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## Signaling Adaptor Protein SH2B1 Enhances Neurite Outgrowth and Accelerates the Maturation of Human Induced Neurons

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**Key Words.** Reprogramming • Neural induction • Neuron • Neural differentiation • SH2B1

### ABSTRACT

Recent advances in somatic cell reprogramming have highlighted the plasticity of the somatic epigenome, particularly through demonstrations of direct lineage reprogramming of adult mouse and human fibroblasts to induced pluripotent stem cells (iPSCs) and induced neurons (iNs) under defined conditions. However, human cells appear to be less plastic and have a higher epigenetic hurdle for reprogramming to both iPSCs and iNs. Here, we show that SH2B adaptor protein 1 $\beta$  (SH2B1) can enhance neurite outgrowth of iNs reprogrammed from human fibroblasts as early as day 14, when combined with miR124 and transcription factors BRN2 and MYT1L (IBM) under defined conditions. These SH2B1-enhanced iNs (S-IBM) showed canonical neuronal morphology, and expressed multiple neuronal markers, such as TuJ1, NeuN, and synapsin, and functional proteins for neurotransmitter release, such as GABA, vGluT2, and tyrosine hydroxylase. Importantly, SH2B1 accelerated mature process of functional neurons and exhibited action potentials as early as day 14; without SH2B1, the IBM iNs do not exhibit action potentials until day 21. Our data demonstrate that SH2B1 can enhance neurite outgrowth and accelerate the maturation of human iNs under defined conditions. This approach will facilitate the application of iNs in regenerative medicine and in vitro disease modeling. STEM CELLS TRANSLATIONAL MEDICINE 2014;3:1–10

### INTRODUCTION

Many human disorders are caused by functional deficiencies in specific cell lineages. Hence, a major goal of regenerative medicine is to replace the lost structures and/or functions that are the consequences of such defects. To this end, great efforts have been devoted to the development of patient-specific tissue engineering and cell therapy-based treatments, as well as cellular models of diseases, both of which have benefited significantly from recent advances in cell reprogramming. Induction of the pluripotent stem cells (iPSCs) by cell reprogramming has been a great leap forward in regenerative medicine research [1]. A number of disease- and patient-specific iPSC lines have been established, including those from patients with amyotrophic lateral sclerosis [2], schizophrenia [3], and Alzheimer's disease [4]. Furthermore, correction of genetic mutations in disease-specific iPSCs can correct phenotypes of sickle cell anemia in mouse models [5]. However, for successful therapeutic application of human diseases, iPSCs need to differentiate into the desired cell type efficiently. Thus, direct reprogramming of somatic cells into lineage-specific cells could sidestep the difficulty of differentiating iPSCs and eliminate the potential hazard of

teratoma formation. Moreover, direct reprogramming, wherein cells are converted directly to the cell lineage of interest without passing through a pluripotent stem cell state, has the potential for (a) delivery of specific cell-based therapies to patients, (b) provision of human experimental models, and (c) high throughput pharmaceutical development. However, to realize the full potential of directed nuclear reprogramming, precise delivery of complex combinations of the optimal reprogramming factors and/or microRNAs would be needed. Somatic cell nuclear transfer and in vitro induction of pluripotency in somatic cells by defined factors reprogram differentiated somatic cells to embryonic stem cell (ESC)-like cells [6–9]. Furthermore, direct conversion of fibroblasts into induced neurons (iNs) indicates that direct lineage conversions are possible between distantly related cell types [10, 11]. Importantly, iNs can be derived from fibroblasts [12–14] and hepatocytes [15]. The reprogramming process both induces neuronal properties and extinguishes prior donor cell identity, and therefore represents a complete and functional lineage switch. In 2010, Vierbuchen et al. converted mouse fibroblasts into neuronal cells by lentiviral infection of the pool of

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Brn2, Ascl1, and Myt1l (BAM) to induce mouse iNs that exhibited both molecular and all principal functional properties of neurons [16]. However, when the BAM factors were introduced into human fibroblasts, the resulting cells remained immature and failed to generate action potentials when depolarized [9]. By further introducing the bHLH factor NeuroD1 to BAM, the generation of functional neuronal cells from human fibroblasts can be achieved [9]. These human iNs expressed a variety of neuronal markers including TuJ1, MAP2, NeuN, neurofilament, and synapsin, and exhibited functional neuronal properties as judged by the measurement of action potentials. However, these human BAM/NeuroD1 iNs formed functional synapses only when cocultured with mature mouse cortical neurons, which potentially provided additional differentiation factors. The first functional synapses were found only after 5 to 6 weeks, suggesting that the full maturation of human iNs is a slow process. Notably, Ambasudhan et al. showed that a combination of miR124, MYT1L, and BRN2 (IBM) is sufficient to directly reprogram postnatal and adult human primary dermal fibroblasts (mesoderm) to functional neurons (ectoderm) under precisely defined conditions without a requirement for helper cells [12]. These IBM iNs exhibited typical neuronal morphology and marker gene expression, fired action potentials, and formed functional synapses [12]. However, formation of functional synapses of IBM iNs still required 4 weeks of incubation. Current technologies for gene delivery into cells are highly stochastic in nature. In the case of induced neurons, their inherently low proliferative potential obviates the need to generate high numbers of reprogrammed neurons from the outset. This problem is further exacerbated because of the low efficiency of reprogramming from more easily accessible human specimens, such as peripheral blood or urine. Given the known critical roles of specific transcription factors, signaling molecules, and microRNAs in neuronal lineage determination during development or cell fate regulation, developing a means to boost the inducibility of nuclear reprogramming and accelerating the maturation process of human iNs are highly desirable.

