Small molecules efficiently reprogram human astroglial cells into functional neurons

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Summary

We have recently demonstrated that reactive glial cells can be directly reprogrammed into functional neurons by single neural transcription factor NeuroD1. Here we report that a combination of small molecules can also reprogram human astrocytes in culture into fully functional neurons. We demonstrate that sequential exposure of human astrocytes to a cocktail of 9 small molecules that inhibit glial but activate neuronal signaling pathways can successfully reprogram astrocytes into neurons in 8-10 days. This chemical reprogramming is mediated through epigenetic regulation and involves transcriptional activation of NEUROD1 and NEUROGENIN2. The human astrocyte-converted neurons can survive for >5 months in culture and form functional synaptic networks with synchronous burst activities. The chemical reprogrammed human neurons can also survive for >1 month in the mouse brain in vivo and integrate into local circuits. Our study opens a new avenue using chemical compounds to reprogram reactive glial cells into functional neurons.

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Authors’ contribution:
L.Z. performed the major part of the experiments and data analysis, as well as participated in writing the manuscript. L.Z., J.Y., H.Y., G.L., and N.M. did the initial drug screening and verification. J.Y. also performed compound removal experiments. H.Y. also performed immunostaining. N.M. worked on epigenetic regulation and in vivo experiments. G.L. contributed to transcriptional regulation during chemical reprogramming. X.C and Y.W worked on histone modification and analysis. L.L., L.C and P.J. contributed on DNA methylation and analyses. G.W. performed calcium imaging, time-lapse imaging and electrophysiology experiments. G.C. and G.W. conceived and supervised the entire project, analyzed the data, and wrote the manuscript.

Additional methods can be found in supplementary material.
Graphical Abstract

Introduction

Regeneration of functional neurons after brain injury remains a major challenge for brain repair. Current efforts largely focus on cell replacement therapy using exogenous cells derived from embryonic stem cells or induced pluripotent stem cells to generate neurons (Sahni and Kessler, 2010; Takahashi et al., 2007; Takahashi and Yamanaka, 2006). Despite great potential, such cell transplantation approaches face significant hurdles such as immunorejection, tumorigenesis and differentiation uncertainty (Lee et al., 2013; Lukovic et al., 2014). Recent studies, including our own, have demonstrated that astroglial cells can be directly converted into functional neurons both in vitro (Guo et al., 2014; Heinrich et al., 2010) and in vivo (Grande et al., 2013; Guo et al., 2014; Liu et al., 2015; Torper et al., 2013). We further demonstrated in a mouse model of Alzheimer's disease that reactive astrocytes can be directly reprogrammed into functional neurons (Guo et al., 2014).

Astrocytes can also be converted first into neuroblast cells and then differentiated into neuronal cells (Niu et al., 2015; Niu et al., 2013; Su et al., 2014). Similar to astrocytes, NG2 glial cells have recently been converted into neurons as well (Heinrich et al., 2014; Torper et al., 2015). However, so far conversion of glial cells into neurons has been largely achieved using viral based expression of transcription factors. In contrast, small molecules have been used to promote neural differentiation (Chambers et al., 2012), facilitate cell reprogramming (Ladewig et al., 2012; Li et al., 2014; Liu et al., 2013), or even directly reprogram fibroblasts into induced pluripotent stem cells (iPSCs) (Hou et al., 2013), neuroprogenitor cells (Cheng et al., 2014), or neurons (Hu et al., 2015; Li et al., 2015). Compared to transcription factor based reprogramming, small molecules offer ease of use and a broader range of downstream applications.

Here, we report a defined combination of small molecules capable of directly reprogramming human astrocytes into functional neurons after sequential administration. We tested a variety of small molecules targeting signaling pathways important for neurogenesis and identified a group of 9 small molecules that can reprogram human astrocytes into neurons. These small molecule-reprogrammed human neurons can survive
for > 5 months in culture and display robust synaptic activities. Injecting the human astrocyte-converted neurons into the mouse brain in vivo revealed that these human neurons can integrate into the local brain circuits. Together, our studies demonstrate the feasibility of chemical reprogramming of human astrocytes into functional neurons.

Results

Reprogramming human astrocytes into neurons by small molecules

We have recently demonstrated that ectopic expression of a single neural transcription factor NeuroD1 can directly reprogram glial cells into functional neurons (Guo et al., 2014). To investigate whether small molecules can replace transcription factors to chemically reprogram glial cells into neurons, we searched literature broadly to identify potential candidate molecules for further functional screening. We selected 20 small molecules as our starting candidate pool based on two major selection criteria: one is to inhibit glial signaling pathways, and the other is to activate neuronal signaling pathways. Some molecules were included because they can modulate DNA or histone structure to increase reprogramming efficiency. The 20 small molecules selected for our initial screening are: SB431542, RepSox, LDN193189, dorsomorphin, DAPT, BMS-299897, CHIR99021, TWS119, Thiazovivin, Y27632, SAG, purmorphamine, TTNPB, RA, VPA, forskolin, BIX 01294, RG-108, ISX9, and Stattic.

