1l*) was shown to induce the generation of neurons from mouse fibroblasts, in which *Ascl1* is the master gene for inducing neuronal fate, while *Brn2* and *Myt1l* enhance the neuronal conversion as the supplementary factors (Vierbuchen et al., 2010). Without *Brn2* and *Myt1l*, *Ascl1* alone induces neurons with low efficiency (Vierbuchen et al., 2010). Accordingly, we first performed a chemical screen for small molecules promoting *Ascl1*-based conversion (Figure 1A) (Tucker et al., 2001; Vierbuchen et al., 2010). By screening about 5,000 small molecules (Table S1A), we found that Forskolin, ISX9, CHIR99021, and SB431542 each increased the number of TAUEGFP-/TUJ1-positive neuronal cells induced by *Ascl1* by more than 2-fold (Figure 1B). In the presence of *Ascl1*, the combination of these four chemical boosters further increased the efficiency of generating induced neurons (iNs) (>10-fold enhancement than *Ascl1* infection alone without compounds) (Figures 1B and 1C). Thus, we identified a combination of four small molecules that robustly facilitate *Ascl1*-mediated induction of neuronal fates from mouse fibroblasts.

To examine whether the exogenous "master genes" are dispensable for inducing neuronal fates, we induced the starting fibroblasts in a transgene-free manner (Figure 1D). Interestingly, in the absence of exogenous *Ascl1*, culture medium containing the four small-molecule cocktail "FICS" (Forskolin, ISX9, CHIR99021, and SB431542) sufficed to induce neuronal fate over an extended duration (21 days) with a >30% yield of TUJ1-positive cells with primitive neuronal-like morphology (Figures 1D and 1F; Table S1B). Each small molecule alone failed to generate neuronal-like cells, suggesting that synergy between the small molecules may be crucial (Figure 1F). These findings suggest that the small-molecule cocktail can initiate neuronal fates from fibroblasts.

Identification of I-BET151-Facilitated Chemical Reprogramming

To improve the transgene-free chemical induction into a more robust system, we performed another chemical screen for additional small molecules that facilitate neuronal reprogramming and maturation. We were hoping to find additional small molecules that could induce neurons with improved neurite outgrowth and, ideally, complex morphologies (Figure 2A) (Vierbuchen et al., 2010). By screening about 1,500 small molecules on "FICS+1" (Figure 2A), we found that an additional small

molecule, I-BET151, dramatically enhanced the reprogramming rate (with a 90% TUJ1-positive cell yield) and neurite outgrowth of the iNs (Figure 2B and Table S1B). Moreover, in the revised cocktail, FICSB, SB431542 (S) was dispensable for generating neurons, although it enhanced the survival and neurite outgrowth of the iNs (Figure 2C and Figure S1A). We then used the small molecule cocktail FICB in further experiments, and the concentration of each small molecule was further optimized (Figure S1B). The FICB combination converted fibroblasts into neurons with a yield up to >90% TUJ1-postive cells (in which 71% were TAUEGFP/TUJ1 double positive and 30% NEUN/TUJ1 double positive) with extensive neurite outgrowth after 16–20 days of induction (Figures 2B and 2C). The FICB-induced cells co-expressed multiple neuronal-specific markers, including MAP2 and NF-H (Figure 2D). Furthermore, the FICB-induced cells seemed to be heterogeneously excitatory and inhibitory, based on detection of both VGLUT1- and GABA-positive neurons (Figure 2D). Taken together, these results suggest that chemical reprogramming into neuronal-like cells has been achieved by using FICB.

Gene Expression Profiling of CiNs Resembles That of Functional Neurons

To further promote the maturation of the induced neuronal-like cells, we used co-culturing of the induced cells with primary astrocytes in maturation medium as reported previously (Chanda et al., 2014; Vierbuchen et al., 2010). After we promoted their maturation for 14–21 days, the induced cells showed more extensively extended neurite outgrowth (Figure 2E). These chemically induced neurons were then referred to as CiNs (Figure 2F). To verify the co-expression of neuronal and functional subtype-specific markers and avoid the noise associated with a mixed cell population, we performed single-cell expression profiling on the CiNs. Co-expression of multiple pan-neuronal and functional synaptic markers was confirmed by these single-cell experiments (Figure 2G and Figure S1C). Both excitatory and inhibitory subtypes of single CiNs were detected. The majority of the CiNs are the excitatory, glutamatergic neurons (about 45.8%), as indicated by the expression of *vGlut*, and the percentage of the inhibitory subtype is about 20.8%, as indicated by the expression of *Gad67* (Figure S1C). Furthermore, we found that the induced cells not only established a neuron-specific transcriptional program but also silenced the expression of the fibroblast-specific genes *Fsp1* and *Col1a1* (Figure 2G and Figure S1C).