Reprogramming of human blood specimens into iPSCs has been reported [17]. It has also been shown that human cord blood CD133(+) cells can be reprogrammed to neuronal-like cells by ectopic expression of Sox2 and c-Myc [18]. However, there is still no report for direct reprogramming of terminally differentiated blood specimens (such as T or B cells) into mature neurons from either mouse or human specimens. We hypothesized that direct enhancement of related signaling pathways involved in the reprogramming processes will increase the efficiency and maturation of human iNs. We have previously shown that the signaling adaptor protein SH2B1 could enhance the neurite outgrowth of PC12 pheochromocytoma cells induced by FGF1 [19]. We further showed that this inducibility is through the signaling pathway of FGFR1-Mek-Erk1/2-Stat3-Egr1 [19]. SH2B1, an adapter/scaffold protein, belongs to a family of adapter proteins including APS (SH2B2) and Lnk (SH2B3) [20, 21]. SH2B1 contains three proline-rich domains, a pleckstrin homology domain, and a C-terminal Src homology (SH2) domain. SH2B1 has been shown to bind to the activated NGF receptor TrkA through its SH2 domain and to promote NGF-induced neurite outgrowth [22–24]. NGF promotes the rapid association of SH2B1 with TrkA and subsequent phosphorylation of SH2B1 on tyrosines as well as serines/threonines. Overexpression of SH2B1 enhances NGF-induced neurite outgrowth in PC12 cells [24, 25]. In addition to NGF signaling, SH2B1 has recently been shown to shuttle between the cytoplasm and the nucleus,

attesting to its novel function in transcriptional regulation [26]. Along this line, a recent study has revealed new functions of SH2B1 in regulating the expression of a subset of NGF-responsive genes as well as the three-dimensional neurite outgrowth [27]. In addition to regulating NGF, SH2B1 has been implicated in promoting FGF1 [19] and glia-derived growth factor (GDNF)-induced neurite outgrowth [28]. Therefore, we combined SH2B1 with miR124, BRN2, and MYT1L (S-IBM) to convert human fibroblasts to functional neurons. Our approach will facilitate the application of iNs in regenerative medicine and in vitro disease modeling.

## MATERIALS AND METHODS

### Production of Viruses

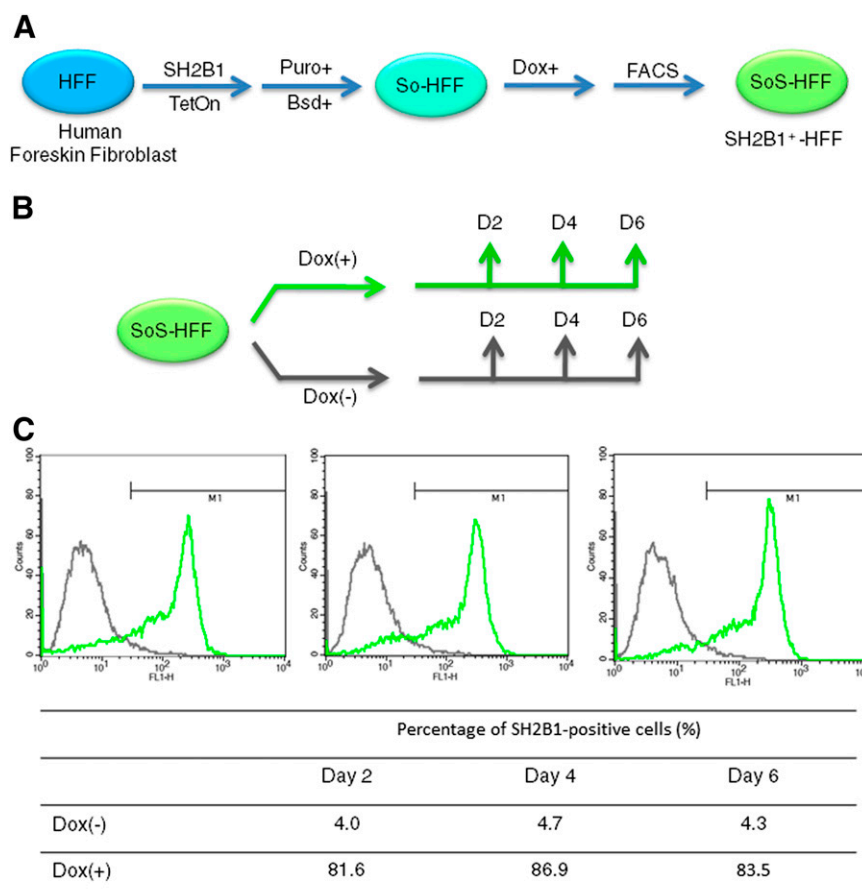
Lentiviral vectors pdlBrn2PkIP and pdlMyt1LPk were provided by Chia-Hsiang Chen (National Health Research Institutes, Miaoli, Taiwan). Doxycycline inducible plasmid pAS4w.1.SH2B1 was constructed using the SH2B1 cDNA provided by Dr. Linyi Chen (National Tsing Hua University, Hsinchu, Taiwan). pLemir-miR-124 vector encoding miR124 sequence was purchased from System Biosciences (Mountain View, CA, <http://www.systembio.com>) and the doxycycline (Dox)-inducible expression system (TetOn, pAS4.1w.Pbsd-aOn) was purchased from National RNAi Core Facility, Academia Sinica (Taipei, Taiwan, <http://mai.genmed.sinica.edu.tw>). Vectors were transfected into 293T cells and viruses were harvested 48 hours and 72 hours after transfection. Viral supernatants were concentrated using an ultracentrifuge for 2 hours at 19,700 rpm. Viral titers were determined using the Global UltraRapid Lentiviral Titer Kit (System Biosciences).

### Cell Culture and Human iN Induction

Human primary foreskin fibroblasts (HFFs; passage 2) were purchased from Animal Technology Institute Taiwan (Miaoli, Taiwan, <http://www.atit.org.tw>) under the government auspices of the Ministry of Economic Affairs Technology Development Program (87-EC-2-A-17-283). The authenticity of their human origin has been certified by the Center for Genomic Medicine, National Cheng Kung University (Tainan, Taiwan). The human fibroblast cells were maintained in fibroblast media (Dulbecco's modified Eagle's medium; Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>) containing 10% fetal bovine serum (HyClone, Logan, UT, <http://www.hyclone.com>), nonessential amino acids, sodium pyruvate, GlutaMAX, 5 mM HEPES, and penicillin/streptomycin (all from Invitrogen). The day before lentiviral infection, human fibroblasts were seeded onto 24-well tissue culture dishes (Corning Enterprises, Corning, NY, <http://www.corning.com>). Next day, cells were infected with filtered viral supernatants in the presence of polybrene (8  $\mu$ g/ml) for 24 hours. Detailed methods for the generation of human iNs were as described previously [12].

