The telomerase inhibitor AZT enhances differentiation and prevents overgrowth of human pluripotent stem cell–derived neural progenitors

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Abstract

Human pluripotent stem cell (hPSC)-based cell-replacement therapy has emerged as a promising approach for addressing numerous neurological diseases. However, hPSC transplantation has the potential to cause human cell overgrowth and cancer, which represents a major obstacle to implementing hPSC-based therapies. Inhibition of the overgrowth of transplanted cells could help reduce the risk for hPSC transplantation–induced tumorigenesis. In this study, we report that the telomerase inhibitor azidothymidine (3′-azido-3′-deoxythymidine, AZT) enhances the differentiation of cortical neurons and maturation of cortical neurons. Of note, AZT-pretreated, hPSC-derived neural progenitors exhibited decreased proliferation and increased differentiation into cortical neurons when transplanted into the mouse brain. In summary, our findings indicate that AZT prevents the overgrowth of hPSC-derived neural precursors and enhances the differentiation of cortical neurons in both cell cultures and hPSC-transplanted mouse brain. We propose that our work could inform clinical applications of hPSC-based cell therapy.

Introduction

Cell replacement therapy is a promising therapeutic approach for human neurological disease (1). Human embryonic stem cells and induced pluripotent stem cells (hPSCs) may provide the unlimited resource for cell replacement therapy. hPSCs have the potential to differentiate...
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into neural stem/progenitor cells (NS/PCs) and be subsequently induced to differentiate into three neural lineages including neurons, astrocytes, and oligodendrocytes (2-6). So far, progresses have been achieved to develop NS/PCs as a promising cell source for regenerative medicine targeting CNS disorders (4,7-11). However, a major hurdle for applying this cell source to human disease is that transplantation of certain hPSC-NS/PCs may lead to tumor-like overgrowth and deterioration of neural function during long-term observations (12). For example, hPSC-NS/PCs presented tumor-like overgrowth in vivo after transplantation (13,14). These overgrowth cells contained a great amount of undifferentiated human-specific NESTIN+ cells and enlarged the host brain.

To apply iPSC-based transplantation therapy in clinical safely, many efforts have been input to prevent the tumor-like overgrowth. Removing remnant immature NS/PCs or differentiate these cells into more mature cell types may help to avoid tumor like over-growth following transplantation. For instance, the physiological medium (BrainPhys basal + serum-free supplements) for adjusting the concentrations of inorganic salts, neuroactive amino acids, and energetic substrates improved maturation and enhanced the proportion of synaptically active (15), which reduced tumor-like overgrowth. Alternative efficient way is to find the key signaling controlling the induction and differentiation of NS/PCs. Inhibition of Notch signaling with a g-secretase inhibitor (GSI) was shown to be able to induce the NS/PCs to develop into a more mature state with limited proliferation in vitro (14,16). In addition, iPSC-derived dopaminergic progenitor cells treated with GSIs prior to transplantation may control the growth of a potentially proliferative cell population in vivo (16). However, the GSIs caused detrimental effects in patients with Alzheimer’s disease and the toxic side effects were main concerns for applying this tool compound in clinical (17).

It is important to find a way to optimize the induction and differentiation of NS/PCs. Azidothymidine (3’-azido-3’-deoxythymidine, AZT), a telomerase inhibitor, could inhibit the telomerase reverse transcriptase (TERT) and interrupted the cell proliferation (18). Our previous study showed that AZT disrupted the proliferation of adult neural stem cells in the SVZ and hippocampus in mice without causing cell damage or apoptosis (19,20). However, the effects of AZT in hPSC-derived neurons have not been explored yet. In this study, we showed that the telomerase inhibitor AZT suppressed the proliferation of hPSC-derived neural progenitors, promoted the differentiation of hPSC-derived cortical neurons and enhanced the maturation of hPSC-derived neurons. Furthermore, we also found that AZT pre-treated hPSC-derived precursors inhibited the proliferation and promoted the differentiation of cortical neurons in vivo.

