Nagai T, Toguchida J, Toyama Y, Muto A and Saya H: IGF2 preserves osteosarcoma cell survival by creating an autophagic state of dormancy that protects cells against chemotherapeutic stress. ***Cancer Res*** 74: 6531-6541, 2014

2. Maruoka R, Takenouchi T, Torii C, Shimizu A, Misu K, Higasa K, Matsuda F, Ota A, Tanito K, Kuramochi A, Arima Y, Otsuka F, Yoshida Y, Moriyama K, Niimura M, Saya H and Kosaki K: The use of next-generation sequencing in molecular diagnosis of neurofibromatosis type 1: a validation study. ***Genet Test Mol Biomarkers*** 18: 722-735, 2014

2:学会発表

1. 佐谷秀行:既存薬を用いた癌幹細胞標的治療の考案。シンポジウム1「ドラッグ・リポジショニング」。第 42 回日本潰瘍学会。10/31/2014、慶應義塾大学芝共立キャンパス、東京
2. 佐谷秀行:骨肉腫幹細胞の性状解析と分化制御による治療戦略の考案。特別講演2。第56回日本小児血液・がん学会学術集会、11/29/2014、岡山コンベンションセンター、岡山

G. 知的財産権の出願・登録状況(予定を含む。)

1.特許取得

なし

2.実用新案登録

なし

3.その他

なし

厚生労働科学研究費補助金（再生医療実用化研究事業）
研究分担報告書

次世代シーケンサーを用いた培養時遺伝子変異蓄積の網羅的解析系の開発

研究分担者　　小崎　健次郎
慶應義塾大学医学部　臨床遺伝学センター　教授

研究要旨
本研究全体の目標は、神経疾患特異的iPS細胞を用いた効率的な創薬システムの構築と高スループット化を行うことである。iPS細胞については継代に応じて細胞に徐々に変異が蓄積することが知られており、創薬研究対象とする患者細胞の性質の明確化・均質化はプロジェクトの成功のために必須のプロセスといえる。遺伝子変異部位の確認を行うために、次世代シーケンサー等による網羅的なスクリーニングを行う。これまでは、点変異（一塩基置換やいわゆるindel等）の検出を中心に解析を進めたが、今年度はcopy number variationを用いてそれ以外の種類の遺伝子変異である欠失・重複を検出するための手法を確立した。

A. 研究目的

本研究全体の目標は、神経疾患特異的iPS細胞を用いた効率的な創薬システムの構築と高スループット化を行うことである。iPS細胞の作成時・継代時・分化誘導時に細胞に徐々に遺伝子変異が蓄積することが知られている。創薬研究を行う場合、患者細胞への遺伝子変異の蓄積の有無および程度を明確化することは、臨床応用を前提として均質な細胞を得るためには必須のプロセスといえる。特にiPS細胞はがん化により大きく性質を変えると懸念されるため、がん関連遺伝子の蓄積についてプロファイリングが必要と考えられる。

本年は次世代シーケンサーを用いて、培養時の遺伝子変異の蓄積を網羅的に把握するための手法を検討した。

B. 研究方法

一般に入手可能な神経線維腫症患者由来の細胞株XとX由来にて著明な増殖能を獲得した細胞株Yをヌードマウスに移植し、増殖した細胞のゲノムDNAを抽出し、4800の既知ヒト疾患関連遺伝子の変異を網羅的に同定した。Yに存在し、Xに存在しない遺伝子変異を抽出するためのプログラム群の導入と比較をおこなった。

TruSightOneキット（イルミナ社）を用いてヒト疾患との関連が報告されいている全4800遺伝子の翻訳領域のゲノムDNAを回収した。次世代シーケンサーMiSeq(イルミナ社)を用いて得られた粗配列を、プログラムBWAを用いて、ヒト参照配列hg19に整列させた。Picardを用いて重複配列を除去し、GATKで再配列してbam形式・vcf形式の出力ファイルを得た。

体細胞変異の有無を検出するため、bam形式の出力ファイルをFishingCNVにより検出した。
Shi Y, Majewski J FishingCNV: a graphical software package for detecting rare copy number variations in exome-sequencing data. Bioinformatics. 2013 , 29(11):1461-2.
http://sourceforge.net/projects/fishingcnv/

正常人に認められる CNV については、平成 25 年度厚生労働省科学研究費補助金難治性疾患克服事業で作成した「正常日本人における染色体欠失・重複のとりまとめ」で編纂したデータを活用した。

VCF ファイル形式のデータをもとに Homozygosity mapping を行い、Loss-of-heterozygosity となっている領域を検出した。H3M2 ソフトウェア（http://sourceforge.net/projects/h3m2/）を用いた。