Electrophysiological Function of CiNs

To examine the electrophysiological properties of the CiNs, we performed whole-cell patch-clamp recordings (Figure 3A). By depolarizing the membrane in current-clamp mode, action potentials (APs) were elicited on the CiNs with extending branches (35.0%, n = 20) after 14–20 days of chemical induction (Figure 3B; Table S3). Furthermore, fast, inactivating inward and outward currents were recorded on the CiNs in the voltage-clamp mode, which may correspond to the opening of voltage-dependent K^+- and Na^+-channels (Figure 3C; Table S3). Accordingly, when the CiNs were re-plated onto a pre-existing monolayer culture of primary astrocytes or primary neurons, the functional membrane properties of the CiNs were significantly enhanced (53.8%, n = 39) (Figures 3D and 3E; Table S4), and spontaneous excitatory postsynaptic currents (EPSCs) could also be recorded (47.6%, n = 21) (Figure 3F and Figure S1D; Table S4). The EPSCs could be blocked by the specific receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 2-amino-5-phosphonovaleric acid (AP5) (Figure 3F). These results suggest that the CiNs have functional membrane properties and that after development by being co-cultured with primary astrocytes or primary neurons the CiNs are capable of forming functional synaptic connections with each other or with the pre-existing primary neurons.

Lineage Tracing to Confirm the Fibroblast-to-Neuron Chemical Reprogramming

To confirm the fibroblast origin of the initial cell culture, we employed a Cre-LoxP lineage tracing system to trace the fate of original fibroblasts expressing a fibroblast-specific gene, *Fsp1* (Bhowmick et al., 2004; Iwano et al., 2002; Madisen et al., 2010; Strutz et al., 1995) (Figure S2A). TdTomato-positive cells were further confirmed by being co-immunostained with another fibroblast marker, COL1A1 (Figures S2B and S2C). After the chemical induction, the tdTomato-positive cells developed extensive branch outgrowth (Figure S2D). We found that tdTomato-positive cells could be chemically reprogrammed into neuronal cells with a comparatively high efficiency (Figures S2E–S2Q). This result provides direct genetic proof of the chemical reprogramming of fibroblasts into neurons.

