

ORIGINAL ARTICLE

Direct conversion of human fibroblasts to induced serotonergic neurons

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Serotonergic (5HT) neurons exert diverse and widespread functions in the brain. Dysfunction of the serotonergic system gives rise to a variety of mental illnesses including depression, anxiety, obsessive compulsive disorder, autism and eating disorders. Here we show that human primary fibroblasts were directly converted to induced serotonergic (i5HT) neurons by the expression of *Ascl1*, *Foxa2*, *Lmx1b* and *FEV*. The transdifferentiation was enhanced by p53 knockdown and appropriate culture conditions including hypoxia. The i5HT neurons expressed markers for mature serotonergic neurons, had Ca^{2+} -dependent 5HT release and selective 5HT uptake, exhibited spontaneous action potentials and spontaneous excitatory postsynaptic currents. Application of serotonin significantly increased the firing rate of spontaneous action potentials, demonstrating the functional utility of i5HT neurons for studying serotonergic neurotransmission. The availability of human i5HT neurons will be very useful for research and drug discovery on many serotonin-related mental disorders.

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INTRODUCTION

Despite their small numbers (~26 000 in the mouse brain¹), serotonergic neurons exert diverse and widespread impact on emotion, feeding, aggression, compulsion, sleep and so on.² Dysfunction of the serotonergic system gives rise to a variety of neurological disorders and mental illnesses, including depression, anxiety, obsessive compulsive disorder, autism and eating disorder.² Increasing evidence suggest that individual variations in genes controlling the development and function of serotonergic neurons may lead to a variety of serotonin-related brain disorders.³ Thus, it would be very useful to generate patient-specific serotonergic neurons for mechanistic studies of serotonergic dysfunctions and drug discovery research on many serotonin-related brain diseases. Recent research on transdifferentiation has shown that fibroblasts can be directly converted by different sets of transcription factors to different types of cells.⁴ *Ascl1* is a key transcription factor for neurogenesis and early neural differentiation.⁵ It serves as a pioneer factor in the transdifferentiation of fibroblasts to induced neurons.⁶ In *Ascl1* knockout mice, there is a profound loss of serotonin, as transcription factors that specify serotonergic neurons such as *Pet1* and *Lmx1b* are not expressed.⁷ *FoxA2* establishes the progenitor domains for the precursors of serotonergic neurons in the ventral hindbrain and activates transcription factors required for the terminal differentiation of serotonergic neurons, such as *Pet1* and *Lmx1b*.⁸ Loss of *FoxA2* at the precursor stage ablates 50% of serotonergic neurons in the hindbrain.⁸ *Lmx1b* is a critical transcription factor in the terminal differentiation of serotonergic neurons. In *Lmx1b*-deficient mice, precursors of serotonergic neurons are generated in normal numbers, but they fail to express the battery of genes (*Tph2*, *Sert*, *Vmat2* and so on) that define a serotonergic neuron.^{9,10} Deletion of *Lmx1b* specifically in serotonergic neurons results in the loss of

these early precursors, confirming the role of *Lmx1b* in the terminal differentiation of serotonergic neurons.¹¹ *Pet1* (FEV in human) expression is restricted to serotonergic neurons.^{12,13} *Pet1*-deficient precursor cells fail to turn on the expression of serotonergic marker genes, such as *Tph2*, *Aadc*, *Vmat2*, *Sert* and *Maob*, resulting in a loss of 70% serotonergic neurons.¹⁴

In this study, we found that Dox-inducible lentivirus-mediated expression of *Ascl1*, *FoxA2*, *Lmx1b* and *FEV* (AFLV) directly converted human fibroblasts to induced serotonergic (i5HT) neurons in 12 days. The transdifferentiation was significantly enhanced by p53 knockdown and suitable culture conditions including hypoxia. The i5HT neurons expressed appropriate markers for serotonergic neurons and exhibited active serotonergic synaptic transmission. This fast and efficient method of generating i5HT neurons would enable research on patient-specific serotonergic neurons for mechanistic study and drug discovery in many mental illnesses involving serotonergic dysfunction.

MATERIALS AND METHODS

Materials

Dorsomorphin dihydrochloride, SB431542, CHIR99021 and Purmorphamine were purchased from Stemgent (Cambridge, MA, USA). Y27632, PD 0332991 isethionate and SU9516 were purchased from Tocris (Bristol, UK). Recombinant human GDNF, BDNF, NGF, bFGF and TGF- β 3 were purchased from PerproTech (Rocky Hill, NJ, USA). cAMP, Ara-C, Ara-A, ascorbic acid and *N*-acetyl-L-cysteine were purchased from Sigma (St. Louis, MO, USA). FUW-tetO-LoxP-hOCT4, shP53pLKO.1, pMD2.G and psPAX2 were purchased from Addgene (Cambridge, MA, USA). Human *Ascl1* (Genebank accession BC031299), *Foxa2* (BC011780), *Lmx1b* (BC113491), *FEV* (BC023511.2), *Nurr1* (CV028069), *Lmx1a* (BC06635), *NeuroD1* (NM_002500), *NeuroD2* (NM_006160) and *Pitx3*(NM_005029) were purchased from Open Biosystems (Pittsburgh, PA, USA) and subcloned by PCR to the EcoRI site on the FUW-tetO-LoxP vector. Human miR-124 (MIMAT0000422) and *Nkx2.2*

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(NM_002509) were amplified from normal human fibroblast genomic DNA and subcloned to the EcoRI site on the FUW-tetO-LoxP vector. FUW-LoxP-M2rtTA was generated by subcloning the BspEI fragment containing the loxP site from FUW-tetO-LoxP-hOCT4 (Addgene) to the BspEI site on the 3' LTR of FUW-M2rtTA (Addgene). All constructs were verified by sequencing.

