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Original Article

Transplantation of human induced cerebellar granular-like cells improves motor functions in a novel mouse model of cerebellar ataxia

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Abstract: Stem cell-based reparative approaches have been applied to cerebellum-related disorders during the last two decades. Direct lineage reprogramming of human fibroblasts into functional granular neurons holds great promise for biomedical applications such as cerebellum regeneration and cellbased disease modeling. In the present study, we showed that a combination of Ascl1, Sox2 and OCT4, in a culture subsequently treated with secreted factors (BMP4, Wnt3a and FGF8b), was capable of converting human fibroblasts from the scalp tissue of patients with traumatic brain injury (TBI) into functional human induced cerebellar granular-like cells (hiCGCs). Morphological analysis, immunocytochemistry, gene expression and electrophysiological analysis were performed to identify the similarity of induced neuronal cells to human cerebellum granular cells. Our strategy improved the efficiency for hiCGCs induction, which gave the highest conversion efficiency 12.30±0.88%, and Ath1^{+}/Tuj1^{+} double positive cells to 5.56±0.80%. We transplanted hiCGCs into the cerebellum of Nmyc$^{TRE/TRE}$: tTS mice, a novel mouse model of cerebellar ataxia, and demonstrated that the hiCGCs were able to survive, migrate, proliferate and promote mild functional recovery after been grafted into cerebellum.

Keywords: lineage reprogramming, cerebellum granular cells, cerebellar ataxia

Introduction

The unique and relatively simple architecture of the three-layered cerebellar cortex and the well-defined afferent and efferent fiber connections have made the cerebellum, one of the most thoroughly studied areas of the CNS, a favored research area and a good candidate for studying the tissue engineering and developmental biology [1, 2]. But so far there is no treatment that can cure or substantially prolong the life span of individuals affected by cerebellar disorders, such as hereditary ataxias, that include the autosomal dominant or recessive spinocerebellar ataxias [3, 4]. One of the hopeful therapies for cerebellar degenerations could be neurotransplantation, which, however, still needs in-depth investigation before it can become a routine method in humans [5].

In 2006, Yamanaka and colleagues demonstrated cells can be reprogrammed into induced pluripotent stem cells (iPSCs) by overexpression of four transcription factors [6]. Since then, accumulating studies demonstrated cell reprogramming could be achieved by using different transcription factor cocktails and many optimizational techniques were gradually developed [7-12]. Rather than switching the fate of differentiated cells going backward and then going forward, there might be a shortcut-directly converting one adult cell type into another without going through the pluripotent state. Recent pioneering works show that this method is feasible. The first successful direct conversion of murine fibroblasts into functional induced neurons was achieved in [13], and this was followed by a number of studies describing similar switches between developmentally remote cell

types [14-20]. Within 2 years, successful direct conversion of mesoderm into ectoderm and endoderm as well as endoderm into ectoderm was reported for murine and human cells. This rapidly evolving field abounds with new questions and aims to exploit these discoveries, for example to induce distinct cellular subtypes and expand progenitor populations, as well as to investigate post-transplantation function in vivo.

In the present study, we directly reprogram human fibroblasts from patients undergoing neurosurgery into functional hiCGCs without going through an intermediate pluripotent stage by using three transcription factors (Ascl1, Sox2 and OCT4) and three important neurotrophic factors for hind brain development (BMP4, Wnt3a, FGF8b). We also demonstrate these cells can express human cerebellar granular neuron markers, and express the voltage-gated ion channels, generate spontaneous action potentials, express functional neurotransmitter receptors, form excitatory postsynaptic currents. We transplanted sorted hiCGCs into the cerebellum of $Nmyc^{TRE/TRE}$: tTS mice and demonstrated that the hiCGCs were able to survive, migrate, proliferate and promote mild functional recovery after been grafted into cerebellum. Moreover, we have confirmed the safety of hiCGCs in vivo by observing long term. This is the first time to evaluate $Nmyc^{TRE/TRE}$: tTS transgenetic mice for cerebellum function and therapeutic effect of cell transplantation.

Materials and methods

Cell cultures of adult human fibroblasts from patient

All specimens were collected with written patient consent and ethical approval. The skin tissue was harvested from patient's scalp in neurosurgery as previously described [18]. Briefly, each tissue was washed with sterile HBSS containing antibiotics (1% penicillin/streptomycin), then dissected blood vessels, hair follicles and subcutaneous fat carefully under an anatomical microscope. Use eye scissors to cut the piece into strips as small as possible, and dissociated the minced tissue pieces with collagenase IV and trypsin (Sigma) in a Falcon tube. Subsequently, cells were filtered and plated in a medium of DMEM (Gibco) supplemented with 10% fetal bovine serum (Hyclone), 1× GlutaMAX (Invitrogen), 0.1 mM non-essential amino acids (Gibco) and 0.5% penicillin and streptomycin (Gibco). Media was changed every other day. Primary fibroblasts were expanded for 3~5 passages to remove other types of cells (such as keratinocytes) and retained fibroblasts only.

Lentivirus infection and iNCs cell generation

Lentiviruses were produced as reported previously [18, 21, 22]. Human cDNAs for Ascl1, Sox2 and OCT4 were sub-cloned into the lentiviral vector pHRST-IRES-GFP. The 293FT cells (Invitrogen) was used to produce sufficient lentivirus for infection. One day before transduction, human fibroblasts were seeded at 5×10^5 cells/2 ml/well of 6-well gelatin-coated plate. Virus supplemented with polybrene (5 mg ml^{-1}) were added into culture medium and incubated for 24 hours. To improve the efficiency of transduction, two rounds of viral infection were performed 24 hours apart or incubated cells with virus for a subsequent 48 hours. After the viral supernatants were removed, medium was replaced with N3 medium (DMEM/F2 (Invitrogen), 500 mg ml^{-1} transferrin (Sigma), 25 mg ml^{-1} insulin (Sigma), 30 nM sodium selenite (Sigma), 20 nM progesterone (Sigma), 100 nM putrescine (Sigma), penicillin/streptomycin (Sigma) supplemented with neurotrophic factors including brain-derived neurotrophic factor (BDNF), glial-cell-derived neurotrophic factor (GDNF), neurotrophin-3 and ciliary neurotrophic factor). Three important neurotrophic factors for hind brain development, bone morphogenetic protein 4 (BMP4), wingless integration 3a (Wnt3a) and fibroblast growth factor 8b (FGF8b), were added into the N3 medium. The culture media was changed every other day.

Immunocytochemical analysis

For immunocytochemistry staining, cells were fixed using 4% paraformaldehyde, washed three times with PBS, and incubated for 45 min in blocking buffer (5% goat serum, 1% BSA, and 0.2% Triton X-100 in PBS) at room temperature. After removing the blocking buffer, cells were incubated overnight with primary antibody at 4°C. The following primary antibodies were used: Vimentin (1:500, abcam), Nestin (1:200, abcam), collagen (1:500, abcam), Pax6 (1:500, abcam), Sox10 (1:200, Santa Cruz), Nanog (1:200, abcam), Tuj1 (1:500, abcam), Map2 (1:500, abcam), GABA (1:100, Millipore), vGluT1

(1:200, abcam), GFAP(1:250, abcam), Synapsin (1:500, Millipore), En1 (1:500, Millipore), Ath1 (1:200, Millipore), Zic1 (1:500, Millipore), Phalloidin (1:250, abcam) and MBP (1:100, abcam). The next day, the slides were washed with PBS and added to fluorescence-labeled secondary antibody in the dark at room temperature for 1 h. The cells were then washed with PBS and stained with DAPI solution (1 μg/ml).