Results

AZT inhibited the proliferation of hPSC-derived neural progenitors

To determine whether AZT affected the proliferation of hPSCs, we differentiated hPSCs to dorsal telencephalic neurons spontaneously (Fig. 1a). The hPSC-derived neural progenitors were examined for neural stem cell markers and new born neuron marker including SOX, NESTIN, and DCX (Supplementary Fig. 1). Most hPSC-derived neural progenitors were positive for these markers. Next, the hPSC-derived neural progenitors were
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treated with 20μM or 100μM AZT respectively from day 22-25. At day 26, the neural progenitors were dissociated and plated for the proliferation assays (Fig. 1a). Intriguingly, 100μM AZT reduced the size of neurospheres significantly (Fig. 1b). The cell proliferation was also measured by EdU and KI67 labelling. We found that 100μM AZT treatment reduced the EdU labeled cells from 16.6% (control) to 9.7% (Fig. 1c and d) and the KI67+ cells from 16.8% (control) to 7.3% (Fig. 1e, f). Furthermore, the neural progenitor markers SOX2 (CON: 42.4%; AZT 20: 17.3%; AZT 100:7.7%) and PAX6 (CON:90.3%; AZT 20: 80.3%; AZT 100:71.6%) were dramatically decreased by AZT treatment at day 28 (Fig. 1g-j). Our results indicated that hPSC pre-treated with AZT for 3-6 days suppressed hPSC-derived neural precursor proliferation effectively. In addition, we tested the effect of AZT on hiPSC-derived neural progenitors (iPS name: DS2U), and the similar results showed that AZT inhibited the proliferation of neural progenitors derived from hiPSC (Supplementary Fig.2a, b and c).

We also compared the effect of AZT with current known tool compounds for enhancing the differentiation of neurons. The effect of AZT and DAPT (GSI N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycinet-butyl ester) were tested in our system. Consistent with the previous report (16), DAPT inhibited the proliferation of neural progenitors. Here, we found AZT showed the similar effect of inhibition with DAPT (Supplementary Fig.2d).

AZT inhibited the proliferation of hPSC-derived neural progenitors through the decrease of telomerase activity

To understand the potential mechanism of AZT suppressed hPSC-derived neural precursor proliferation, we firstly tested whether AZT suppressed hPSC-derived neural precursor proliferation was due to the AZT caused cell death. We measured the cell death by TUNNEL and CCK assay and we showed that AZT treatment did not induce the cell death in vitro (Fig. 1k, l), which suggested that the AZT suppressed cell growth was not through killing hPSC-derived cells.

To explore whether AZT inhibited the activity of telomerase in hPSC, we tested the telomerase activity in AZT treated hPSC-derived neural progenitors. Cells with AZT treatment showed a significantly decrease of telomerase activity (Supplementary Fig.3a). Furthermore, we found that AZT inhibited the expression pattern of telomerase associated genes hTEP1, hTER, TERF1, and TERF2IP, as well as DNA binding and mitotic cell cycle associated genes DDIT3, CCNA1, and CDKN1A, compared to the controls (Supplementary Fig.3b).

Taken together, our results showed that AZT significantly inhibited hPSCs-derived cell proliferation through inhibiting telomerase activity, but not inducing cell death.

AZT promoted the differentiation and maturation of hPSC-derived cortical neurons

Cell differentiation and maturation were critical aspects for disease modeling and cell therapy of hPSC-derived cortical neurons. We investigated whether AZT affected the differentiation of hPSC-derived cortical neurons. The dorsal telencephalic progenitors were treated with 100μM AZT for 6 days and were plated for differentiation analysis. The TBR2+ cortical progenitors were dramatically increased from 25.4% to 56.75% at day 28 (Fig. 2a, b).
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In addition, the markers of cortical layers: TBR1 (CON: 50.1%; AZT: 74.2%), CTIP2 (CON: 38.5%; AZT: 66.2%), and SATB2 (CON: 13.9%; AZT: 39.2%) were also significantly increased by AZT treatment (Fig. 2c-h). Our results suggested that AZT significantly enhanced the differentiation of cortical neurons. We also examined whether AZT promoted the generation of astrocytes. However, we only found a small population of hESC-derived GFAP positive cells (less than 1%) at day 80 by following previously methods (5) (Supplementary Fig. 4), which were consistent with the previous reported works that human glial cells require long time to differentiation. Thus, the effect of AZT on the differentiation of astrocytes was still unclear.