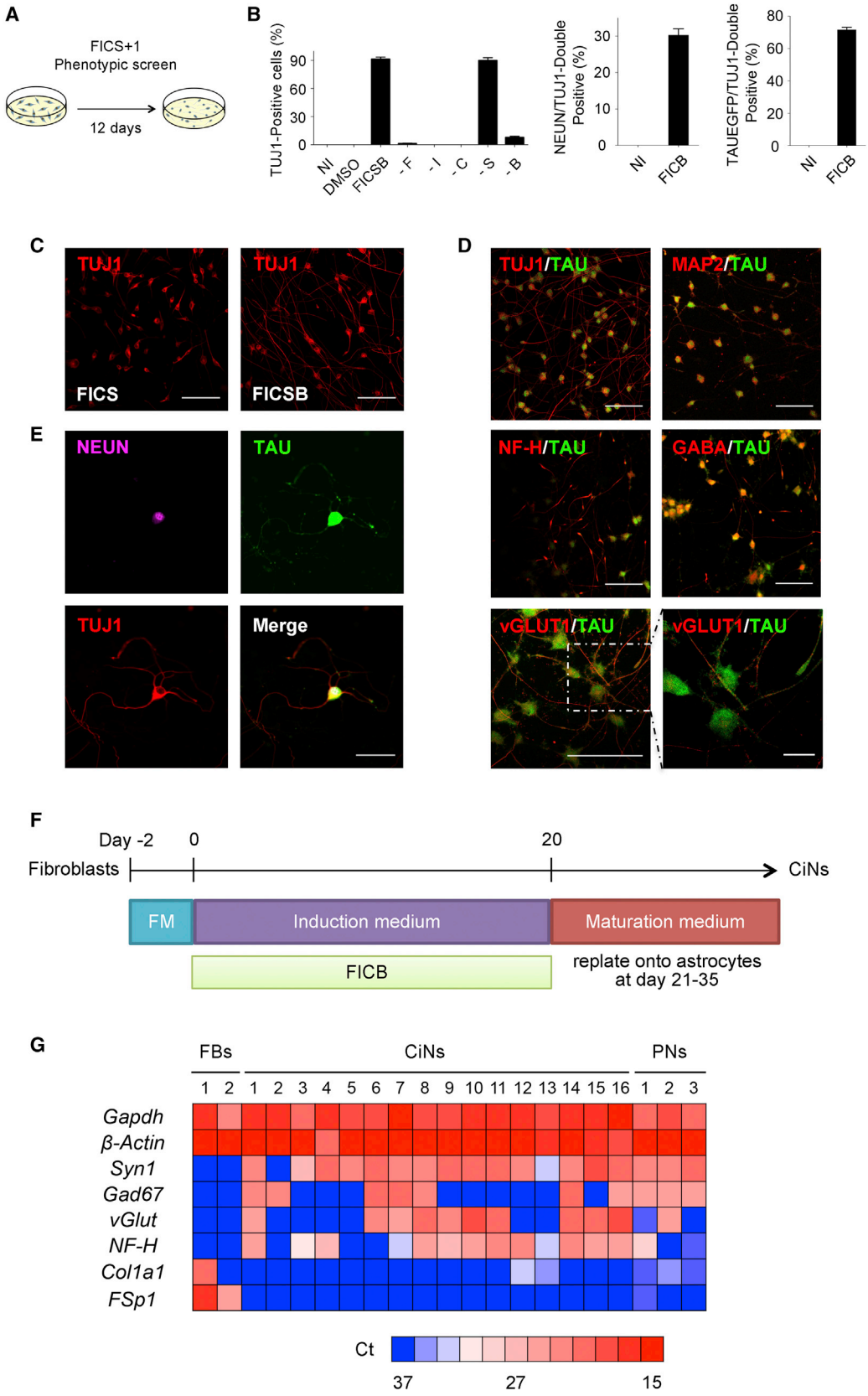
CiN Generation Is Direct without an Intermediary Proliferative Stage

To further understand the reprogramming process, we treated the cells with 5-bromodeoxyuridine (BrdU) with the small-molecule induction and throughout the culture period for chemical reprogramming (Figure S3A) (Vierbuchen et al., 2010). The results showed that the vast majority (about 80%) of the TUJ1-positive iNs did not incorporate BrdU (Figures S3B and S3C), indicating a direct cell fate reprogramming bypassing an intermediary proliferative stage.

Small Molecules Directly Induced Neuronal-Master Transcription Factors and Disrupted the Fibroblast-Specific Program during CiNs Generation

To explore the roles of the small molecules in chemical reprogramming, we first investigated their biological activity by replacing the individual small molecules with functional analogs. We found that Forskolin (a cyclic AMP, or cAMP, agonist), CHIR99021 (a glycogen synthase kinase 3 beta, or GSK3, inhibitor), and I-BET151 (a BET family bromodomain inhibitor) could be substituted by other cAMP agonists, GSK3 inhibitors, and BET inhibitors, respectively (Figure S1E), suggesting that these activities correspond to their functional targets in chemical reprogramming of fibroblasts into CiNs.

