Selective Ablation of Tumorigenic Cells Following Human Induced Pluripotent Stem Cell-Derived Neural Stem/Progenitor Cell Transplantation in Spinal Cord Injury

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ABSTRACT

Tumorigenesis is an important problem that needs to be addressed in the field of human stem/progenitor cell transplantation for the treatment of subacute spinal cord injury (SCI). When certain “tumorigenic” cell lines are transplanted into the spinal cord of SCI mice model, there is initial improvement of motor function, followed by abrupt deterioration secondary to the effect of tumor growth. A significant proportion of the transplanted cells remains undifferentiated after transplantation and is thought to increase the risk of tumorigenesis. In this study, using lentiviral vectors, we introduced the herpes simplex virus type 1 thymidine kinase (HSVtk) gene into a human induced pluripotent stem cell-derived neural stem/progenitor cell (hiPSC-NS/PC) line that is known to undergo tumorigenic transformation. Such approach enables selective ablation of the immature proliferating cells and thereby prevents subsequent tumor formation. In vitro, the HSVtk system successfully ablated the immature proliferative neural cells while preserving mature postmitotic neuronal cells. Similar results were observed in vivo following transplantation into the injured spinal cords of immune-deficient (nonobese diabetic–severe combined immune-deficient) mice. Ablation of the proliferating cells exerted a protective effect on the motor function which was regained after transplantation, simultaneously defending the spinal cord from the harmful tumor growth. These results suggest a potentially promising role of suicide genes in opposing tumorigenesis during stem cell therapy. This system allows both preventing and treating tumorigenesis following hiPSC-NS/PC transplantation without sacrificing the improved motor function.

SIGNIFICANCE STATEMENT

Given the nature of stem cell therapy, it is impossible to be certain that a tumorigenic transformation will not occur following transplantation. However, clinicians/scientists are duty-bound to tackle the issue from multiple angles, working toward clinical implementation of this novel technology. Previous studies, aiming to tackle tumorigenesis after the transplantation has been performed, involved the eradication of all of the transplanted cells and the loss of the improved motor function as a result. This new approach, however, allows us to both prevent and treat tumorigenesis that occur post-transplantation without sacrificing the improved motor function which is definitely an improvement worth noting.

INTRODUCTION

Given the extraordinary speed at which stem cell research has evolved over the last decade, its application in regenerative medicine based on cell transplantation for the treatment of spinal cord injury (SCI) has been receiving increasing interest. Positive reports of functional recovery resulting from the transplantation of human induced pluripotent stem cell-derived...
neural stem/progenitor cells (hiPSC-NS/PCs) and other forms of neural stem cells (NSCs) into the spinal cords of subacute SCI model nonobese diabetic–severe combined immune-deficient (NOD/SCID) mice and nonhuman primates have indicated new possibilities for the use of this methodology in a clinical setting [1–9]. The first in-human clinical trial of hiPSC-NS/PC transplantation for the treatment of subacute SCI in Japan is expected to begin within the next 3 years, and public interest is increasing [10, 11].

However, the issue of safety is one of the greatest concerns for both clinicians and patients in the clinical application of any novel treatment, and the matter of tumorigenesis is an issue that is commonly referenced in association with stem cell therapy [12–14]. The gold standard for the treatment of any spinal cord tumor is the multimodal approach involving chemotherapy, radiotherapy, and surgical resection, which also applies in the case of tumorigenesis following stem cell transplantation. However, concerns related to this approach include the adverse iatrogenic effects associated with this treatment and the possibility of the patient being left with an increased degree of disability due to the resulting damage. Several strategies are being explored in an effort to minimize the risk of tumorigenesis before cells are transplanted. These strategies include the selection of target cells using surface antigen markers [15], the use of glucose-depleted culture media [16], and pretreatment with gamma secretase inhibitors [17]. However, although tumorigenesis following iPSC-NS/PC transplantation is not frequently reported [8, 9], it is difficult to completely eliminate uncertainty given the pluripotent nature of iPSCs. When certain lines of hiPSC-NS/PCs (e.g., 253G1, produced through retroviral transduction) are transplanted into the injured spinal cord of murine SCI models, significant improvements in motor function are observed over an initial period of 6 weeks post-transplantation, which is then followed by an abrupt deterioration secondary to the effect of tumor growth [17–20]. One interesting observation recorded for this cell line is a high proportion of immature Nestin-positive cells that remain in the spinal cord long after the initial improvements in motor function are achieved. Given that the motor function appears to reach a plateau around 6 weeks following transplantation, one could argue that any residual NS/PCs that maintain “stemness” not only contribute little to functional recovery but also increase the risk of tumorigenesis in the future.