Next, we asked whether AZT promoted the maturation of hPSC-derived neurons. After 6 days AZT treatment (3 days before and after cell plating on day 26), the percentage of TUJ-1+ (a marker for neurons) and MAP2+ (a marker for mature neurons) cells were increased by AZT treatment at day 35 (Fig. 3a, b, c). The length of neurites (CON: 88.22μm; AZT 20: 107.40μm; AZT 100: 118.80μm), numbers of primary branches (CON: 2.94; AZT 20: 4.25; AZT 100: 4.47) and average length of the primary neuritis (CON: 67.33μm; AZT 20: 67.83μm; AZT 100: 96.57μm) were significantly increased (Fig. 3d, e, f), which indicated the increased maturation in neural morphology in AZT treated group. To further test the maturation of the neurons, we measured vGLUT1 (a marker for glutamatergic transporter) and Synaptophysin (a pre-synaptic marker) at day 95. The vGLUT1+ puncta and vGLUT1+ puncta among Synaptophysin+ puncta were significantly increased by AZT treatment (Fig. 3g, i, h, j). The density of Synaptophysin+ puncta was also significant enhanced by AZT treatment (Fig. 3k). In addition, we tested whether AZT affected the generation and maturation of ventral GABAergic neurons. The iPSC-derived ventral progenitors were treated with AZT (our established protocol (21), iPS name: ihtc). Similar to cortical progenitors, the percentage of Ki67+ cells significantly decreased and MAP2+ positive neurons significantly increased (Supplementary Fig. 5). Thus, AZT also promoted the maturation of GABAergic neurons significantly.

Electrophysiological characteristics correlated well with the maturation status of neurons. Thus, we examined the electrophysiological activities of AZT treated neurons. The whole-cell clamp was performed on 7-10 days-plated neurons. The inward and outward currents were recorded by using voltage-clamp steps from -80 mV to 60 mV (Fig. 3m). The membrane input conductance (CON: 6.94 nS; AZT 10.82 nS) and amplitude of Na+ currents (CON: 470.6pA; AZT 823.2pA) & K+ currents (CON: 18.49pA; AZT 55.96pA) were enhanced by AZT treatment (Fig. 3n, o, p). Current-clamp recordings show that current injection (10 pA) elicited significantly more and larger action potentials (APs) in AZT-treated neurons (CON: 13.1mV; AZT: 31.7mV), suggesting that these neurons are functionally more mature than the neurons from the control group (Fig. 3q, r, s). Taken together, AZT promoted the maturation of hPSC-derived neurons in vitro.

AZT pre-treated hPSCs did not show cell overgrowth after transplantation in vivo

Cell overgrowth in post transplantation caused the high risk for tumor formation in human and was a major concern for hPSC-based cell therapy. The effective inhibition of cell overgrowth post
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transplantation is essential for translating cell therapy to clinic. Therefore, we tested whether AZT inhibited the cell overgrowth \textit{in vivo}. We pretreated hPSC-derived neural progenitors with AZT prior to transplantation, and measured the cell proliferation and differentiated progeny 4 weeks post injection (Fig. 4a) (four mice in each group). The brain weight among each group did not show significant differences and no tumor formation was observed for all hPSCs transplanted mouse brains (Supplementary Fig. 6a). The transplanted hPSC-derived neurons survived in mice brains, and most cells showed a dispersed distribution (Fig. 4b). Among dispersed cells, KI67 was rarely detected in HN+ (human nuclei, a marker for human nuclei) cells (less than 1%) (Fig. 4b) and no significant difference was found between two groups. However, 5% area of grafts showed the rosette structure, indicating immature neural progenitors (Fig. 4c). Thus, we analyzed the cell proliferation among the rosette structures. The percentage of PH3 (a marker expressed at the late stage of G2 phase and the entire course of M phase during cell division) in HN+ cells was reduced to 62.3% with the AZT treatment in comparison with 80.8% in control group (Fig. 4c, d). In addition, the KI67+ cells dramatically decreased upon AZT treatment (Fig. 4e, f). The proportion of PROX1+ (a marker for hippocampal granule cells) cells were increased from 22.03% to 45.11% (Fig. 4g, h), which indicated more grafted cells became postmitotic upon AZT treatment. For 10 weeks post-transplantation observation (four mice in each group), we found most rosette structures were barely observed and the percentage of KI67+ cells among HN+ cells in AZT pre-treated group was dramatically decreased to less than 1% (Fig. 4, i), while there were still 2.9% KI67 positive cells in control group. Altogether, the AZT pre-treated hPSC-derived neurons showed the decrease of proliferation in mice brain post transplantation, which suggested AZT may effectively improve the safety of hPSC-based cell therapy.

