

SV40T reprograms Schwann cells into stem-like cells that can re-differentiate into neurocytes

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ABSTRACT Background: The specific effect of SV40T on neurocytes has seldom been investigated by the researchers. We transfected Schwann cells (SCs) that did not have differentiation ability with MPH 86 plasmid containing SV40T, in order to explore the effects of SV40T on Schwann cells. Methods: SCs were transfected with MPH 86 plasmid carrying the SV40T gene and cultured in different media, and also co-cultured with neural stem cells (NSCs). In our study, SCs overexpressing SV40T were defined as SV40T-SCs. The proliferation of these cells was detected by WST-1, and the expression of different biomarkers was analyzed by qPCR and immunohistochemistry. Results: SV40T induced the characteristics of NSCs, such as the ability to grow in suspension, form spheroid colonies and proliferate rapidly, in the SCs, which were reversed by knocking out SV40T by the Flip-adenovirus. In addition, SV40T up-regulated the expressions of neural crest-associated markers Nestin, Pax3 and Slug, and down-regulated S100b as well as the markers of mature SCs MBP, GFAP and Olig1/2. These cells also expressed NSC markers like Nestin, Sox2, CD133 and SSEA-1, as well as early development markers of embryonic stem cells (ESCs) like BMP4, c-Myc, OCT4 and Gbx2. Co-culturing with NSCs induced differentiation of the SV40T-SCs into neuronal and glial cells. Conclusions: SV40T reprograms Schwann cells to stem-like cells at the stage of neural crest cells (NCCs) that can differentiate to neurocytes.

KEY WORDS: Schwann cells, SV40T, cell transformation, cell reprogramming, inverse differentiation, neural crest cells

Introduction

Reprogramming, or the de-differentiation of mature cells to the totipotent stem-cell like state via genetic or epigenetic mediation (Takahashi and Yamanaka, 2006), is a potent tool for achieving tissue engineering and repair in various degenerative diseases, especially neurological disorders. Schwann cells (SCs) are glial cells that have the ability to repair damaged peripheral nerve myelin sheath (Baron-Van Evercooren *et al.*, 1997, Hyung *et al.*, 2018), and in theory can be reprogrammed for potential clinical applications.

Although the Simian virus 40 T antigen (SV40T) is routinely used to construct immortalized cell lines (Kaiser *et al.*, 2017), it can induce cell transformation depending on the host cell type (Wang *et al.*, 2014). For example, forced expression of SV40T in brain tissues leads to the loss of terminal differentiation markers on the

Abbreviations used in this paper: EMT, epithelial mesenchymal transition; ESCs, embryonic stem cells; NCC, neural crest cells; NSCs, neural stem cells; SCs, Schwann cells; SV40T, Simian virus 40 T antigen.