One major difference between the targeting of ordinary primary tumors and stem cell-derived tumors resides in the fact that it is possible to tag or mark the cells prior to transplantation. There have been major breakthroughs in the world of cancer research with the introduction of suicide gene therapy (gene-directed enzyme prodrug therapy), promising a safer and more targeted approach to cancer treatment. In this approach, a therapeutic transgene that is introduced into a tumor through a viral vector converts the nontoxic prodrug into cytotoxic products, thereby inducing cell death. Clinical application of suicide genes has been investigated in several cancers, including cancers of the brain, spinal cord, colon, liver, lung, prostate, ovary, and breast, with many encouraging findings [21–32]. Current limitations arise from the difficulties in infecting all target cells with the transgene [31]. There are many advantages in targeting only the transplanted cells, and suicide genes may play a role in achieving this aim. Several options are available when selecting the appropriate suicide gene for ablating tumorigenic cells derived from transplanted cells [18, 19, 31]. We recently reported the use of the inducible caspase-9 (iCaspase9)/CID system to address post-transplantation tumorigenesis and demonstrated the possibility to eradicate transplanted cells using suicide genes [18]. Although this system has the potential to dramatically improve the safety of stem cell transplantation, there is one unfortunate downside, in that the improvement of motor function associated with the transplantation also tends to deteriorate after triggering the suicide gene because all of the transplanted cells, including mature functional cells, undergo apoptosis.

The herpes simplex virus type 1 thymidine kinase (HSVtk) gene is another well-known and widely used suicide gene for which several clinical trials are underway [33–40]. Unlike cellular thymidine kinase, HSVtk exhibits broad substrate specificity, including pyrimidines, purines, and their analogs. Ganciclovir (GCV), the prodrug of HSVtk, can be converted to cytotoxic GCV-triphosphate by HSVtk, thereby killing HSVtk-expressing cells. The HSVtk/GCV system causes a delay in S and G2 phases, resulting in apoptosis due to delayed proliferation [31]. This system is therefore cell cycle dependent, which implies that, when applied to our SCI model along with hiPSC-NS/PC transplantation, immature mitotic neuronal cells as well as proliferative and clonogenic tumor cells can be ablated while sparing mature postmitotic neuronal cells; whereas, the iCaspase9/CID system was designed to target all transplanted cells, the HSVtk/GCV system can selectively target cells that are actively multiplying, given its cell cycle-dependent nature.

Although the use of suicide genes to eradicate tumor cells has been widely investigated in the past, the potential of this strategy to prevent tumor formation following stem cell transplantation has rarely been exploited. Therefore, the aim of this study was to use of the HSVtk gene to selectively ablate undifferentiated cells, which increase the risk of tumorigenesis, while preserving fully differentiated cells, which contribute to functional recovery.

**MATERIALS AND METHODS**

**Lentiviral Vector Preparation**

HSVtk cDNA that had been modified by humanizing codon usage and eliminating all CpG dinucleotides was purchased from InvivoGen (San Diego, CA) and then cloned into the tetracycline (Tet)-inducible lentiviral vector CSIV-RFA-TRE-EF-KT (Fig. 1A) [41]. Recombinant lentiviral vector production and titer determination were performed as described previously [42].