Efficiency calculation

The following method was used to calculate the efficiency of neuronal induction, which were previously used by Vierbuchen *et al.* [13]. The total number of GFP/Tuj1-double positive cells with a granular morphology and absence of fibroblast-like morphology, which were thought to be human induced cerebellar granular-like cells, was quantified. The total number of Ath1/Tuj1-double positive cells was also quantified. We determined this number in at least 15 randomly selected 10× visual fields. The conversion efficiency of hiCGCs was determined by dividing the number of GFP/Tuj1-double positive neurons formed by the number of fibroblasts plated before infection. The Ath1/Tuj1-double positive cells were thought to be more specific cerebellum granular-like cells. Data were presented as mean ± s.d.

Western blotting

Cells were harvested and lysed in RIPA Lysis Buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate and 0.1% SDS), supplemented with 1× phosphatase inhibitor cocktail (Pierce) and 1× protease inhibitor cocktail (Roche). 25 to 100 μg cell lysates proteins were heat for 5 min at 65°C in 2× SDS sample buffer, and separated by electrophoresis on 5% or 10% polyacrylamide gel and transferred onto a polyvinylidine difluoride (PVDF) membrane (Millipore). Antibodies used for western blotting were anti-Ascl1 (1:200, Santa Cruz), anti-Sox2 (1:500, abcam), anti-OCT4 (1:200, Abnova), anti-Ath1 (1:500, Millipore), anti-NeuN (1:500, Millipore), anti-Pax6 (1:500, abcam) and anti-p53 (1:500, abcam).

Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated by using Trizol (Invitrogen), according to the manufacturer's instructions (TRIzol ® Reagent). For each sample, 1~2 μg of RNA was used for reverse transcription performed with random primers and Super-Script III Reverse Transcriptase (Invitrogen). Each cDNA was diluted 1:20, and 2 μl was used for each real-time reaction. Quantitative real-time PCR was performed with SYBR Green in a two-step cycling protocol on 7700 Fast Real-Time PCR System (Applied Biosystems). All the qPCR reactions were done in triplicate, and the expression data were averaged upon normalization to GAPDH expression. Data were quantified with the ΔΔCt method. The primer sequences are listed in previous publications [21, 23, 24].

Electrophysiology

The electrophysiological recordings were performed from hiCGCs in voltage-clamp and current-clamp mode at room temperature. The internal solution for voltage-clamp recordings contained (in mM): 140 CsCl, 10 NaCl, 0.1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 1 EGTA (pH 7.2, 310 mOsm). The internal solution for current-clamp recordings contained (in mM): 140 KCl, 0.5 EGTA, 5 HEPES and 3 Mg-ATP (pH 7.3, 300 mOsm). The external bath solution contained (in mM): 142 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose and 10 HEPES (pH 7.3, 320 mOsm). Whole-cell recordings were made in current-clamp mode with a Multiclamp 700B amplifier (Axon instruments). Electrodes had resistances of 2-4 MΩ when filled with intracellular solution in mM: 150 K-gluconate, 10 HEPES, 0.2 EGTA, 4 KCl, 2 NaCl, 14 phosphocreatine, 2 Mg-ATP, and 0.3 Na-GTP (pH 7.35, ~305 mOsm). In voltage clamp mode, cells were held at a membrane potential between -60 mV and -70 mV. For the initiation voltage-gated currents, we used voltage steps from -90 to +50 mV in 10 mV increments. In current clamp mode, cells were recorded for up to 25 min to examine possible spontaneous firing. Data were filtered at 10 kHz, digitized at 20 kHz, and analyzed with Clampfit 9.2 (Axon instruments).

Transplantation and function examination

$Nmyc^{TRE/TRE}$: tTS mice were offered by Shanghai Research Center for Model Organisms, bred and maintained in our animal facilities. These transgenetic mice were produced based on the tTS-dox system [25].

30 transgenetic mice were equally randomized into 3 groups after identification of genotypes. Group A served as an untreated control; Group B was transplanted with hiCGCs; Group C was shame operation group. 10 C57BL/6 mice were treated as wild type control.

The hiCGCs were collected at a density of 1×10^4 cells/μl for transplantation, which were labeling PSA-NCAM-APC and collected by fluorescence activated cell sorting (FACS). Mice (8 weeks, 25-30 g) were anesthetized with an intraperitoneal administration of ketamine (100 mg/kg) and xylazine (16 mg/kg), and fixed on a stereotaxic frame. Cell suspension (5 μl per lobe) was injected into the cerebellum (bregma-6.2 mm, midline, depth bregma-3 mm) with a glass microcapillary (inner diameter 60 μm). The injection speed was 0.5 μl/min. After microinjection, the mice were placed under a lamp and warmed for 4 h. Then they were immunosuppressed with cyclosporine A (10 mg/kg) each day for 7 days.

Three weeks after transplantation, the mice were evaluated of motor and non-motor function.

Beam walking test. The apparatus of this test was consisted of a 120 cm long wooden beam (2 cm diameter), fixed 80 cm above the ground with a large 40 cm^2 platform at stop end. An animal was considered to have learned the task when it would spontaneously cross the entire 120 cm length of beam to the 'safe' platform. The total time taken to cross the beam and times of sideslip were recorded along with the time spent moving.

Passive avoidance learning. Passive avoidance learning test based on negative reinforcement was used to examine the long-term memory. The measurements were conducted in a Passive Avoidance System, which had two compartments (light and dark boxes) and was connected to a computer. A step through 'Trials to Criterion' procedure was used in the training session on the first day. The animal was given a foot shock (0.05 mA, 2 seconds) whenever it entered the dark box compartment. The session ended after the animal stayed in the light box compartment for more than 300 seconds. All animals were tested for memory retention 24 and 48 hours after the learning trials. In this stage, no electrical shock was applied, when the mouse entered the dark box compartment during this retention test. Mice were directly taken back to their home cage after they entered the dark box compartment within 300 seconds or if they stayed in the light box compartment for 300 seconds.

Four weeks after transplantation, the mice were anesthetized and perfused with PBS (pH 7.4) followed by 4% paraformaldehyde. The brains were collected and post-fixed for 24 h at 4°C. After being embedded in low melting point agarose, the brains were cut into 15 μm consecutive sagittal sections. The sections were immersed into 80°C antigen retrieval solution for 25 min and washed three times in PBS. Then, the sections were permeabilized and blocked using TBS (0.05 M Tris, 150 mM NaCl, and 0.5% Triton X-100) supplemented with 0.1% sodium azide, 1.0% BSA, and 5.0% normal goat serum for 1 h. Sections were incubated with primary antibodies for 48 h on a shaker at 4°C, washed in TBS three times, and incubated with fluorescence-labeled secondary antibody at room temperature for 2 h. Finally, the sections were washed with PBS and stained with DAPI solution (2 μg/ml).