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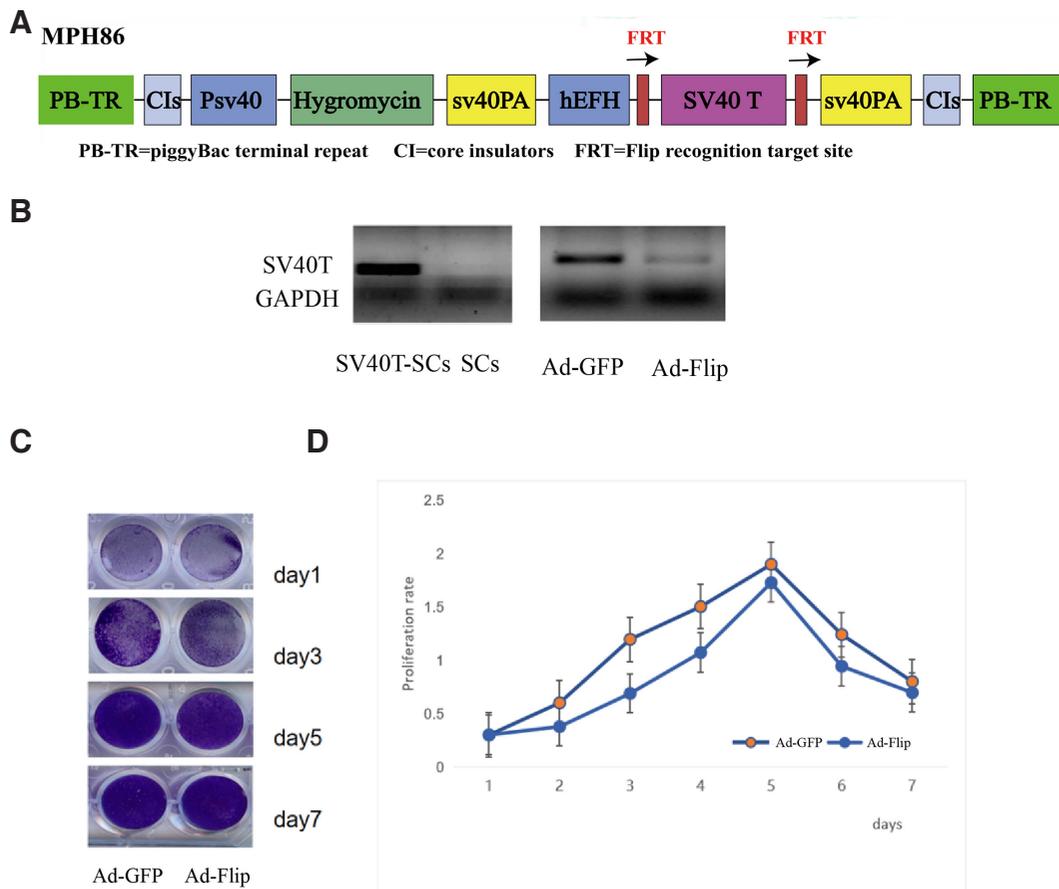


Fig. 1. The SCs were transfected with SV40T and exhibited higher proliferative activity than that of primary SCs. Representative images showing (A) MPH86 plasmid structure, (B) expression of SV40T in SCs, SV40T-SCs and cells in the Ad-Flip and Ad-GFP groups, (C) cells in the Ad-Flip and Ad-GFP groups were stained with crystal violet, (D) percentage of proliferating cells in the Ad-Flip and Ad-GFP groups was different at day 3 and 4.

astrocytes, and the cells acquire an early-differentiated phenotype (Stevanovic *et al.*, 1993). Moreover, SV40T significantly improved reprogramming efficiency (Mali *et al.*, 2008).

We constructed mouse sciatic nerve SCs by transfecting the primary cells with the MPH 86 plasmid carrying the SV40T gene (Wang *et al.*, 2014). In our study, SCs overexpressing SV40T were defined as SV40T-SCs. We found that SV40T induced the expression of Sox2 and c-Myc, which are the important transcription factors that have a role in the reprogramming of somatic cells (Yamanaka, 2007). Reprogramming induces differentiated cells into reverting to stem-like cells (Jopling *et al.*, 2011).

The ensuing SV40T-overexpressing SCs showed significant changes in their morphology, proliferation rates, phenotypic markers etc. compared to primary SCs. These characteristics of SV40T-overexpressing SCs are similar to stem-like cells at the stage of neural crest cells (NCCs). We then co-cultured SV40T-overexpressing SCs with neural stem cells to induce the cells to re-differentiate (Rhee *et al.*, 2019). Finally, the SV40T-overexpressing SCs re-differentiate into neuronal and glial cells. Based on our findings, we surmised that SV40T-mediated reprogramming led to the reverse differentiation of SCs.