### Immunostaining and Antibodies

Primary antibodies used in this study are as follows: mouse anti-TuJ1 (1:1000; Covance, Princeton, NJ, <http://www.covance.com>), mouse anti-MAP2 (1:1000; Millipore, Billerica, MA, <http://www.millipore.com>), mouse anti-NeuN (1:1000; Millipore), rabbit anti-Synapsin 1 (1:2000; Millipore), rabbit anti-GABA (1:1000; Sigma-Aldrich, St. Louis, MO, <http://www.sigmaaldrich.com>), guinea pig anti-vesicular glutamate transporter 1 (vGluT1, 1:2000; Synaptic Systems, Göttingen, Germany, <http://www.sysy.com>), mouse



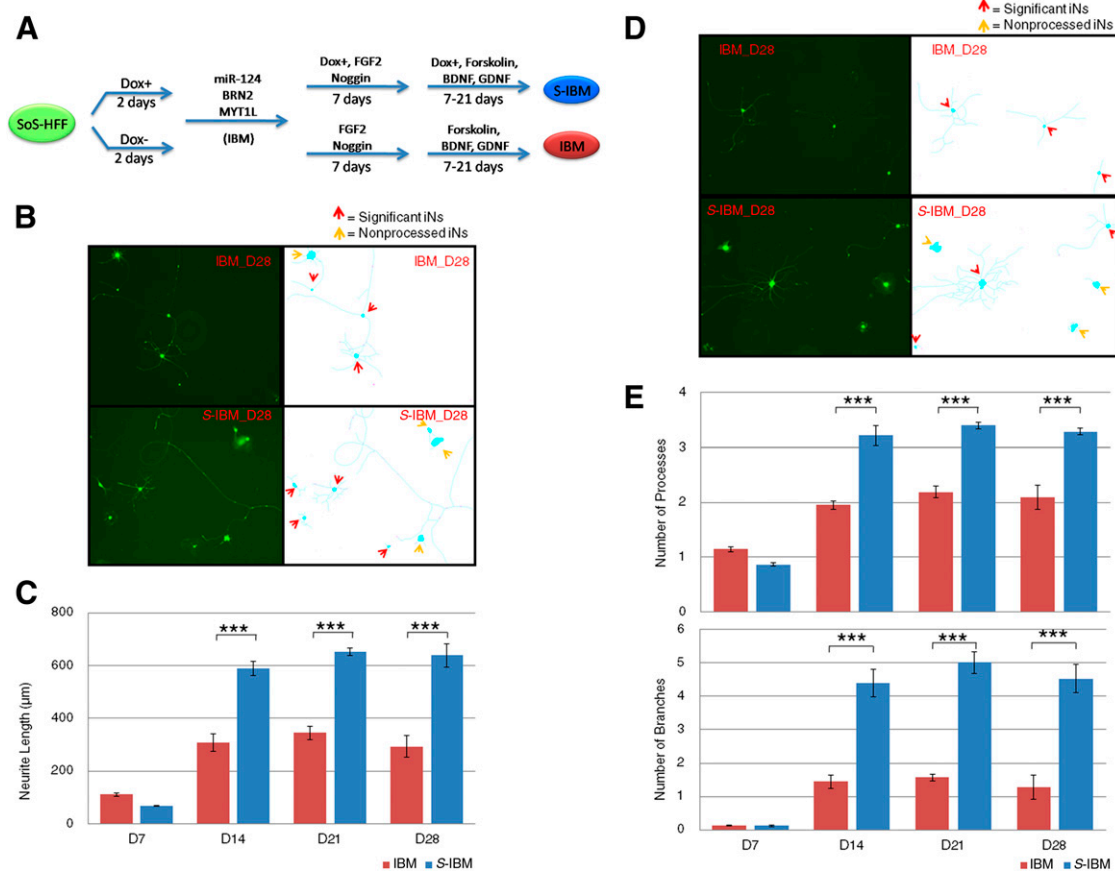
**Figure 1.** Generation of SH2B1-positive HFFs whose SH2B1 expression can be induced by doxycycline. **(A):** Schematic drawing showing the generation of SH2B1-positive HFFs by FACS. So-HFF indicates HFFs that were transfected with doxycycline-inducible SH2B1 plasmid and TetOn plasmid. These two plasmids were selected by puromycin and blasticidin, respectively. SoS-HFFs indicates So-HFFs that were cultured in the presence of doxycycline for 2 days and collected for SH2B1GFP-positive cells using FACS. **(B):** Schematic drawing showed the protocol for the induction of SH2B1-GFP expression. SoS-HFFs were cultured either with or without doxycycline and collected for FACS analyses on days 2, 4, and 6. **(C):** A representative figure and summary table showed the percentage of SH2B1-positive cells. The GFP(+) cells in the Dox(-) culture ranged from 4.0%–4.7%, and increased to 81.6%–86.9% in the Dox(+) culture during day 2 to day 6 following the stimulation by doxycycline. Abbreviations: Bsd, blasticidin; Dox, doxycycline; D2–D6, days 2–6; FACS, fluorescence activated cell sorting; HFF, human foreskin fibroblast; Puro, puromycin; SH2B1, SH2B adaptor protein 1 $\beta$ ; So-HFF, human foreskin fibroblasts transfected with doxycycline-inducible SH2B1 plasmid and TetOn plasmid.

anti-tyrosine hydroxylase (TH, 1:1000; Abcam, Cambridge, U.K., <http://www.abcam.com>), rabbit anti-vesicular glutamate transporter 2 (vGluT2, 1:1000; Abcam), rabbit anti-GFAP (1:800; Millipore). Alexa Fluor 488- and Alexa Fluor 594-conjugated secondary antibodies were purchased from Invitrogen. iNs were fixed in 4% paraformaldehyde for 30 minutes, permeabilized with 0.1% Triton X-100 for 15 minutes, and blocked in 1% bovine serum albumin in phosphate-buffered saline for 1 hour. Cells were incubated with primary antibody overnight at 4°C, washed, and incubated with Alexa Fluor (Invitrogen) secondary antibody for 1 hour at room temperature. Cells were counterstained with Hoechst 33258 to identify nuclei.

### Cellular Imaging Using High-Content Imager and Analysis

For neurite analyses, cells were seeded in tissue culture-treated 24-well plates (Corning; 3524) at a density of 1,300 cells per  $\text{cm}^2$  corresponding to approximately 2,500 cells per well of a 24-well plate in 500  $\mu\text{l}$  of medium. For neuronal marker staining, cells were seeded in 24-well plates at density of 5,200 cells per  $\text{cm}^2$

corresponding to approximately 10,000 cells per well of a 24-well plate in 500  $\mu\text{l}$  of medium. At specified time points, fluorescent images were taken using ImageXpress Micro automated microscopy (Molecular Devices, Sunnyvale, CA, <http://moleculardevices.com>) either manually or laser-based, autofocus with a Nikon 4 $\times$  objective (ELWD S Fluor/0.20 NA) and an image acquisition time of 500 milliseconds or as specified. Neurite detection and analysis were performed with MetaXpress (Molecular Devices) using the Neurite Outgrowth analysis module. Neuronal marker stainings were analyzed by the Multi Wavelength Cell Scoring analysis module (Molecular Devices). Cell bodies were specified as pixel blocks of maximum width 40  $\mu\text{m}$ , minimum area 200  $\mu\text{m}^2$ , and pixel intensities 1,000 units above the local background. Neurites were specified as linear objects with maximum width 20  $\mu\text{m}$  and pixel intensities 90 units above the local background of the object being measured. We concluded that our automated neurite detection methods can reliably report the effects on neurite induction and are sufficient to study quantitatively the neurite outgrowth. Cell morphological features from each image measured by MetaXpress software include (a) percentage of cells with significant neurite