Results

Conversion of human fibroblasts into hiCGCs with transcription factors and regional specific neurotrophic factors

We collected the scalps of patients with traumatic brain injury (TBI) under informed consent. After three passages, we checked the morphology and antigenic expression pattern of cultured cells, which exhibited a typical fibroblast-like morphology and a reliable fibroblast marker Vimentin but no Nestin staining. If the cells express Vimentin while not Nestin, we could consider they are indeed fibroblasts. Moreover, the cultured cells expressing collagen I without the neuroepithelial marker Pax6, the neural crest marker Sox10 and the stem cell marker Nanog, confirming their fibroblast identity and not derived from neural crest derivatives or stem cells. Besides, the absence of neuronal marker Tuj1 and granular neuron marker Ath1 showed the starting cells contain no cerebellar neurons (**Figure 1**).

We select three transcription factors (Ascl1, Sox2 and OCT4), cloned into the lentiviral vectors harbouring a GFP reporter, and then we virally transduced fibroblasts with different unique combinations of these three vectors. The cells in the colonies proliferated more rapidly than the original fibroblasts.

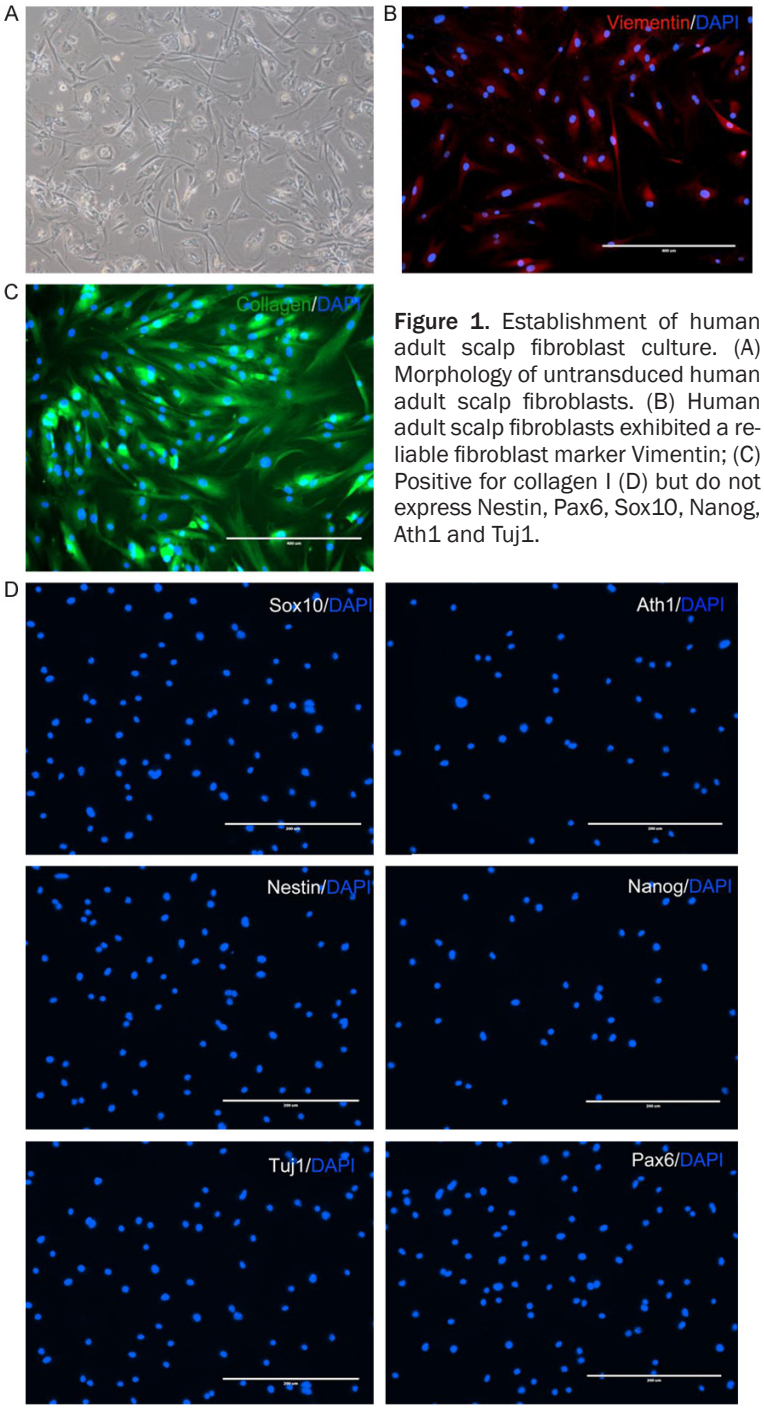

Figure 1. Establishment of human adult scalp fibroblast culture. (A) Morphology of untransduced human adult scalp fibroblasts. (B) Human adult scalp fibroblasts exhibited a reliable fibroblast marker Vimentin; (C) Positive for collagen I (D) but do not express Nestin, Pax6, Sox10, Nanog, Ath1 and Tuj1.

After two rounds of viral infection, the cells were cultured in N3 neural induction medium containing BMP4, Wnt3a and FGF8b. About 7 days later, transduced GFP$^+$ cells presented simple neuronal morphology with small cell body and mono- or bipolar neurite. By 14 days after transduction, we can observe those GFP$^+$ cells exhibited more complex neuronal morphologies similar to that of human neuronal cell with long fine processes resembling neuronal axons. After 21 days, cells with more mature neuronal morphologies were positive for neuronal markers, including Tuj1 (also known as βIII-tubulin), Map2 and Synapsin. The most striking result of culturing cells in N3 medium was formation of the signature "T-shaped" axons of the cerebellar granule neurons. The GFP$^+$/Tuj1$^+$ neuronal cells were quantified at indicated time points after transduction, the number of double-positive cells peaked at 28 days (**Figure 2A-D**).

Although most of our combinations can induce a neuron-like phenotype, including expression of Tuj1 and Map2, we observed that the combination of Ascl1, Sox2 and OCT4, improved the conversion efficiency nearly threefold than Ascl1+Sox2, Ascl1+OCT4, OCT4+Sox2 and much more than Sox2, OCT4 or Ascl1 alone. Thus, based on number of cells and morphological criteria, the combination of Ascl1, Sox2 and OCT4 was the most efficient for iNCs induction, which gave the highest conversion efficiency 12.30±0.88% (**Figure 2C**).

In contrast, the cells cultured in neural induction media without viral infection were not Tuj1-positive though some morphologic change occurred, and the lentiviral expression of GFP alone in human fibroblasts was not capable for reprogramming in spite of treating identically. Both of the two controls confirmed that human fibroblasts lack spontaneous neuronal differentiation potential without the presence of transcription factors.