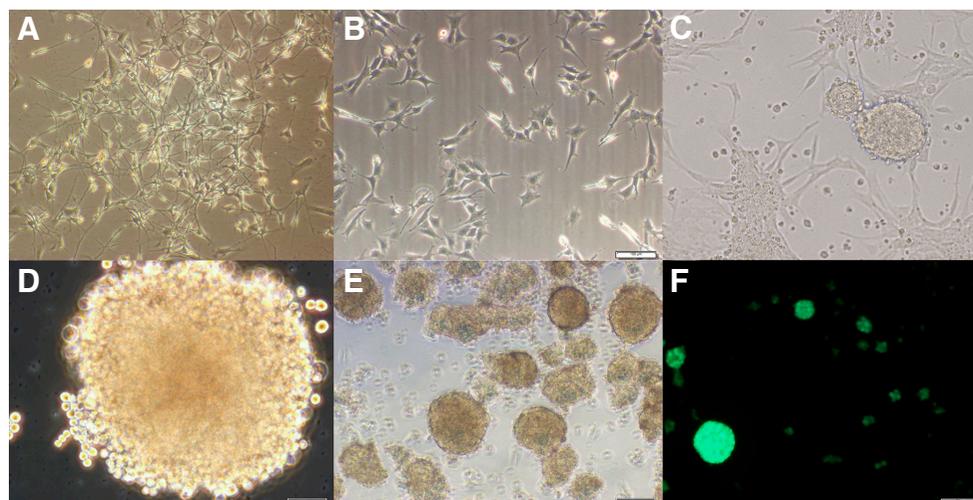


Fig. 2. SV40T-SCs grow with stem cell-like growth characteristics. Based on one-week culturation, representative images showing (A) primary SCs forming long cell processes in NSC medium (magnification 100x), (B) adherent growth of SV40T-SCs with short processes in complete DMEM (magnification 200x), (C) spherical colonies of SV40T-SCs in suspension in complete DMEM (magnification 40x), (D) a round shape, and formed spherical colony of SV40T-SCs in suspension (magnification 400x), (E) round shape, and formed spherical colonies of SV40T-SCs grown in suspension in NSC medium (magnification 200x), and (F) NSCs grown in suspension in NSC medium (magnification 200x).

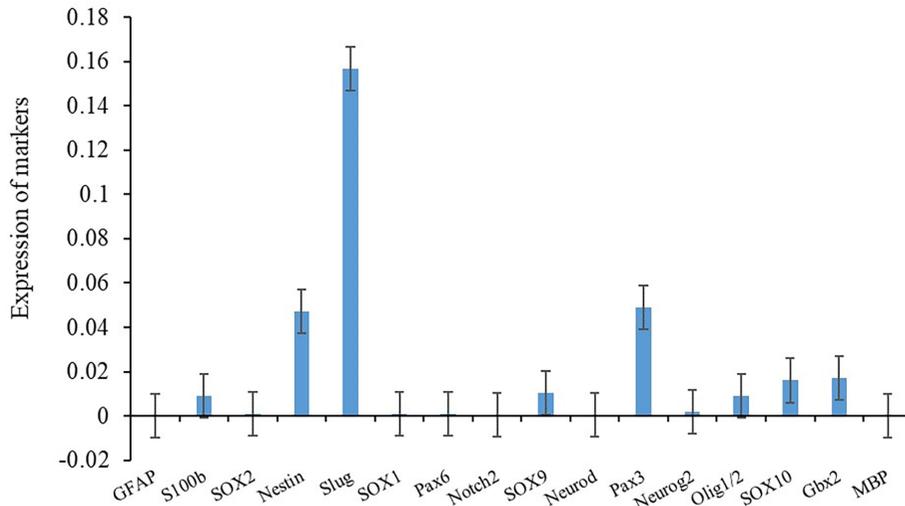


Fig. 3. The expression level of the neural crest markers and the NSC marker in SV40T-SCs. SV40T-SCs highly expressed Nestin, Slug and Pax3.

Results

SV40T reprogrammed the SCs into NSC-like cells

The SCs transfected with MPH 86 plasmid carrying the SV40T stably expressed SV40T gene (Fig. 1B). As shown in Fig. 1B, SV40T expression was significantly decreased in the Ad-Flip group. Furthermore, SV40T knockdown significantly decreased the proliferation rates of cells compared to that of Ad-GFP-infected SCs at days 3 and 4 (Fig. 1 C,D).