**Lentiviral Transduction of 253G1-hiPSCs and Cell Viability Assay**

253G1-hiPSCs [43] (provided by Prof. Shinya Yamanaka at CiRA, Kyoto University) were transduced with the Tet-inducible HSVtk lentiviral vector at a multiplicity of infection (MOI) of 2–10. Almost 100% transduction efficiency was observed based on examining humanized Kusabira-Orange 1 fluorescent protein (hK01) [44] expression under a fluorescence microscope. Single hKO1-positive iPSCs were sorted using the FACSAria III system (BD Biosciences, San Jose, CA) and then expanded. 253G1-hiPSCs expressing Tet-inducible HSVtk (HSVtk-hiPSCs) were dissociated into single cells, seeded in 96-well plates at a density of 5 x 10^3 cells/200 μl per well with
Neural Induction of HSVtk-hiPSCs

Neural induction was performed as described previously [19] with slight modifications. To produce HSVtk-hiPSC-NS/PCs, embryoid bodies (EBs) were generated from HSVtk-hiPSCs grown in suspension in bacterial culture dishes without fibroblast growth factor 2 (FGF-2) for 4 weeks. The EBs were then dissociated into single cells using TrypLE Select (Thermo Fisher Scientific, Yokohama, Japan) and cultured in suspension at 1 × 10⁵ cells per milliliter in media containing a hormone mix- 

Neural Differentiation Analysis

Dissociated fourth-passage HSVtk-hiPSC-NS/PCs were plated in poly-γ-ornithine/fibronectin-coated 8-well chamber slides (Thermo Fisher Scientific) at a density of 8.0 × 10⁴ cells per milliliter and cultured in medium without growth factors at 37°C under 5% CO₂ and 95% air for 28 days in total. Four sets were prepared for analysis. Cells in the chambers of two of the four sets were treated with 2 μg/ml DOX and 3 μg/ml GCV during the final 7 days (GCV[+]). The other two sets were treated only with 2 μg/ml DOX (GCV[−]). Differentiated cells were fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS) and stained with the following primary antibodies: anti-Nestin (mouse immunoglobulin G [IgG], 1:200; Merck KGaA, MAB5326), anti-Ki67 (rabbit IgG, 1:200; Abcam, Cambridge, U.K., ab15580), and anti-β-III Tubulin (mouse IgG2b, 1:300; Sigma–Aldrich, St. Louis, MO, T8660). Nuclei were stained with Hoechst 33258 (10 μg/ml; Sigma–Aldrich). All in vitro images were obtained using a confocal laser scanning microscope (LSM 700; Carl Zeiss, Jena, Germany). One hundred cells stained with Hoechst 33258 were randomly counted from each well, and Nestin+, Ki67+, and β-III Tubulin-positive cells were counted.

Lentiviral Transduction of 253G1-hiPSC-NS/PCs

Primary neurospheres differentiated from 253G1-hiPSCs were dissociated and transduced with the Tet-inducible HSVtk lentiviral vector at an MOI of 2. Then, secondary neurospheres were dissociated and transduced with the lentiviral vector expressing ffLuc (a Venus [an improved yellow fluorescent protein [45]] and firefly luciferase fusion gene) under the control of the human elongation factor 1α subunit promoter (EF-1α) at an MOI of 2 [46]. These secondary neurospheres were pas- 

SCI Model and iPSC-NS/PC Transplantation

Female 8-week-old immunodeficient NOD/SCID mice (19–22 g, n = 32) were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Laminectomy was performed at the 10th thoracic spinal vertebra to expose the dorsal surface of the dura mater. Contusive SCI was induced at the level of Th10 using an IH impactor (60 kdyn; Precision Systems and Instrumentation, Fairfax Station, VA). Nine days after the injury, 5 × 10⁵/2 μl HSVtk-hiPSC-NS/PCs were transplanted into the lesion epicenter in 16 mice using a glass pipette at a rate of 1 μl/minute with a 25-μl Hamilton syringe and a stereotaxic microinjector (KS 310; Muromachi Kikai, Tokyo, Japan). Using the same method, eight mice were injected with 5 × 10⁵/2 μl 253G1-hiPSC-NS/PCs (fourth passage neurospheres that were not transduced with the Tet-inducible HSVtk lentiviral vector; Control group), and eight mice were injected with PBS (PBS group). All experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Keio University (Assurance No. 13020) and the Guide for the Care and Use of Laboratory Animals (NIH). All surgeries were conducted under anesthesia, and efforts were made to minimize animal suffering, with selected humane endpoints.