**Figure 2.** SH2B1 enhances the length of neurite outgrowth and the numbers of processes and branches of human iNs induced by IBM. **(A):** Schematic showing the experimental protocol. **(B):** A representative figure showing that after 28 days of infection, SH2B1, miR124, BRN2, and MYT1L (S-IBM)-infected human fibroblasts were more efficiently converted to iNs than IBM iNs. Red arrows indicate the successfully reprogrammed iNs. **(C):** Within 28 days of infection, S-IBM iNs showed twofold more total neurite outgrowth than IBM iNs at days 14, 21, and 28.  $n = 3$ ;  $***, p < .001$ . **(D):** A representative figure showing that after 28 days of infection, S-IBM iNs showed significantly more neuronal processes and branches than IBM iNs. Red arrows indicate the successfully reprogrammed iNs. **(E):** Within 28 days of infection, S-IBM iNs showed significantly more neuronal processes and branches than IBM iNs at days 14, 21, and 28.  $n = 3$ ;  $***, p < .001$ . Abbreviations: BDNF, brain-derived neurotrophic factor; Dox, doxycycline; D7–D28, days 7–28; FACS, fluorescence activated cell sorting; FGF, fibroblast growth factor; GDNF, glia-derived growth factor; HFF, human foreskin fibroblast; IBM, transcription factors BRN2 and MYT1L; iNs, induced neurons; SH2B1, SH2B adaptor protein 1 $\beta$ ; S-IBM, SH2B1-enhanced iNs; SoS-HFF, SH2B1GFP-expressing cells.

growth, (b) number of cells, (c) total neurite outgrowth, (d) mean neurite outgrowth length per cell, (e) normalized mean neurite outgrowth per cell, (f) mean number of processes per cell, (g) mean branches per cell, and (h) mean cell body area.

### Calcium Imaging

Cells were loaded with Fura2-AM (5  $\mu$ M; Invitrogen) in Hanks' balanced salt solution (HBSS; Invitrogen) for 30 minutes in a 37°C incubator. After two washes with HBSS, cells were imaged using an ultrafast external filter wheels (excitation 340/387 nm and emission 520/35 nm). All images were converted to TIFF files and analyzed off-line with Metamorph (Molecular Devices) or Simulator AF6000 (Leica, Heerbrugg, Switzerland, <http://www.leica.com>). Photobleaching was corrected by fitting the prestimulation baseline to a linear curve.

### Electrophysiology

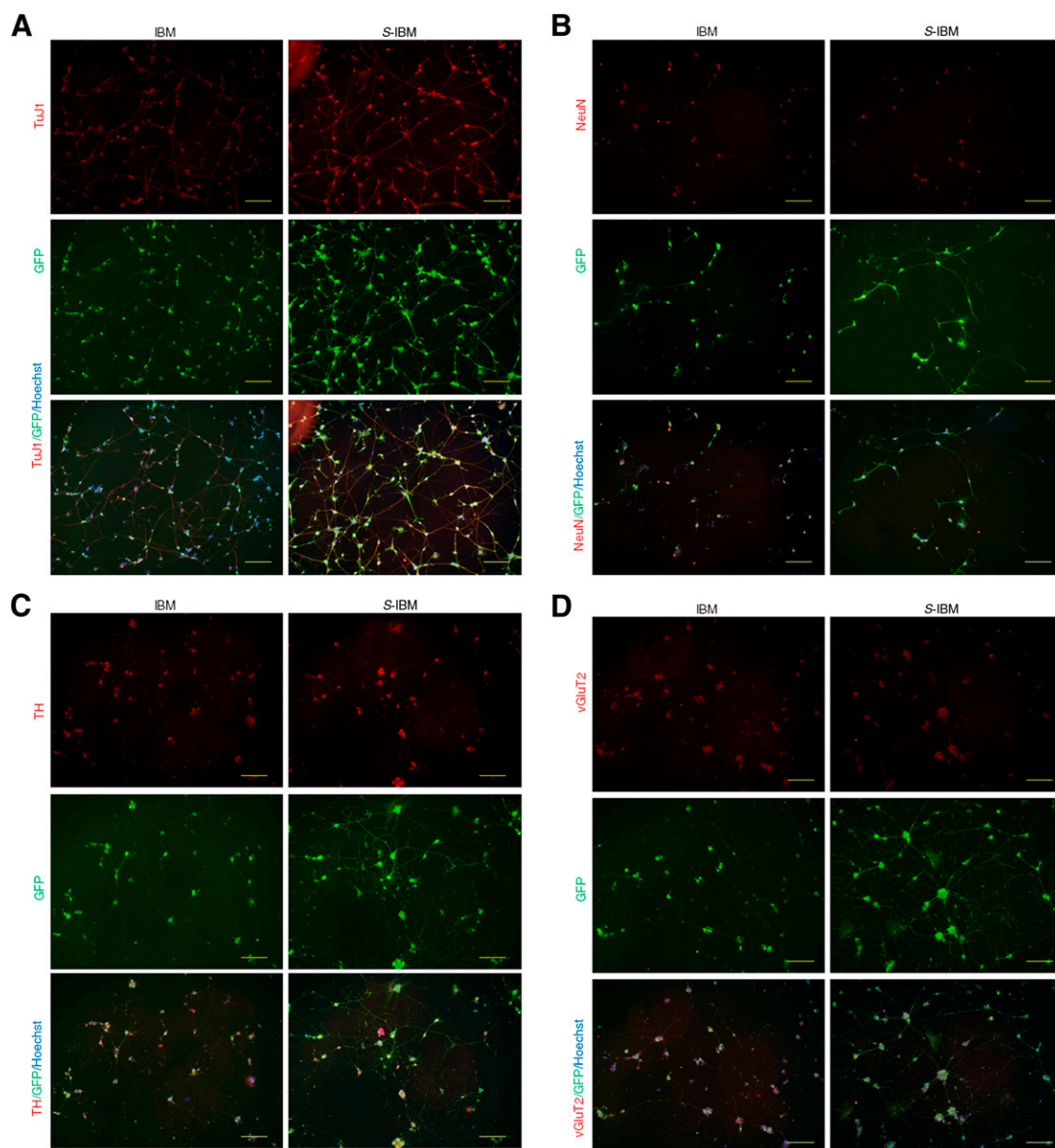
Prior to recording from differentiated cells, green fluorescent protein (GFP)-positive reprogrammed cells were identified and