The primary SCs were adherent in Neurobasal medium containing B27, and characterized by the long processes of neurocytes (Fig. 2A). However, the SCs did not proliferate in complete DMEM. In contrast, SV40T-SCs grew rapidly in both media. SV40T-SCs were in adherent growth with short synapses, round, triangular, diamond-shaped or striped morphologies (Fig. 2B), and formation of spherical colonies (Fig. 2C). In the NSC medium, however, these cells grew in suspension attaining a round shape, and formed spherical colonies (Fig. 2 D,E). The NSCs were used as controls (Fig. 2F). Thus, forced expression of SV40T induced characteristics of stem cells in the primary SCs.

Consistent with the phenotypic changes, SV40T increased the expression levels of the neural crest markers Slug and Pax3, as well as the NSC marker Nestin in the SCs. In contrast, the SC

marker S100b (Shen *et al.*, 2017), and the markers of mature SCs MBP, GFAP and Olig1/2 were weakly expressed in these cells (Fig. 3). Furthermore, the SV40T-SCs were positive for the NSC markers Nestin, Sox2, CD133 and SSEA-1, as well as the embryonic stem cell (ESC) markers BMP4, c-Myc, OCT4 and Gbx2 (Fig. 4). Taken together, SV40T reprograms the primary SCs to the early stages of neuronal differentiation, NSC-like cells.

SV40T-SCs re-differentiate into neuronal and glial cells following co-culture with NSCs

The SV40T-SCs were labeled with RFP (Fig. 5A), and the NSCs with GFP (Fig. 5B). After 7 days of co-culture in 10% FBS-supplemented DMEM, the SCs showed significantly elongated cell processes (Fig. 5I). Nestin (neural stem marker), beta-III tubulin (neuron cell marker) and S100b (Schwann cell marker) were also up-regulated in the co-cultured SV40T-SCs, and these cells corresponded to neuron-like morphology, neuronal morphology with triangular shape and long axons, and SC morphology, respectively (Fig. 6). Thus, the SV40T-SCs can differentiate into neuronal and glial cells.

Discussion

To determine the biological role of SV40T in the primary SCs, we transfected the cells with MPH86 plasmid carrying the SV40T gene. The transgenic cells grew rapidly in the NSC medium and complete DMEM, and formed spheroid colonies in suspension. In addition, SV40T significantly up-regulated Nestin, Pax3 and Slug, which are the major markers of NSCs and NCCs. However, the SC marker S100b, as well as the mature SCs markers MBP, GFAP and Olig1/2, were markedly lower following SV40T expression. Furthermore, SV40T also induced the expression of various NSC and ESC-related factors like Nestin, Sox2, CD133, SSEA-1, BMP4, c-Myc, OCT4 and Gbx2. As transcription factors, Sox2 and c-Myc played critical roles in the reprogramming of somatic cells (Yamanaka, 2007). We surmised, therefore, that SV40T reprograms the SCs to stem-like cells (Jopling *et al.*, 2011). To