\textbf{AZT pretreatment enhanced the differentiation of cortical neurons from hPSC-derived cortical progenitors \textit{in vivo}}

The hPSC-based cell treatment also required that hPSCs can differentiate into certain types of neurons, such as cortical neurons. Therefore, we evaluated the differentiation of grafted cortical neurons \textit{in vivo} 4 weeks post transplantation. Most transplanted human cells co-expressed HN and TUJ1 (Fig. 5a). AZT pre-treatment significantly reduced the percentage of PAX6 (CON: 48.4%; AZT: 9.45%) and FOXG1 (CON: 50.1%; AZT: 29.0%) in the graft area (Fig. 5b, c, d, e), which indicated the decrease of neural precursor cells. In contrast, the cortical layer markers, TBR1 (CON: 49.2%; AZT: 66.3%) and CTIP2 (CON: 52.9%; AZT: 73.1%), were significantly increased in AZT pre-treated group, which suggested that AZT promoted the differentiation of cortical neurons post transplantation (Fig. 5f, g, h, i). Taken together, AZT pre-treatment significantly suppressed hPSC-derived neural precursor proliferation and promoted neural differentiation \textit{in vivo}.

\textbf{Discussion}

In this study, we discovered the effects of a small molecule AZT, the telomerase inhibitor, on cell proliferation and neural differentiation of human hPSC-derived neurons. AZT inhibited the proliferation of hPSC-derived neural progenitors, promoted the differentiation of hPSC-derived cortical neurons and enhanced the maturation of
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hPSC-derived neurons (Fig. 6). AZT pretreatment also significantly reduced cell proliferation post transplantation, but increased mature neurons in vivo (Fig. 6).

Few studies have been reported to study the function of telomerase in regulating the proliferation and neural differentiation of human hPSC-derived neural cells. AZT interrupted the cell proliferation by inhibiting the telomerase reverse transcriptase (TERT). Previous work showed that AZT disrupted the proliferation of adult neural stem cells in the SVZ and hippocampus in mice without causing cell damage or apoptosis (19,20). Our study showed that AZT reduced the undifferentiated cells from hPSC-derived neural progenitors and promoted neural differentiation and maturation effectively both in vitro and in vivo. However, the mechanism of AZT promoting neural differentiation is still not clear. AZT incorporated into DNA in place of thymidine, terminated the early DNA synthesis and inhibited reverse transcriptase activity during viral DNA synthesis. AZT is also an effective telomerase inhibitor. It may be due to AZT caused the inhibition of cell proliferation or the important role of telomerase in neural differentiation. More efforts are needed to understand how AZT regulating the neural differentiation and the function of telomerase in hPSCs derived neural differentiation.

Previous studies showed that inhibiting Notch signaling promoted neural differentiation of NPCs derived from human iPSCs (14,16). γ-Secretase inhibitors (GSIs) inhibited Notch signaling in human-induced pluripotent stem cell-derived neural progenitors. A marked reduction in the percentage of dividing cells and increased neural maturation were detected in GSI-treated samples in vitro. Grafts from hPSCs pretreated with GSIs were significantly smaller. GSIs not only reduced the graft volume, but also altered the composition of the graft. GSI-treated samples developed into mature neural grafts. Improved cell quality and safety, particularly with respect to the risk of tumor-like overgrowth, is crucially important for any clinical application of hiPSC-NS/PCs. However, the application of GSI caused detrimental effects in AD patients (22). The toxic side effects for GSI were mainly ascribed to the inhibition of the physical functions of γ-secretase, such as Notch signaling pathway. In clinical trials, the severe side effect of GSI treatment was the worsening the memory in patients (22). It also increased the risk of skin cancer, which also likely resulted from the Notch signal inhibition (23). In comparison with GSI, AZT is a safe and efficacious antiretroviral drug that is recommended to reduce risk of HIV vertical transmission in pregnant women by medical guidelines (24,25). AZT is a widely used drug to prevent or treat HIV/AIDS and commonly used in pregnancy clinically and appears to be safe for infant, suggesting it could be safe to the developmental organs. Moreover, we treated AZT in cell culture system before transplantation and this in vitro application would not be harmful. However, with the AZT treatment, telomerase may have the potential to be translocated from the nucleus to mitochondria after induction by high dosage of AZT to cause the potential mitochondria damage (26). This effect might bring concerns for using as an anti-proliferative tool for the regenerative medicine in clinic. Beside cell transplantation therapy, dedifferentiation in central nervous system for neural regeneration may also cause cell overgrowth, treatment of redifferentiating cells with AZT
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may reduce the risk for tumor generation.