Derivation of the induced serotonergic neurons

Primary human fibroblasts MRC5 and CCD-19Lu (both from ATCC, Manassas, VA, USA) and IMR90 (from Coriell, Camden, NJ, USA) were cultured in DMEM containing 10% FBS and 2 mM L-glutamine. All cell cultures were done without antibiotics and regularly tested for the absence of mycoplasma by PCR. Lentiviral production and fibroblast infections were performed as described previously.¹⁵ In brief, lentiviruses were produced by cotransfecting 293FT cells (Invitrogen, Carlsbad, CA, USA) in 10 cm dishes with 10 µg FUW-tetO-LoxP-cDNA (hAscl1, hFoxa2, hLmx1b, hFEV, Lmx1a, Pitx3, NeuroD1, NeuroD2, Nlx2.2, Nurr1 and miRNA-124) or shP53pLKO.1 or 10 µg FUW-LoxP-M2rtTA with 2.5 µg pMD2.G and 7.5 µg psPAX2 using Lipofectamine 2000. Viruses were collected from 16 to 60 h after transfection and titered for p24 levels using an ELISA kit (ZeptoMetrix Corporation, Buffalo, NY, USA). After the fibroblasts were thawed and passaged once more, they were plated at $5.4 \times 10^4 \text{ cm}^{-2}$ and infected 1 day later for 16 h with the indicated combinations of lentiviruses (M2rtTA, hASCL1 and hLmx1b each at MOI 20, hFoxa2, hFEV and hp53shRNA each at MOI 10) in the presence of 8 µg ml⁻¹ polybrene. Virus-containing media was removed after 16 h and replaced with DMEM. After 24 h, the media were changed to neural induction media (DMEM/F12, 1 × N2 supplements, 1 × NEAA, 1 × B27, 10 µM ROCK inhibitor Y27632, 1 µM PD 0332991, 20 ng ml⁻¹ BDNF and 20 ng ml⁻¹ GDNF) containing 1 µg ml⁻¹ doxycycline (stopped at day 7). Dorsomorphin (0.5 µM) and 2.5 µM SB431542 were added during day 2 to day 7. The media was changed every other day for the duration of the culture period.

Serotonin release

i5HT neurons cultured in six-well plates were incubated at 37 °C in 0.5 ml Hank's balanced salt solution (HBSS) for 30 min, or in 0.5 ml HBSS for 15 min and then 56 mM KCl was added for another 15 min, or in 0.5 ml HBSS without Ca²⁺ and without Mg²⁺, but with 2 mM EDTA for 15 min and then 56 mM KCl was added for another 15 min. The HBSS solutions were taken out from the wells and concentrated by Amicon ultra-0.5 ml 3k centrifugal filter units (Millipore, Billerica, MA, USA). The amounts of 5HT in HBSS solutions were measured by reverse phase HPLC (ESA Model 582 with ESA MD150 × 3.2 column, at 0.6 ml min⁻¹ flow rate in MD-TM mobile phase) coupled with electrochemical detection (ESA Coulochem III, E1: -250 mV, 2 µA; E2: 350 mV, 2 µA). Cells in the wells were lysed in 0.5 N NaOH to measure protein levels, which were used to normalize serotonin release.

Serotonin uptake

i5HT neurons cultured in six-well plates were rinsed with 1 ml prewarmed uptake buffer (10 mM HEPES, 130 mM NaCl, 1.3 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 10 mM glucose, pH 7.4) three times. Cells were incubated for 5 min at 37 °C with 1 ml uptake buffer containing 5 µM serotonin without or with 10 µM citalopram. After the cells were washed at least three times in uptake buffer, they were lysed in 0.1 M perchloric acid with 1 mM EDTA and 0.1 mM sodium bisulfite. Cleared cell lysates were concentrated by Amicon ultra-0.5 ml 3k centrifugal filter units (Millipore) and analyzed for serotonin on HPLC coupled with electrochemical detection (E1: -250 mV, 2 µA; E2: 350 mV, 2 µA). The pellets of cellular proteins were dissolved in 0.5 N NaOH to measure protein contents, which were used to normalize serotonin uptake. The amount of endogenous serotonin in i5HT neurons without any treatment was also measured.

Immunocytochemistry

Cells grown in 12-well plates were fixed *in situ* with 4% paraformaldehyde in PBS for 20 min, permeabilized with 0.1% Triton X-100 in PBS for 20 min at room temperature (RT), blocked in 3% BSA in PBS for 60 min at RT, and then incubated in primary antibody overnight at 4 °C, secondary antibody for 2 h at RT, DAPI for 20 min at RT. The sources, catalog numbers and dilutions of the antibodies used in this study are listed in Supplementary Table 2. Fluorescence images were taken on Zeiss Axio Observer Inverted Microscope with lenses corrected for plastic culture plates. The 5HT⁺, Tuj1⁺ and DAPI⁺ cells were counted from at least five randomly selected images at 10 × magnification for each condition.

Real time quantitative RT-PCR

Total RNA was extracted using RNeasy Mini kit (QIAGEN, Germantown, MD, USA). First-strand complementary DNA was synthesized with iScript cDNA synthesis kit according to manufacturer's protocol (Bio-Rad 170-8890, Hercules, CA, USA). An equal volume mixture of the products was used as templates for PCR amplification. Reactions were performed in a 25 µl volume with iQ SYBR Green Supermix (Bio-Rad) and 200 nM each of forward and reverse primers shown in Supplementary Table 3 using iCycler and iQ software (Bio-Rad). Each sample was run in duplicate. PCR conditions included an initial denaturation step of 4 min at 95 °C, followed by 40 cycles of PCR consisting of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. Average threshold cycle values from the duplicate PCR reactions for a gene of interest were normalized against the average threshold cycle values for GAPDH from the same complementary DNA sample.

Statistical analyses

The data were expressed as mean ± s.e.m. Unpaired, two tailed Student's *t*-tests were performed to evaluate whether two groups were significantly different from each other.