Linuxオペレーティングシステムの環境下で32コアCPU・メインメモリー200GB超を有するサーバーを用いてパフォーマンスを検討した。得られた変異について、variant toolsを用いてアノテーションを行い、包括的に比較した。
San Lucas FA, Wang G, Scheet P, Peng B. Integrated annotation and analysis of genetic variants from next-generation sequencing

studies with variant tools. San Lucas FA1, Wang G, Scheet P, Peng B. Bioinformatics. 28:421-2, 2012.
http://varianttools.sourceforge.net

(倫理面への配慮)

入手可能なヒト細胞株を用いた検討のため、ゲノム指針の適応とならない。患者検体を用いた解析はヒトゲノム指針に従い、慶應義塾大学医学部倫理委員会の承認を得て解析を行った

C．研究結果

1検体あたりの計算時間はFishingCNVで、約28コア使用時1分で終了した。計算速度は、使用するCPUコア数に完全に依存した。欠失ないし重複部位を図示することが可能であった。Fishing CNV法で示唆される欠失部位とH3M2で示唆されるloss-of-heterozygosityの領域はオーバーラップしていた。

D．考察

iPS細胞の臨床応用を考える場合、細胞の質の担保は極めて重要である。ゲノム全体の変化を検出する目的から、次世代シーケンサーが用いられる。一般に点変異や数塩基程度の欠失・重複の検出のためのアルゴリズムについては標準化がなされつつある。一方、数百塩基から数百万塩基の欠失・重複の評価については標準化がなされていない。

本研究では、FishingCNV法を導入した。バッチで行われる実験に対して、バッチ間差を主成分分析により除去するアルゴリズムである。疾患エクソームに対してもFishingCNVが有効であることが示された。H3M2がloss-of-heterozygosityの検出に有効であった。

E．結論

数百塩基から数百万塩基の欠失・重複の評価については標準化の検出にFishingCNV法・H3M2法を適用した。本研究の実施を通じて、ハイスループットスクリーニングのためのパイプラインを構築することができた。スループットに応じて、使用するプログラムを適宜組み合わせて使用することが望ましい。

F．研究発表

1．論文発表

Numasawa-Kuroiwa Y, Kosaki K, Okano H, et al., Involvement of ER stress in dysmyelination of Pelizaeus-Merzbacher Disease with PLP1 missense mutations shown by iPSC-derived oligodendrocytes. ***Stem Cell Reports*** 2(5): 648-661, 2014

2．学会発表

なし

G．知的財産権の出願・登録状況

1．特許取得

なし

2．実用新案登録

なし

3．その他

なし

Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

【研究代表者：岡野栄之】

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
岡野栄之、山中伸弥	幹細胞と疾患iPS細胞の研究最前線	岡野栄之、山中伸弥	実験医学増刊 再生医療2015	羊土社	日本	2015	121(273)-126(278)
尾崎正大、岡野栄之	脊椎損傷に対する再生医療	川﨑洋志	脳神経系の再生医学-発生と再生の融合的新展開-	診断と治療社	日本	2015	125-130

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kano SI, Yuan M, Cardarelli RA, Maegawa G, Horiuchi Y, Higurashi N, Gaval-Cruz M, Wilson AM, Tristan C, Kondo MA, Chen Y, Koga M, Obie C, Ishizuka K, Seshadri S, Srivastava R, Kato TA, Sedlak TW, Lee Y, Rapoport JL, Hirose S, Okano H, Valle D, O'Donnell P, Sawa A, Kai M	Clinical utility of neuronal cells directly converted from fibroblasts of patients for neuropsychiatric disorders: studies of lysosomal storage diseases and channelopathy.	Curr Mol Med.	[Epub ahead of print]		2015

Itakura G, Kobayashi Y, Nishimura S, Iwai H, Takano M, Iwanami A, Toyama Y, Okano H, Nakamura M	Controlling Immune Rejection Is a Fail-Safe System against Potential Tumorigenicity after Human iPSC-Derived Neural Stem Cell Transplantation.	PLoS One	23	10(2)	2015
Nori S, Okada Y, Nishimura S, Sasaki T, Itakura G, Kobayashi Y, Renault-Mihara F, Shimizu A, Koya I, Yoshida R, Kudoh J, Koike M, Uchiyama Y, Ikeda E, Toyama Y, Nakamura M, Okano H	Long-Term Safety Issues of iPSC-Based Cell Therapy in a Spinal Cord Injury Model: Oncogenic Transformation with Epithelial-Mesenchymal Transition.	Stem Cell Reports	10;4(3)	360-73	2015