In conclusion, we firstly identified a small molecule AZT played an important role in reducing proportion of dividing cells and increasing neural maturation (Fig. 6). AZT also prevented the tumor-like overgrowth of transplanted cells by inhibiting cell proliferation and promoting the neural differentiation, which may lead the safe and long-lasting functional recovery in vivo. AZT would be a powerful tool to sculpt the hPSC derived neural differentiation process and improve the safety of hPSC transplantation therapy.

Experimental procedures

Cell culture

Human PSCs (H9, passages 35 to 67, WiCell Agreement No.16-W0060; DS2U, passages 56 to 62, which was kind gifts of Dr. Anita Bhattacharyya(27), Waisman Center and WiCell Research Institute) were used for in vivo and in vitro studies. hPSCs were cultured under feeder free conditions that coating with vitronection (Thermo Fisher Scientific), and maintained in the Essential 8 medium (Thermo Fisher Scientific) as previously described (28). Cells were passaged every 4-5 days at 80% density by using EDTA (Lonza) for 1 minute.

Derivation of cortical neurons from hPSCs

To induce cortical neurons, we used the previously described procedure (21,29). Briefly, hESC clones were detached by Dispase (Thermo Fisher Scientific) to form embryoid bodies (EBs), and cultured in flask with neural induction media (NIM) (Day 0). At day 7, EBs was attached on 6-well plates. Rosette structures can be observed during day 10-16. At day 16, rosettes was detached by using a 1 ml pipette manually. Neuroepithelial cells were transferred into a new flask and floating cultured with NIM. At day 28, neurospheres were dissociated into single cells by using TripLE (Thermo Fisher Scientific) and plated on matrigel (BE Biosciences) & poly-l-ornithine (Sigma) pre-coated coverslips at a density of 50,000 cells/coverlip. Neurons were treated with AZT during Day 22 to 25. After plating, AZT was still remaining in the differentiated medium until day 28.

Transplantation

SCID (severe combined immune deficient) mice were purchased from the Model Animal Research Center (MARC) of Nanjing University. Mice were cared for following local ethics legislation for animal experimentation. Animal welfare and experimental procedures were conducted with the approval of the Institutional Animal Care and Use Committee of MARC in Nanjing Medical University. For cell transplantation, day 42 neurospheres were broken into small clusters and suspended in 10~20 μl NIM with B27 (1:50, Thermo Fisher Scientific) & Penicillin (1:100, Thermo Fisher Scientific) as previously described (4,8). The P0 SCID mice were randomly divided into 2 groups and anesthetized on ice for 5 second. The mouse was injected with approximately 10,000 AZT-treated cells (neurospheres were treated with AZT during Day 22 to 29) or non-treated cells separately in basal forebrain using a pulled glass micropipette. After injection, mice were put on pre-warmed cushion for 15 minutes and then were put back to the cages. Four weeks or ten weeks after transplantation, animals were perfused with 4% paraformaldehyde.
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TUNEL Assay & Edu Cell Proliferation Assay

For TUNEL assay, cortical neurons on coverslips were fixed in 4% paraformaldehyde. By using Roche In Situ Cell Death Detection Kit (Roche), apoptotic cells could be detected. Edu assay was performed by using Click-iT® EdU Alexa Fluor® Imaging Kit (Thermo Fisher Scientific). At day 28, we treated cells with Edu solution and incubated cells for 4 hours, then fixed cells for Edu detection. DNA was stained with Hoechst.

Cell Counting Kit-8 assay

Transfer cell suspension (100ul/well, about 2,000 cells) in a 96-well plate. Add 10ul of the CCK-8 (Beyotime C0037) solution to each well of the plate. Incubate cells in the incubator for 0.5-4 hours. Measure the absorbance at 450 uM using a microplate reader.

Immunocytochemistry

Cortical neurons on coverslips were fixed in 4% paraformaldehyde for 30 minutes and washed with phosphate buffered saline 3 times. Brain tissues were fixed in 4% paraformaldehyde, frozen and sliced into 35-μm-thick slices with Leica CM1900 rapid sectioning cryostat. Immunostaining was performed as previously described methods(4). Nuclei were stained with Hoechst. The primary antibodies used in this study included KI67(1:500, rabbit IgG, Zymed), SOX2(1:1000, rabbit IgG, Abcam), PAX6(1:1000, rabbit IgG, DSHB), TUJ-1(1:1000, mouse IgG, Sigma-Aldrich), TBR2(1:500, rabbit IgG, Abcam), TBR1(1:500, rabbit IgG, Abcam), CTIP2(1:500, rat IgG, Abcam), SATB2(1:500, mouse IgG, Abcam), VGLUT1(1:500, mouse IgG, Synaptic Systems), SYNAPTOPHYSIN(1:500, rabbit IgG, Abcam), PH3(1:1000, rabbit IgG, Millipore), PROX1(1:2000, rabbit IgG, Abcam), HN(1:500, mouse IgG, Millipore) and FOXG1(1:1000, rabbit IgG, Abcam). Coverslips were observed from Nikon TS100 fluorescence microscope.