We mainly used human cortical astrocytes (HA1800, ScienCell, San Diego, CA, USA) in primary cultures for chemical reprogramming. Human astrocytes were isolated, passaged, and maintained in culture medium with 10% fetal bovine serum (FBS) to reduce possible contamination of progenitor cells, because FBS stimulates differentiation of progenitors. For initial testing, we applied a group of small molecules together to human astrocyte cultures, but massive cell death was observed after 2 days of drug treatment. To reduce cell death, we added fewer small molecules at different time points with different concentrations. After testing hundreds of different conditions (see Excel file “Small molecule screening table” in the supplementary material), we found a cocktail of 9 small molecules (LDN193189, SB431542, TTNPB, thiazovivin, CHIR99021, VPA, DAPT, SAG, and purmorphamine) capable of reprogramming human astrocytes into neurons when added in a stepwise manner (Fig. 1A). This set of 9 small molecules is hereafter briefed as master conversion molecules (MCMs). Specifically, human astrocytes were first treated with LDN193189 (0.25 μM), SB431542 (5 μM), TTNPB (0.5 μM), and thiazovivin (Tzv, 0.5 μM) for 2 days. SB431542 is an inhibitor of TGFβ/activin receptors, which are involved in inhibiting neuronal fate and promoting glial fate (Rodriguez-Martinez and Velasco, 2012). Similarly, LDN193189 is an inhibitor of BMP receptors, which are important for astrogial differentiation (Gross et al., 1996). TTNPB is an agonist of retinoic acid receptors, which are crucial in neural patterning (Maden, 2002). We used the combination of LDN193189, SB431542, and TTNPB to initiate the reprogramming process by inhibiting glial signaling pathways and activating neuronal signaling pathways simultaneously. Tzv, an inhibitor of Rho-associated kinase (ROCK), promotes cell survival and improves reprogramming efficiency (Lin et al., 2009; Watanabe et al., 2007). Tzv was included throughout the 8 days of reprogramming period. After initial two days of priming with LDN193189, SB431542, and TTNPB, they were replaced with
CHIR99021 (1.5 μM), DAPT (5 μM), and VPA (0.5 mM). CHIR99021 is an inhibitor of glycogen synthase kinase 3 (GSK3). Inhibition of GSK3 signaling promotes neuroprogenitor homeostasis and neural induction (Hur and Zhou, 2010; Li et al., 2012). DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester), a γ-secretase inhibitor that indirectly inhibits notch signaling pathway, promotes neural differentiation (Borghese et al., 2010). VPA (valproic acid) is a histone deacetylase inhibitor that enhances reprogramming efficiency (Huangfu et al., 2008). VPA was only included in the reprogramming medium for 2 days because longer exposure increased cell death, whereas CHIR99021 and DAPT were present from day 3-6. In day 7-8, we used SAG (0.1 μM) and purmorphamine (Purmo, 0.1 μM), two agonists for activating sonic hedgehog (Shh) signaling pathway, to complete the reprogramming process. Shh signaling is a key determinant of neural patterning. SAG and Purmo have been used to induce neuronal differentiation (Qu et al., 2014). At day 9, we removed SAG and Purmo in the medium, and replaced with neurotrophic factors (BDNF, NT3, and IGF-1) to promote neuronal maturation after astrocyte-neuron conversion. The successful reprogramming strategy is illustrated in Fig. 1A.

The human astrocytes in our cultures were immunopositive for astrocyte markers GFAP (79.3 ± 4.9%) and Glt1 (astrocyte-specific glutamate transporter, 82.5 ± 4.3%) with no neurons detected (Fig. 1B-C). We found little contamination of neural stem cells in our human astrocyte cultures as shown by immunostaining with Sox2, Musashi, and Nestin (Fig. S1A-B), likely due to the presence of 10% FBS in our culture medium. This was further confirmed after culturing human astrocytes for one month in neural differentiation medium supplemented with growth factors (BDNF, NT3, and NGF) (Fig. S1C-H). In control medium without small molecules (1% DMSO), few neurons were detected (Fig. 1D); however, after sequential exposure to small molecules, we found a large number of cells immunopositive for neuronal markers such as Doublecortin (DCX), β3-tubulin (Tuj1), MAP2, and NeuN (Fig. 1E-F). The human astrocyte-converted neurons survived 4-5 months in our cultures, and developed robust axon and dendrites (Fig. 1G). To visualize the conversion process from astrocytes to neurons, we infected human astrocytes with 1 μl retroviruses encoding EGFP so that a small number of EGFP-positive astrocytes were observed lively during time-lapse imaging (Fig. S2). Compared to controls (Fig. S2A), small molecule treatment clearly changed astrocytes from flat, polygonal morphology to neuronal morphology with long neurites at D8-D10 (Fig. S2B-D). We further used GFAP::GFP retrovirus to label the astrocytes (91 ± 6.7% of GFAP::GFP-infected cells were GFAP+) and confirmed the astrocyte-neuron conversion after small molecule treatment (Fig. 1H, 68.7 ± 4.2% NeuN+; n = 5 batches; see also Fig. S2E-G). Similar results were obtained using LCN2::GFP retrovirus (88.5 ± 3% of LCN2::GFP-labeled cells were GFAP+) to trace astrocyte-neuron conversion (Fig. S2H-J, 54.4 ± 5.3% NeuN+, n = 3 batches). The conversion efficiency obtained through lineage tracing experiments was similar to the overall conversion efficiency induced by small molecule treatment (Fig. 1I-J; control, 3.3 ± 0.5% Tuj1+; n = 4 batches; MCM, 67.1 ± 0.8% Tuj1+, n = 4 batches; p < 0.0001, Student’s t test).

To investigate whether human astrocytes from different origins can be chemically reprogrammed into neurons, we obtained human midbrain and spinal cord astrocytes from

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ScienCell. Interestingly, human midbrain astrocytes were efficiently reprogrammed into neurons (Fig. 1K-M, Fig. S3A-F), whereas human spinal cord astrocytes were not (data not shown), suggesting that our protocol is more suitable for astrocytes with human brain origin. To confirm this, we purchased human brain astrocytes from Gibco and found that they could also be reprogrammed into neurons (Fig. S3G-I). To test whether human astrocytes might de-differentiate into neuroprogenitor cells, we monitored Sox2, Nestin, Pax6 and Ki67 signals during chemical reprogramming process from D0 to D10, and compared to neuroprogenitor cells (Fig. S4). While Sox2 showed some increase during reprogramming, it never reached the level of neuroprogenitor cells (Fig. S4A, G). Nestin and Pax6 did not show much increase during small molecule treatment (Fig. S4B-C, H-I). Ki67-labeled proliferating cells decreased significantly after small molecule treatment (Fig. S4D, J), suggesting that there were no expansion of progenitor cells during chemical reprogramming. In addition, when we labeled human astrocytes with BrdU before chemical treatment, many converted neurons were BrdU positive (Fig. S4E, K); however, when we labeled our cell culture with BrdU at day 10 after small molecule treatment, essentially all converted neurons were negative for BrdU (Fig. S4F, K), suggesting that glia-to-neuron conversion occurred during the presence of small molecules. Taken together, we have developed a successful strategy using a defined combination of small molecules to chemically reprogram human astrocytes into neurons.