To better understand the process of cell fate reprogramming, we carried out RNA-seq analysis to examine the change of global expression profiling after treating cells with small molecules for 48 hr and 19 days. As demonstrated by hierarchical clustering, the induced cells grouped closely to the primary neurons but distinctly from the fibroblasts (Figure S4A). Based on selection of genes differently expressed at least 3-fold among



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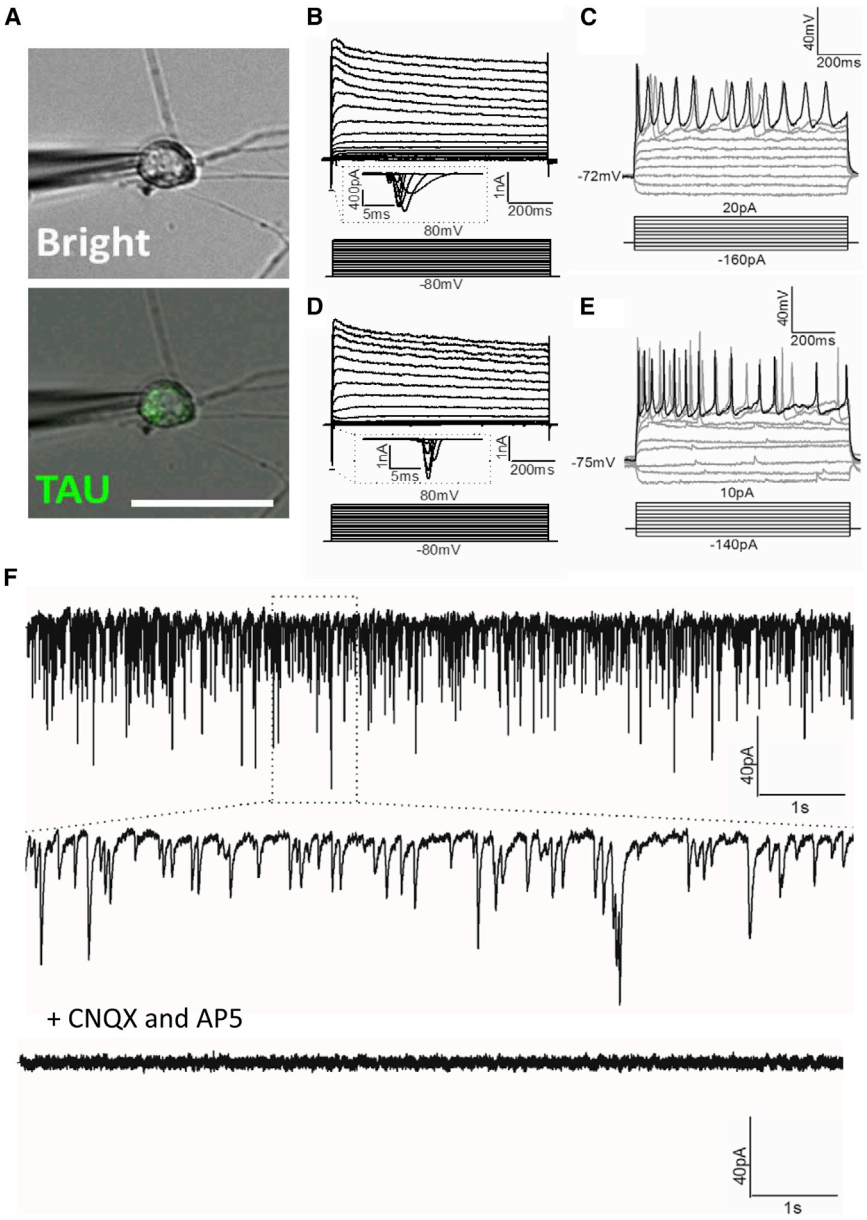

Figure 3. Electrophysiologically Functional Properties of the CiNs

(A) Patch-clamp recordings were performed 14–20 days after chemical induction. Scale bars represent 50 μm.

(B) Whole-cell current-clamp recording of CiNs. APs were elicited by current injection. One exemplary AP trace was highlighted.

(C) Whole-cell voltage-clamp recording of CiNs, with inward and outward currents recorded.

(D and E) After co-culture with primary neurons, the functional membrane properties of the CiNs were significantly enhanced. One exemplary AP trace was highlighted.