Maekawa M, Yamada K, Toyoshima M, Ohnishi T, Iwayama Y, Shimamoto C, Toyota T, Nozaki Y, Balan S, Matsuzaki H, Iwata Y, Suzuki K, Miyashita M, Kikuchi M, Kato M, Okada Y, Akamatsu W, Mori N, Owada Y, Itokawa M, Okano H, Yoshikawa T	Utility of Scalp Hair Follicles as a Novel Source of Biomarker Genes for Psychiatric Illnesses.	Biol Psychiatry	[Epub ahead of print]		2014
Kondo T, Funayama M, Tsukita K, Hotta A, Yasuda A, Nori S, Kaneko S, Nakamura M, Takahashi R, Okano H, Yamanaka S, Inoue H	Focal Transplantation of Human iPSC-Derived Glial-Rich Neural Progenitors Improves Lifespan of ALS Mice.	Stem Cell Reports	12;3(2)	242-9	2014
Okano H	Stem cell research and regenerative medicine in 2014: first year of regenerative medicine in Japan.	Stem Cells Dev.	15;23(18)	2127-28	2014

Matsuzaki Y, Mabuchi Y, Okano H	Leptin Receptor Makes Its Mark on MSCs.	Cell Stem Cell	7;15(2)	112-4	2014
Numasawa-Kuroiwa Y, Okada Y, Shibata S, Kishi N, Akamatsu W, Shoji M, Nakanishi A, Oyama M, Osaka H, Inoue K, Takahashi K, Yamanaka S, Kosaki K, Takahashi T, Okano H	Involvement of ER Stress in Dysmyelination of Pelizaeus-Merzbacher Disease with PLP1 Missense Mutations Shown by iPSC-Derived Oligodendrocytes.	Stem Cell Reports	24;2(5)	648-61	2014
Yoshida T, Ozawa Y, Suzuki K, Yuki K, Ohyama M, Akamatsu W, Matsuzaki Y, Shimmura S, Mitani K, Tsubota K, Okano H	The use of induced pluripotent stem cells to reveal pathogenic gene mutations and explore treatments for retinitis pigmentosa.	Mol Brain	16;7(1)	45	2014
Bamba Y, Shofuda T, Kanematsu D, Nonaka M, Yamasaki M, Okano H, Kanemura Y	Differentiation, polarization, and migration of human induced pluripotent stem cell-derived neural progenitor cells co-cultured with a human glial cell line with radial glial-like characteristics.	Biochem Biophys Res Commun.	16;447(4)	683-8	2014

【研究分担者：佐谷秀行】

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Shimizu T, Sugihara E, Yamaguchi-Iwai S, Tamaki S, Koyama Y, Kamel W, Ueki A, Ishikawa T, Chiyoda T, Osuka S, Onishi N, Ikeda H, Kamei J, Matsuo K, Fukuchi Y, Nagai T, Toguchida J, Toyama Y, Muto A, Saya H	IGF2 preserves osteosarcoma cell survival by creating an autophagic state of dormancy that protects cells against chemotherapeutic stress.	Cancer Res	74	6531-6541	2014

Maruoka R, Takenouchi T, Torii C, Shimizu A, Misu K, Higasa K, Matsuda F, Ota A, Tanito K, Kuramochi A, Arima Y, Otsuka F, Yoshida Y, Moriyama K, Niimura M, Saya H, Kosaki K	The use of next-generation sequencing in molecular diagnosis of neurofibromatosis type 1: a validation study.	Genet Test Mol Biomarkers	18	722-735	2014

【研究分担者：小崎健次郎】

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Numasawa-Kuroiwa Y, Okada Y, Shibata S, Kishi N, Akamatsu W, Shoji M, Nakanishi A, Oyama M, Osaka H, Inoue K, Takahashi K, Yamanaka S, Kosaki K, Takahashi T, Okano	Involvement of ER stress in dysmyelination of Pelizaeus-Merzbacher Disease with PLP1 missense mutations shown by iPSC-derived oligodendrocytes.	Stem Cell Reports	2(5):	648-661	2014

Ⅳ. 研究成果の刊行物・別刷

PLOS | ONE

RESEARCH ARTICLE

Controlling Immune Rejection Is a Fail-Safe System against Potential Tumorigenicity after Human iPSC-Derived Neural Stem Cell Transplantation

Go Itakura[1,2], Yoshiomi Kobayashi[1], Soraya Nishimura[1,2], Hiroki Iwai[1,2], Morito Takano[1], Akio Iwanami[1], Yoshiaki Toyama[1], Hideyuki Okano[2]*, Masaya Nakamura[1]*

1 Department of Orthopaedic Surgery, Keio University School of Medicine, 35 Shinanomachi, Shinjuku, Tokyo, 160-8582, Japan, **2** Department of Physiology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku, Tokyo, 160-8582, Japan

* hidokano@a2.keio.jp (HO); masa@a8.keio.jp (MN)

Citation: Itakura G, Kobayashi Y, Nishimura S, Iwai H, Takano M, Iwanami A, et al. (2015) Controlling Immune Rejection Is a Fail-Safe System against Potential Tumorigenicity after Human iPSC-Derived Neural Stem Cell Transplantation. PLoS ONE 10(2): e0116413. doi:10.1371/journal.pone.0116413

Academic Editor: Alysson Renato Muotri, University of California, San Diego, UNITED STATES

Received: August 3, 2014

Accepted: December 9, 2014

Published: February 23, 2015

Data Availability Statement: All relevant data are within the paper.