RESULTS

Transdifferentiation of human fibroblasts to induced serotonergic neurons

In an effort to identify transcription factors that can directly convert human fibroblasts to induced serotonergic (i5HT) neurons, we tested a variety of transcription factors involved in the genesis of serotonergic neurons^{3,16,17} or the direct conversion of fibroblasts to induced neurons.⁴ Of these factors (details later), we found that the combined expression of human AFLV generated a large amount of i5HT neurons. Using the protocol in Figure 1a, we transduced human primary fibroblast MRC5 cells with Dox-inducible lentiviruses expressing AFLV and a constitutively active lentivirus for p53 shRNA (AFLVp). After Dox-initiated reprogramming, morphology of the cells changed within days to neuron-like cells (Figures 1b–e). When cells were stained at day 12 for serotonin (5HT), the neuronal marker β3-tubulin (Tuj1), and DAPI, we found that 49.2 ± 2.1% of total cells (DAPI⁺) were Tuj1⁺ neurons and 24.4 ± 0.9% of total cells were 5HT⁺ neurons (Figures 1f–h and n–q). Removal of Ascl1 from the transcription factors generated no neuron (Figures 1i and n–q), demonstrating the requirement for Ascl1 in the transdifferentiation of human fibroblasts to neurons. Removing FoxA2 (Figure 1j) or Lmx1b (Figure 1k) failed to generate any i5HT neurons (Figure 1o), whereas omitting FEV (Figure 1l) produced significantly fewer i5HT neurons (Figure 1o) but more Tuj1⁺ neurons (Figure 1p). FEV appeared to be somewhat toxic to cells; the number of DAPI⁺ cells increased significantly in the absence of FEV (Figure 1q). When we did not include p53 knockdown (Figure 1m), the efficiencies (Figure 1n) and yields (Figures 1o and p) in generating i5HT neurons and Tuj1⁺ neurons were significantly reduced. The results suggest that attenuation of p53 significantly improves reprogramming of human fibroblasts to i5HT neurons.

We also tested other transcription factors implicated in the development of serotonergic neurons^{3,16,17} or transdifferentiation of fibroblasts to induced neurons.⁴ Replacing Lmx1b (Figure 2a) with Lmx1a (Figure 2b) significantly decreased the number of 5HT⁺ neurons and slightly reduced the number of Tuj1⁺ cells (Figures 2i–l). In the presence of AFLVp, the addition of NeuroD1, which has been shown to enhance the conversion of human fibroblasts to induced neurons,¹⁸ significantly reduced the number of 5HT⁺ cells, but did not significantly affect the number of Tuj1⁺ cells or DAPI⁺ cells (Figures 2c and i–l). The addition of NeuroD2, which helps the generation of induced neurons by Ascl1 and Myt1l,¹⁹ abolished the production of 5HT⁺ cells and significantly decreased the number of Tuj1⁺ cells and DAPI⁺ cells (Figures 2d and i–l). The addition of miR124, which improves the derivation of human iN cells by Ascl1 and Myt1l,¹⁹ almost