Motor Function Analysis

Hindlimb motor function was evaluated using the Basso mouse scale (BMS) locomotor rating scale on a weekly basis. Skilled
investigators who were blinded to the treatment conducted the behavioral analysis. The gait performance of the mice in each group was also analyzed through treadmill gait analysis (DigiGait system; Mouse Specifics, Framingham, MA) and the rotarod performance test at 91 days after SCI.

Bioluminescence Imaging
A Xenogen-IVIS spectrum cooled charge-coupled device optical macroscopic imaging system (PerkinElmer, Waltham, MA) was used for bioluminescence imaging (BLI) to confirm the survival of the transplanted hiPSC-NS/PCs. Monitoring was performed once a week following cell transplantation. In brief, α-luciferin (VivoGlo Luciferin; Promega, Madison, WI) was administered via intraperitoneal injection at a dose of 300 mg/kg body weight. Animals were placed in a light-tight chamber, and photons emitted from luciferase-expressing cells were collected with integration times of 5 seconds to 2 minutes, depending on the intensity of bioluminescence emission. BLI signals were quantified in maximum radiance units (photons per second per centimeter squared per steradian) and presented as log10 (photons per second) values.

Induction of Apoptosis
All mice were fed an autoclaved rodent diet with added DOX (0.0625%) starting 42 days after transplantation for a period of 22 days. Mice in the GCV(+) group, Control, and PBS groups (n = 8 each) were treated with intraperitoneal injection of GCV (50 mg/kg) starting 43 days after transplantation for 21 days. GCV was not administered to the mice in the GCV(−) group (n = 8).

Administration of BrdU
All mice were given water with 1 mg/ml bromodeoxyuridine (BrdU) starting 77 days after transplantation for a period of 7 days.

Histological Analysis
Animals were anesthetized and transcardially perfused with 0.1 M PBS containing 4% PFA 85 days after transplantation. Their spinal cords were subsequently removed, embedded in Optimal Cutting Temperature compound (Sakura Finetek, Torrance, CA). The samples were examined under an inverted fluorescence microscope (BZ 9000; Keyence, Osaka, Japan) or a confocal laser scanning microscope (LSM 700). For quantification of BrdU-, Ki67-, Nestin-, SOX1-, pan-ELAVL-, NeuN-, APC-, and GFAP-positive cells, five regions within the area 2 mm rostral and caudal to the lesion epicenter were randomly selected and counted under ×40 magnification.

Statistical Analysis
All data are presented as the means ± SEM. The in vitro cell viability assay was repeated six times, and the neural differentiation analysis was repeated three times. Mann–Whitney U test was used to detect any significant differences between groups with respect to the IHC results. One-way analysis of variance (ANOVA) followed by Tukey–Kramer test for multiple comparisons was used to detect significant differences in stride length, stance angle, and rotarod score between the four groups. Two-way repeated-measures ANOVA followed by Tukey–Kramer test was used for comparing BMS scores and IVIS photon counts. Fisher’s exact test was used to compare the rate of tumorigenesis in GCV (+) and GCV(−) mice. For all statistical analyses, the significance level was set at p < .05. Microsoft Excel 2016 and IBM SPSS Statistics (ver. 24) were used for all calculations.

Results

Establishment of 253G1 hiPSCs with Tet-Inducible HSVtk Expression
We used a lentiviral vector with the Tet-inducible (Tet-on) HSVtk expression system to transduce 253G1 hiPSCs (Fig. 1A) because we found that high-level constitutive expression of HSVtk was cytotoxic in hiPSCs (Fig. 1B). Twelve independent 253G1 hiPSC clones stably expressing Tet-inducible HSVtk (HSVtk-iPSCs) were established. HSVtk expression with DOX and cell death in the presence of GCV was confirmed in each clone. As all clones showed comparable sensitivity to GCV, clone #1 was selected for further study (Fig. 1B).