Funding: This work was supported by grants from the Japan Science and Technology (JST)-California Institute for Regenerative Medicine (CIRM) collaborative program; a Grant-in-Aid from the Zenkyoren (National Mutual Insurance Federation of Agricultural Cooperatives) Research Foundation; a Grant-in-Aid from the General Insurance Association of Japan to MN; Grants-in-Aid for the Research Center Network for Realization of Regenerative Medicine and the Centers for Clinical Application

Abstract

Our previous work reported functional recovery after transplantation of mouse and human induced pluripotent stem cell-derived neural stem/progenitor cells (hiPSC-NS/PCs) into rodent models of spinal cord injury (SCI). Although hiPSC-NS/PCs proved useful for the treatment of SCI, the tumorigenicity of the transplanted cells must be resolved before they can be used in clinical applications. The current study sought to determine the feasibility of ablation of the tumors formed after hiPSC-NS/PC transplantation through immunoregulation. Tumorigenic hiPSC-NS/PCs were transplanted into the intact spinal cords of immunocompetent BALB/cA mice with or without immunosuppressant treatment. *In vivo* bioluminescence imaging was used to evaluate the chronological survival and growth of the transplanted cells. The graft survival rate was 0% in the group without immunosuppressants versus 100% in the group with immunosuppressants. Most of the mice that received immunosuppressants exhibited hind-limb paralysis owing to tumor growth at 3 months after iPSC-NS/PC transplantation. Histological analysis showed that the tumors shared certain characteristics with low-grade gliomas rather than with teratomas. After confirming the progression of the tumors in immunosuppressed mice, the immunosuppressant agents were discontinued, resulting in the complete rejection of iPSC-NS/PC-derived masses within 42 days after drug cessation. In accordance with the tumor rejection, hind-limb motor function was recovered in all of the mice. Moreover, infiltration of microglia and lymphocytes was observed during the course of tumor rejection, along with apoptosis of iPSC-NS/PC-generated cells. Thus, immune rejection can be used as a fail-safe system against potential tumorigenicity after transplantation of iPSC-NS/PCs to treat SCI.

Research on Specific Disease/Organ from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan and the JST; Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS); Keio Gijuku Academic Development Funds; a Grant-in-Aid for the Global Center of Excellence (COE) program from the MEXT to Keio University; and a Grant-in-Aid for Scientific Research on Innovative Areas (Comprehensive Brain Science Network) from the MEXT to HO. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: HO is a paid scientific consultant to San Bio, Inc. and Daiichi Sankyo Co., Ltd. The remaining authors declare that they have no competing financial interests or other potential conflicts of interest associated with this manuscript. There are no restrictions on the sharing of data or materials and the authors fully adhere to PLOS ONE's policy on the sharing of data and materials.

Introduction

Enormous progress has been made in the field of regenerative medicine centered on cell transplantation therapy, largely owing to advances in stem cell biology. For example, we recently reported the efficacy of human induced pluripotent stem cell (hiPSC)-derived neural stem/progenitor cell (hiPSC-NS/PC) transplantation for the treatment of spinal cord injury (SCI) in rodents and a non-human primate, the common marmoset [1–4]. However, concerns about the potential tumorigenicity of iPSCs and their progeny must be addressed before these cells can be used in clinical practice.

To pursue the issue of iPSC-NS/PCs safety, the cells must be thoroughly characterized. To do this, the expression of cell surface markers and differentiation-associated genes, genome copy number variation, and DNA methylation status should be analyzed using flow cytometry, microarray technology, and related techniques [4]. Furthermore, the tumorigenicity of iPSC-NS/PCs requires careful evaluation by grafting the cells into immunodeficient mouse models. However, even these quality control measures prior to clinical cell transplantation cannot completely exclude the possibility of late-onset tumorigenesis. Thus, a fail-safe strategy against tumorigenesis is essential. Studies using the Herpes Simplex Virus type 1 thymidine kinase (HSV/TK) system for the selective ablation of stem cell-derived tumors reported a reduced cancer risk after the transplantation of mouse embryonic stem cells (ESCs) and iPSCs into animal models [5,6]. Furthermore, an inducible caspase 9 system is already in clinical use, although it has not been applied to stem cells [7]. However, because the HSV/TK system is accompanied by issues of genomic insertion, the establishment of an anti-tumor system with higher safety remains of utmost importance.