**Small molecule-converted human neurons are fully functional**

We next investigated whether the chemically reprogrammed neurons are functional. We found that the small molecule-converted neurons survived for a long time (>5 months) and showed robust synaptic puncta along dendrites (Fig. 2A). Similarly, neurons reprogrammed from the midbrain human astrocytes and the human astrocytes of Gibco also survived more than 2 months in culture with many synaptic puncta along dendrites (Fig. S3F, I). Patch clamp recordings revealed significant sodium and potassium currents in astrocyte-converted neurons, which gradually increased during neuronal maturation (Fig. 2B-E; 2-month: $I_{Na} = 1889 \pm 197$ pA, $n = 10$; $I_K = 2722 \pm 263$ pA, $n = 10$). These neurons were capable of firing repetitive action potentials (Fig. 2F). More importantly, small molecule-converted neurons showed robust spontaneous synaptic events, including both excitatory postsynaptic currents (EPSCs; frequency = 0.66 ± 0.14 Hz; amplitude = 24.8 ± 8.2 pA, $n = 15$) (Fig. 2G-H), and inhibitory postsynaptic currents (IPSCs; frequency = 0.48 ± 0.21 Hz; amplitude = 23.3 ± 6.3 pA, $n = 2$) (Fig. 2I). It is noteworthy that 3 months after initial small molecule treatment, the human astrocyte-converted neurons showed large periodic burst activities which were abolished by TTX or DNQX (Fig. 2J-L), suggesting that these neurons formed functional networks and started to fire synchronously together. In support of this notion, we performed dual whole-cell recordings and demonstrated that two adjacent neurons showed synchronous burst activities (Fig. 2M). Furthermore, we employed Fura-2 Ca$^{2+}$ ratio imaging and recorded synchronized Ca$^{2+}$ spikes in the chemically reprogrammed neurons (Fig. 2N), indicating that these neurons have been functionally networked together. Therefore, human astrocytes can be chemically reprogrammed into fully functional neurons with defined small molecules.
Small molecules reprogram human astrocytes into forebrain glutamatergic neurons

To characterize the neuronal properties after small molecule-induced reprogramming, we examined neuronal markers expressed from anterior to posterior nervous system. We found that the majority of human astrocyte-converted neurons were immunopositive for forebrain marker FoxG1 (97.1 ± 1.1%, Fig. 3A, n = 3 batches), but negative for hindbrain and spinal cord markers HoxB4 and HoxC9 (Fig. 3B-C, n = 3 batches). We next performed a series of immunostaining with a variety of cortical neuron markers. We found that the human astrocyte-converted neurons were largely immunonegative for cortical superficial layer marker Cux1 (Fig. 3D), but positive for deep layer markers Ctip2 (Fig. 3E, 71.4 ± 3%, n = 5 batches) and Otx1 (Fig. 3F). The human astrocyte-converted neurons were also immunopositive for forebrain neuronal marker Tbr1 (Fig. 3G, 86.4 ± 3.4%, n = 3 batches), as well as hippocampal neuronal marker Prox1 (Fig. 3H). Fig. 3I shows the quantitative results. Therefore, our chemically reprogrammed neurons are mainly forebrain deep layer neurons or hippocampal neurons.

We further investigated neuronal subtypes based on neurotransmitters they contain. We found that the majority of small molecule-reprogrammed neurons were immunopositive for glutamatergic neuron marker VGluT1 (Fig. 3J). A small fraction of the converted neurons were immunopositive for GABAergic neuron marker GAD67 (Fig. 3K). On the other hand, the astrocyte-converted neurons were largely immunonegative for cholinergic marker VAChT (Fig. 3L), dopaminergic marker TH (Fig. 3M), or spinal motor neuron marker Isl1 (Fig. 3N). The quantitative analyses of the neuronal subtypes were shown in Fig. 3O (VGluT1, 88.3 ± 4%, n = 4 batches; GAD67, 8.2 ± 1.5%, n = 4 batches). These results suggest that the glutamatergic neurons are the major subtype using our small molecule reprogramming protocol. Different small molecules may be required to reprogram human astrocytes into other neuronal subtypes.

Activation of endogenous neural transcription factors during chemical reprogramming

To understand the molecular mechanisms of chemical reprogramming, we first employed PCR Array (Qiagen) to investigate gene profile changes. At day 4 after small molecule treatment, we found a dramatic increase, up to 300-fold, in the transcriptional levels of several neural transcription factors including NGN1/2, NEUROD1, and ASCL1, as well as immature neuronal marker DCX (Fig. 4A). At day 8, the most significant change at the transcriptional level was the immature neuronal gene DCX, which showed 2000-fold increase (Fig. 4B), suggesting that the majority of newly converted cells are immature neurons by the end of small molecule treatment. In contrast, the glia-related genes were generally downregulated (Fig. 4A-B). We then performed quantitative real-time PCR experiments to examine the time course of transcriptional changes of NGN2, NEUROD1 and astrogial genes GFAP and ALDH1L1 during chemical reprogramming process (Fig. 4C-F). Interestingly, we found that NGN2 transcription peaked at day 4 (Fig. 4C) while NEUROD1 peaked at day 6 during small molecule treatment (Fig. 4D), consistent with their sequential expression during early brain development. As for glial genes, the GFAP transcriptional level was significantly reduced over 200-fold at D4 (Fig. 4E), coinciding with the activation of neural transcription factors (Fig 4C-D). Similarly, the transcriptional level of another astrocytic gene ALDH1L1 was also downregulated (Fig 4F). In contrast,
control experiments without small molecule treatment showed little transcriptional changes (Fig. S5A-F). Therefore, our small molecule treatment activates neural transcriptional factors and in the mean time inhibits astrocytic genes.