The HSVtk/GCV System Induces Apoptosis in Immature iPSC-NS/PC-Derived Cells While Preserving Mature Differentiated Neurons In Vitro
The established HSVtk-iPSCs underwent neural induction to form HSVtk-iPSC-NS/PCs, followed by neural differentiation over a period of 4 weeks. Cells in the GCV(+) group were treated with GCV after 21 days of induced differentiation, followed by immunocytochemical analysis. DOX was administered to both the GCV(+) and GCV(−) groups. The number of Nestin-positive undifferentiated neural cells and Ki67-positive proliferating cells decreased significantly following GCV administration (60.0%–18.5%, p < .01 and 30.0%–3.1%, p < .05, respectively; Fig. 2A, 2B). The number of β-III Tubulin-positive postmitotic neuronal cells, however, did not show any significant changes following GCV administration (61.8%–54.7%, p > .05), confirming that the HSVtk/GCV system was effective only in cells undergoing mitosis. This finding is consistent with the hypothesis that selective ablation of undifferentiated cells while...
preserving the postmitotic neurons was possible through this system (Fig. 2A, 2B).

GCV Administration Prevents Tumor Formation Following hiPSC-NS/PC Transplantation In Vivo

The lentiviral vector with the Tet-inducible HSVtk expression system was used to transduce 253G1-hiPSC-NS/PCs to generate HSVtk-hiPSC-NS/PCs. HSVtk-hiPSC-NS/PCs were also transduced with the lentiviral vector with ffLuc (a Venus and firefly luciferase fusion gene) [46] and then transplanted into the injured spinal cords of 16 NOD/SCID mice at 9 days postinjury. Eight of these mice were administered GCV at 6 weeks after transplantation (GCV[–] group), and the remaining mice were not treated with GCV (GCV[+] group; Fig. 3). All mice were fed the autoclaved rodent diet with added DOX. Luminescence began to increase in both groups 2 weeks after transplantation. In the GCV(–) group, luminescence continued to increase throughout the 12-week period. In the GCV(+) group, however, luminescence began to gradually decrease 2 weeks after GCV administration. There was a significant difference in the increase in IVIS photon count between the two groups at 10 weeks after transplantation (GCV[–] +2,346%; GCV[+] +717%; p < .05; Fig. 4A, 4B). Luminescence did not disappear completely following GCV administration, suggesting that some of the transplanted cells had matured and were no longer mitotic and that these cells had become resistant to GCV. There was no significant difference between the change in luminescence of the GCV(–) and Control groups (Supporting Information Fig. 1), suggesting that the tumorigenic nature of the cells was not altered by HSVtk gene transduction and that GCV was not the direct cause of cell death in the GCV(+) group.

All mice were sacrificed 85 days after transplantation, and their spinal cords were extracted on the same day. H&E staining and immunohistochemistry revealed a visible mass adjacent to the site of transplantation in six of the eight mice in the GCV(–) group (75%), but there was no obvious mass

Figure 2. Neural differentiation and cell death in the presence of ganciclovir (GCV). Herpes simplex virus type 1 thymidine kinase-human induced pluripotent stem cell-derived neural stem/progenitor cells were dissociated into single cells, seeded on coverslips and allowed to differentiate over 4 weeks in the presence (GCV[+]) or absence (GCV[–]) of DOX and GCV during the final week. The cells were immunostained with anti-Nestin, anti-Ki67, and anti-β-III Tubulin antibodies. The nuclei were stained with Hoechst 33258. The percentages of Nestin-, Ki67-, and β-III Tubulin-positive cells were plotted in a histogram (A) with representative staining results from each group (B).

* p < .05; ** p < .01; scale bar = 50 μm.
formation in the spinal cord of any GCV(+) mouse (p < .01; Fig. 4C, Supporting Information Fig. 2).