Previous reports suggested that the optimal timing of cell transplantation for SCI is at the subacute phase, when the inflammatory response has subsided, but before the formation of the glial scar is complete (generally 2–4 weeks after SCI in non-human primates and rodents) [8,9]. Given the limitations of this therapeutic time window, autologous transplantation of iPSC-NS/PCs for SCI is technically challenging at present [4,10,11]. Furthermore, vigorous validation and quality control of each iPSC lines and its derivatives are necessary for their clinical use. This would involve the expansion, derivation, and quality control of patient-specific iPS-NSCs, and is therefore too time-consuming and expensive to treat acute and sub-acute SCI patients. Therefore, clinical application of iPS-NSCs for SCI will presumably necessitate allogeneic procedures in the foreseeable future.

Compared with other organ systems, the central nervous system (CNS), including the spinal cord, is regarded as a relatively "immune-privileged" site, signifying that the CNS is immunologically tolerant [12–16]. Moreover, the ability of NS/PCs to modulate the immune response by secreting immunosuppressive cytokines (e.g., transforming growth factor-β1) has been described both *in vitro* and *in vivo* [17–19]. However, as evidenced by the grafting of rat NS/PCs into the lesioned rat spinal cord, the T-cell-mediated immune response can still be induced in the host following the transplantation of allogeneic cells [20]. Therefore, to prevent the chronic rejection of grafted cells and to promote their long-term engraftment, combinatorial immunosuppressive/cell transplantation therapy is required for a certain period of time following SCI.

The present study explored the xenotransplantation of tumorigenic hiPSC-NS/PCs into a mouse spinal cord with or without immunosuppressant agents. Furthermore, upon transplantation of the tumorigenic hiPSC-NS/PCs, we investigated whether the resultant stem cell-derived tumors could be eliminated by immune rejection following the withdrawal of the immunosuppressants.

Materials and Methods

Cell culture, neural induction, and lentivirus transduction

Cell culture and neural induction of hiPSCs (hiPSC clone 253G1[21], Caucasian, 36 years old, female, human dermal fibroblast) were performed as previously described [1,2,22,23], with slight modifications. hiPSCs (253G1) were grown on gelatin-coated (0.1%) culture dishes and irradiated murine embryonic fibroblasts (MEFs), maintained in standard ES cell medium, and used for EB formation as described previously. Thirty days after their formation, EBs were enzymatically dissociated into single cells and cultured in suspension in serum-free media hormone mix (MHM) medium for 12 days to allow neurospheres to form. The recombinant human immunodeficiency virus type 1 (HIV-1)-based lentivirus was prepared and transduced into hiPSC-derived neurospheres according to previously published methods [23]. Briefly, primary hiPSC-derived neurospheres were first dissociated and infected with a fusion HIV-1-based lentiviral vector (kindly gifted by Dr. Hara, Brain Science Institute, RIKEN, Japan) expressing ffLuc (Venus fused to firefly luciferase) [24] under the control of the elongation factor (EF) promoter (pCS II-EF-dVenus-Luc2). This vector enabled the grafted cells to be detected as strong bioluminescent ffLuc signals in live mice and as fluorescent Venus signals by using an anti-green fluorescent protein (GFP) antibody in fixed spinal cord sections because the Venus protein was originally modified from GFP. The primary hiPSC-derived neurospheres were passaged into secondary and tertiary neurospheres, including hiPSC-NS/PCs, and used for transplantation into the murine spinal cord, as described below.

Cell transplantation

Female 8-week-old immunocompetent BALB/cA mice (20–22 g, n = 27) were anesthetized with an intraperitoneal (i.p.) injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). After laminectomy at the 10^{th} thoracic spinal vertebra, the dorsal surface of the dura mater was exposed. hiPSC-NS/PCs (5×10^5 cells/2 μl) were transplanted into the spinal cord with a glass micropipette at a rate of 1 μl/min using a 25 μl Hamilton syringe and a stereotaxic microinjector (KDS 310, Muromachi Kikai Co., Ltd., Tokyo, Japan). For immunosuppression, the female BALB/cA mice were randomized to receive FK506 (Prograf; Astellas Pharma US, Inc., Northbrook, IL, USA) plus anti-mouse CD4 monoclonal antibody (anti-mCD4 mAb; BioXcell, West Lebanon, NH, USA). FK506 was administered by subcutaneous injection at a dose of 5 mg/kg once daily beginning on the first day of cell transplantation, and anti-CD4 mAb was administered by i.p. injection at a dose of 10 mg/kg beginning 2 days before cell transplantation and continuing once per week after transplantation (with immunosuppressant (IS) group n = 5, IS off group n = 16).

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 (National Institutes of Health, Bethesda, MD, USA). All surgery was performed under anesthesia, and all efforts were made to minimize animal suffering and were used humane endpoints.