**Epigenetic regulation during chemical reprogramming**

We next investigated whether epigenetic regulation was involved in our chemical reprogramming. DNA methylation in gene promoter affects the accessibility of transcriptional factor binding and hence becomes a rate-limiting factor in reprogramming of pluripotent stem cells (Papp and Plath, 2013; Yao and Jin, 2014). We performed methylated DNA immunoprecipitation followed by sequencing (MeDIP-seq) to examine the methylation level of genes of interest before and after small molecule treatment. As expected, the promoter region of GFAP gene was initially unmethylated in human astrocytes before small molecule treatment (D0), but a clear increase of methylation was detected after 8 days of small molecule treatment (Fig. 4G). This increased methylation was further confirmed by targeted bisulfite sequencing (BS-seq) (Fig. 4H). Notably, this GFAP promoter region contains the transcription factor binding sites for STAT3 and AP1, which have been shown to play a critical role in the activation of GFAP gene (Cheng et al., 2011; Condorelli et al., 1994). BS-seq data revealed that the flanking sites of STAT3 and AP1 binding region were hypermethylated (Fig. 4H), which could explain why GFAP transcription was significantly downregulated after small molecule treatment (Fig. 4E)(Xu et al., 2015). Our MeDIP-seq also revealed an increase of DNA methylation at the GFAP transcription start site (TSS) after small molecule treatment, which was also confirmed by BS-seq (Fig. 4I). In contrast to glial gene GFAP, neuronal gene NEFM, a midsized neurofilament gene specific to neurons, showed a decrease of methylation signal at the promoter region after small molecule treatment (Fig. 4J-K), suggesting the activation of neuronal genes. We also investigated epigenetic regulation of transcription factor NGN2, an important gene involved in neuronal differentiation. MeDIP-seq analyses indicated that the methylation level of the NGN2 promoter region was quite low before and after small molecule treatment (data not shown), consistent with previous report (Hirabayashi et al., 2009). Alternative to DNA methylation, histone modification can also regulate gene expression. Therefore, we further investigated histone modification of NGN2 promoter region and transcription start site (Fig. 4L-O). Consistent with the application of HDAC inhibitor VPA during our chemical reprogramming process, we observed a significant increase of histone acetylation at D8 (Fig. 4M). Interestingly, the H3K4me3 level significantly increased at the promoter region (Fig. 4N), whereas H3K27me3 level significantly decreased at the transcription start site at D8 (Fig. 4O), consistent with transcriptional activation of NGN2 induced by small molecule treatment. Together, our results suggest that both transcriptional and epigenetic regulations are involved in our chemical reprogramming process.

To corroborate with our transcriptional and epigenetic analyses, we further performed immunostaining to examine the protein expression changes during chemical reprogramming process (Fig. 5). We found that the Ascl1 expression level first showed a significant increase after 2-day treatment with LDN193189, SB431542, and TTNPB (Fig. 5A and G). The expression level of Ngn2 showed a peak at D4 after small molecule treatment (Fig. 5B and
H; in the presence of CHIR99021, DAPT, and VPA). Compared to Ascl1 and Ngn2, the expression of NeuroD1 appeared to be delayed, with a peak level reached at D6 after small molecule treatment (Fig. 5C and I), consistent with our transcriptional studies (Fig. 4C-D). In addition, immunostaining experiments also revealed that some cells started to show neuronal marker such as DCX at D4-D6 (Fig. 5D), and NeuN+ neurons appeared at D8-D10 (Fig. 5E and J), which is after the peak expression of NeuroD1. In contrast to the increase of neuronal markers, astrocytic protein GFAP showed a significant decrease after small molecule treatment (Fig. 5F and K), consistent with epigenetic silencing and transcriptional downregulation of the GFAP gene. Control astrocytes cultured for 10 days without small molecule treatment did not show much change in the expression level of neural transcription factors, neuronal protein NeuN, or astrocytic protein GFAP (Fig. S6). These experiments suggest that our small molecule strategy has successfully activated endogenous neural transcription factors, which may play an important role in the reprogramming of astrocytes into neurons.

**Functional role of each individual compound during chemical reprogramming**

To dissect out the precise contribution of each single molecule toward reprogramming, we performed a series of experiments by withdrawing each individual compound from our cocktail pool (Fig. 6). Compared to the sequential exposure to 9 molecules in total, removing DAPT resulted in a most significant reduction of the number of converted neurons (Fig. 6A-C). Similarly, removing CHIR99021 or SB431542 or LDN193189 also significantly reduced the reprogramming efficiency (Fig. 6D-F). Removing VPA or SAG +Purmo slightly reduced the reprogramming efficiency (Fig. 6G-H). Interestingly, removing Tzv or TTNPB did not have a significant effect on the astrocyte-neuron reprogramming (Fig. 6I-J). Fig. 6K illustrates the summarized data of drug withdrawing experiments. While it is not a surprise that Tzv had no effect since it mainly acts as a cell survival factor, it is quite unexpected to find that removing TTNPB had no effect. We included TTNPB because it is an agonist of retinoic acid receptors, which were found to play an important role in neural differentiation. The lack of contribution of TTNPB suggested that retinoic acid may not be a necessary factor in reprogramming astrocytes into neurons. On the other hand, the inhibition of Notch signaling, GSK-3β, and BMP/TGFβ signaling pathways appeared to be important for reprogramming astrocytes into neurons. To ensure that these signaling pathways are indeed inhibited during our small molecule treatment, we performed a series of immunostaining against phosphorylated SMAD1/5/9, Notch intracellular domain (NICD), and phosphorylated GSK3β (Fig. S5G-I). Our results showed that the BMP/TGFβ, Notch, and GSK3β signaling pathways were significantly inhibited (Fig. S5G-I) after small molecule treatment, suggesting a close link between the inhibition of these signaling pathways and the astrocyte-to-neuron conversion.

**In vivo integration of human neurons in the mouse brain after reprogramming**

We further investigated whether the human astrocyte-converted neurons can survive in the mouse brain in vivo. To distinguish the human astrocyte-converted neurons from pre-existing mouse neurons inside the brain, we used EGFP-lentiviruses to infect human astrocytes before small molecule treatment so that human astrocyte-converted neurons were mostly labeled by EGFP (Fig. 7A). At 14 days after initial small molecule treatment, we
harvested the cells, which contained both converted neurons and non-converted astrocytes, and injected into the lateral ventricles in neonatal mice (Fig. 7A). At 7 days post cell injection (DPI), we found a cluster of EGFP-labeled cells inside the lateral ventricle, which were all immunopositive for human nuclei (HuNu, Fig. 7B), suggesting that these cells were originated from the injected human cells. Importantly, we found that many EGFP-labeled human cells were immunopositive for neuronal markers DCX (Fig. 7B), MAP2 (Fig. 7C), and NeuN (Fig. 7D), suggesting that the human astrocyte-converted neurons can survive in the mouse brain in vivo. Even one month after cell injection, we were still able to identify clusters of EGFP-labeled neurons in brain areas adjacent to the lateral ventricles such as thalamus and striatum (Fig. 7E), suggesting that the human astrocyte-converted neurons might have migrated out of the lateral ventricles and integrated into the local neural circuits. In supporting this notion, we found many synaptic puncta along the dendrites of EGFP+ human neurons (Fig. 7F), suggesting that these grafted human neurons have established synaptic connections with host neurons. Together, these in vivo experiments demonstrate that our small molecule-reprogrammed human neurons not only can survive in the mouse brain but also can integrate into the local neural circuits.