**GCV Administration Induces Apoptosis in Immature hiPSC-NS/PC-Derived Cells While Preserving Mature Differentiated Neurons Following Transplantation**

BrdU was orally administered to all mice 7 days before sacrifice to label the proliferating cells that remained in the spinal cord on the final week of observation. Immunohistological evaluation revealed a significant decrease in the percentage of Nestin-, Ki67-, and BrdU-positive cells in the spinal cord of the GCV(+) mice compared with that in the GCV(−) mice (GCV[−]: 36.9%, 15.4%, and 20.8%, respectively; GCV[+]: 4.3%, 1.0%, and 1.7%, respectively; p < .05 for all; Fig. 5A, 5B). The percentage of SOX1-positive immature neural cells also decreased significantly in the GCV(+) mice (GCV[−]: 32.0%; GCV[+]: 2.2%;...
The percentage of pan-ELAVL (Hu)-positive neuronal cells increased in the GCV(+) mice (GCV[−]: 42.6%; GCV[+]: 74.1%; p < .05). The percentage of NeuN-positive mature neuronal cells, APC-positive oligodendrocytes, and GFAP-positive astrocytes cells, representing the more mature cells derived from hiPSC-NS/PCs, did not decrease significantly in the GCV(+) mice (GCV[−]: 19.5%, 5.3%, and 1.7%, respectively; GCV[+]: 23.0%, 5.1%, and 6.9%, respectively; p > .05; Fig. 5C, 5D). The human-specific presynaptic marker (hSyn)-positive cells also appear to have been preserved following the ablation process (Supporting Information Fig. 3). These findings support the in vitro results showing that the HSVtk/GCV system can be used to ablate immature hiPSC-NS/PC-derived cells while preserving mature postmitotic cells.

**GCV Administration Protects the Improved Motor Function Gained from Transplantation**

253G1-hiPSC-NS/PCs without lentiviral transduction were also transplanted into the injured spinal cord at the thoracic level in eight NOD/SCID mice (Control group), and PBS was injected into the injured spinal cords of another eight NOD/SCID mice (PBS group) at 9 days after SCI. The hindlimb motor function of the mice in the four groups was compared through weekly assessment using the BMS. The average BMS scores of the GCV(−), GCV(+), and Control groups were significantly higher than that of the PBS group at 21 days after SCI (PBS: 2.4; Control: 3.4; GCV[−]: 3.4; GCV[+]: 3.2; p < .05). The average BMS scores of the Control, GCV(−), and GCV(+) mice all reached at least 4.0 at their peak (Control: 4.0; GCV[−]: 4.2; GCV[+]: 4.4). The average BMS scores of the Control and GCV(−) groups started to decline after they had peaked (Control: 56 days after SCI; GCV[−]: 49 days after SCI), to the point that there was no significant difference compared with the average BMS score of the PBS group on the final day of observation (PBS: 2.6; Control: 3.4; GCV[−]: 3.3; p > .05). However, the BMS score of the GCV(+) group did not show the same trend and remained above 4.0 throughout the entire observation period. Significant differences in the average BMS scores between the GCV(−) and GCV(+) groups were observed at 77 days after SCI (GCV[−]: 3.4; GCV[+]: 4.3; p < .05; Fig. 6A).

Treadmill gait analysis (DigiGait system) was performed at 91 days after SCI. Only the mice in the GCV(+) group showed any significant improvement in stride length (PBS: 3.4 cm; Control: 3.5 cm; GCV[−]: 3.6 cm; GCV[+]: 4.3 cm; p < .01) or stance angle (PBS: 36.1°; Control: 28.6°; GCV[−]: 25.8°; GCV[+]: 15.7°; p < .01) compared with the PBS group (Fig. 6B, 6C). As for the rotarod performance test, performed at 91 days after SCI, only the mice in the GCV(+) group showed any...
significant improvement in riding time compared with the PBS group (PBS: 20 seconds; Control: 47.4 seconds; GCV[−]: 47.0 seconds; GCV[+]: 57.5 seconds; \(p < .05\); Fig. 6D).