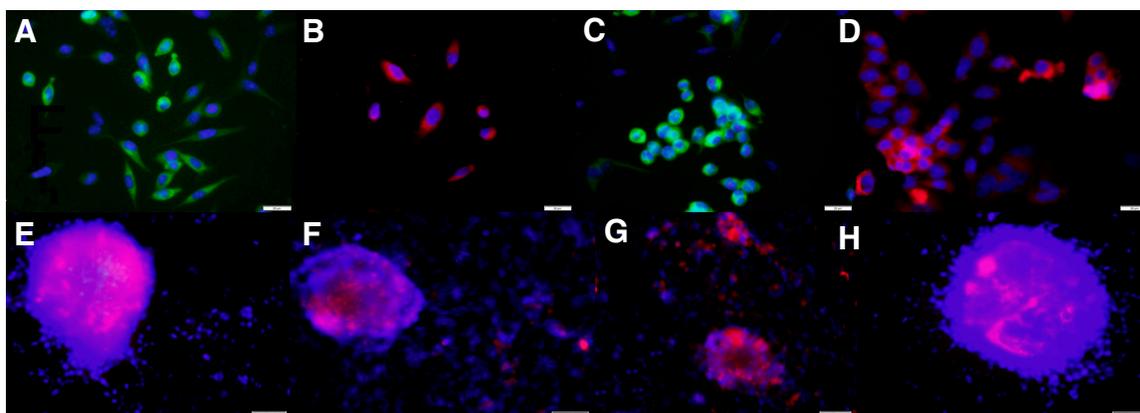
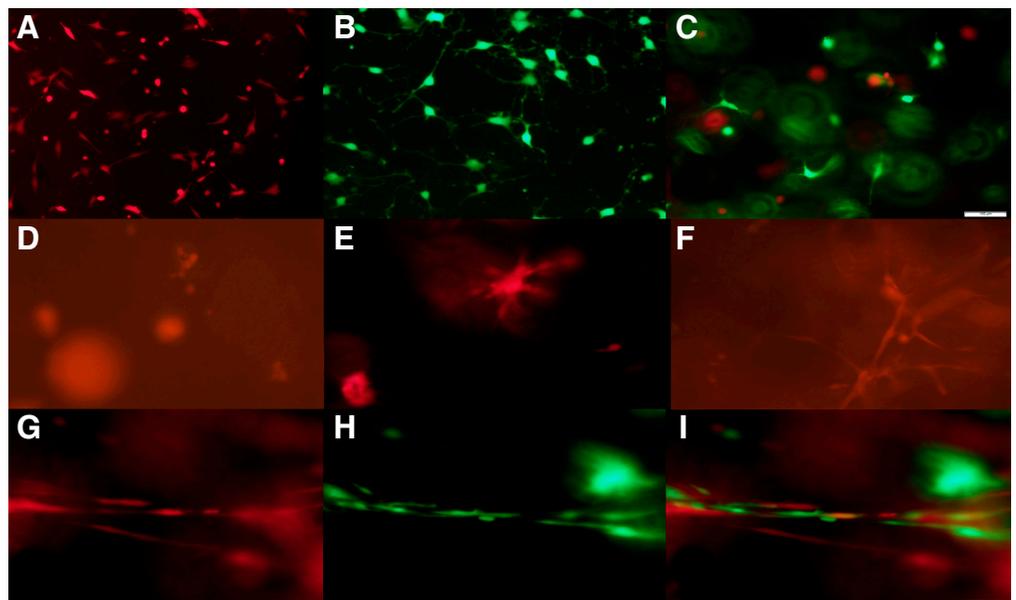


Fig. 4. SV40T-SCs expressed markers of early neural differentiation. Representative images showing expression of Nestin (A), Sox2 (B), CD133 (C), SSEA-1 (D), BMP4 (E), c-Myc (F), OCT4 (G), and Gbx2 (H) (magnification 200x).

Fig. 5. SV40T-SCs differentiated to neuronal and glial cells in the presence of NSCs. Representative images showing (A) RFP-labeled SV40T-SCs in complete DMEM (magnification 100x), (B) GFP-labeled NSCs and in complete DMEM (magnification 100x), (C) co-cultured SV40T-SCs and NSCs on day 1 (magnification 100x), (D-G) RFP-labeled SV40T-SCs on day 1 (D), 3 (E), 5 (F) and 7 (G) of co-culture with NSCs (magnification 200x), (H) GFP-labeled NSCs on day 7 of co-culture with SV40T-SCs (magnification 400x), and (I) the merging of G and H (magnification 400x).



test this hypothesis, we co-cultured the SCs with NSCs, and observed a significant lengthening and intertwining of the synapses, along with morphology and immune-phenotypic characteristics of various neurocytes. Therefore, the SV40T-SCs are possibly stem-like cells at the stage of NCCs that can differentiate into the terminal neural cells.