We have also attempted to reprogram mouse astrocytes into neurons using our small molecule strategy both in vitro and in vivo. However, we did not succeed after many repeats, suggesting that mouse and human astrocytes are significantly different in response to the same set of small molecules (Han et al., 2013). Nevertheless, we did find that the small molecule-treated mouse astrocytes in vivo expressed much more Nestin signal than the vehicle control (Fig. S7A-B). Therefore, we isolated the cortical tissue surrounding the small molecule injection areas and cultured in vitro. Interestingly, the small molecule-treated cortical tissue gave many more neurospheres than the vehicle control (Fig. S7C-H). These neurospheres could dissociate into neural stem cells and gave rise to neurons, astrocytes, and oligodendrocytes (Fig. S7I-J). These data suggest that our small molecule cocktail was capable of stimulating cellular plasticity within the brain tissue, but not sufficient to achieve in vivo reprogramming of mouse astrocytes.

**Discussion**

We demonstrate here that human astrocytes can be chemically reprogrammed into functional neurons with a cocktail of 9 small molecules added in a sequential manner. Importantly, these chemically reprogrammed human neurons are fully functional, demonstrated by long-term survival in cell cultures and robust synaptic events and synchronous burst activities. These chemically reprogrammed human neurons can also survive in the mouse brain in vivo and integrate into local circuits. Mechanistically, the cocktail of small molecules may act through epigenetic silencing of glial genes and transcriptional activation of neural transcription factors such as NGN2 and NEUROD1. The successful reprogramming of human astrocytes into functional neurons with chemically synthesized compounds may potentially lead to a novel drug therapy for brain repair.
Identification of small molecules capable of reprogramming astrocytes into neurons

To identify the small molecules for astrocyte-neuron reprogramming, we searched for small molecules that play crucial roles in neurodevelopment and neurodifferentiation (Chambers et al., 2009; Chambers et al., 2012; Huangfu et al., 2008; Ladewig et al., 2012; Li et al., 2011; Liu et al., 2013; Sirko et al., 2013; Zhang et al., 2011). We tested a variety of small molecules (20 in total) targeting signaling pathways critical for neurodevelopment including noggin, BMP, TGFβ, GSK3β, Wnt, retinoic acid, Notch, SHH, cAMP, DNA methylation, and histone deacetylation or methylation. After testing hundreds of different conditions, we identified a group of 9 small molecules (LDN193189, SB431542, TTNPB, Tzv, CHIR99021, DAPT, VPA, SAG, and Purmo) capable of reprogramming human astrocytes into neurons. Importantly, adding these 9 small molecules together would cause severe cell death, suggesting that some signaling pathways cannot be inhibited simultaneously. A successful strategy is to add fewer small molecules in a sequential manner. By withdrawing each individual molecule from the 9-molecule pool, we found that DAPT plays the most significant role in chemical reprogramming, followed by CHIR99021, SB431542, and LDN193189. Coincidentally, some of the small molecules identified in our study appear to be important in inducing neural differentiation from human stem cells (hSCs) (Chambers et al., 2009; Chambers et al., 2012; Li et al., 2011), but the neuronal fate is quite different. Our astrocyte-converted neurons are deep layer cortical neurons or hippocampal neurons, possibly because our human astrocytes are cortical origin and thus bear cortical lineage trace. In contrast, Chambers et al. found that when hSCs were treated with 5 small molecules (LDN193189 + SB431542 + CHIR99021 + DAPT + SU5402), they were differentiated into spinal cord neurons (Chambers et al., 2012). A different study reported that treating hSCs with 3 small molecules (CHIR99021, SB431542, and compound E, a γ-secretase inhibitor similar to DAPT used in our study) induced differentiation into self-renewing neuroepithelial cells that can be further differentiated into midbrain and hindbrain neurons (Li et al., 2011). These studies, together with our own, suggest that different combinations of small molecules, sometimes with only 1-2 compound difference, may result in different neuronal fate. An alternative explanation for these different neuronal subtypes is the different cell types to start with for reprogramming. This is supported by our own observation that our small molecule protocol only works for human astrocytes with brain origin but not work for human spinal cord astrocytes, nor for mouse astrocytes. It is interesting to note that after completing our studies, two recent articles reported using small molecules to reprogram human or mouse fibroblasts into neurons (Hu et al., 2015; Li et al., 2015). Comparing these two studies with our own, CHIR99021 emerged as an indispensable small molecule for chemical reprogramming into neurons, and transcriptional activation of NeuroD1 and Ngn2 was also observed after chemical reprogramming fibroblasts into neurons (Li et al., 2015). On the other hand, comparing to fibroblasts, we found that glial cells can be more efficiently reprogrammed into neurons (67%) in a short time (10 days) and survive for >5 months in culture. Another point worth of mentioning is that our chemically reprogrammed neurons are forebrain glutamatergic neurons, which share close lineage with cortical astrocytes that they come from. It can be challenging to reprogram fibroblasts into a specific subtype of neurons in a particular brain region.
Mechanisms of small molecule-mediated astrocyte-neuron reprogramming

The development of central nervous system is under precise temporal and spatial control by both intrinsic genetic programs and external signals such as FGF, TGFβ, SHH, BMP, Notch, RA and Wnt proteins (Hur and Zhou, 2010; Miller and Gauthier, 2007). We have tested various chemical compounds that may activate or inhibit these signaling pathways during our search for small molecules to convert astrocytes into neurons. One surprising finding is that RA, which plays a critical role in neural stem cell proliferation and differentiation, appears to be dispensable for astrocyte-neuron conversion. Another unexpected finding is that SHH, one of the major organizing signals in the brain and spinal cord development (Dessaud et al., 2008; Sirko et al., 2013), also seems to be not absolutely required for astrocyte-neuron conversion. Therefore, the mechanism of reprogramming astrocytes into neurons clearly differs from that of neurodevelopment or neurodifferentiation. One possible explanation is that neural development or differentiation starts from neural stem/progenitor cells, whereas our reprogramming process starts from astrocytes, which are the progeny of neural stem cells. RA and SHH may be upstream of astroglial fate determination, and therefore not required for astrocyte-neuron reprogramming. On the other hand, it is important to note that our current astrocyte-neuron reprogramming strategy mainly results in glutamatergic neurons. It is possible that RA and SHH may be important for the conversion of astrocytes into other subtypes of neurons such as dopaminergic or GABAergic neurons.