**DISCUSSION**

The aim of this study was to explore the possibility of using the HSVtk gene as a tool for improving the safety of hiPSC-NS/PC transplantation. The insertion of the Tet-inducible HSVtk gene did not affect the differentiation capability of hiPSC-NS/PCs [17], and the observed improvements in motor function were on par with those observed when using the same line of cells (253G1) without the inserted gene [17, 20]. Histological evaluation of the tumors in the GCV[−] group revealed that the tumor was composed of undifferentiated neoplastic cells with embryonal appearances resembling blastemal tissue (BLT) which was previously reported as iPSC-NS/PCs with arrested development [47]. Therefore, it is speculated that the tumor arose from BLT.

Tumorigenic Ki67-positive cells, potentially tumorigenic proliferative BrdU-positive cells, and immature Nestin- and SOX1-positive cells were ablated, while mature pan-ELAVL-, NeuN-, APC-, GFAP-, and hSym-positive cells were preserved. The ablation of the proliferative cells exerted a protective effect on motor function that was gained through transplantation, sheltering the spinal cord from the effect of tumor growth. While both the iCaspase9/CID and HSVtk/GCV systems have been clinically validated for human use [48], they induce apoptosis in very different ways [49]. The iCaspase9/CID system, which was designed to ablate all transplanted cells, risks ablation of the positive attributes of cell transplantation as well, including ablation of mature neuronal cells, resulting in subsequent deterioration of motor function [18]. The cell cycle-dependent property of the HSVtk/GCV system used in our experiment [49, 50] enabled us to selectively target proliferative/tumorigenic cells without harming the beneficial effects of transplantation.

In the current experiment, we used a relatively well-known suicide gene in a slightly unorthodox manner, by addressing
tumorigenesis from a preventative standpoint. Similar to previous reports [51], the HSVtk/GCV system successfully prevented the accumulation of proliferative cells while preserving the mature cells in vitro. Following transplantation, the early ablation of these immature proliferative cells resulted in the prevention of tumorigenesis, allowing the spinal cord to regenerate to its full potential. Although there was no further evidence of tumorigenesis in the GCV (+) mice during the final weeks of observation, if any of the remaining cells were to undergo oncogenic changes, they would become proliferative and, hence, sensitive to GCV again. Therefore, the HSVtk/GCV system can theoretically also be used as a curative measure every time a tumorigenic episode occurs, provided the suicide gene itself does not undergo any mutation. Our results suggest that this system has the potential to be used as both a preventative and curative measure against tumorigenesis following hiPSC-NS/PC transplantation. Most importantly, we were successful in protecting the motor function gained through transplantation. As previously stated, when 253G1-hiPSC-NS/PCs are transplanted into the injured spinal cord of NOD/SCID mice in the subacute phase, there is initial improvement of motor function, followed by abrupt deterioration secondary to the formation of a tumorigenic mass [20]. In the current experiments, we observed the usual improvements in hindlimb motor function following the NS/PC transplantation in all three groups (GCV [+], GCV [−], and Control), but subsequent deterioration was observed only in the GCV (−) and Control groups.