(F) Spontaneous EPSCs were recorded from CiNs co-cultured with primary neurons and blocked by 20 μM CNQX and 50 μM AP5.

fibroblasts, CiNs, and primary neurons (Caiazzo et al., 2011; Du et al., 2014), the general degree of overlapping specifically expressed genes showed similarities between the induced cells and the primary neurons (Figure S4B). Compared to the fibroblasts, the cells at 19 days post-treatment enriched the expression of multiple neuronal-specific genes, including genes involving in neuronal morphogenesis and maintenance, ion channels, and functional synaptic components (*Mapt*, *Gap43*, *Stmn3*, *Stmn4*, *Syn1*, *Syp*, and *Syt1*) (Figures S4C and S4D). Furthermore, the expression of fibroblast hallmark genes (*Fap*, *Des*, *Twist2*, and others) was downregulated (Figure S4D) (Caiazzo et al., 2011; Kim et al., 2011), indicating loss of the original fibroblast features in CiNs. Consistently, by selecting genes that were >10-fold differentially expressed for analysis, expression of the fibroblast-enriched gene program was disrupted in the small-molecule-treated cells, while neuron-enriched genes were upregulated (Figure 4A). After 19 days of chemical induction, 60.6% of the neuron-enriched genes were upregulated by at least 2-fold, while only 1.8% were downregulated. Furthermore, 80% of the fibroblast-enriched genes were also downregulated by at least 2-fold, whereas only 6.1% were upregulated, suggesting that the chemical induction has the dual effect of activating global neuron-enriched genes and suppressing global fibroblast-enriched genes (Figure 4A). GO (Gene Ontology) analysis showed that

Figure 2. Efficiently Generating CiNs

(A) Diagram of the process to identify small molecule candidates that facilitate reprogramming.

(B) Quantification of TUJ1-positive cells, NEUN/TUJ1 double positive cells and TAUEGFP/TUJ1 double positive cells induced by FICSB or by withdrawing individual chemicals from FICSB.

(C) TUJ1-stained cells 14 days after treatment with the FICS or FICS+I-BET151.

(D) Representative TUJ1-, MAP2-, NF-H-, GABA-, and vGLUT1-positive induced cells. The bottom right panel is a higher magnification (2×) of the area marked in the bottom left panel.

(E) Mature TAUEGFP-positive CiNs expressed TUJ1 and NEUN 2 weeks after co-culture with primary neurons.

(F) Diagram of generating CiNs.

(G) Single-cell analysis of the CiNs. FBs, fibroblasts; PNs, primary neurons.

Scale bars represent 100 μm (C and D, upper, middle, and lower left), 50 μm (E), and 20 μm (D, lower right). See also Figures S1 and S2.