Both epigenetic and transcriptional regulations appear to be involved in our chemical reprogramming process. Our epigenetic analyses revealed a significant increase of DNA methylation in the promoter region of GFAP gene, particularly at the flanking sites of two transcription factors STAT3 and AP1 binding region, consistent with previous studies on epigenetic regulation of astroglial fate (Cheng et al., 2011; Condorelli et al., 1994). The epigenetic silencing of GFAP promoter through DNA methylation may explain the downregulation of GFAP transcriptional level after small molecule treatment. On the other hand, regulation of NGN2 appears to be mediated by histone modification, as shown by an increase of H3K4 methylation in its promoter region and a decrease of H3K27 methylation at the transcription start site. Our results are consistent with previous finding regarding the regulation of NGN2 through histone modification (Hirabayashi et al., 2009). The activation of NGN2 promoter is consistent with our transcriptional analyses showing >200-fold increase of the NGN2 transcriptional level after small molecule treatment. Therefore, our chemical reprogramming is mediated by epigenetic silencing of glial genes and transcriptional activation of neural transcriptional factors.

Conclusion

Our studies demonstrate the proof-of-principle that human astrocytes can be chemically reprogrammed into neurons. Importantly, our chemical reprogramming protocol is effective for human astrocytes, but not mouse astrocytes. Among human astrocytes, our protocol is effective for brain astrocytes, but not spinal cord astrocytes. Therefore, different glial cell lineages may be sensitive to different sets of small molecules, suggesting that different neurological disorders may require different chemicals to regenerate specific subtypes of neurons. Another challenge ahead is how to effectively deliver small molecules across the...
brain-blood-barrier to the injured or diseased brain areas. After delivered to the brain, how to chemically reprogram reactive glial cells with less effect on normal glial cells also needs to be resolved. Regardless of the challenges, chemical reprogramming of human astrocytes into functional neurons provides a novel approach to regenerate neurons for future brain repair.

**Experimental Procedures**

**Human astrocyte culture**

Human astrocytes were purchased from ScienCell (HA1800, California) or Gibco (N7805-100). Human astrocytes were primary cultures obtained from human fetal brain tissue. They were isolated and maintained in the presence of 10% fetal bovine serum (FBS), which will essentially cause any progenitor cells to differentiate. Human astrocytes were subcultured when they were over 90% confluent. For subculture, cells were trypsinized by TrypLE™ Select (Invitrogen), centrifuged for 5 min at 900 rpm, re-suspended, and plated in a culture medium consisting of DMEM/F12 (Gibco), 10% fetal bovine serum (Gibco), penicillin/streptomycin (Gibco), 3.5 mM glucose (Sigma), and supplemented with B27 (Gibco), 10 ng/mL epidermal growth factor (EGF, Invitrogen), and 10 ng/mL fibroblast growth factor 2 (FGF2, Invitrogen). Cells were maintained at 37°C in humidified air with 5% CO₂.

**Reprogramming human astrocytes into neurons**

The astrocytes were cultured on poly-D-lysine (Sigma) coated coverslips (12 mm) at a density of 50,000 cells per coverslip in 24-well plates (BD Biosciences). The cells were cultured in human astrocyte medium until 90% confluence. At day 0 before reprogramming, half of the culture medium was replaced by N2 medium consisting of DMEM/F12 (Gibco), penicillin/streptomycin (Gibco) and N2 supplements (Gibco). The following day (Day 1), the culture medium was completely replaced by N2 medium supplemented with small molecules, or with 1% DMSO in control group. For most of the experiments using 9 molecules for reprogramming (MCM treatment), astrocytes were treated with TTNPB (0.5 μM, Tocris #0761), SB431542 (5 μM, Tocris #1614), LDN193189 (0.25 μM, Sigma #SML0559) and Thiazovivin (0.5 μM, Cayman #14245) for 2 days. At day 3, the culture medium was replaced with a different set of small molecules including CHIR99021 (1.5 μM, Tocris #4423), DAPT (5 μM, Sigma #D5942), VPA (0.5 mM, Cayman #13033) and Thiazovivin (0.5 μM). At day 5, VPA was withdrawn by replacing medium containing only CHIR99021 (1.5 μM), DAPT (5 μM) and Thiazovivin (0.5 μM). At day 7, medium was replaced containing SAG (0.1 μM, Cayman #11914), purmophamine (Purmo, 0.1 μM, Cayman #10009634) and Thiazovivin (0.5 μM). At day 9, medium was completely replaced with neuronal differentiation medium (NDM) including DMEM/F12 (Gibco), 0.5% FBS (Gibco), 3.5 mM glucose (Sigma), penicillin/streptomycin (Gibco), and N2 supplement (Gibco). 200 μl neuronal differentiation medium was added into each well every week to keep the osmolarity constant. To promote synaptic maturation of converted neurons, brain-derived neurotrophic factor (BDNF, 20 ng/mL, Invitrogen), Insulin-like growth factor 1 (IGF-1, 10 ng/mL, Invitrogen) and neurotrophin 3 (NT-3, 10 ng/mL, Invitrogen) were added.
in neuronal differentiation medium at day 9 and were refreshed every four days until day 30 (Song et al., 2002).

To examine whether our human astrocytes contain any neural stem cells, we cultured human astrocytes in neuronal differentiation medium supplemented with BDNF 20 ng/ml, NT3 10 ng/ml and NGF 10 ng/ml for 1 month. The growth factors were refreshed every 3-4 days. The human neuroprogenitors (NPCs) derived from human pluripotent stem cells were gift from Dr. Fred Gage. The NPCs were cultured in poly-L-ornithine and laminin-coated coverslips with neuronal proliferation medium including DMEM/F12, penicillin/streptomycin, B27 supplement, N2 supplement and FGF2 (20 ng/ml) (Gibco).