We found that a high expression level of HSVtk was cytotoxic in hiPSCs. This may be due to the rapid proliferation property or peculiar nucleotide metabolism of stem cells. We demonstrated that the Tet-inducible system, although requiring DOX as well as GCV for the induction of cell death, is a feasible solution for overcoming the cytotoxic effect of HSVtk expression; and provides added safety by preventing undesired expression of the gene. Several issues with the HSVtk/GCV system still need to be addressed before clinical application. The success of this system in vivo depends on the ability of the prodrug to reach the transplanted cells past the blood–spinal cord barrier (BSCB). GCV is relatively poor at crossing the BSCB [32, 52–59], which means that a relatively high dose of GCV over a long period (50 mg/kg per day, intraperitoneal administration for 3 weeks) is required to fully ablate proliferating cells. Although our results demonstrate that the HSVtk/GCV system can induce apoptosis in intraspinal hiPSC-NS/PCs without causing any obvious detrimental health issues in our mice, GCV is known to cause renal impairment, hepatic dysfunction, and pancytopenia [60, 61]. Therefore, even though GCV can be safely administered to humans with careful application [48, 62], methods to improve its pharmacokinetics should be sought. For example, there have been reports that simultaneous administration of (E)-5-(2-bromovinyl)-2′-deoxyuridine enhances the effects of GCV, which could help reduce the required dose and administration period of the prodrug [63]. Alternative methods for introducing the HSVtk gene to hiPSCs should also be considered, as lentiviral transduction is not ideal in the clinical setting. Recent advances in the development of genome editing technologies based on programmable nucleases have increased the precision and safety of genome editing in eukaryotic cells [64]. The clustered regularly interspaced short palindromic repeat-associated nuclease Cas9 is a promising tool for achieving safe gene editing that is appropriate for clinical application [65]. The extent to which the “bystander effect” (BSE) played a role in the ablation of transplanted cells in vivo is difficult to quantify [24, 53, 60]. We speculate that the transfer of toxic metabolites of GCV via gap junctional intercellular communication (GJIC) would enhance the effects of the prodrug in ablating the immature proliferative cells. The host neurons and fully differentiated neurons that originated from transplanted cells are nonproliferative and will, therefore, no longer be susceptible to the BSE. Increased inflammatory infiltrations, especially in immune competent animals, could potentially affect the mature neuronal cells that are adjacent to the ablated cells [66]. However, a previous report utilizing the same HSVtk/GCV system in the treatment of intracranial gliomas demonstrated that tumoricidal BSE does not injure normal brain tissues [67]. In our study, mature neural cells and motor function were both preserved following GCV administration, suggesting that the adverse effects of the BSE were negligible. BSE is often a desired property of the HSVtk/GCV system in strategies designed to ablate tumors where complete transduction of tumorigenic cells with the transgene is difficult. In this study, however, we transduced the iPSC-NS/PCs prior to transplantation. Furthermore, studies to reduce GJIC through drugs such as Dieldrin [68] could help elucidate the effects of BSE in this strategy.

CONCLUSION

We successfully prevented tumorigenic transformation following hiPSC-NS/PC transplantation for the treatment of subacute-phase SCI in NOD/SCID mice through the use of the HSVtk/GCV suicide gene system. Intraperitoneal administration of the pro-drug (GCV) induced selective apoptosis of immature proliferating cells within the spinal cord. Mature postmitotic cells were unaffected by the prodrug, thus helping maintain the improvement of motor function gained by transplantation. To the best of our knowledge, this is the first time that this gene has been introduced to hiPSCs to serve as a safety-lock system in preventing tumorigenesis following transplantation into the spinal cord of SCI model mice. We believe that the ability of this system to prevent and remove tumors while preserving the beneficial effects of transplantation provides a better alternative for combatting tumorigenesis following stem cell transplantation. Along with the production of “safe” iPSC-NS/PCs and development of an effective screening system for potentially tumorigenic cells prior to transplantation, this strategy could help abolish the remaining risks of tumorigenesis in the treatment of SCI with iPSC-NS/PCs.

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**AUTHOR CONTRIBUTIONS**

K.K.: conception and design, administrative support, provision of study material or patients, collection of data, data analysis and interpretation, manuscript writing; T.I. and M.M.: conception and design, data analysis and interpretation; K.S.: collection of data, data analysis and interpretation; S.I.: provision of study material or patients, manuscript writing; R.F.: data analysis and interpretation, manuscript writing; K.Y.: provision of study material or patients; F.R.M. and S.S.: data analysis and interpretation; M.N. and H.O.: conception and design, financial support, administrative support, provision of study material or patients, data analysis and interpretation, manuscript writing, final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

H.O. is a compensated scientific consultant of San Bio, Co., Ltd. and K Pharma, Co., Ltd. N.M. is a compensated scientific consultant of K Pharma, Co., Ltd. The other authors indicated no potential conflicts of interest.

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