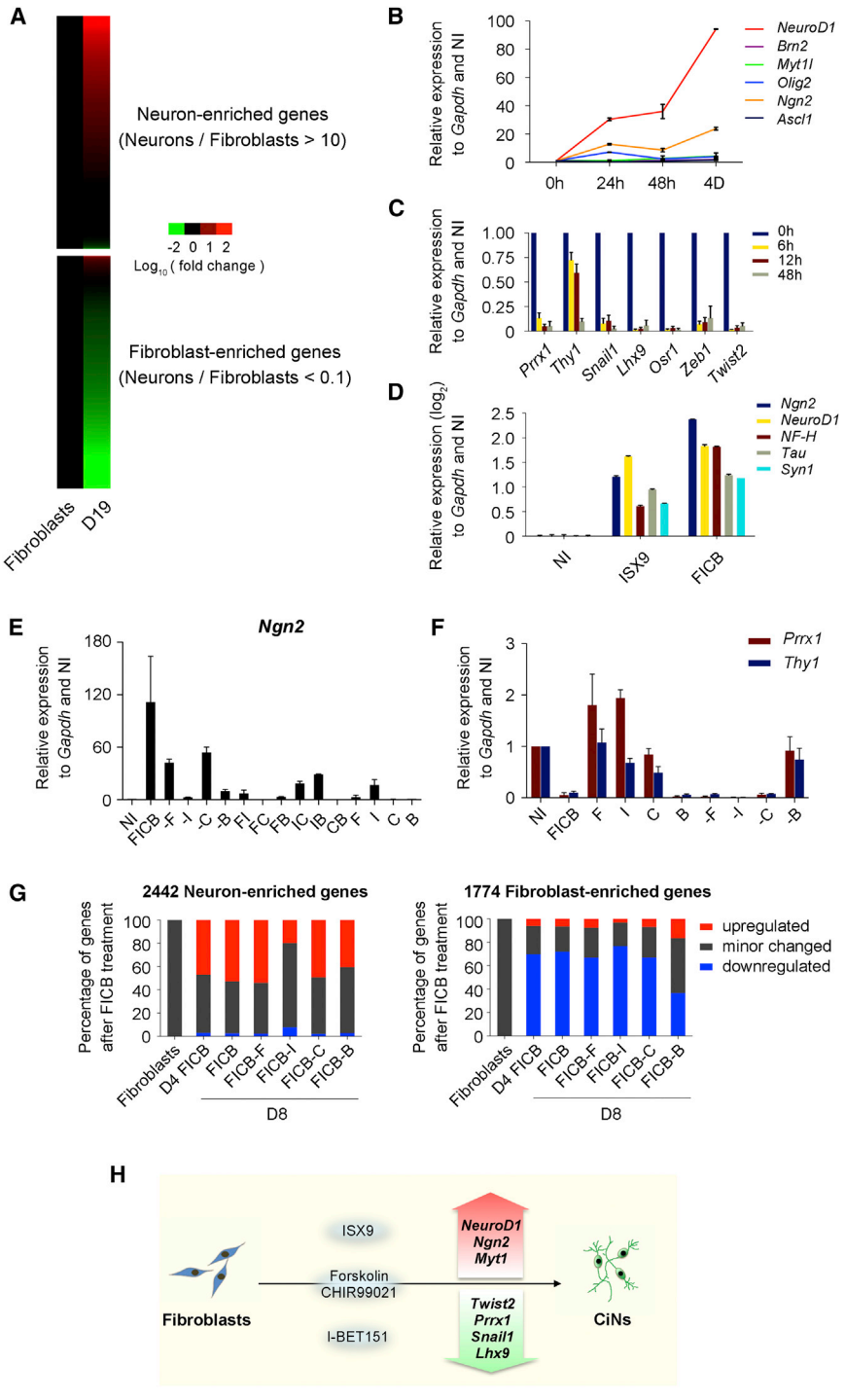

Figure 4. Roadmap of Chemical Reprogramming

(A) Heat map showing global expression change of neuron-enriched genes and fibroblast-enriched genes after 19-day induction by FICB.

(B and C) The activation of master neuronal genes (B) and the suppression of fibroblast genes (C) were validated by real-time qPCR.

(D) ISX9 was necessary to activate some neuronal genes.

(E and F) The effect of individual chemicals on the expression of Ngn2 and fibroblast genes at 48 hr.

(G) The effect of individual chemicals on global gene expression. "Up-regulated" represents genes whose expression level was upregulated by more than 2-fold compared to fibroblasts, while "down-regulated" represents genes whose expression level was downregulated by more than 2-fold compared to fibroblasts.

(H) Schematic representation of the roles of small molecules in the process of CiN generation.

The data are presented as the mean ± SEM. See also Figures S3 and S4.

the upregulated genes after chemical induction were mainly enriched in synaptic transmission, neuron differentiation, neuron development, ion transport, axonogenesis, ion channel activities, and other crucial biological process in neural development (Figure S4E). The downregulated genes mainly participate in biological processes such as the cell cycle, cell division, and others (Figure S4E). Taken together, these results indicate that the cells, by small-molecule induction, gained a transcriptional profile resembling that of primary neurons and disrupted the program of the original cells from the single-cell and global experimental perspectives.

By RT-qPCR analysis, we found that the activation of the neural-fate-determining factors and the downregulation of fibroblast-fate-determining factors were induced within 24 hr (Figures 4B and 4C). ISX9 is necessary to activate multiple neuron-specific genes, including the neural-fate mastering gene *NeuroD1* (Figures 4D). During the first 48 hr of chemical induction, the expression of *NeuroD1* and *Ngn2*, another neural-fate mastering gene, was dramatically induced (Figures 4B and 4E), suggesting that these two transcription factors may be involved in the first step of chemical reprogramming. Interestingly, *Ascl1*, the master transcription factor most frequently used to induce neuronal fate conversion, was not activated in the early stage of reprogramming (Figure 4B).