Data and statistical analysis

Cell counting was performed by taking images at several randomly chosen fields per coverslip and analyzed by Image J software. The fluorescence intensity was analyzed by Image J software. Data were represented as mean ± SEM. Student's t test was used for the comparison between two groups of data. One-way ANOVA and post hoc tests were used for statistical analyses of data from multiple groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References


### Highlights

- A cocktail of small molecules reprogram human astrocytes into functional neurons.
- Human astrocyte-converted neurons survive >5 months with synchronous activities.
- Chemical reprogramming is mediated through epigenetic & transcriptional regulation.
- Human astrocyte-converted neurons can integrate into mouse brain in vivo.
Figure 1. Sequential exposure to a defined group of small molecules converts human astroglial cells into neuronal cells

(A) Schematic illustration of our strategy to convert cultured human astrocytes into neurons using a cocktail of small molecules. Note that different subsets of small molecules were used at different reprogramming stages.

(B, C) Quantitative analysis of the human astrocyte cultures (HA1800, ScienCell). The majority of cells in our human astrocyte cultures were immunopositive for astrocytic marker GFAP (79.3 ± 4.9%), astrocytic glutamate transporter GLT-1 (82.5 ± 4.3%), and to a lesser degree S100β (39.3 ± 1.8%). No cells were immunopositive for neuronal markers NeuN, MAP2 or Doublecortin (DCX). HuNu, human nuclei, marker for human cells. N = 3 batches.

(D) Control human astrocyte cultures without small molecule treatment had very few cells immunopositive for neuronal markers DCX (green), β-III tubulin (Tuj1, red) or MAP2 (cyan).

(E) Sequential exposure of human astrocytes to small molecules resulted into a massive number of neuronal cells, which were immunopositive for DCX (green), Tuj1 (red) and MAP2 (cyan). MCM stands for master conversion molecules, including the 9 small molecules for reprogramming together. Analyzed at 14 days after initial small molecule treatment.

(F) At 30 days post initial small molecule treatment, human astrocyte-converted neurons developed extensive dendrites (MAP2, green) and were immunopositive for mature neuronal marker NeuN (red).

(G) Small molecule-converted human neurons survived for 4 months in culture and showed robust dendritic trees (MAP2, green) as well as extensive axons (SMI312, red).

(H) Astroglial lineage tracing with GFAP::GFP retrovirus showing GFP⁺ cells were immunopositive for neuronal marker NeuN (red) after small molecule treatment. N = 5 batches.

(I and J) Small molecule treatment achieved high conversion efficiency after 8 days exposure to MCM (67.1 ± 0.8%, Tuj1⁺ neurons/total cells labeled by DAPI, n = 4 batches).
(K) Chemical reprogramming of human midbrain astrocytes into neurons. At 1-month post initial small molecule treatment of human midbrain astrocytes (ScienCell), most cells were immunopositive for neuronal marker NeuN (red) and MAP2 (green).

(L) Control human midbrain astrocyte cultures without small molecule treatment had very few cells immunopositive for NeuN (red) or MAP2 (green) at 1-month culture in neuronal differentiation medium.

(M) Quantitative analysis revealed a large number of NeuN-positive neurons converted from human midbrain astrocytes at 1-month post small molecule treatment (199.7 ± 9.2 per 40x field), whereas control group only had a few NeuN+ cells (5.6 ± 1.4 per 40x field). N = 4 batches. Scale Bars: 50 μm for panel B; 20 μm for other images. *** P < 0.001, Student's t test.

Data are represented as mean ± SEM.
Figure 2. Functional analyses of human astrocyte-converted neurons induced by small molecule treatment

(A) Long-term survival of small molecule-induced human neurons (5 months in culture) and massive number of synaptic puncta (SV2, red) along the dendrites (MAP2, Green). Scale bar: 20 μm.

(B-D) Representative traces showing Na\(^+\) and K\(^+\) currents recorded from 1-month (B) and 2-month (C) old human neurons induced by small molecules. Panel D shows the blockade of Na\(^+\) currents by TTX (2 μM).

(E) Quantitative analyses of peak Na\(^+\) and K\(^+\) currents in 2-week to 3-month old neurons converted from human astrocytes by small molecules.

(F) Representative trace of repetitive action potentials recorded in small molecule-induced human neurons at 75 days post initial drug treatment.

(G and H) Representative traces showing spontaneous synaptic events in 2-month old converted human neurons. Holding potential = −70 mV. (H) Expanded trace from (G).

(I) Inhibitory GABAergic events revealed in human astrocyte-converted neurons when holding potential was held at 0 mV (2-month old). The events were blocked by GABA\(_A\) receptor antagonist bicuculline (BIC, 10 μM).

(J-K) Representative traces showing spontaneous burst activities in 3-month old small molecule-induced human neurons. HP = −70 mV. (K) Expanded view of a burst in (J).

(L) The burst activities were blocked by TTX (2 μM). The majority of synaptic events at −70 mV were blocked by glutamate receptor antagonist DNQX (10 μM), suggesting that they were glutamatergic events.

(M) Dual whole-cell recordings illustrating that small molecule-converted human neurons formed robust synaptic networks and fired synchronously.
(N) The Ca\(^{2+}\) ratio imaging further illustrating that the small molecule-converted human neurons were highly connected and showed synchronous activities. Data are represented as mean ± SEM.
Figure 3. Characterization of the human astrocyte-converted neurons induced by small molecules

(A-C) Immunostaining with anterior-posterior neuronal markers revealed that the small molecule-converted human neurons were positive for forebrain marker FoxG1 (A), but negative for hindbrain and spinal cord marker HOX B4 (B) and HOX C9 (C).

(D-F) Immunostaining with cortical neuron markers revealed that small molecule-induced human neurons were negative for superficial layer marker Cux1 (D), but positive for deep layer marker Ctip2 (E) and Otx1 (F).

(G-H) The small molecule-converted human neurons were also immunopositive for general cortical neuron marker Tbr1 (G) and hippocampal neuron marker Prox1 (H).

(I) Quantitative analyses of small molecule-induced human neurons (FoxG1, 97.1 ± 1.1%, n = 3 batches; Cux1, 3.1 ± 1.9%, n = 4 batches; Ctip2, 71.4 ± 3%, n = 4 batches; Otx1, 87.4 ± 3.2%, n = 3 batches; Tbr1, 86.4 ± 3.4%, n = 3 batches; Prox1, 73.4 ± 4.4%, n = 4 batches).

Scale bars: 20 μm.