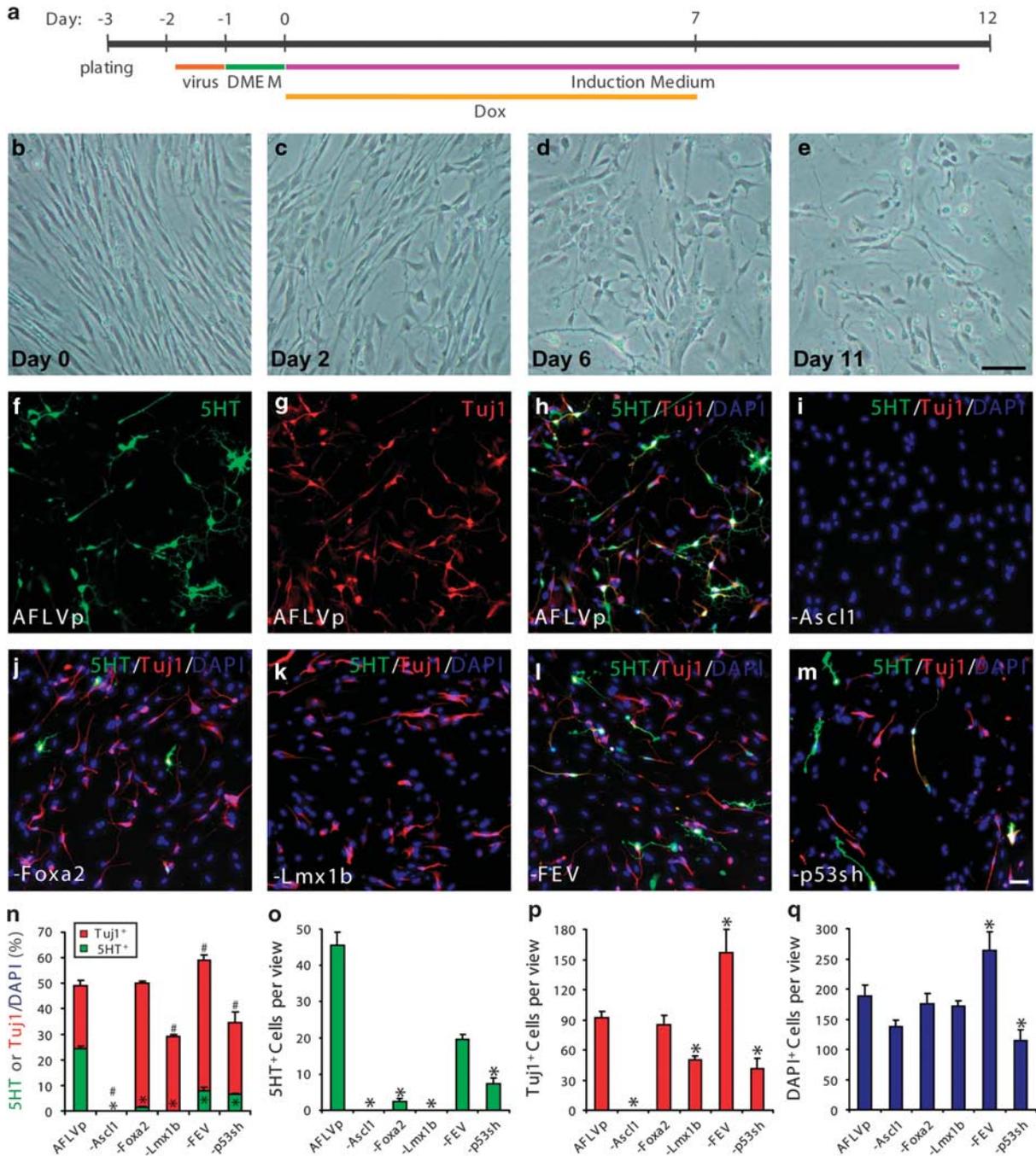


Figure 1. Reprogramming of human fibroblasts to induced serotonergic (i5HT) neurons. (**a–h**) Using the protocol in (**a**), MRC5 cells were transduced with lentiviruses expressing Ascl1, Foxa2, Lmx1b, FEV and p53 shRNA (AFLVp). Phase contrast images at days 0 (**b**), 2 (**c**), 6 (**d**) and 11 (**e**) were shown. Cells at day 12 were stained for 5HT (**f**), Tuj1 (**g**) and DAPI (**h**, merged with **f** and **g**). (**i–m**) MRC5 cells transduced with AFLVp minus Ascl1 (**i**), Foxa2 (**j**), Lmx1b (**k**), FEV (**l**) or p53 shRNA (**m**) were stained for 5HT, Tuj1 and DAPI. (**n–q**) Quantification at day 12 of the percentage of 5HT⁺ neurons or Tuj1⁺ neurons among all cells (DAPI⁺) (**n**) or the average number of 5HT⁺ (**o**), Tuj1⁺ (**p**) or DAPI⁺ (**q**) cells per view. *[#]*P* < 0.05, vs 5HT⁺ or Tuj1⁺ for AFLVp, respectively, cells from at least five random views in each of the three wells from three independent experiments were counted. Bars = 50 μm.

completely abrogated the generation of 5HT⁺ cells without significantly affecting the number of Tuj1⁺ cells or DAPI⁺ cells (Figures 2e and i–l). Surprisingly, the addition of Nkx2.2, which controls the production of 5HT neurons in posterior rhombomeres,²⁰ markedly reduced the number of 5HT⁺ cells, as well as the number of Tuj1⁺ and DAPI⁺ cells (Figures 2f and i–l).

We also tried Nurr1 and Pitx3, which drive the terminal specification of midbrain DA neurons.²¹ Nurr1 significantly reduced the production of 5HT⁺ cells without significantly affecting the number of Tuj1⁺ and DAPI⁺ cells (Figures 2g and i–l). Pitx3 almost completely abolished the production of 5HT⁺ and Tuj1⁺ neurons (Figures 2h–l).

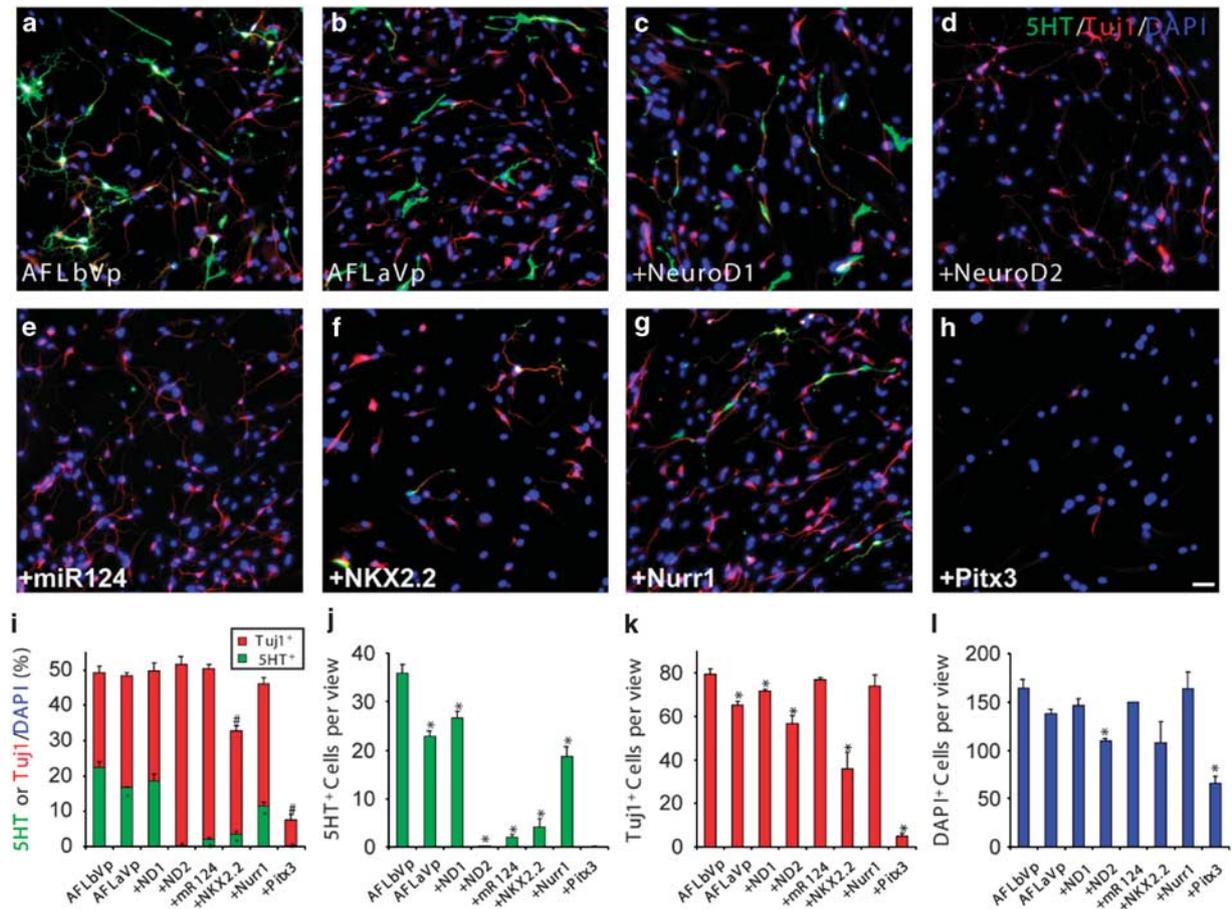


Figure 2. Other transcription factors tested. (a–h) Immunostaining of MRC5 cells reprogrammed with *Ascl1*, *FoxA2*, *Lmx1b*, *FEV* and *p53* shRNA (AFLbVp) (a), *Ascl1*, *FoxA2*, *Lmx1a*, *FEV* and *p53* shRNA (AFLaVp) (b), AFLbVp plus *NeuroD1* (c), AFLbVp plus *NeuroD2* (d), AFLbVp plus *miR124* (e), AFLbVp plus *NKX2.2* (f), AFLbVp plus *Nurr1* (g) or AFLbVp plus *Pitx3* (h). (i–l) Quantification of the percentage of 5HT⁺ neurons or Tuj1⁺ neurons among all cells (DAPI⁺) (i) or the average number of 5HT⁺ (j), Tuj1⁺ (k) or DAPI⁺ (l) cells per view. *[#]*P* < 0.05, vs 5HT⁺ or Tuj1⁺ for AFLbVp, respectively, cells from at least five random views in each of the three wells from three independent experiments were counted. Bars = 50 μm.