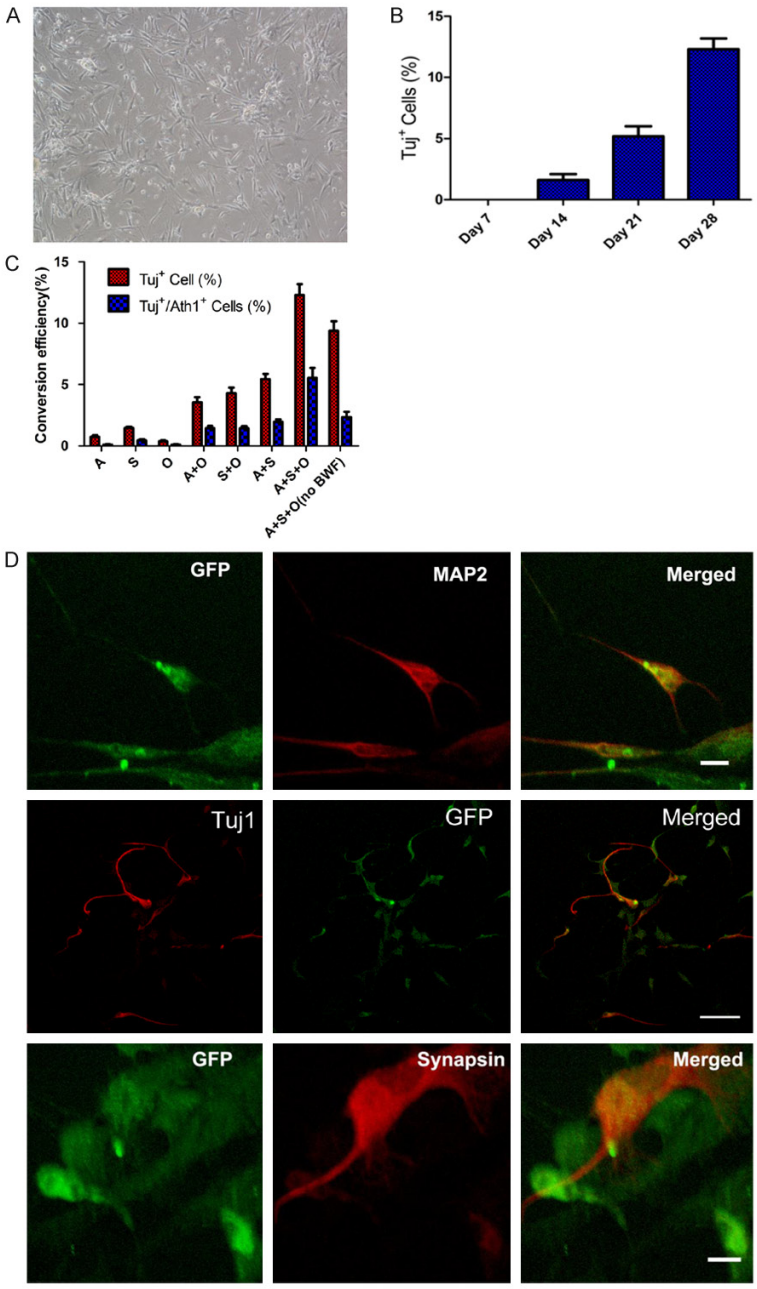

Figure 2. Induction of human induced cerebellar granular-like cells (hiCGCs) from patient-derived fibroblasts. A. Ascl1, Ngn2, and Sox2 transduced fibroblasts exhibited a mature granular morphology resembling neurons at 21 days after transduction. B. Quantification of GFP/Tuj1 double-positive hiCGCs. C. The percentage of Tuj1/GFP and Ath1/Tuj1 double-positive hiCGCs reflects the hiCGCs conversion efficiency. D. Immunocytochemistry of Tuj1, Map2 and Synapsin.

hiCGCs express human neural subtype and cerebellum specific markers

Ath1 is one of the master gene in granule neuron differentiation [26-28]. The expression of both Ath1 and Tuj1 could identify cerebellar granule neuron. The Ath1$^+$/Tuj1$^+$ neuronal cells were quantified at indicated time points after transduction, the number of double-positive cells peaked at 28 days. The addition of BMP4, Wnt3a and FGF8b improved the efficiency for hiCGCs induction, which gave the highest conversion efficiency 5.56±0.80% (**Figure 2C**). En1 and Zic1 are two transcription factors that are expressed in the rhombic lip (RL) and have overlapping patterns of development and cooperate in cerebellar development [29, 30]. The expression of En1 and Zic1 could illustrate the successful induction of fibroblasts into cerebellar neurons (**Figure 3A-C**).

It would be of high interest if the presented combination leads to a distinct neuronal subtype. We sought to characterize the neurotransmitter phenotype of hiCGCs. Been further cultured more than 30 days, we detected vGluT1-positive puncta outlining GFP-positive cells, indicating the presence of excitatory, glutamatergic neurons. In addition, we found our cells labelled with antibodies against GABA, the major inhibitory neurotransmitter in brain. 1.0% of transfected cells expressed GABA, and 5.3% were vGluT1-positive, whereas we were unable to detect tyrosine hydroxylase, choline acetyltransferase, or serotonin expression (**Figure 3D**, **3E**).

Nevertheless, there were none glial fibrillary acidic protein (GFAP)-positive or MBP-positive cells in hiCGCs cultures (**Figure 3F**, **3G**), revealing that human fibroblasts could be not converted into astroglia or oligodendroglia under this culture condition .