(J) MCM-converted human neurons were immunopositive for VGluT1.

(K) A small portion of MCM-converted human neurons were GAD67-positive.

(L-N) MCM-converted neurons were immunonegative for cholinergic neuronal marker vesicular acetylcholine transporter (VACHT) (L), dopaminergic neuronal marker tyrosine hydroxylase (TH) (M), or spinal motor neuron marker Isl1 (N).

(O) Quantitative analyses of small molecule converted human neurons (VGluT1, 88.3 ± 4%, n = 4 batches; GAD67, 8.2 ± 1.5%, n = 4 batches). Scale bars: 20 μm.

Data are represented as mean ± SEM.
Figure 4. Transcriptional and epigenetic regulation during chemical reprogramming of human astrocytes into neurons

(A-B) PCR array revealed substantial transcriptional activation of neural transcription factors (NGN1/2, NEUROD1, and ASCL1) and immature neuronal gene DCX at day 4 (A) or day 8 (B) after small molecule treatment. Note that DCX increased >2000-fold at D8 compared to the control. The genes showing significant change in PCR array assay were presented (P < 0.05, Mann-Whitney t test).

(C-F) The time course of transcriptional changes revealed by quantitative real-time PCR analyses. Neural transcriptional factors NGN2 (C) and NEUROD1 (D) showed a peak transcription at D4 and D6, respectively; whereas astroglial genes GFAP (E) and ALDH1L1 (F) were significantly downregulated. * P < 0.05, ** P < 0.01, *** P < 0.001; Two-way ANOVA followed with Dunnett’s test. N = 3 batches.

(G-I) Epigenetic regulation of GFAP promoter and transcription start site during chemical reprogramming. MeDIP-seq revealed a significant increase of methylation in the GFAP promoter region (G, box region) after 8 days of small molecule treatment, which was confirmed by subsequent BS-seq (H). Note that the hypermethylated sites were located in the flanking region of two important transcription factor-binding sites, STAT3 and AP1, which will significantly inhibit the transcription of GFAP. BS-seq also showed a significant increase of the methylation level at GFAP transcription start site (TSS) and 5’ UTR regulatory region (I), further suggesting an inhibition of GFAP transcription through DNA methylation.

(J-K) MeDIP-seq and BS-seq revealed a significant decrease of methylation at the promoter region of a neuronal gene NEFM (neurofilament-M), suggesting transcriptional activation of neuronal genes during chemical reprogramming of human astrocytes into neurons.

(L-M) CHIP-qPCR revealed a significant increase of histone acetylation in the NGN2 promoter region after small molecule treatment, likely caused by HDAC inhibitor VPA.
The methylation level of H3K4 increased significantly in the NGN2 promoter region (N), whereas H3K27 methylation at the NGN2 transcription start site showed a significant decrease (O), indicating epigenetic activation of NGN2 through histone modification. Data are represented as mean ± SEM.
Figure 5. Increase of the protein expression level of neural transcription factors during chemical reprogramming

(A-C) Representative images illustrating the gradual activation of endogenous neural transcription factors Ascl1 (A), Ngn2 (B), and NeuroD1 (C) at different days of small molecule treatment.

(D-E) Representative images showing the gradual increase of neuronal signal DCX (D) and NeuN (E) during the conversion process from D0 to D10.

(F) Representative images showing the decrease of astrocytic marker GFAP from D0 to D10. Scale bars: 20 μm

(G-I) Quantitative analyses of the protein expression level of Ascl1 (G), Ngn2 (H), and NeuroD1 (I). Note that Ascl1 significantly increased at day 2 by 3-fold, while Ngn2 peaked at day 4 and NeuroD1 peaked at day 6. N = 3 batches.

(J) Quantified data showing a significant increase of NeuN from day 6 to day 10. N = 3 batches.

(K) Quantified data showing a significant decrease of GFAP from D0 to D10. N = 3 batches. Data are represented as mean ± SEM.
Figure 6. Evaluating the essential role of each individual small molecule during astrocyte-neuron reprogramming

(A) Human astrocytes treated with 1% DMSO as a control. NeuN, green; MAP2, red.

(B) A defined combination of 9 small molecules induced a massive number of neurons (14 days post initial small molecule treatment, the same for the following removal experiments).

(C-F) Individual removal of DAPT (C), CHIR99021 (D), SB431542 (E) or LDN193189 (F) from the 9 small molecule pool significantly reduced the number of converted neurons.

(G) Removal of sonic hedgehog agonists SAG and Purmo together slightly reduced the number of converted neurons.

(H) Removal of VPA also slightly reduced the neuronal number.

(I-J) Removal of Tzv (I) or TTNPB (J) did not affect the neuronal conversion. Scale bars: 20 μm.

(K) Quantitative analyses showing that DAPT is the most potent reprogramming factor, followed by CHIR99021, SB431542, and LDN193189. * P < 0.05; ** P < 0.01; *** P < 0.001; one-way ANOVA followed with Sidak's multiple comparison test. N = 3 batches. Data are represented as mean ± SEM.
Figure 7. In vivo survival and integration of small molecule-converted human neurons in the mouse brain

(A) Schematic drawing showing the transplantation of small molecule-converted human neurons into the mouse brains at postnatal day 1.

(B) GFP-positive cells were identified around lateral ventricles at 7 days post cell injection (7 DPI). Many GFP-positive cells were also positive for DCX (red), and all of the GFP-positive cells were immunopositive for human nuclei (HuNu, Blue), indicating their human cell identity. N = 6 mice.

(C) At 11 DPI, some GFP-positive cells were immunopositive for MAP2 (red), indicating the survival and growth of human neurons in the mouse brain in vivo. N = 6 mice.

(D) Some GFP-positive human neurons, which were immunopositive for NeuN (red) and HuNu (cyan), migrated into the adjacent striatum areas and extended long neurites at 11 DPI.

(E) Human neurons, labeled by NeuN (red) and HuNu (blue), survived for more than 1 month inside the mouse brain and were surrounded by mouse neurons (NeuN positive but HuNu negative). N = 2 mice.

(F) GFP-positive human neurons were innervated by surrounding neurons as indicated by many synaptic puncta (SV2, red) along the GFP-positive neurites (inset), suggesting the synaptic integration of the transplanted human neurons into the local neural circuit. N = 2 mice. Scale bars: 20 μm.