Optimization of culture conditions for the generation of i5HT neurons

We tested a variety of small-molecule compounds and neurotrophic factors by adding each of them to the basal media, which was DMEM/F12 plus B27 and N2 (Figure 3a). The addition of the Rock inhibitor Y27632 (10 μM)²² (Figure 3b), the SMAD inhibitor dorsomorphin (DM, 0.5 μM)²³ (Figure 3c) or SB431542 (SB, 5 μM)²⁴ (Figure 3d) or the CDK4/6 inhibitor PD0332991 (PD, 1 μM)²⁵ (Figure 3g) improved both the conversion efficiency and morphology, whereas BDNF (20 ng ml⁻¹) (Figure 3e) and GDNF (20 ng ml⁻¹) (Figure 3f) slightly improved the morphology but not the conversion efficiency (Figure 3j for conversion efficiency and Supplementary Figures S1b–d for conversion yield). Strikingly, induction media with all the six agents (Figure 3h) increased the conversion efficiency from 23.6 ± 2.6 to 49.3 ± 2.1% for Tuj1⁺ neurons and from 5.7 ± 0.5 to 24.7 ± 1.1% for 5HT⁺ neurons (*P* < 0.05) (Figure 3j). We substantiated the results by removing each of the six agents from induction media and found that taking away any one of them indeed reduced the conversion efficiency (Supplementary Figure S1e) and yield (Supplementary Figures S1f–h). Representative images for these conditions are shown in Supplementary Figures S2a–h. Furthermore, we tested many other agents, including the selective GSK3β inhibitor CHIR99021,²⁶ the Smoothed agonist Purmorphamine,²⁷ the antioxidant vitamin C

or NAC, cAMP, bFGF, NGF or TGFβ3.¹⁵ None of them had any favorable effect (Supplementary Figures S1i–l, Supplementary Figures S2i–p). Replacing DMEM in IM with neurobasal markedly reduced the efficiency and yield in the generation of 5HT⁺ neurons (Supplementary Figures S1i–l, Supplementary Figure S2q). A previous study has shown that hypoxia (5% O₂) improves the direct conversion of human fibroblasts to induced neurons.²⁸ We found that the conversion of MRC5 cells to i5HT neurons was significantly more efficient at 5% O₂ (Figure 3h) than at 21% O₂ (Figure 3i) in induction media (Figure 3k and Supplementary Figures S1m–p). The selective CDK4/6 inhibitor PD-0332991 significantly reduced the number of DAPI⁺ cells (Supplementary Figure S1d) without affecting the number of 5HT⁺ (Supplementary Figure S1b) or Tuj1⁺ neurons (Supplementary Figure S1c), suggesting that it blocks the proliferation of unconverted mitotic cells. We tested other mitotic inhibitors such as the CDK2 inhibitor SU9516, or the DNA synthesis inhibitor AraA or AraC. Replacing PD in induction medium with any of these mitotic inhibitors significantly reduced conversion efficiency (Supplementary Figure S1q) and yield (Supplementary Figures S1r–t and Supplementary Figures S2r–t). After 30 days of culture in induction medium, there was no significant reduction in the number of 5HT⁺ cells (Supplementary Figure S3b), but the number of Tuj1⁺ cells (Supplementary Figure S3c) and the total number of cells (DAPI⁺, Supplementary Figure