Neuron morphology analysis

Neurons that expressed MAP2 were randomly selected for morphology analysis. The longest neuritis’ pixel length was measured by Image J. Numbers of primary branches were counted manually. At least 60 neurons were analyzed randomly in every group.

Electrophysiology

Day 30 hPSC-driven neurons were cultured under feeder free system on matrigel pre-coated coverslips. Neurons were observed under an inverted microscope with a 60X water-immersion objective in differential interference contrast mode. Whole-cell patch clamp recordings were made with Olympus microscope (BX51WI), MultiClamp 700B and Digidata 1550B (Axon). Na+/K+ currents were recorded in voltage-clamp mode at a holding potential of -80 mV with step-voltage changes (15 steps, from -80 mV to 60 mV). Action potentials were recorded in current-clamp mode with membrane potentials be maintained around -60 mV. And a series of step currents (15 steps, with 10 pA increment) were injected to elicit action potentials. The pipettes were pulled by pipette puller P-1000 (Sutter). The resistance of pipettes ranged from 8-10 MΩ were filled with internal solution (in mM): 130 K-Gluconate, 10 KCl, 10 EGTA, 2 MgCl₂, 0.3 NaGTP, 2 Na₂ATP, 10 HEPES (280 mOsm, pH=7.3). The bath solution contained (in mM): 145 NaCl, 1 CaCl₂, 5
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KCl, 1 MgCl₂, 5 HEPES, 5 glucose (280 mOsm, pH=7.3). Data analysis was performed using Clampfit software (Axon).

Quantification and Statistical analysis
The quantification of fluorescent images was analyzed by Image J (v1.50i). At least 9 fields of each coverslip were chosen randomly and counted manually. Three coverslips in each group were counted. For brain slices, at least 3 fields were chosen. Statistical analyses were performed using Graphpad Prism (v 6.01). One-way ANOVA analysis and student’s tests were performed to statistically analyze the results. P<0.05 was considered to be significant. All graphical data were presented as mean±SEM.

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Author contributions
Y.H., YY.C. and Y. L. designed the experiments; KH.F., LP.S., SY.C, F.Y., Y.S., M.X. and Y.H. performed the experiments; Y.H., KH.F., YY.C. and Y. L. analyzed the data; YY.C. and Y. L. wrote the paper.

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**Figure legends**

**Figure 1. AZT inhibited the proliferation of hPSC-derived neural progenitors**

(a) A brief time line of human stem cell differentiation for generating cortical neurons. (b) The Bright-field of neurospheres. 100μM AZT treatments significantly reduced the diameter of neurospheres. Bar=250μm (c, d) Examine of EdU incorporation in adherent cells at day 28. 100μM AZT treatments significantly reduced the percentage of EdU incorporated cells (EdU/HO). (e, f) Immunostaining of adherent cells at day 28 for KI67. 100μM AZT treatments significantly reduced the percentage of KI67 positive cells (KI67/HO). (g, h) Immunostaining of adherent cells at day 28 and day 35 for SOX2. 20μM and 100μM AZT treatments significantly reduced the percentage of SOX2 positive cells (SOX2/HO) at day 28. AZT treatments abolished SOX2 expression in cells at day 35. (i, j) Immunostaining of adherent cells at day 28 for PAX6. 100μM AZT treatments significantly reduced the percentage of PAX6 positive cells (PAX6/HO). (k) Labeling of adherent cells at day 28 for TUNEL. Neither 20μM nor 100μM AZT treatments increased the percentage of TUNEL positive cells (TUNEL/HO). (l) Cell counting kit-8(CCK8) assay of adherent cells at day 28. Neither 20μM nor 100μM AZT treatments increased the OD value of CCK8 test. CON: n=5; 20μM AZT: n=5; 100μM AZT: n=6. *P<0.05; **P<0.01. Bar=100μm.