Bioluminescence imaging

The Xenogen-IVIS spectrum cooled charge-coupled device optical macroscopic imaging system (Caliper Life-Sciences, Hopkinton, MA, USA) was used for bioluminescence imaging (BLI) to confirm the survival of the transplanted hiPSC-NS/PCs. Monitoring was performed at a frequency of 1–2 times per week after cell transplantation. Briefly, D-luciferin (Promega, Madison, WI, USA) was administered via i.p. injection at a dose of 300 mg/kg body weight.

Animals were placed in a light-tight chamber, and photons emitted from the luciferase-expressing cells were collected with integration times of 5 s to 2 min, depending on the intensity of the bioluminescence emission. BLI signals were quantified in maximum radiance units (photons per second per centimeter squared per steradian (p/s/cm^2/sr)) and presented as log10 (photons per second).

Histological analysis

Animals were anesthetized and transcardially perfused with 0.1 M phosphate buffered saline containing 4% paraformaldehyde. The spinal cords were removed, embedded in Optimal Cutting Temperature compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan), and sectioned in the sagittal plane on a cryostat (Leica CM3050 S, Leica Microsystems, Buffalo Grove, IL, USA). Sections were stained with hematoxylin-eosin (HE), Hoechst 33258 dye (10 μg/mL; Sigma Chemical Co., St. Louis, MO, USA), and the following primary antibodies: anti-GFP (rabbit IgG, 1:200; Frontier Institute Co., Ltd., Hokkaido, Japan), anti-β-tubulin isotype III (mouse IgG, 1:1,000; Sigma Chemical Co.), anti-glial fibrillary acidic protein (anti-GFAP, rabbit IgG, 1:200; Dako, Carpinteria, CA, USA), anti-Oligo-1 (mouse IgG, 1:200; R&D Systems, Minneapolis, MN, USA), anti-human-specific nestin protein (rabbit IgG, 1:200; described previously [25,26]), anti-Ki-67 (rabbit IgG, 1:200; Novocastra, Newcastle upon Tyne, UK), anti-CD11b (rat IgG, 1:200; BD Pharmingen, San Diego, CA, USA), anti-terminal deoxynucleotidyl transferase (anti-TdT; included in the Apop Tag Plus Fluorescein In Situ Apoptosis Detection Kit; Chemicon, Temecula, CA, USA), and anti-CD3 (rat IgG, 1:100; AbD Serotec, Raleigh, NC, USA). Samples were examined on an inverted fluorescence microscope (BZ 9000; Keyence Co., Osaka, Japan) or a confocal laser scanning microscope (LSM 700, Carl Zeiss, Jena, Germany). To quantify the human nuclear antigen (HNA)-, Ki-67-, CD11b-, CD3-, NKp46-, and TdT-positive cells, three representative mid-sagittal sections were selected and five regions within 1 mm rostral and caudal to the lesion epicenter were automatically captured at 200× magnification. The numbers of marker-positive cells were counted in each section (n = 2 per group).

Flow cytometric analysis

Isolated peripheral blood leukocytes were analyzed by triple immunofluorescence staining, followed by flow cytometry. The following primary antibodies were purchased from eBiosciences (San Diego, CA, USA): anti-allophycocyanin (APC)-labeled CD3 (clone 145-2C11), anti-fluorescein isothiocyanate (FITC)-labeled CD4 (clone GK 1.5), anti-phycoerythrin (PE)-labeled CD8 (clone 53–6.7), Armenian Hamster IgG Isotype Control APC (clone eBio299Arm), Rat IgG2b K Isotype Control FITC (eB149/10H5), and Rat IgG2a K Isotype Control PE (eBR2a). The cells were stained with a mixture of the primary antibodies at 4°C for 30 min. Flow cytometry was performed on a fluorescence- activated cell sorting (FACS) Calibur instrument (BD Biosciences, San Jose, CA, USA).

Statistical analysis

All data are presented as the mean value ± the standard error of the mean. Friedman's test followed by Dunn's post-hoc test was used to determine significant differences in the BLI analysis. For all statistical analyses, the significance level was set at $p < 0.05$.

Results

Characterization of lentivirally transduced hiPSC-NS/PCs

hiPSC-derived NS/PCs generated from hiPSC clone 253G1 (253G1-NS/PCs) were cultured and labeled with the ffLuc gene (Venus fused to luciferase) [24] via lentiviral transduction (Fig. 1A, B). Differentiation assays revealed that the 253G1-NS/PCs differentiated into β-III tubulin-positive neurons and GFAP-positive astrocytes *in vitro*, but not into Oligo-1-positive oligodendrocyte progenitor cells (Fig. 1C). Sufficient fluorescence was detected from the 253G1-NS/PCs *in vitro* via fluorescence microscopy (Fig. 1D) for their subsequent identification *in vivo*. To examine the sensitivity of BLI, we used the Xenogen-IVIS system (Caliper Life-Sciences) to detect the luminescence intensity of the 253G1-NS/PCs at various cell numbers (ranging from 1.5×10^5 to 1.2×10^6 cells per well) in the presence of D-luciferin. Quantitative analysis of bioluminescent ffLuc signals revealed that the luminescence intensities were in direct proportion to the hiPSC-NS/PC numbers *in vitro* ($r^2 = 0.99$) (Fig. 1D, E).