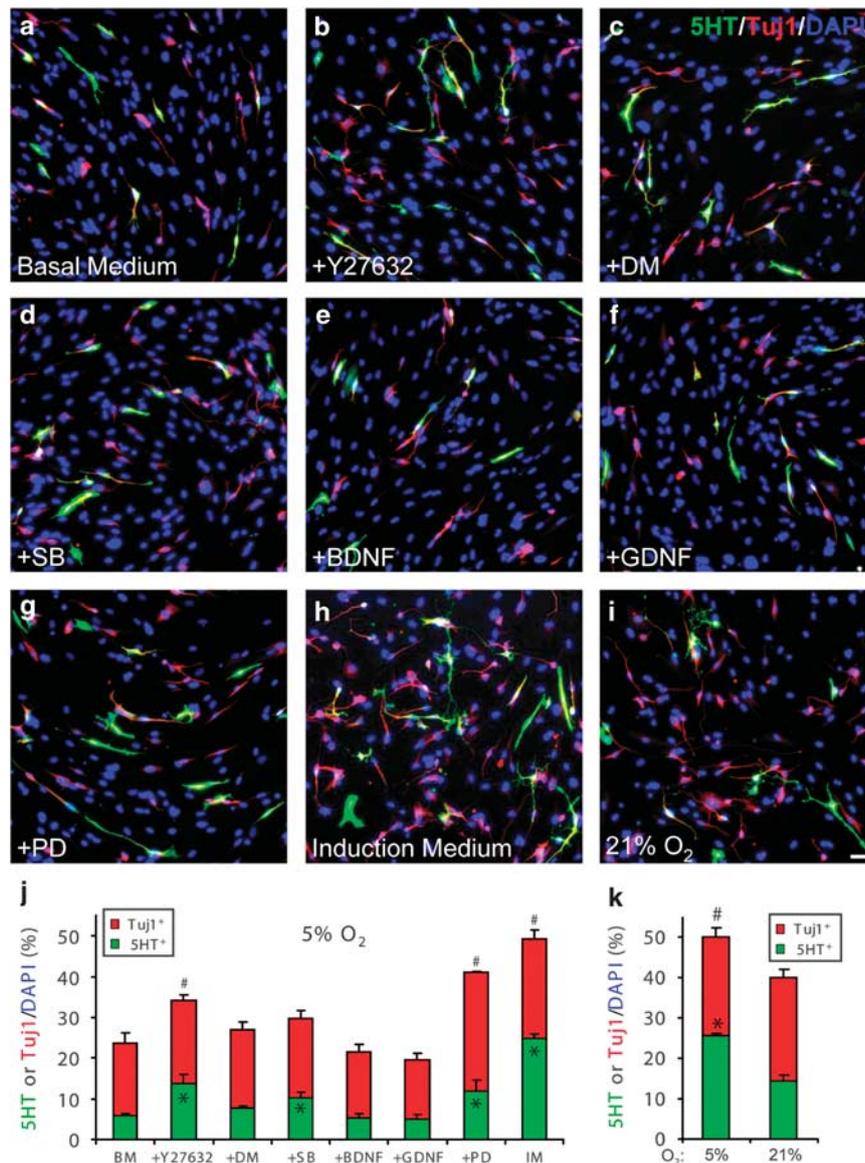


Figure 3. Optimal culture conditions for the conversion of human fibroblasts to induced serotonergic neurons. (a–h) Representative images and quantification (j) of 5HT⁺ or Tuj1⁺ neurons at day 12 in basal medium (BM, DMEM/F12 plus B27 and N2) (a) plus ROCK inhibitor Y27632 (b), dorsomorphin (DM) (c), SB431542 (SB) (d), brain-derived neurotrophic factor (BDNF) (e), glial cell line-derived neurotrophic factor (GDNF) (f), PD0332991 (PD) (g) or induction media (IM) with all of the above additives (h). (i and k) Representative image (i) and quantification (k) of i5HT neurons converted in 5% or 21% O₂. *[#]*P* < 0.05, vs 5HT⁺ or Tuj1⁺ for the first bar, cells from at least five random views in each of the three wells from three independent experiments were counted. Bars = 50 μm.

S3d) decreased significantly, thereby increasing conversion efficiency (Supplementary Figures S3a, e–h).

i5HT neurons expressed appropriate markers for serotonergic neurons

We co-stained i5HT neurons with antibodies against serotonin and various serotonergic markers and neuronal markers. As these markers are expressed only in mature serotonergic neurons, we performed the staining when the i5HT neurons were cultured *in vitro* for 28 days or longer. As shown in Figure 4a, the i5HT neurons coexpressed serotonin and tryptophan hydroxylase 2 (TPH2, the rate-limiting enzyme for the synthesis of 5HT in the brain). They also coexpressed 5HT with L-aromatic amino-acid decarboxylase (AADC, the enzyme for the second step of 5HT

synthesis) (Figure 4b), vesicular monoamine transporter 2 (VMAT2, for the sequestration of 5HT in synaptic vesicles) (Figure 4c), serotonin transporter (SERT, for the reuptake of released serotonin) (Figure 4d), aldehyde dehydrogenase 1a1 (ALDH1A1, for the degradation of 5HT) (Figure 4e), as well as markers for mature neurons such as MAP2 (Figure 4f), NeuN (Figure 4g) and syntaxin 1 (Figure 4h). Separate channels of merged images are shown in Supplementary Figure S4. Real-time quantitative RT-PCR assays showed that endogenous AFLV were strongly induced at day 6 and day 25 (Figures 4i and j). Genes responsible for the synthesis (TPH1, TPH2 and AADC), vesicular sequestration (VMAT2), reuptake (SERT) and degradation (ALDH1A1, MAO-A and MAO-B) of serotonin, as well as serotonin autoreceptors (HTR1a and HTR1b) were all significantly induced (Figures 4i and j). The expression levels of neuronal genes such as MAP2, TUBB3