**Figure 2. AZT promoted the differentiation of hPSC-derived cortical neurons**

(a, b) Immunostaining of adherent cells at day 28 for TBR2 and TUJ-1. 100μM AZT treatments significantly enhanced the percentage of TBR2 positive neurons. (c, d) Immunostaining of adherent cells at day 35 for TBR1 and TUJ-1. 100μM AZT treatments significantly enhanced the percentage of TBR1 positive neurons. (e, f) Immunostaining of adherent cells at day 55 for CTIP2 and TUJ-1. 100μM AZT treatments significantly enhanced the percentage of CTIP2 positive neurons. (h, i) Immunostaining of adherent cells at day 85 for SATB2 and TUJ-1. 100μM AZT treatments significantly enhanced the percentage of Satb2 positive neurons. CON: n=4; 100μM AZT: n=6. *P<0.05. Bar=100μm.

**Figure 3. AZT promoted dendritic and synaptic maturation of neurons**

(a) Immunostaining of adherent cells at day 35 for MAP2 and TUJ-1. (b,c) 20μM and 100μM AZT treatments significantly enhanced the percentage of TUJ-1 positive cells and MAP2 positive neurons (MAP2/HO and MAP2/TUJ-1) at day 35. (d) Representative image for MAP2 immunostaining in adherent cells at day 35. Cells equally expressed MAP2 were subjected to morphological analysis. (e) 100μM AZT-treated cells significantly increased length of the longest neurites in D95 neurons. (f) 20μM and 100μM AZT-treated neurons showed more primary branches at day 95 compared to control group neurons. (g, h)
AZT enhances the differentiation of hPSC-derived neurons

Immunostaining of adherent cells at day 95 for vGLUT1, TUJ-1 and SYNAPTOPHYSIN. (e, f, g) (i) 100μM AZT-treated neurons showed significantly increased vGLUT1 puncta. (j) 20μM and 100μM AZT-treated neurons enhanced vGLUT1 and SYNAPTOPHYSIN co-labeled puncta significantly. (k) 100μM AZT-treated neurons showed significantly the increase of SYNAPTOPHYSIN puncta. (l) 100μM AZT-treated neurons showed significantly enhance of average length of the primary neuritis. (m) Na+/K+ currents were recorded in voltage-clamp mode by step depolarizations from -80 to 60 mV from a holding potential of -80 mV. (n) Membrane input conductance by calculating linear function of injected currents response against membrane potential. (o) Peak INa showed slightly enhancement in AZT pre-treated group than control group. (p) Peak IK showed significantly increase in AZT pre-treated group than control group. (q) Representative current-clamp recordings in control group and AZT pre-treated neurons. (r) The numbers of action potentials in response to current injection. CON: n=4; 20μM AZT: n=4; 100μM AZT: n=5. *P<0.05. Fig A: Bar=100μm; Fig D: Bar=50μm; Fig g, and h: Bar=25μm; SYN: SYNAPTOPHYSIN.

Figure 4. Characterization of human iPSCs derived grafts in mouse brain under AZT pretreatment

(a) Schematic diagram of iPSCs-derived neural progenitors’ transplantation to mouse brain. (b) Immunostaining of in non-rosette grafted area. (c, d) Immunostaining of grafted cells for the proliferative marker PH3 4 weeks post transplantation. Compared with control group, percentage of PH3 positive cells in grafts from 100μM AZT pretreated cells significant decreased. (e, f) Immunostaining of grafted cells for the proliferative marker KI67 4 weeks post transplantation. Compared with control group, percentage of KI67 positive cells in grafts from 100μM AZT pretreated cells significant decreased. (g, h) Immunostaining of grafted cells for PROX1 (a hippocampal granule cells marker) 4 weeks post transplantation. Compared with control group, percentage of PROX1 positive cells in grafts from 100μM AZT pretreated cells significant increased. (i, j) Immunostaining of grafted cells for the proliferative marker KI67 at 10 weeks post transplantation. CON: n=3; AZT: n=4. *P<0.05. Bar=100μm.