Next, we determined the roles of the small molecules in regulating these endogenous cell-fate-determining factors. By removing each small molecule from the cocktail, we found that ISX9 was essential to induce the master neural genes (Figures 4D–4F), suggesting that the small-molecule cocktail induces the neural-fate-determining program in an ISX9-dependent, synergistic manner. Interestingly, we found that I-BET151 was the core small molecule needed to suppress the endogenous fibroblast-fate-determining program (Figures 4F and 4G), and it accounts for an efficient disruption of the fibroblast core transcriptional network by

chemicals. These results indicated that cell fate was manipulated by small molecules through synergistic activation of the target cell fate regulatory program and repression of the original cell fate program (Figure 4H).

DISCUSSION

In this study, we identified a cocktail of small molecules that induces an efficient direct lineage reprogramming across germ layers from fibroblasts into functional neurons. The CiNs possess neuronal properties in terms of gene expression pattern and electrophysiological functional capabilities. Taking together the CiNs and the chemically induced pluripotent stem cells (CiPSCs) (Hou et al., 2013), these findings demonstrate that somatic cell fates can be converted by the manipulation of cell signaling pathways and endogenous cell-fate-determining programs with only small molecules, without the need for exogenous transgenes or other cell-fate-specific factors such as microRNAs.

Although master transcription factors are considered the major determinants of specific cell identities (Xu et al., 2015), our findings show that a small molecule cocktail is sufficient to activate the expression of such genes. As shown in our study, ISX9, an isoxazole, which has been shown to facilitate neural differentiation via a neurotransmitter-evoked Ca^{2+} signal (Schneider et al., 2008), is necessary to induce the activation of neuronal genes in fibroblasts, and this process was further enhanced by other small molecules (Figures 4D and 4E). For induction of CiNs, *Ngn2* and *NeuroD1* were the first-wave responsive genes in the reprogramming process, and they were activated within 6 hr (Figure 4B). Recently, *Ngn2*, the neuron-fate-determining proneural gene in development, has been demonstrated to be capable of establishing neuronal cell fate with additional transcriptional factors or small molecules (Bertrand et al., 2002; Liu et al., 2013). Other neural factors were sequentially activated in the following days (Figure 4A and Figure S4D). This staged gene activation suggests that small-molecule-driven fibroblast-to-neuron direct reprogramming is a hierarchical transcriptional activation process, in which the efficient activation of cell-fate-determining genes by small molecules may initiate and stabilize an auto-regulatory loop of the neuronal-specific transcriptional program and may further stimulate the expression of downstream regulatory genes to establish neuronal functional properties.

Interestingly, the addition of I-BET151 into the small molecule cocktail significantly improved the reprogramming process (Figures 2B and 2C), and we further found that I-BET151 was the key small molecule for disruption of the fibroblast core transcriptional network (Figure 4F). I-BET151 is reported to competitively bind at the BRD domain of BET family proteins (Seal et al., 2012). BRD4, a BET family protein, was reported to specifically bind to the activated chromatin domains and maintain the cell-fate-specific gene expression pattern (Wu et al., 2015). Inhibition of BRD4 can disrupt cell fate maintenance and alter the gene expression pattern of the initial cell type (Chiang, 2014; Di Micco et al., 2014), consistent with our finding that I-BET151 directly disrupts the fibroblast-specific gene expression program in early stage reprogramming (Figures 4D and 4F). Moreover, our results are also consistent with a "mutual repression model" of cell fate conversion (Wang et al., 2011) and the previous finding that cell fate reprogramming could be facilitated by knockdown of key transcription factors in the initial cell type (Hanna et al., 2008). Furthermore, it was found very recently that BRD4 binds to super-enhancers of core genes to maintain the pluripotent cell fate of ESCs, and inhibition of BRD4 results in loss of the core program of ESCs and their commitment to a neuroectodermal lineage (Di Micco et al., 2014). It is possible that small molecules that are targeting protein complexes at super-enhancers or that are related to active gene expression may contribute to cell lineage reprogramming as a general eraser of initial cell identities in the future.