During embryonic development, the notochord and adjacent mesenchymal cells induce the ectoderm on its surface to form a neural plate, and its central axis forms a depression known as the nerve groove or fold. The junction of the top of this fold and the peripheral ectoderm is called neural crest. The NCCs are pluripotent stem cells that arise from the triploblasts in the third week of human embryonic development, migrate all over the embryo, settle in elected sites, and finally differentiate into various cell types (Le Douarin and Dupin, 2018). The neural crest undergoes the process of delamination mediated by an epithelial-to-mesenchymal transition (EMT), before migrating by intricate but featured paths (Szabó and Mayor, 2018). EMT is controlled by PAX3, which plays a key role in the development of embryonic neurocytes. PAX3 and *Msx1* induce *Snail1* and *Snail2* to down-regulate the expression of adhesion proteins, thereby allowing the NCCs to migrate and differentiate into mesenchymal cells (Kim *et al.*, 2014). The transcription factor *GBX2* antagonizes *OTX2*, which inhibits *PAX6* expression and induces the production of hindbrain neural progenitor cells. *Sox2* gene is widely expressed in the neural endoderm at the beginning of the formation of gut embryo, and is induced by *chordin* to promote the differentiation of neurocytes (Stevanovic *et al.*, 1993). We detected high levels of the above

factors in the SV40T-SCs, which supports our aforementioned hypothesis. The differentiation potential of these cells needs to be verified by further experiments.

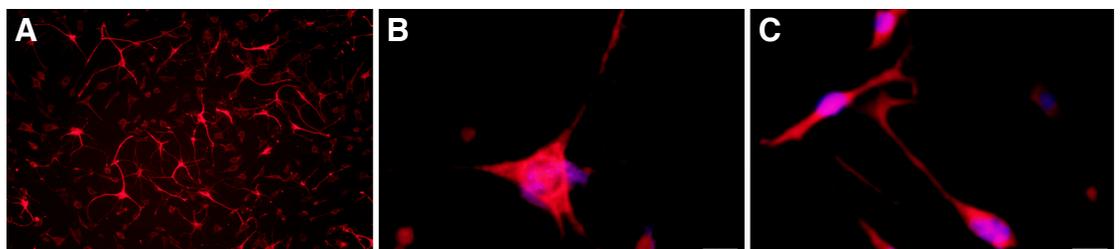
SV40T functions as an ATPase and DNA helicase, and is also involved in the phosphorylation of protein serine/threonine residues, and ribosylation and acylation of ADP. SV40T also activates ribosomal genes in host cells, induces DNA synthesis, and modifies initiation factors of protein synthesis. Previous studies have suggested a key role of SV40T in oncogenic transformation as well (Moens *et al.*, 2001). SV40T alters cell cycle and proliferation by inhibiting the expression of p53 and RB proteins (Takahashi and Yamanaka, 2006). It forms a complex with and inactivates p53 protein (Dobbelstein and Roth, 1998), which is known to promote MDM2-mediated ubiquitination and degradation of Slug (Wang *et al.*, 2009). The latter is essential for stem cell maintenance and de-differentiation. Therefore, SV40T regulates cell cycle through p53 and inhibits p53-mediated degradation of Slug, which down-regulates E-cadherin and induces EMT (Leong *et al.*, 2007).

Oct4, Sox2 and c-myc induce cellular reprogramming (Takahashi and Yamanaka, 2006), and were upregulated in the SV40T SCs. Previous studies have shown that SV40 T can improve reprogramming efficiency (Mali *et al.*, 2008, Tan *et al.*, 2017). The reprogramming of Schwann cells by SV40T may involve cell cycle regulation and Slug activation.

Conclusions

SV40T reprograms Schwann cells to stem-like cells at the stage of NCCs that can differentiate to neurocytes.

Fig. 6. The expression of neuronal or glial cells markers. Representative images showing expression levels of (A) Nestin (magnification 100x), (B) β -III tubulin (magnification 400x), and (C) S100b (magnification 400x) in the co-cultured SV40T-SCs.



Materials and methods

Antibodies and Chemicals

The antibodies - Nestin, CD133, SSEA-1, Sox2, Oct4, c-Myc, Gbx2 and BMP4 - were purchased from Abcam (Cambridge, MA, USA) and Cell Signaling Technology (Beverly, MA, USA). All chemicals were purchased from either Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Pittsburgh, PA), other than those specifically indicated.