targeted for whole-cell electrophysiological recordings. Whole-cell patch-clamp recordings were carried out at room temperature within 1 hour with an EPC-10 patch-clamp amplifier and Patchmaster software (HEKA Elektronik, Lambrecht/Pfalz, Germany, <http://heka.com>). Data were digitized at 10 kHz with a 3-kHz low-pass filter and stored on computer. Coverslips containing cells were placed in a recording chamber and incubated with normal Tyrode's solution containing 136.5 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl<sub>2</sub>, 0.53 mM MgCl<sub>2</sub>, 5.5 mM glucose, and 10 mM HEPES (pH 7.4). Patch electrodes were filled with solution containing 140 mM KCl, 1 mM MgCl<sub>2</sub>, 3 mM Na<sub>2</sub>ATP, 0.1 mM EGTA, and HEPES 5.5 mM (pH 7.2). For all electrophysiological recordings, low-resistance recording pipettes (3–5 M $\Omega$ ) were pulled from capillary glass (World Precision Instruments, Sarasota, FL, <http://www.wpiinc.com>). All chemicals (Sigma-Aldrich) were prepared with deionized distilled water.

### Statistical Analyses

Data are expressed as mean  $\pm$  SD. Independent *t* test was used for comparison of two groups. One-way analysis of variance





**Figure 3.** Human iNs infected with IBM or S-IBM exhibited neuronal markers and markers for neurotransmission. **(A):** TuJ1-stained human iNs 28 days after infection of human fibroblasts with IBM (left) and S-IBM (right). GFP-positive cells indicate miR124-infected cells. **(B):** NeuN-stained human iNs 28 days after infection of human fibroblasts with IBM and S-IBM. GFP-positive cells indicate miR124-infected cells. **(C):** TH-stained human iNs 28 days after infection of human fibroblasts with IBM and S-IBM. GFP-positive cells indicate miR124-infected cells. **(D):** S-IBM iNs expressing vGluT2. **(E):** S-IBM iNs expressing GABA. **(F):** S-IBM iNs expressing synapsin. **(G):** Within 28 days of infection, S-IBM iNs showed significantly higher expression levels of TuJ1, NeuN, TH, and GABA activities than IBM iNs at days 14, 21, and 28, and the expression levels of vGluT2 and synapsin remained unchanged. The percentages were the ratios of the numbers of marker-positive cells divided by the numbers of cells remaining in the culture plates following the induction protocol. Data were collected from three independent experiments; \*,  $p < .05$ ; \*\*,  $p < .005$ . Scale bars = 200  $\mu\text{m}$ . Abbreviations: BDNF, brain-derived neurotrophic factor; D, day; Dox, doxycycline; FACS, fluorescence activated cell sorting; GFP, green fluorescent protein; GDNF, glia-derived growth factor; IBM, transcription factors BRN2 and MYT1L; iNs, induced neurons; SH2B1, SH2B adaptor protein 1 $\beta$ ; S-IBM, SH2B1-enhanced iNs; SoS-HFF, SH2B1GFP-expressing cells; TH, tyrosine hydroxylase.

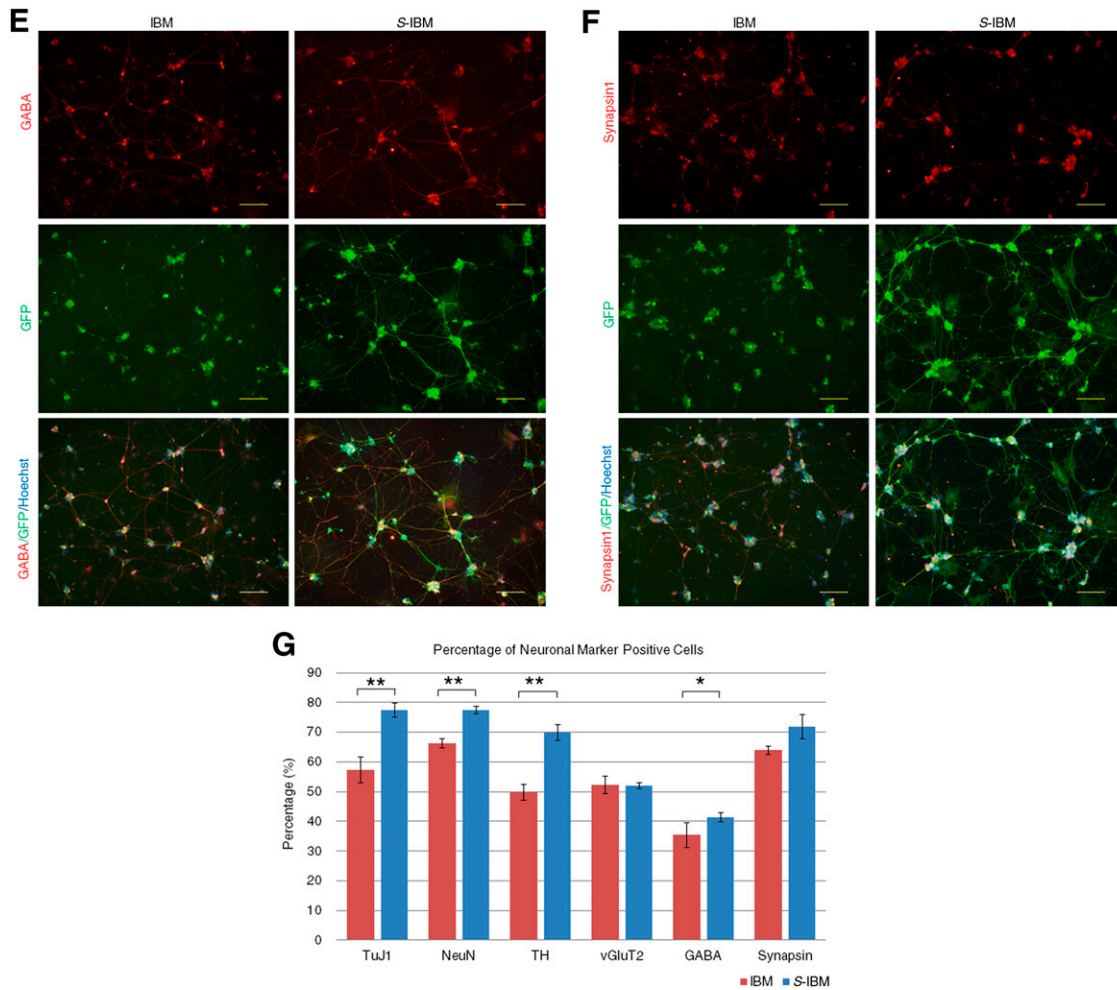
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was used for comparison of multigroups. The data were considered statistically significant at  $p < .05$ .