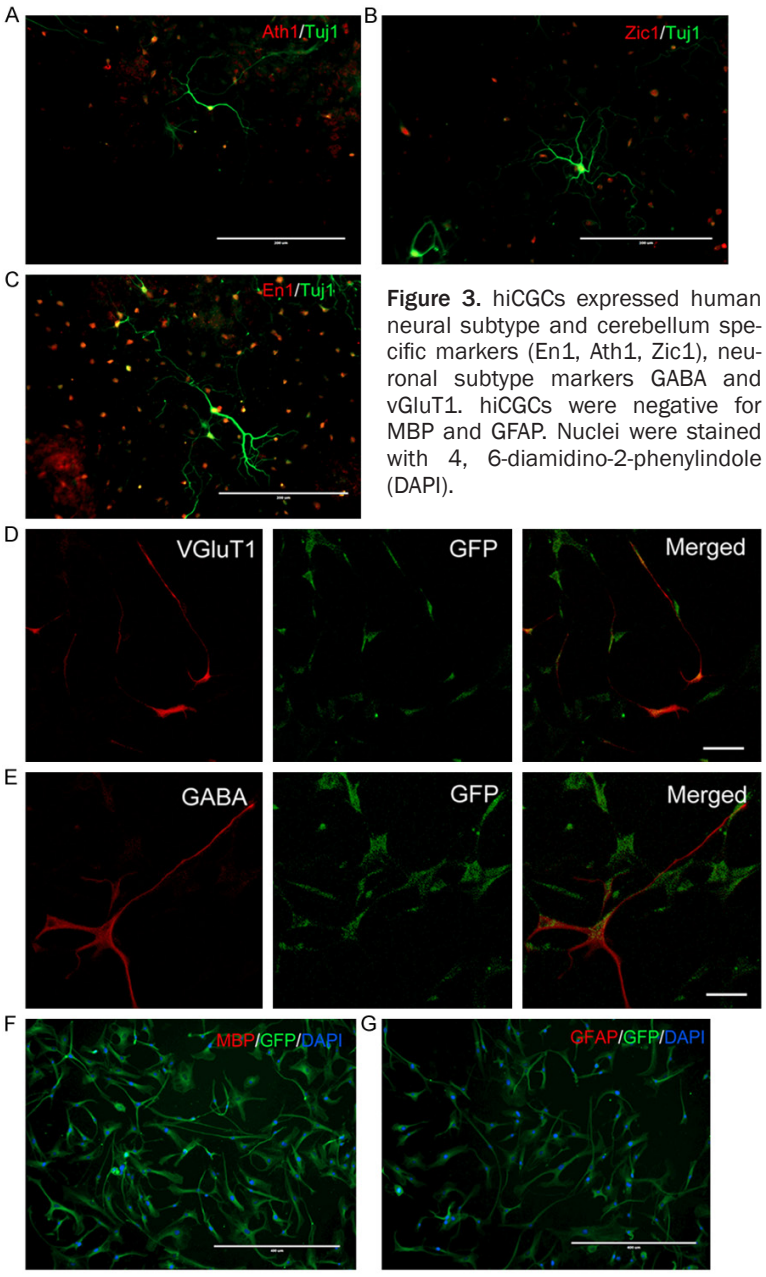

Figure 3. hiCGCs expressed human neural subtype and cerebellum specific markers (En1, Ath1, Zic1), neuronal subtype markers GABA and vGluT1. hiCGCs were negative for MBP and GFAP. Nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI).

Characterization of protein and gene expression in hiCGCs

We then tested the expression status of introduced foreign genes and some markers of neural lineages by western blot analysis of hiCGCs. The results showed a tendency toward up-regulation of Ascl1, OCT4 and Sox2 from day 7 to day 28. Nevertheless, at day 28, all the three transcripts had a lower expression level than before. So, transcription factors might play an initiate role in reprogramming process. Protein levels of NeuN and Pax6 with a disparity in expression time and quantity reflected a conversion of fibroblasts to neurons. The expression of Ath1 could identify the cerebellar granule neuron conversion. These results were also confirmed by immunofluorescence analysis. We also analyzed the P53 protein expression level, its consistent low level showed a probably safety (**Figure 4A**). qRT-PCR analysis corroborated these results (**Figure 4B**).

Functional characterization of hiCGCs

To test whether iNCs exhibit functional membrane properties, whole cell patch clamp recordings were performed after days 30 of initial induction. About 75% (n=12 out of 16 cells recorded) of human iNCs fired mature action potentials in response to depolarizing current injection, and expressed the voltage-gated inward Na^+ and outward K^+ currents (**Figure 5A**, **5B**). Na^+, Ca^{2+} current: voltage-clamp, holding at -70 mV and ramp to +60 mV during 100ms, the first peak should be the sodium current, which is large and quick and can be blocked by TTX (1 μM), the second peak should be the calcium current, and it was blocked by $NiCl_2$ (300 mM) (**Figure 5C**). We further evaluated spontaneous excitatory postsynaptic currents. At -80 mV, AMPAR mediated currents were recorded by electric stimulation the nearby afferents, which could be bolcked by AMPAR antagonist NBQX (10 μM). And at +40 mV, with NBQX added, NMDAR mediated currents were recorded which can be blocked by NMDAR antagonist AP-V (50 μM) (**Figure 5D**). Both of these two receptors are glutamate receptors, while cerebellum granular neurons are glutamatergic. With NBQX and AP-V in the perfusion bath, GABAR mediated excitory currents (EPSCs) were recorded at 0 mV by electric stimulation the nearby afferents.

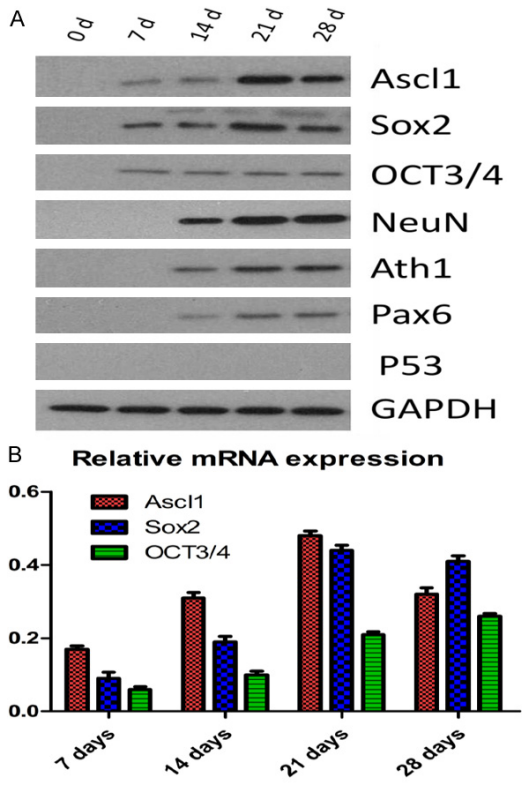

Figure 4. Characterization of hiCGCs. A. Western blot analysis of lentiviral-mediated genes and neural marker genes. B. Quantitative real-time polymerase chain reaction analysis of the three transcription factor expression hiCGCs harvested at multiple time points during the direct reprogramming process.

Whole-cell patch-clamp recordings confirmed that hiNCs exhibited functional membrane properties of mature neurons in vitro.

Cerebellum atrophy model mice and hiCGCs transplantation

Fluorescence-activated cell sorting (FACS) was performed using PSA-NCAM, a marker for immature neurons or neuronal progenitors [31]. The percentage of $GFP^+/PSA\text{-}NCAM^+$ cells was 9.11% (**Figure 6A**), which were used for $Nmyc^{TRE/TRE}$: tTS mice transplantation (**Figure 6B**).

Low walking speed of $Nmyc^{TRE/TRE}$: tTS mice, reverse after hiCGCs transplantation. Cerebellar degeneration influences the animal's ability to adjust motor movements required to successfully complete the beam walking test. The time required by wild type mice to cross the beam was shorter than $Nmyc^{TRE/TRE}$: tTS mice, and the sideslip number of wild type mice was also less than transgenetic one (**Figure 7A**, **7B**; $P<0.05$). Transplantation of hiCGCs into $Nmyc^{TRE/TRE}$: tTS mice resulted in improvement of the across time ($P<0.05$) but not the sideslip number ($P>0.05$) when compared with the control group (**Figure 7A**, **7B**).

hiCGCs transplantation partially ameliorate memory in passive avoidance learning. Implantation of hiCGCs caused a mild enhancement in the passive avoidance learning and memory in hiCGCs group compared with control group. The total staying time in the dark electrical foot shock box and latency to enter the dark room of transplanted group were not different to control groups ($P>0.05$) (**Figure 7C**, **7D**). The statistical analysis showed a significant difference of times to enter dark room during 5-minute periods in all groups ($P<0.05$) (**Figure 7E**). The hiCGCs transplantation improved the development of memory deficit caused by cerebellar degeneration. There was statistical significant difference between pretraining and test groups ($P<0.05$) (**Figure 7D**, **7E**). The results showed that there was no obvious learning ability deficit of $Nmyc^{TRE/TRE}$: tTS mice.