Survival of hiPSC-NS/PCs grafted into the spinal cord of immunocompetent BALB/cA mice

ffLuc-Transduced 253G1-NS/PCs were transplanted into the intact spinal cord of immunocompetent BALB/cA mice with immunosuppressants (FK506 plus anti-CD4 mAb; With-IS group, n = 21) and without immunosuppressants (Without-IS group, n = 6). The survival rate of the grafted 253G1-NS/PCs was then examined using *in vivo* BLI. The bioluminescent ffLuc signals emanating from the transduced 253G1-NS/PCs disappeared between 14 and 21 days after transplantation in the Without-IS mice (Table 1, Fig. 2A, B). This result suggests that immune rejection of the xenografted 253G1-NS/PCs occurred within 3 weeks in all of the mice without immunosuppressants, even though the spinal cord is a so-called immune-privileged site.

Graft survival rate was 100% in the With-IS (FK506 plus anti-cluster of differentiation (CD4) monoclonal antibody) group, versus 0% in the Without-IS group. Immune rejection of transplanted human induced pluripotent stem cell-derived neural stem/progenitor cells (hiPSC-NS/PCs) was achieved by discontinuing the immunosuppressant treatment.

In the Without-IS group, three mice died by day 28 and one mouse was sacrificed by day 72. In the With-IS group, two mice died by day 138 and one mouse was sacrificed by day 123. In the IS off group, six mice died (four mice by day 28, a further one mouse by day 123, and a further one mouse by day 148) and five mice were sacrificed (three mice by day 123 and a further two mice by day 200).

The phylogenetic difference between mice and humans leads to a reduced affinity of murine T-cell receptors (TCRs) for human major histocompatibility complex (MHC) molecules. Therefore, immune recognition by an indirect pathway involving the host's antigen-presenting cells and CD4-positive T-cells apparently participates in the elimination of the xenografted cells. Human iPSC-NS/PC rejection after cell transplantation is largely owing to a T-cell-mediated, donor-specific immune response [16,27–29]; therefore, we transplanted 253G1-NS/PCs in the presence of FK506 and anti-CD4 mAb immunosuppressants, as noted above, to avoid the immune rejection of the xenografts. Monitoring of the number of lymphocytes in the peripheral blood indicated that CD4-positive T-cells were depleted immediately after administration of the immunosuppressants but recovered at 28 days after drug discontinuation (Fig. 3).

Consistently, the long-term survival of the grafted 253G1-NS/PCs was achieved in all of the With-IS group mice through the use of immunosuppressants. The graft survival rate was 100% at 100 days after transplantation (n = 5/5 surviving animals), as well as at 200 days after transplantation (n = 2/2 surviving animals) (Table 1).