## RESULTS

To address whether SH2B1 induction could accelerate the maturation of human iNs, we established a lentivirus-mediated gene

delivery system to achieve Dox-inducible SH2B1 expression in human fibroblasts (Fig. 1A). HFFs were infected with SH2B1 and TetOn lentiviruses to establish Dox-inducible stable cell lines after puromycin and blasticidin selection (So-HFFs; Fig. 1A). SH2B1GFP-expressing cells (SoS-HFFs) were then enriched by fluorescent activated cell sorting. Dox-induced SH2B1GFP expression in So-HFF for 2, 4, and 6 days was determined by flow cytometry (Fig. 1B).



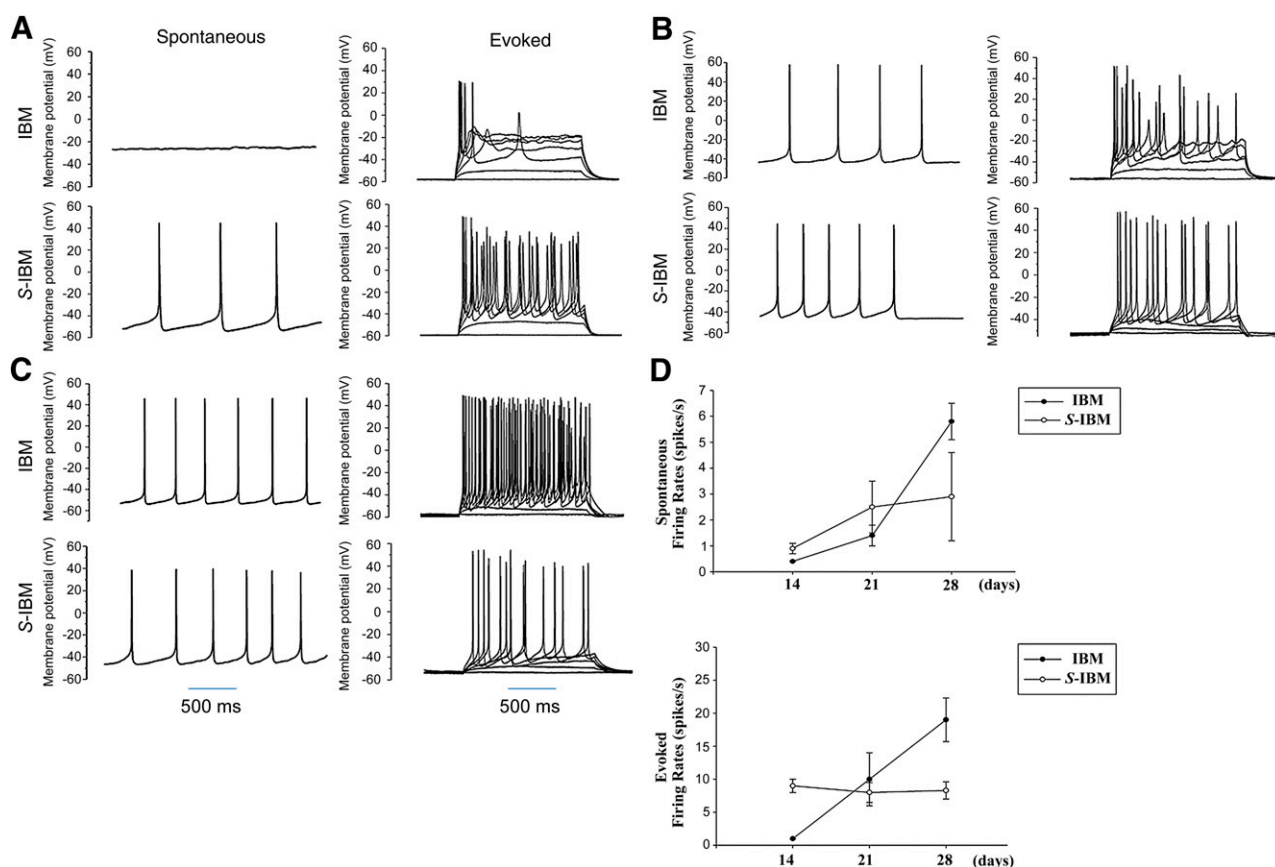
**Figure 3.** Continued from previous page.

Next, we induced ectopic SH2B1 expression by Dox during the generation of human iNs by IBM following a protocol outlined in Figure 2A. SoS-HFFs were maintained in a feeder-free culture system, and SH2B1 was induced by Dox during the whole reprogramming procedure to drive neuronal maturation. Infected cells were monitored by the presence of GFP coexpressed with the miR124 (pLemir). It was important to note that miR124-GFP expression is significantly higher than SH2B1GFP expression. Confocal microscopy observation revealed that the vast majority of GFP-positive cells manifested typical neuronal morphology in the S-IBM-infected cultures at day 14 (days after induction) (Fig. 2B, 2D). We further quantified and compared the neurite outgrowth of S-IBM and IBM-infected iNs, and found that S-IBM iNs exhibited at least twofold more neurite outgrowth than IBM-iNs (Fig. 2C). In addition, within 14 days of transduction with S-IBM, most of GFP-positive fibroblast cells exhibited significantly more neuronal processes and branches than IBM-infected cells (Fig. 2E). A characteristic neuronal morphology, consisting of increased neurite length (Fig. 2B), multiple neuritic extensions, and elaborate branches, was more obvious with S-IBM iNs than with IBM iNs at 28 days after infection (Fig. 2D). The majority of S-IBM cells displayed positive immunoreactivity for the neuronal markers TuJ1 (Fig. 3A, 3G) and NeuN (Fig. 3B, 3G). Furthermore, 69.9%  $\pm$

2.6% of S-IBM cells exhibited TH activities (Fig. 3C, 3G); 52.1%  $\pm$  1.0% of S-IBM cells exhibited vGluT2 activities (Fig. 3D, 3G).