Figure 5. AZT pre-treatment promoted cortical differentiation in iPSCs-derived grafts

(a) Representative view of immunostaining for TUJ-1 in HuNuc positive grafts. Level of TUJ-1 expression was significantly higher in grafts than host area. (b, f) Immunostaining of grafted cells for PAX6: the early stage marker of neural progenitors 4 weeks post transplantation. Compared with control group, the percentage of PAX6 positive cells in grafts from 100μM AZT pretreated cells significant decreased. (c, g) Immunostaining of grafted cells for the early stage forebrain marker: FOXG1 4 weeks post transplantation. Compared with control group, percentage of FOXG1 positive cells in grafts from 100μM AZT pretreated cells significant decreased. (d, h) Immunostaining of grafted cells for the marker of deeper cortical layer TBR1 10 weeks post transplantation. Compared with control group, percentage of TBR1 positive cells in grafts from 100μM AZT pretreated cells significant increased. (e, j) Immunostaining of grafted cells for the marker of upper cortical layer CTIP2 10 weeks post transplantation. Compared with control group, percentage of CTIP2 positive cells in grafts from 100μM AZT pretreated cells significant increased. CON: n=4; AZT: n=4. *P<0.05.
AZT enhances the differentiation of hPSC-derived neurons

Bar=100μm.

**Figure 6. Schematic depicts the procedure of this study.**
AZT, a telomerase inhibitor, inhibits the proliferation of hPSC-derived neural progenitors, promotes the differentiation of hPSC-derived cortical neurons and induces the maturation of hPSC-derived neurons both *in vitro* and *in vivo*. 
Figure 1

(a) Day 0 7 14 21 28

hESCs/hPSCs → Embryoid body → Neurosphere → Neurons

(b) Neurosphere

Vehicle AZT 20μM AZT 100μM

(c) CON AZT 20μM AZT 100μM

(d) Diameter of Neurospheres (μM)

(e) EdU / HO

(f) EdU / HO

(g) KI67 / HO

(h) KI67 / HO

(i) SOX2 / HO

(j) SOX2 / HO

(k) PAX6 / Tuj-1 / HO

(l) PAX6 / HO

(n.s.)

Cell Counting Kit-8 Test OD value
Figure 2

(a) HO TBR2 TUJ-1 Merge

(b) TBR2 / TUJ-1 (%)

(c) HO TBR1 TUJ-1 Merge

(d) TBR1 / TUJ-1 (%)

(e) HO CTIP2 TUJ-1 Merge

(f) CTIP2 / TUJ-1 (%)

(g) HO SATB2 TUJ-1 Merge

(h) SATB2 / TUJ-1 (%)

Legend:

- CON
- AZT

Scale bars: 100 μm

* p < 0.05
Figure 3

(a) HO/TUJ-1/MAP2

(b) TUJ-1/HO

(c) MAP2/HO

(d) MAP2

(e) Length of the longest neurites (μm)

(f) Numbers of primary branches per neuron

(g) HO/TUJ-1/VGLUT1

(h) HO/SYN/VGLUT1

(i) VGLUT1 puncta / 100μm

(j) VGLUT1 & SYNAPTOPHYSIN colabeled puncta / 100μm

(k) SYNAPTOPHYSIN puncta / 100μm

(l) Average length of the primary neurites (μM)

(m) Action potential Amplitude (mV)

(n) membrane input conductance (nS)

(o) INa (pA)

(p) IK (pA)

(q) Numbers of AP's Fired

(r) Action potential Amplitude (mV)
Figure 4

a. Diagram illustrating the timeline for stem cell culture, AZT treatment, cell transplant, and morphology observation.

b. Images showing HO/HN/Ki67 staining under CON and AZT conditions.

c. Images showing HO, HN, PH3, and merge staining under CON and AZT conditions for week 4.

d. Graph showing PH3/HN in rosettes area under CON and AZT conditions for week 4.

e. Images showing HO, HN, Ki67, and merge staining under CON and AZT conditions for week 4.

f. Graph showing Ki67/HN in rosettes area under CON and AZT conditions for week 4.

g. Images showing HO, HN, PROX1, and merge staining under CON and AZT conditions for week 4.

h. Graph showing PROX1/HN in rosettes area under CON and AZT conditions for week 4.

i. Images showing HO, HN, Ki67, and merge staining under CON and AZT conditions for week 10.

j. Graph showing Ki67/HN in rosettes area under CON and AZT conditions for week 10.
Figure 5

(a) HO, HN, TUJ-1, Merge

(b) HN, PAX6, Merge

(c) PAX6 / HN

(d) HN, FOXG1, Merge

(e) FOXG1 / HN

(f) HN, TBR1, Merge

(g) TBR1 / HN

(h) HN, CTIP2, Merge

(i) CTIP2 / HN
hPSCs

Neural precursors

In dish

Transplantation

Control

+AZT

Azidothymidine (AZT)

Inhibit proliferation

Promote maturation

Figure 6
The telomerase inhibitor AZT enhances differentiation and prevents overgrowth of human pluripotent stem cell derived neural progenitors
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