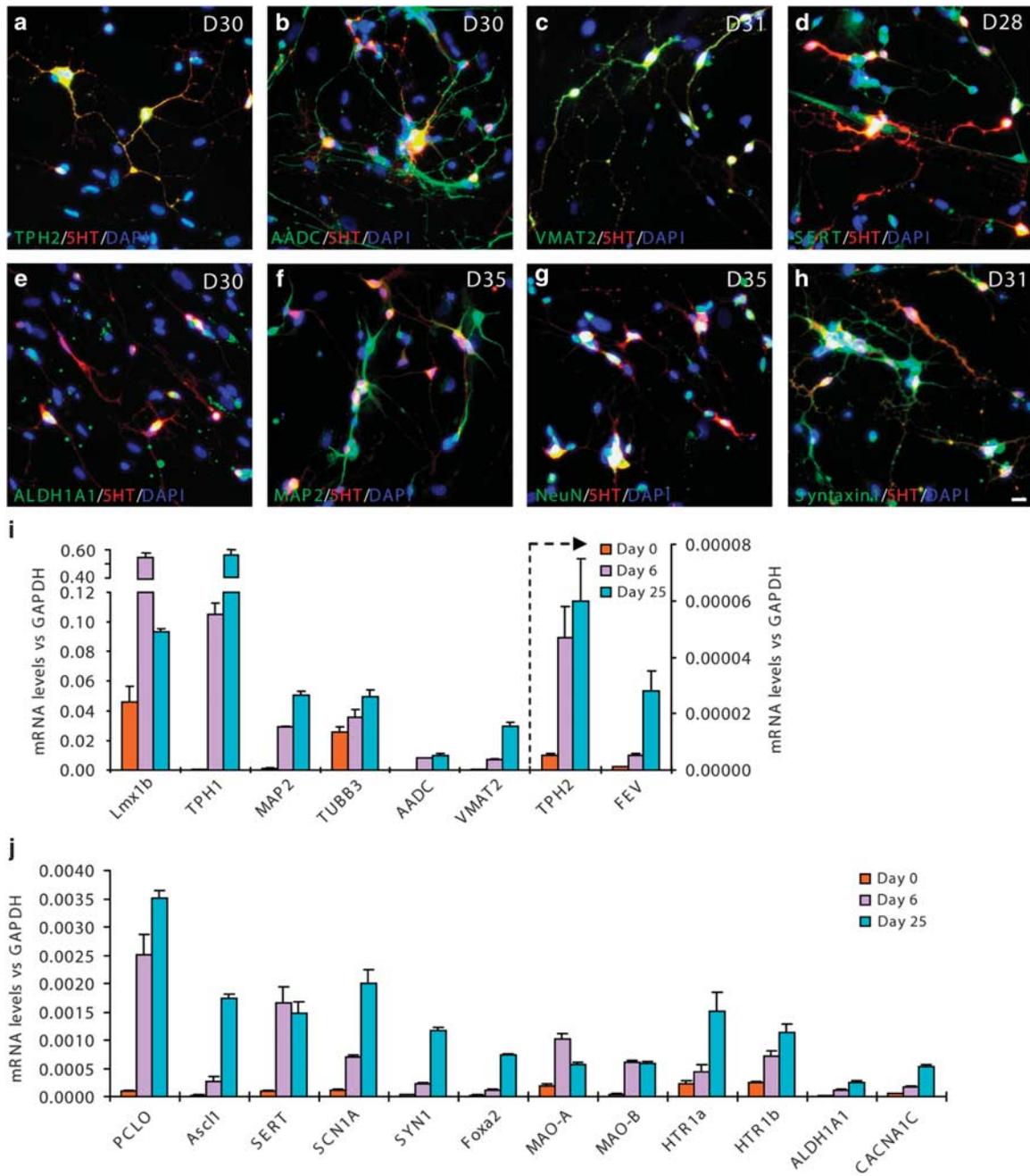


Figure 4. The iSHT neurons express appropriate markers for serotonergic neurons. (a–h) Costaining of iSHT neurons with antibodies against 5HT and serotonergic markers including AADC (a), ALDH1A1 (b), VMAT2 (c), TPH2 (d), SERT (e), or markers for mature neurons MAP2 (f) and NeuN (g), or synaptic markers syntaxin-1 (h). Bars, 20 μ m. (i and j) Induction of endogenous genes at different time points after the addition of DOX. Arrow, values were according to the Y axis on the right. $N=4$ from three independent experiments.

(β 3 tubulin), SYN1 (synapsin 1), PCLO (Piccolo Presynaptic Cytomatrix Protein), Cacna1c (CaV1.2 voltage-dependent calcium channel) and Scn1a (NaV1.1 voltage-dependent sodium channel) were significantly increased (Figures 4i and j). These results indicate that the transcription program of the cell was converted to that of serotonergic neurons, consistent with the morphological changes from fibroblasts to neurons (Figures 1b–e). All five transgenes (AFLVp) were greatly silenced in iSHT neurons (Supplementary Figure S5). This is consistent with the results that DOX was only needed during the first week of transdifferentiation (Figure 1a).

To substantiate our findings, we used the same method to generate iSHT neurons from another line of primary human fetal fibroblasts IMR90 (Supplementary Figures S6a–d) and the primary adult human fibroblasts CCD-19Lu (Supplementary Figures S6e–h). The conversion efficiency and yield of 5HT⁺ and Tuj1⁺ neurons were very similar between the fetal fibroblasts MRC5 and IMR90 (Supplementary Figures S6i–l). The conversion efficiency (Supplementary Figure S6i) and yield (Supplementary Figure S6j) of 5HT⁺ neurons from the adult fibroblast CCD-19Lu were much lower, although the generation of Tuj1⁺ cells was comparable to the situation in fetal fibroblasts (Supplementary Figures S6i and k).

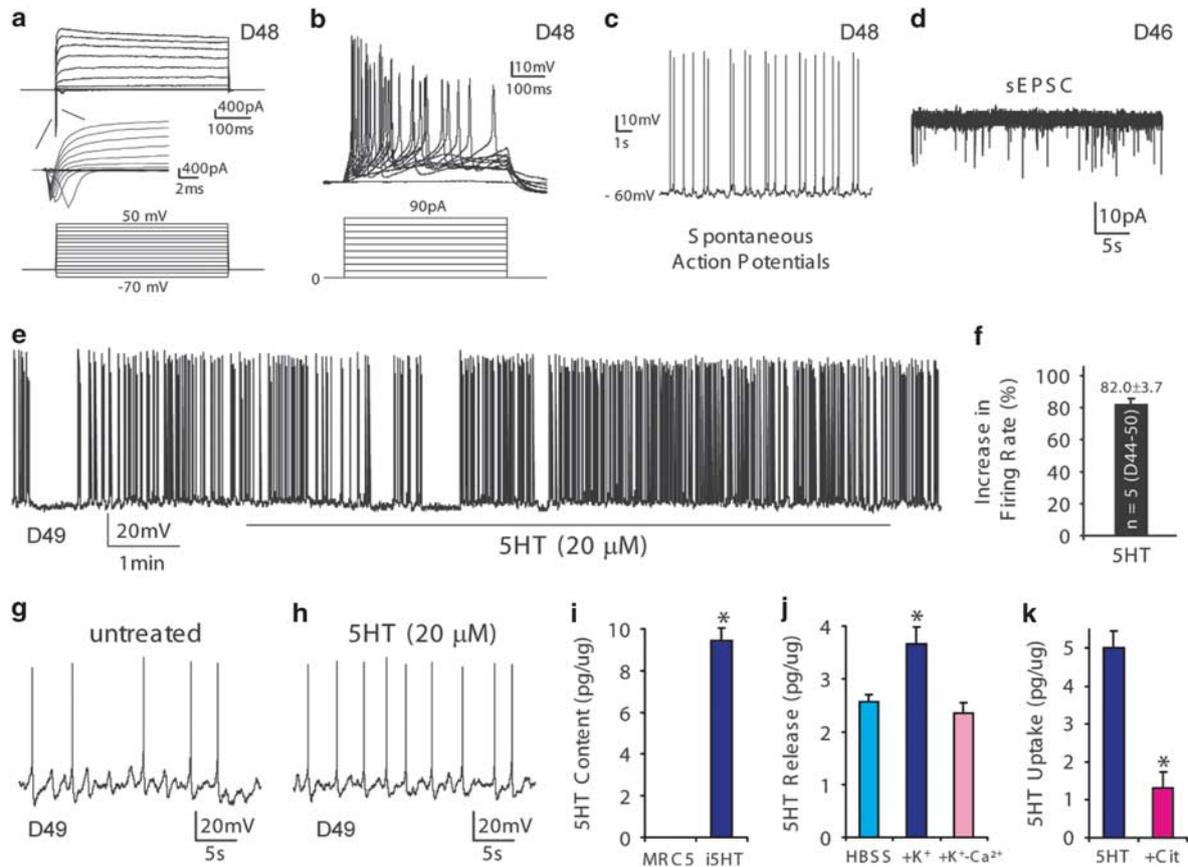


Figure 5. The i5HT neurons were functional. (a–d) Electrophysiological recordings showed that the i5HT neurons had voltage-gated Na⁺ and K⁺ currents (a), evoked action potentials in response to current injections (b), spontaneous action potentials (c) and spontaneous excitatory postsynaptic currents (d). (e–h) The frequency of spontaneous action potentials increased in response to serotonin (20 μM) treatment, as shown in a representative recording (e), the statistical summary (f), and time-expanded segments of untreated (g) and treated (h) conditions. (i) Serotonin content in the i5HT neurons and the parental MRC5 fibroblasts. **P* < 0.05, *n* = 3 independent experiments. (j) Serotonin released from i5HT neurons in Hank's balanced salt solution (HBSS), HBSS plus KCl (56 mM), or Ca²⁺-free HBSS with KCl (56 mM) **P* < 0.05, vs the other bars, *n* = 3 independent experiments. (k) Serotonin uptake in the absent or present of the selective SERT inhibitor citalopram (10 μM). **P* < 0.05, *n* = 3 independent experiments.