We next examined whether the S-IBM iNs manifested properties of neurotransmitters by testing for their respective markers. Immunostaining revealed that 61.9%  $\pm$  3.4% S-IBM iNs were positive for the neurotransmitter GABA (Fig. 3E, 3G). To functionally characterize S-IBM iNs, we examined their electrophysiological properties at day 28 when 41.3%  $\pm$  1.6% displayed synapsin immunoreactivity (Fig. 3F, 3G). High-quality micrographs with either low- or high-magnification images are shown in supplemental online Figures 1 and 2. Notably, S-IBM cells exhibited significantly higher expression levels of the neuronal markers TuJ1, NeuN, TH, and GABA activities than IBM cells (Fig. 3G). In contrast, the levels of vGluT2 and synapsin activities were not discernible between the S-IBM and IBM iNs.

We then compared the spontaneous and stimulus-evoked firing of action potentials in the cells infected with IBM or S-IBM at days 14, 21, and 28. As shown in Figure 4A–4D, the spontaneous (left) firings can be detected from the S-IBM iNs (lower) at day 14. The S-IBM iNs exhibited a waveform of neuronal action potentials with peak amplitudes of  $+52 \pm 8$  mV. On the contrary, three of four IBM iNs (upper) did not fire action potentials spontaneously. Only one of four cells had repetitive spontaneous



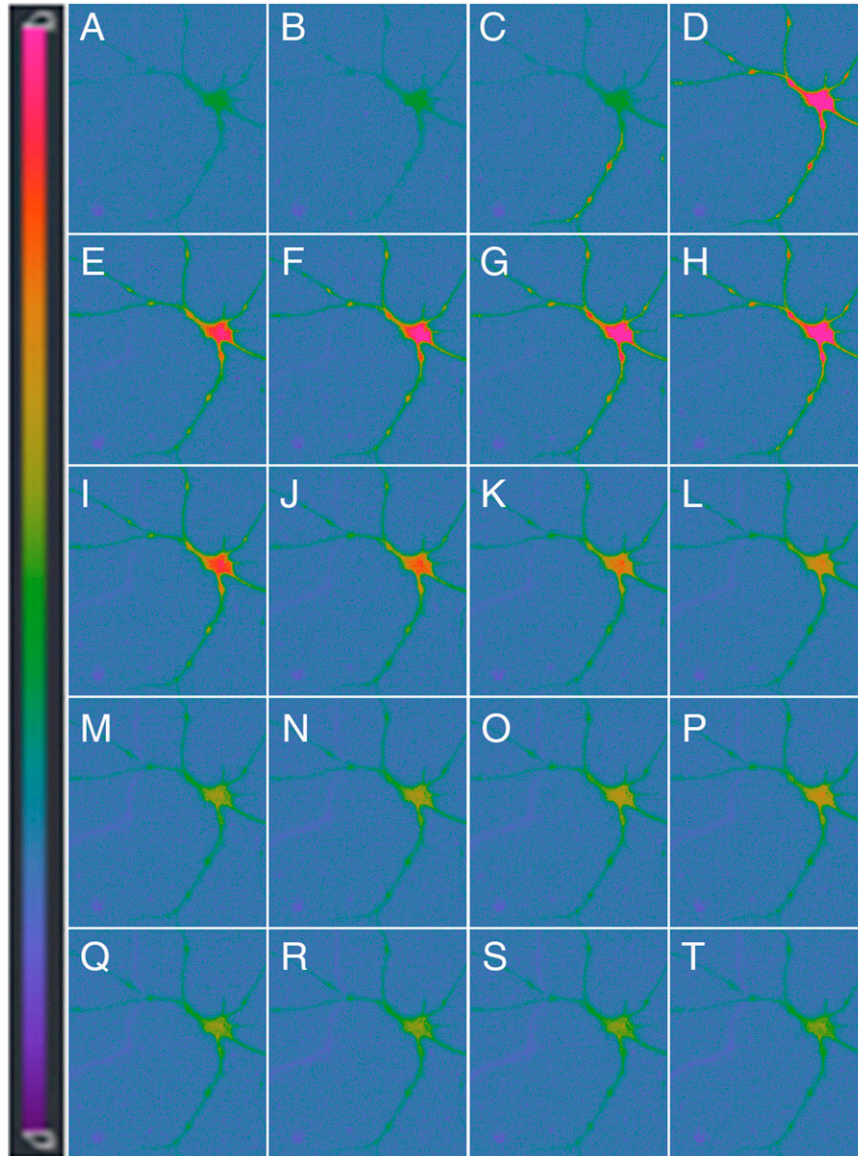
**Figure 4.** Electrophysiological characterization of S-IBM and IBM iNs. Human foreskin fibroblasts were infected with three factors (IBM; upper panel) or three factors plus SH2B1 (S-IBM; lower panel) for 14 (A), 21 (B), and 28 (C) days. Traces of spontaneous (left) and evoked (right) action potential recorded in current-clamp configuration. For the measurement of evoked action potential firing in current clamp, membrane potentials were held at  $-60$  mV. Recordings of membrane potential in response to five current steps (in 10-pA increments) from holding membrane potential were applied. The spontaneous and evoked activities of the iNs are illustrated in (D) and (E). For the measurement of evoked firing, cells were recorded at  $-60$  mV, and then a +50-pA current was injected to elicit spikes. For the measurement of spontaneous firing, the cell membrane potential was held at either  $-50$  or  $-60$  mV without current stimulation. Data were collected from three independent experiments. Abbreviations: IBM, transcription factors BRN2 and MYT1L; iNs, induced neurons; SH2B1, SH2B adaptor protein 1 $\beta$ ; S-IBM, SH2B1-enhanced iNs; SoS-HFF, SH2B1GFP-expressing cells.