The mice were sacrificed 28 days after transplantation, and the immunofluorescence was performed to confirm that the injected cells survived and integratied into the host cerebellar tissue. In these mice, the transfected cells could clearly be detected throughout the whole cerebellum. These cells were either spherical-shaped or elongated. From the injection site, it was possible to detect GFP^+ cells traveling around the exterior of the cerebellum, in between the lobes, until finally penetrating through the molecular layer into the granular layer (**Figure 8A**). The vast majority of the cells were located in the granular layer of the cerebellar cortex.

Phalloidin, a bicyclic heptapeptide, binds specifically at the interface between F-actin subunits, locking adjacent subunits together [32, 33]. The properties of phalloidin make it a useful tool for investigating the distribution of F-actin in cells by labeling phalloidin with fluorescent analogs and using them to stain actin filaments for light microscopy. The mice were processed by immunohistochemical detection of the injected cells, as well as counter-stained with Phalloidin immunoreactivity to identify the

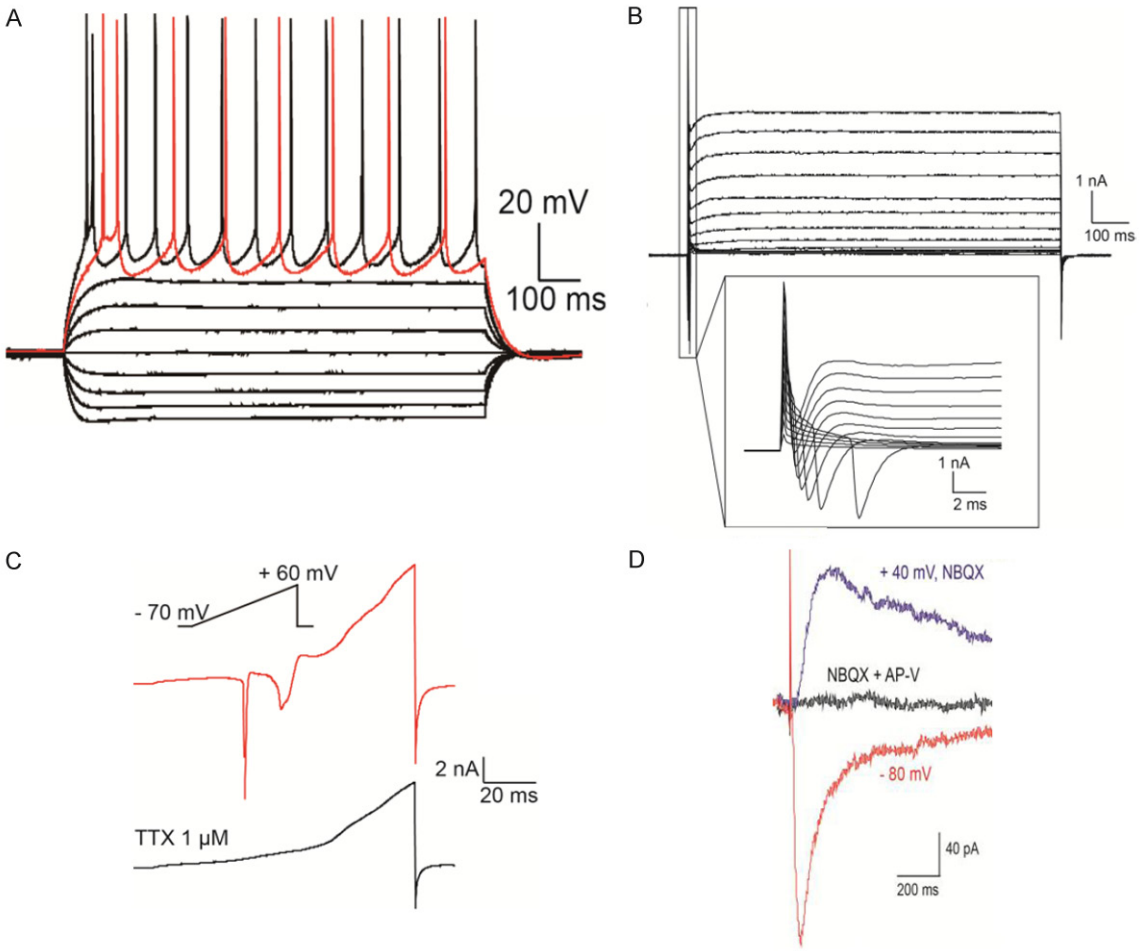

Figure 5. Membrane properties of human hiCGCs. A. Action potential firing characteristics of human hiCGCs, increased from -200 to +200 pA in 50 pA increments. B. In voltage-clamp mode, both voltage-gated sodium (INa) and delayed outwardly rectifying potassium currents (Ik) were observed. C. Na^+, $Ca2^+$ current: voltage-clamp, holding at -70 mV and ramp to +60 mV during 100ms, the first peak should be the sodium current, which is large and quick and can be blocked by TTX (1 μM). D. AMPAR, NMDAR current: voltage-clamp, with 100 μM picrotoxin in bath solution to block GABAR mediated inhibitory current. At -80 mV, AMPAR mediated currents were recorded by electric stimulation the nearby afferents, which could be bolcked by AMPAR antagonist NBQX (5 μM). And at +40 mV, with NBQX added, NMDAR mediated currents were recorded which can be blocked by NMDAR antagonist AP-V (50 μM). Both of these two receptors are glutamate receptors.

cell division situation. Clusters of grafted cells were localized close or in physical contact with the surviving host cells (**Figure 8B**). Since surviving implanted cells mainly expressed the marker of Phalloidin, we postulated that the grafted cells not even survived in host brain, but also participated in host karyokinesis.

In theory, one major advantage of skipping the pluripotent stage is the avoidance of tumor formation, so it is essential to confirm the hiCGCs were safe from tumorigenesis. We injected hiCGCs into the brains of C57BL/6 mice to see whether there happens tumorigenesis, no neural tumors occurred (n=10 out of 10 mice recorded). In addition, normal karyotype of hiCGCs compared was confirmed.

Discussion

Cerebellar disorders represent a wide group of diseases that may have a detrimental impact on patients since the cerebellum not only plays a key role in motor coordination control but is also involved in cognitive and affective functions [34, 35]. The unique and relatively simple architecture makes the cerebellum a good candidate for studying the intrinsic and environmental parameters influencing regenerative processes triggered by the injected cells [2, 5,