This is consistent with many previous studies showing the difficulty in reprogramming adult cells than the fetal cells.^{29–31}

i5HT neurons have active synaptic transmission

To test whether the i5HT neurons are functional, we performed electrophysiological recordings on neurons maintained for at least 46 days. Recordings at earlier time showed immature electrophysiological output (Supplementary Figure S7 and Supplementary Table 1). The i5HT neurons exhibited voltage-dependent Na⁺ and K⁺ currents (Figure 5a), evoked action potentials (Figure 5b), spontaneous action potentials (Figure 5c) and spontaneous excitatory postsynaptic currents (Figure 5d). More strikingly, the firing frequency of spontaneous action potentials was significantly increased by the application of 5HT (20 μM) (Figures 5e–h), suggesting that these neurons have serotonin receptors that act to enhance neuronal excitability. Using HPLC coupled with electrochemical detection,¹⁵ we found that serotonin can be robustly detected in the i5HT neurons, but not the parental MRC5 fibroblasts (Figure 5i). Spontaneous 5HT release was measured by incubating the i5HT neurons in HBSS for 30 min (Figure 5j). Serotonin release was greatly increased in response to membrane depolarization induced by high concentration of KCl (56 mM). This increase was completely blocked in Ca²⁺-free HBSS (Figure 5j). The i5HT neurons exhibited strong 5HT

uptake, which was significantly blocked by the selective inhibitor of serotonin transporter, citalopram (10 μM) (Figure 5k). Together, these results showed that the i5HT neurons have active serotonergic synaptic transmission.

DISCUSSION

Through a targeted screen based on existing knowledge on the development of serotonergic neurons in the mouse brain, we identified a combination of four transcription factors (AFLV) that converted primary human fibroblasts to induced serotonergic (i5HT) neurons in 12 days (Figure 1). The conversion efficiency and yield were significantly increased by p53 knockdown (Figure 1). Previous studies have demonstrated that the reprogramming of fibroblasts to iPSCs is significantly enhanced by attenuation of the p53 pathway.^{32–37} It is still unclear how p53 knockdown facilitate cellular reprogramming to pluripotency and to i5HT neurons in this case. Further studies are needed to understand the impact of p53 on reprogramming in general.

Consistent with previous studies that have shown the critical roles of Ascl1 in the transdifferentiation of fibroblasts to induced neurons,⁴ no neuron (including i5HT neuron) was generated in the absence of Ascl1 (Figure 1). When FoxA2 was not included, there was a dramatic reduction in the number of 5HT⁺ neurons, whereas the number of Tuj1⁺ neurons was not significantly affected. This

corroborates with the pivotal role of FoxA2 in specifying precursors of serotonergic neurons in the ventral hindbrain; deletion of FoxA2 in mice reduces hindbrain serotonergic neurons by 50%.⁸ Removing Lmx1b from the reprogramming factors completely abrogated the production of i5HT neurons, without significantly affecting the production of 5HT⁻/Tuj1⁺ neurons, which accounted for approximately half of the neurons (Figure 1). This is consistent with the important role of Lmx1b in the terminal differentiation of serotonergic neurons during development, as genetic ablation of Lmx1b in mice renders precursors of serotonergic neurons unable to turn on the battery of genes that specify serotonergic neurons.^{9,10} Although Lmx1b and Pet1 (which is FEV in human) are both controlled by FoxA2 in the development of mouse serotonergic neurons,⁸ the omission of FEV in the reprogramming factors had a quite different effect compared with the removal of Lmx1b (Figure 1). There was only a partial loss of 5HT⁺ neurons in the absence of FEV, but the number of Tuj1⁺ neurons and the number of DAPI⁺ cells increased significantly (Figure 1). It suggests that FEV is toxic, but facilitates the transdifferentiation of fibroblasts to i5HT neurons.

In screening for medium additives that can enhance the transdifferentiation, we found that the combination of ROCK inhibitor, double SMAD inhibitors dorsomorphin and SB431542, neurotrophic factors BDNF and GDNF, as well as the mitotic inhibitor PD0332991 markedly increased the efficiency (Figure 3j) and yield (Supplementary Figure S1b) of 5HT⁺ neurons. The total number of Tuj1⁺ neurons was not significantly increased, but the significant decrease of DAPI⁺ cells (by PD0332991) (Supplementary Figure S1d) markedly increased the apparent conversion efficiency for Tuj1⁺ neurons (Figure 3j). Other mitotic inhibitors, such as SU9516, AraA or AraC, had significant toxicity on i5HT neurons (Supplementary Figures S1q–t). Previous studies have shown that SB431542³⁸ and dorsomorphin³⁹ enhance transdifferentiation of human fibroblasts to induced neurons. Although each of the media additives increased the efficiency in converting fibroblasts to i5HT neurons, the combined addition of all of them in induction media achieved a much bigger synergistic effect on reprogramming efficiency (Supplementary Figure S1a) and yield (Supplementary Figures S1b and c).

The i5HT neurons expressed appropriate markers for serotonergic neurons (Figure 4), exhibited active serotonergic synaptic transmission (Figures 5e and f), and showed Ca²⁺-dependent release of serotonin (Figure 5j) and selective uptake of serotonin (Figure 5k). The results show that the i5HT neurons possess characteristics very similar to serotonergic neurons *in vivo*. Thus, the method would be very useful for the generation of patient-specific serotonergic neurons for a variety of studies on many serotonin-related mental illnesses.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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