Cell culture

Primary SCs were obtained from ATCC (Manassas, VA) and were cultured in serum-free Neurobasal medium supplemented with B27. Neural stem cells (NSCs) derived from rat hippocampus gyrus were cultured in DMEM/F12 N2 medium supplemented with B27, 20ng/ml hFGF2, 0.1mM β -ME, 100 IU/ml Penicillin and 100ug/ml Streptomycin.

Primary SCs were transfected with MPH 86 plasmid carrying the SV40T and Hygromycin resistance genes (Fig. 1A) using Lipofectamine as per the manufacturer's instructions. The stably transfected cells were screened using 100 μ g/ml Hygromycin, and the drug-resistant clones were further expanded. SV40T-overexpressing cells were confirmed by Touchdown (TD) PCR, and transduced with the Flip adenovirus (Ad-Flip) or green fluorescent protein (GFP) adenovirus (Ad-GFP). The expression of SV40T was detected by TD-PCR 24h after viral infection. In our study, SCs overexpressing SV40T were defined as SV40T-SCs.

Proliferation assay

The SV40T-SCs were cultured in both serum-free Neurobasal medium and the 10% FBS-supplemented NSC culture medium, and their morphology and growth rates were recorded. In addition, cells transduced with Ad-Flip and Ad-GFP were cultured in complete DMEM for one week and the proliferation rates were analyzed by crystal violet staining and WST-1 (He *et al.*, 2010, Wang *et al.*, 2014). The growing cells were plated into 96-well culture plates at 20% confluence. Wells without seeding any cells were used as background controls. At the indicated time points, 4 ml of the premixed WST-1 (BD Clontech, Mountain View, CA) were added to each well and incubated at the 37 °C CO₂ incubator for 1 hr. 100 μ l of the WST-1-containing culture medium were taken for absorbance reading at 440 nm using a plate reader. The obtained values of the absorption coefficients at 440 nm [a (440)] were subjected to background reading subtractions. Each assay condition was performed in triplicate.

RNA isolation and Absolute quantitative polymerase chain reaction (AQ-PCR) Analysis

Total RNA was isolated using TRIZOL reagent (Life Technologies) and reverse transcribed using hexamer primers and M-MuLV reverse transcriptase (New England Biolabs, Ipswich, MA). The qPCR primers (Additional file 1) were designed with the Primer 3 program. AQ-PCR Analysis was performed using CFX-96 Connect (Bio-Rad, CA). All samples were tested in triplicate and the standard sample provided in the kit was used as the negative control.

Immunofluorescence

The SV40T-SCs cultured in 24-well plates, fixed with methanol, permeabilized with 1% NP-40, and blocked with 10% donkey or goat serum. The cells were then incubated with antibodies against Nestin, CD133, SSEA-1, Sox2, Oct4, c-Myc, Gbx2 and BMP4, washed, and probed with Texas Red or Green-labeled secondary antibody as appropriate. After counterstaining with DAPI, the slides were examined under a fluorescence microscope.

SCs and rat hippocampal gyrus NSCs co-culture

The SV40T-SCs and NSCs were transfected with RFP and GFP, respectively, using the piggyback transposon vector system. The cells were mixed at equal densities (1×10^6) in 100 μ l complete DMEM and seeded into 6-well plates pre-coated with rat-tail glue. The cells were co-cultured for 1-2 weeks, and the morphological changes were observed under a

fluorescence microscope. On day 7 of culture, the cells were harvested, sorted by flow cytometry and re-seeded into 24-well plates. After 24h, the cells were fixed with methanol, and the expression of Nestin, S100b and beta-III tubulin was detected by immunofluorescence.

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Authors' contributions

ZL-Zhang and TC-He conceived, designed, and supervised all aspects of the project with input from RF-Li. RF-Li performed most experiments. GX-Nan performed, analyzed, and wrote the cell culture experiments. D-Wang, C-Gao and J-Yang participated in writing the manuscript. RF-Li, TC-He and ZL-Zhang wrote the manuscript with input from all authors.

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