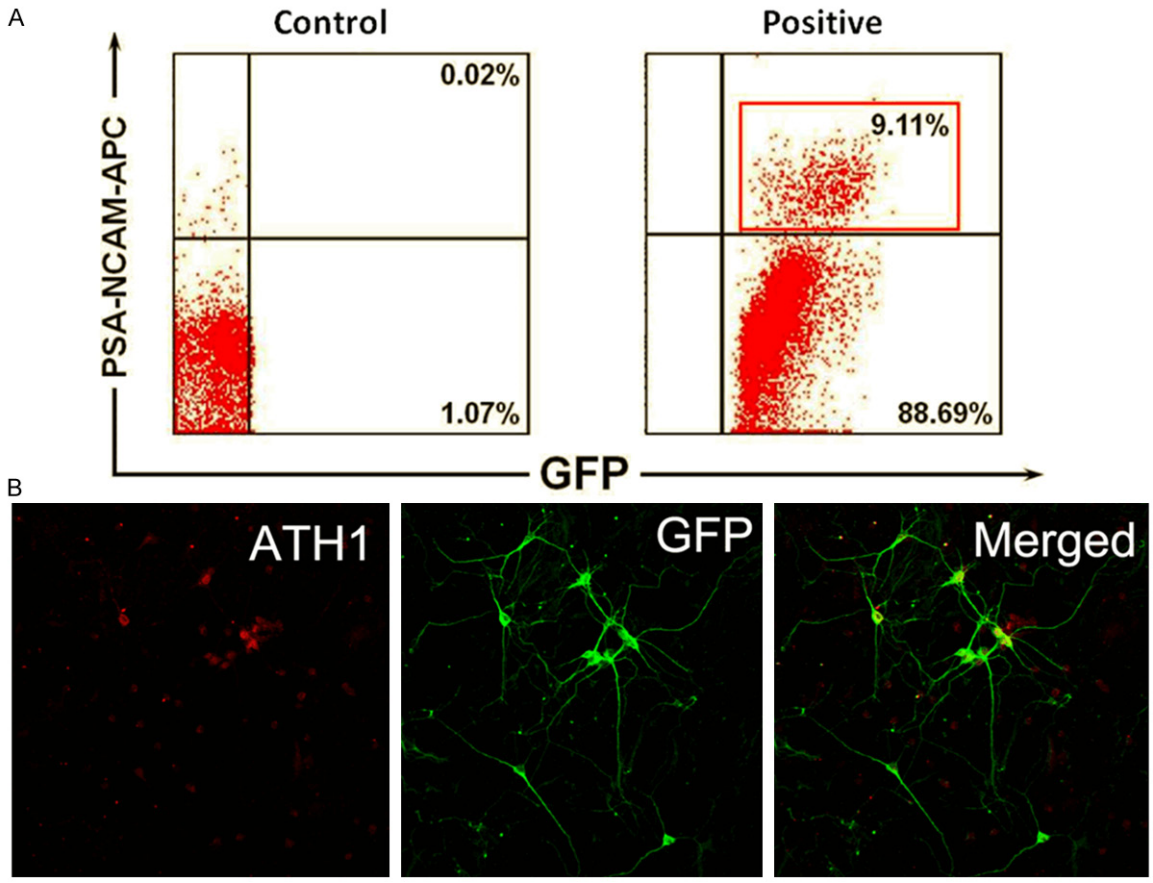

Figure 6. The cells were analyzed by flow cytometry for PSA-NCAM. A. There were 9.11% cells that were positive for PSA-NCAM. B. GFP+/PSA-NCAM+ cells expressed human cerebellum granular neuron specific marker Ath1.

36]. Thus, this central nervous system (CNS) structure is trilaminar and organized in a point-to-point manner representing a favorable ground for investigating whether neural replacement could be an effective strategy for reestablishing neural circuits.

As the adult mammalian central nervous system possesses very limited regenerative capacity [37-39], obtaining a feasible source of transplantable neural cells that possess identical genetic background with patients' cells is of great promise for neurological disease therapy. Many studies demonstrated that defined sets of transcription factors could reprogram fibroblasts directly into functional neurons [13-19]. Derivation of induced cerebellar cells from non-neural lineages could provide a new tool for the treatment cerebellum neurodegenerative diseases; however, no such direct conversion strategy has been reported yet.

Recently, several combination strategies of transcriptional factors, including Ascl1, Sox2, Brn4, Klf4, and c-Myc, had been used to reprogram somatic cells into neuronal cells [13, 40, 41]. Most recently, direct reprogramming of mouse and human fibroblasts into multipotent neural stem cells with only Sox2 had been reported [17]. Here we show that expression of three transcription factors (Ascl1, Sox2 and OCT4), combined with three important neurotrophic factors for hind brain development (BMP4, Wnt3a, FGF8b) [34, 35], can rapidly and efficiently convert human fibroblasts into functional cerebellar granular-like neurons without going through an intermediate pluripotent stage.

In the present study, we hypothesized that Sox2 and Ascl1 are thought to establish a profile of homeodomain transcription factors that is necessary for the subsequent reprogramming of human cells. It is speculated that the Sox2-Ascl1 complex regulates the structure of chromatin, thus allowing binding of OCT4 to their specific target sites and leading to full activation of the neuronal genesis program.