spikes, albeit with much smaller peak amplitude (+10 mV). In addition to spontaneous firings, the stimulus-evoked repetitive firings (right) exhibited in the S-IBM iNs at day 14 (Fig. 4A, lower right). However, in the IBM iNs, only a single evoked spike could be detected at day 14 (Fig. 4A, upper right). Figure 3B indicates that repetitive firings can be detected from iNs infected with S-IBM or IBM in spontaneous settings by day 21 (Fig. 4B). Similar to spontaneous firings, the stimulus-evoked repetitive firings exhibited in the IBM or S-IBM iNs at day 21. At day 28, the repetitive firings can be detected from iNs infected with S-IBM or IBM in both spontaneous and evoked settings (Fig. 4C). We next quantified the firing rates of spontaneous and stimulus-evoked firings in the iNs induced with IBM or S-IBM at days 14, 21, and 28. As shown in Figure 4D, there was no apparent difference in the spontaneous firing rate between the IBM and S-IBM iNs at a later stage. However, the higher ability to generate evoked-spikes observed in IBM iNs at day 28 (Fig. 4E) suggests that these cells are more sensitive to stimulation. Previous studies demonstrated that a majority of juvenile Aplysia bag cell neurons, in contrast to adult neurons, rarely exhibited more than one action potential [29]. Our data suggest that IBM iNs, similar to juvenile neurons, are less prone to excessively repetitive firing in response to a current

injection at day 14 (Fig. 4E). Finally, the membrane potentials during resting were detected by whole-cell current-clamp mode. The reprogrammed iNs recorded from day 14 showed significant difference in resting membrane potentials between the IBM and S-IBM iNs, with less negative resting membrane potentials in IBM iNs ( $-22 \pm 4$  mV) than in S-IBM iNs ( $-45 \pm 9$  mV). The resting membrane potentials recorded at days 21 and 28 in both IBM and S-IBM iNs were close to the resting membrane potential of regular neurons (approximately  $-65$  mV). S-IBM iNs at day 14 had more negative resting membrane potentials and higher ability to generate action potentials, suggesting that SH2B1 could accelerate the S-IBM-induced neuronal differentiation and maturation.

Electrical cell activity leads to increases in intracellular  $Ca^{2+}$  levels and activation of signaling pathways that are important for the regulation of processes in neurons [30]. We then tested whether S-IBM iNs neuronal networks were functionally capable of generating  $Ca^{2+}$  transients; we preloaded the cells with the calcium indicator Fura2-AM, and the neurons were earmarked using GFP fluorescence.  $Ca^{2+}$  transients detected in S-IBM iNs showed the changes in Fura2-AM signal over time. Representative figures showing the  $Ca^{2+}$  influx before (Fig. 5A) and after (Fig. 5B–5T) glutamate stimulation are provided.





**Figure 5.** SH2B adaptor protein  $1\beta$ -enhanced induced neurons showed calcium influx activity upon glutamate stimulation at day 28. Images before (A) and after (B–T) glutamate injection were taken at consecutive intervals of 3 seconds.

## DISCUSSION

It has been suggested that human cells appear to be less plastic and have a higher epigenetic hurdle for reprogramming to both iNs and iPSCs [11]. Human ESC-derived neurons require amounts of time to develop synaptic competence that are similar to those of human iNs [31, 32]. Thus, a long maturation time may be an inherent property of human cells, which may be due to the fact that human brain development is orders of magnitude slower than that of rodents [11]. In this study, we demonstrate that a combination of SH2B1, miR124, BRN2, and MYT1L (*S*-IBM) could induce and enhance rapid reprogramming of adult human fibroblasts into functional neurons. Although other combinations of transcription factors have recently been reported to produce neuronal-like cells [9] and dopaminergic neurons [13, 14] from human fibroblasts, an important development with our combined *S*-IBM approach is that we could enhance the length and

the numbers of processes and branches of neurite outgrowth. In addition, *S*-IBM could accelerate the maturation of human iNs from 28 days to 14 days based on the electrophysiological data (Fig. 4A–4D). Along these lines, the maturation of *S*-IBM iNs requires a much shorter duration. One possible mechanism is that the iN-defined culture condition contains fibroblast growth factor (FGF) and GDNF, where SH2B1 has been implicated in promoting FGF- [19] and GDNF-induced neurite outgrowth [28]. Functional analysis demonstrated that SH2B1 promotes GDNF-induced neurite outgrowth in both PC12 cells and cultured mesencephalic neurons [28]. Therefore, SH2B1 is an important signaling molecule involved in FGF- and GDNF-induced neuronal differentiation. However, overexpression of SH2B1 alone is insufficient to convert human fibroblasts to neurons (data not shown). In line with these observations, SH2B1 is critical for promoting neuronal maturation in *S*-IBM iNs. The reprogramming platform of *S*-IBM iNs described here provides an efficient method for

expedient conversion of adult human fibroblasts into functional neurons with proper morphological, immunohistochemical, and electrophysiological characteristics.

After central nervous system (CNS) injury, excessive accumulation of glutamate can overstimulate glutamate receptors and subsequently trigger intracellular calcium overload, which initiates a series of downstream lethal events including oxidative stress, mitochondrial dysfunction, and inflammation [33]. Glutamate receptor-mediated neuronal excitotoxicity is one of most important factors responsible for CNS injury. In the present study, we took advantage of the effects of glutamate accumulation and  $\text{Ca}^{2+}$  imaging techniques to show glutamate-induced increase of intracellular  $\text{Ca}^{2+}$  in S-IBM iNs (Fig. 5; supplemental online Fig. 3). We also provided a movie clip (supplemental online Video 1) to demonstrate the real-time imaging of  $\text{Ca}^{2+}$  influx upon the glutamate stimulation. Glutamate activates three classes of ionotropic receptors, namely, NMDA, AMPA, and kainite receptors, among which the NMDA receptors are mainly responsible for glutamate-induced excitotoxicity because of their high  $\text{Ca}^{2+}$  permeability [34]. Overall, S-IBM iNs recapitulate key features of neurons in CNS, making this S-IBM approach particularly applicable for CNS disease modeling using patient-derived iNs.

## CONCLUSION

Our data demonstrate that SH2B1 can enhance neurite outgrowth and shorten the maturation period by at least 7–14 days of human iNs under defined conditions. SH2B1 could also enhance the reprogramming efficiency by approximately 10%. We anticipate that this approach may facilitate robust generation

of functional neurons from more easily accessible but refractory to-be-induced specimens, such as blood, for personalized regenerative medicine, drug screening, or in vitro disease modeling.

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## AUTHOR CONTRIBUTIONS

Y.-C.H.: conception and design, data analysis and interpretation, manuscript writing; S.-L.C.: collection and/or assembly of data, data analysis and interpretation, manuscript writing; Y.-J.W.: collection and/or assembly of electrophysiology data, data analysis and interpretation; Y.-H.C.: generation of lentivirus constructs; D.-Y.W.: collection and/or assembly of data, data analysis and interpretation; L.C.: generation of SH2B1 constructs; C.-H.C.: generation of lentivirus constructs; H.-H.C.: collection and/or assembly of electrophysiology data, data analysis and interpretation; I.-M.C.: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript.

## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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