Although the small molecules identified in our study are not specific to the neural lineage, the pathways that they target have been reported to be involved in directed neural differentiation in vitro and even in neural development in vivo. CHIR99021 is traditionally used to induce neuronal development from pluripotent stem cells as a GSK3 inhibitor (Chambers et al., 2009), and it has been reported as a chemical booster that enhances transcription-factor-based neuronal conversion (Ladewig et al., 2012). Forskolin is a diterpene adenylate cyclase activator and is commonly used to increase the level of cyclic AMP (cAMP) (Seamon et al., 1981), and cAMP-responsive element binding (CREB), a downstream target of Forskolin, has been reported to regulate neuronal specification and promote axonal regeneration (Seamon et al., 1981; Dworkin and Mantamadiotis, 2010). The dual functions of these small molecules in direct differentiation and lineage reprogramming suggest that developmental cues and development-relevant signaling pathways could be instructive in developing small molecule combinations that facilitate cell lineage reprogramming.

Overall, our study suggests a blueprint for engineering cell identity using chemical agents in regenerative medicine. The development of CiNs and CiPSCs (Hou et al., 2013) together suggests a general strategy for developing small molecule cocktails that can manipulate cell fates by replacing reported lineage reprogramming genes, activating desirable cell-type-specific master gene expression, and silencing initial cell-type-specific master gene expression. To move toward therapeutic approaches, we anticipate that it will be possible to employ this type of chemical reprogramming strategy to manipulate human somatic cell fates. In the future, it may well be possible to identify other small molecule cocktails capable of inducing other functional cell types by direct lineage reprogramming and to define more precisely the mechanistic elements underlying cell fate maintenance and reprogramming. Our findings also may suggest an unanticipated level of somatic cell plasticity and indicate that somatic cell identity is much more flexible than previously appreciated.

EXPERIMENTAL PROCEDURES

Generation of CiNs

Small molecules (from Tocris) (Table S1B) were dissolved and diluted in DMSO according to the manufacturer's instructions and then processed to use at the following final concentrations: ISX9, 20 μM; SB431542, 10 μM; Forskolin, 100 μM; CHIR99021, 20 μM; and I-BET151, 0.5–2 μM. The passaged fibroblasts were seeded onto a Matrigel-coated plate (BD; 1:30 in pre-cold PBS) and were not chemically induced until confluent (preferentially seeded at a density of 100,000 cells per well of a 6-well plate and induced after 4 days'

culture). The neuronal induction medium contained Neurobasal Medium, N2 and B27 supplements, GlutaMAX, penicillin-streptomycin (all from Life technologies), and bFGF (100–250 ng/ml; Origene). Culture medium (neuronal induction medium plus the small molecules) was refreshed every 3–4 days during the chemical induction period. For the proliferation assay, BrdU was added at a final concentration of 10 μM and refreshed in medium. Note: cell death was found in some fibroblast batches for inducing neurons (mainly due to the toxicity and high dose of I-BET151). To enhance the survival rate and facilitate reprogramming in these batches, ROCK inhibitor Y27632 (2 μM) or Fasudil (2 μM) can be used at the early stage of induction (from day 0 to day 4–8), or P38 MAPK inhibitors SB203580 (1 μM) or BIRB796 (1 μM) can be used. After chemical treatment for 20 days, induction medium was changed to maturation medium (the neuronal induction medium plus Forskolin, 10 μM; BDNF, 20 ng/ml; and GDNF, 20 ng/ml). The cells were dissociated by using 0.25% trypsin (note: gently dissociate within 1 min to reduce the damage to the induced neuron-like cells) or mechanistically detached by pipette (note: gently handle to reduce the damage to the induced neuron-like cells) from the culture plate, and then re-plated to co-culture with primary astrocytes/pre-existing primary neurons (preferentially cultured 7 days) at day 21–35 for further maturation. (Note: see also Supplemental Experimental Procedures.) Animal experiments were approved and performed according to the Animal Protection Guidelines of Peking University, China.

ACCESSION NUMBERS

The GEO accession number for RNA-seq data in this study is GEO: GSE68715.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes four figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2015.06.003.

AUTHOR CONTRIBUTIONS

H.D. and X.L. conceptualized and projected the study. H.D. and Y.Z. supervised the project. X.L. developed the methodology and designed the experiments. X.L. and X.H.Z. performed most of the experiments and analyzed the data. Y.T.M. and others helped to perform experiments, validated the results, and provided technical supports. J.Z.J. and Z.C. contributed to electrophysiological assays. X.L., Y.Z., and H.D. wrote the manuscript.

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