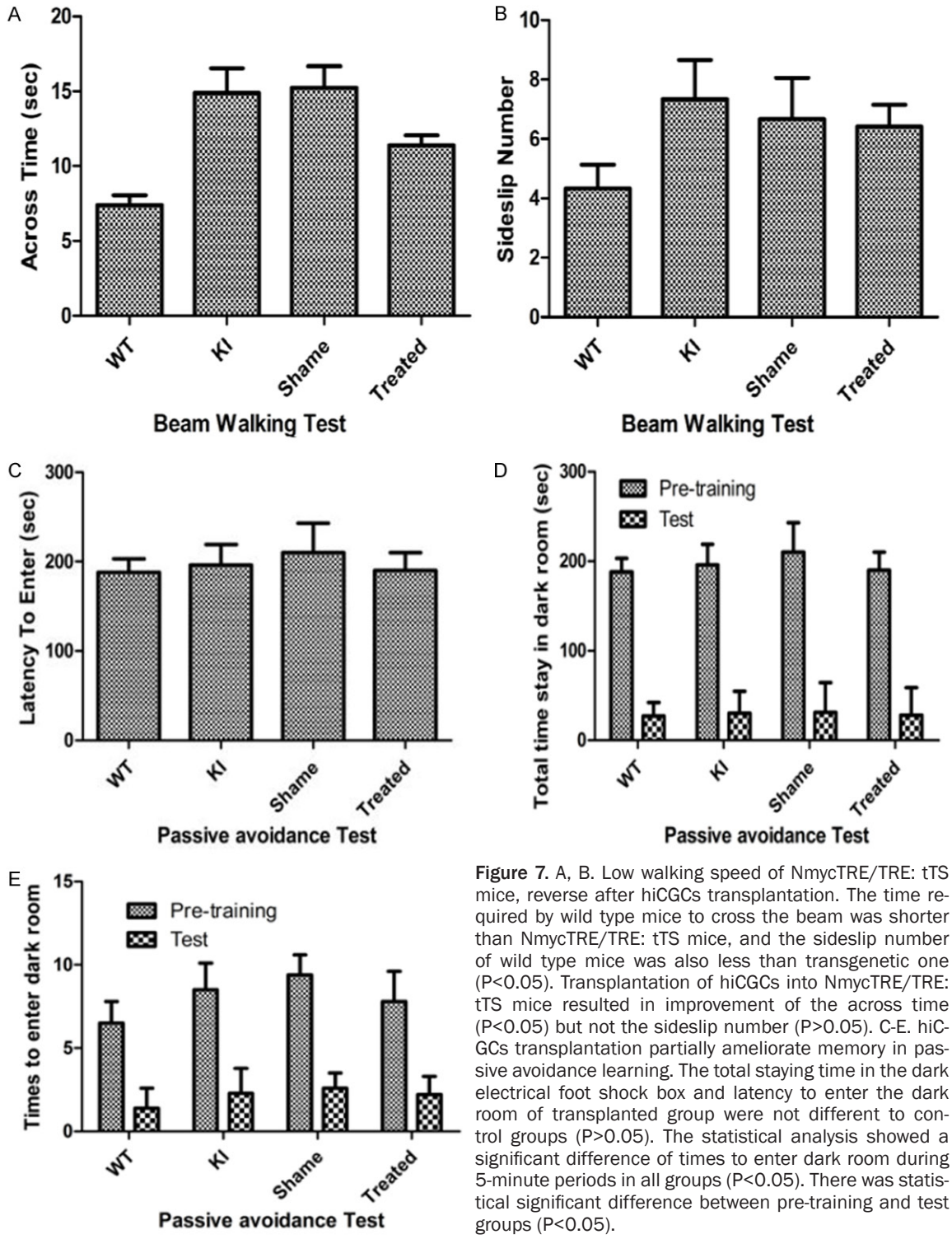

Figure 7. A, B. Low walking speed of NmycTRE/TRE: tTS mice, reverse after hiCGCs transplantation. The time required by wild type mice to cross the beam was shorter than NmycTRE/TRE: tTS mice, and the sideslip number of wild type mice was also less than transgenetic one ($P<0.05$). Transplantation of hiCGCs into NmycTRE/TRE: tTS mice resulted in improvement of the across time ($P<0.05$) but not the sideslip number ($P>0.05$). C-E. hiCGCs transplantation partially ameliorate memory in passive avoidance learning. The total staying time in the dark electrical foot shock box and latency to enter the dark room of transplanted group were not different to control groups ($P>0.05$). The statistical analysis showed a significant difference of times to enter dark room during 5-minute periods in all groups ($P<0.05$). There was statistical significant difference between pre-training and test groups ($P<0.05$).

Our study showed that addition of neurotrophic factors BMP4, Wnt3a and FGF8b could commit the neural stem cells to a cerebellar fate. While the factors Ascl1, Sox2 and OCT4 were sufficient to induce neuronal but not cerebellum granular features, the addition of neurotrophic factors generated mature $Tuj1^+/Ath1^+$ cells with efficiencies of up to 5.56±0.80%.

Our findings indicate that the patient-specific induced cerebellar granular-like cells were similar to human granular cells in morphology, spe-

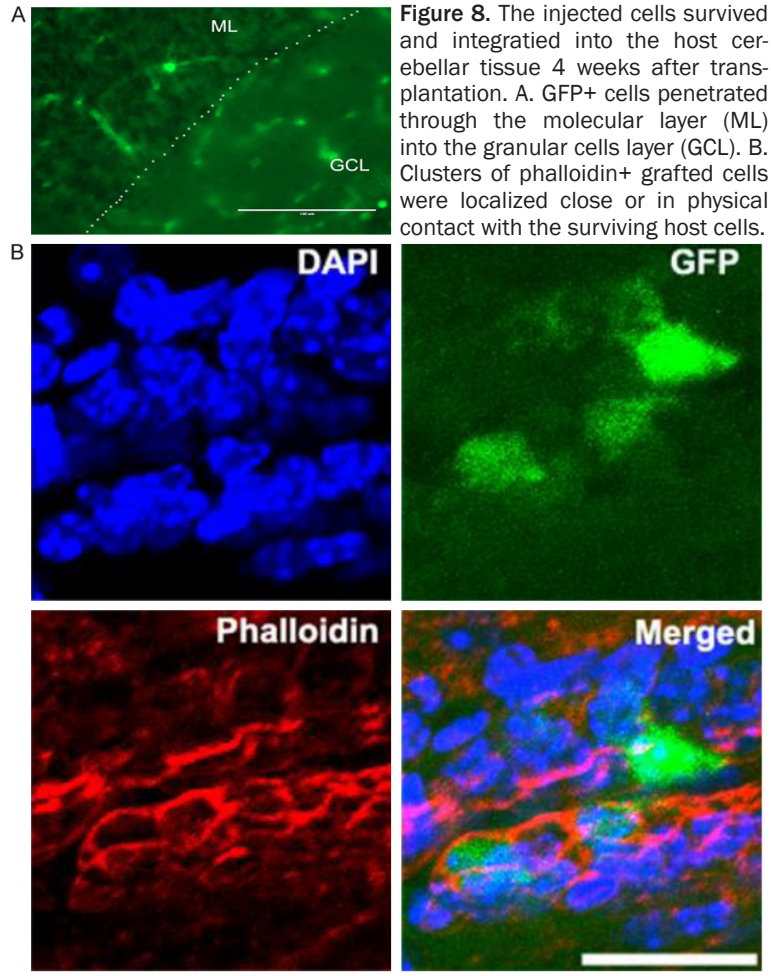

Figure 8. The injected cells survived and integratied into the host cerebellar tissue 4 weeks after transplantation. A. GFP+ cells penetrated through the molecular layer (ML) into the granular cells layer (GCL). B. Clusters of phalloidin+ grafted cells were localized close or in physical contact with the surviving host cells.

cific surface antigens, gene expression and electrophysiological signals, such as expressing the voltage-gated ion channels, generating spontaneous action potentials, expressing functional neurotransmitter receptors and forming excitatory postsynaptic currents. Recently, direct conversion of human fibroblasts to dopaminergic neurons opens new possibilities for regenerative therapies for neurodegenerative disease and related disorders [42, 43]. In our study, some hiCGCs expressed markers of glutamatergic neurons, which were consistent with cerebellum granular cells and considered to play a critical role in modulating neuropsychiatric behaviors and indicate that manipulations of this neuronal subtype may be a promising avenue for therapeutic interventions, but no other neurotransmitter phenotypes were detected. Our data suggest the intriguing possibility that additional combinations of neural transcription factors might also be able to generate functional neurons whose phenotypes remain to be explored. One of the next important steps will be to generate human cerebellum neurons of other specific neuronal subtypes.

An ultimate goal of hiCGCs will be using the direct reprogramming strategy for cell therapy, while how hiCGCs might impact on the overall functional aspects of the host brain will need to be tested further in long-term in vivo transplantation studies. In our study, we transplanted hiCGCs into the cerebellum of $Nmyc^{TRE/TRE}$: tTS mice and demonstrated that the hiCGCs were able to survive, migrate, proliferate and promote partially functional recovery after been grafted for a relative long time. Unlike those studies directing reprogramming of fibroblasts into iNSCs or iNPCs, our protocol generated only neurons without GFAP-positive astroglia or MBP-positive oligodendroglia, which means the hiCGCs were not tumorigenic. Moreover, we have confirmed the safety of hiCGCs in vivo by observing 5 months, neither teratoma nor neural tumor emerged. To our knowledge, this is the first time that $Nmyc^{TRE/TRE}$: tTS mice are treated with cell transplantation and evaluated for functional recovery and long term safety.

Our findings not only represent a successful generating of patient-specific neurons from somatic cells, but also optimize the transdifferentiation strategy by precisely directing these cells conversion to cerebellum regional specific granular neurons, and prove their function in vivo. It will now be of great interest to decipher the molecular mechanism of this fibroblast to neuron conversion. Although still at early stages, the accelerated research in the field of stem cell technology gives hope that efficient cell-based therapy in cerebellar diseases will become a reality in the near future.

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Disclosure of conflict of interest

None.

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