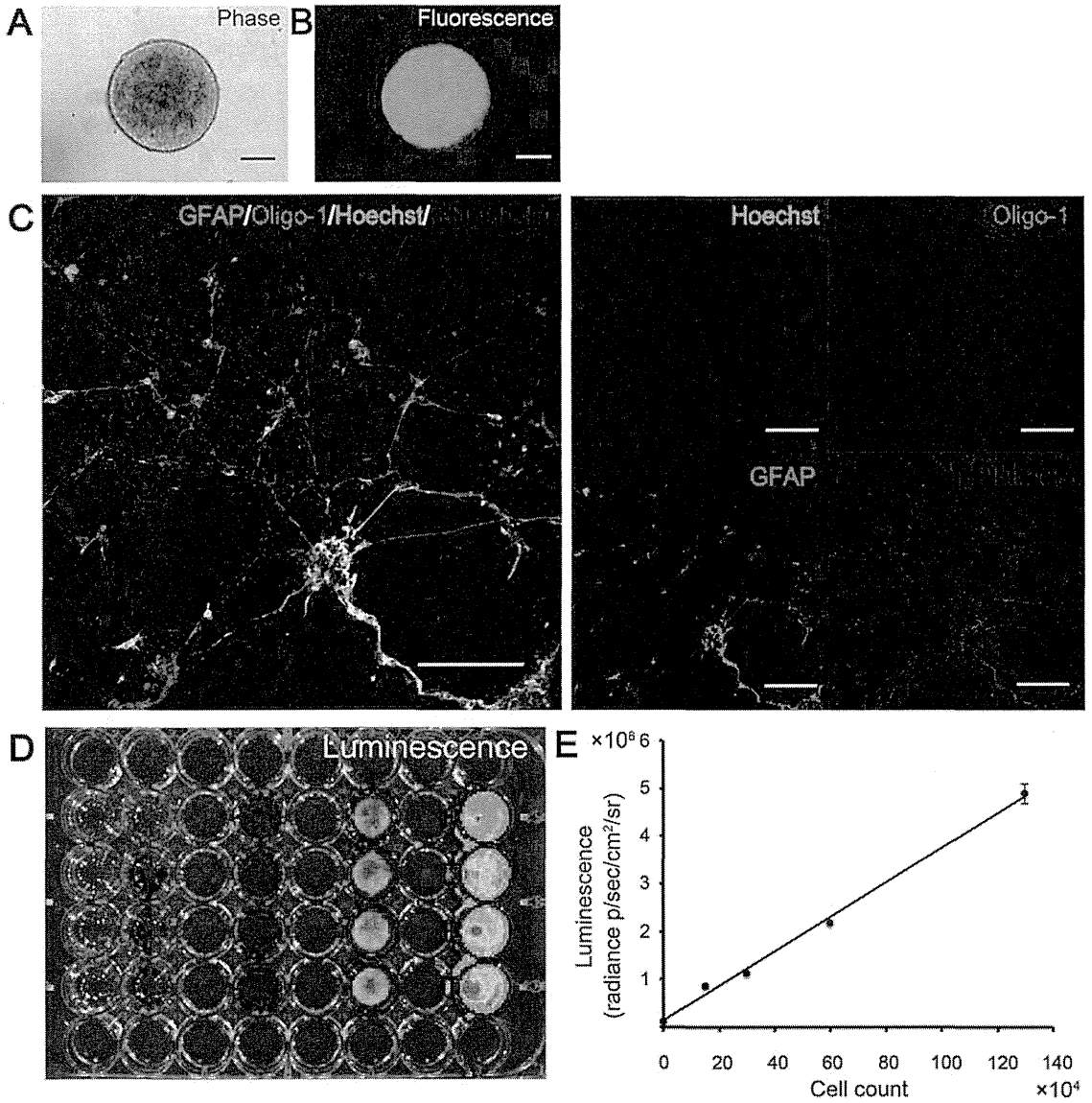

Fig 1. Detection of bioluminescence and fluorescence signals in lentivirally transfected 253G1-NS/PCs *in vitro*. Phase-contrast (A) and fluorescence (B) images of a neurosphere derived from tumorigenic 253G1 induced pluripotent stem cells. Neural stem/progenitor cells (NS/PCs) differentiated into β-III tubulin-positive neurons and glial fibrillary acidic protein (GFAP)-positive astrocytes *in vitro* (C). Bioluminescence imaging was used to detect bioluminescence signals in various numbers of 253G1-NS/PCs (0, 1.5×10^5, 3×10^5, 6×10^5, and 1.2×10^6 cells per well) (D). A direct linear correlation was found between cell numbers and photon counts *in vitro* (E). Scale bars in A–C, 1,000 μm.

doi:10.1371/journal.pone.0116413.g001

Histological analysis of hiPSC-NS/PC-derived tumors

The HE-stained spinal cord of a representative mouse in the With-IS group is shown in Fig. 4A. A number of HNA-positive (Fig. 4B) tumor cells occupied the entire spinal cord. The resultant tumors showed a biphasic pattern including high and low cell density areas. In the high cell density area, the tumor cells were characterized by small cell bodies with bland nuclei

Table 1. Fate of hiPSC-NS/PCs transplanted into the mouse spinal cord.

		Graft survival						
Group	**Immunosuppressant**	**0 d**	**28 d**	**72 d**	**123 d**	**138 d**	**148 d**	**200 d**
Without-IS	**None**	6/6	0/3	0/2	0/2	0/2	0/2	0/2
		(100%)	(0%)	(0%)	(0%)	(0%)	(0%)	(0%)
With-IS	**FK506 + anti-CD4**	5/5	5/5	5/5	3/3	2/2	2/2	2/2
		(100%)	(100%)	(100%)	(100%)	(100%)	(100%)	(100%)
IS off	**FK506 + anti-CD4**	16/16	12/12	12/12	5/8	2/8	0/6	0/5
	(Discontinued at 100 d)	(100%)	(100%)	(100%)	(62.5%)	(25%)	(0%)	(0%)

doi:10.1371/journal.pone.0116413.t001

and elongated fibers. On the other hand, the tumor cells in the low cell density area were characterized by minute vacuoles and microcysts (Fig. 4A). Histologically, the tumors resembled low-grade glioma rather than teratomas (Fig. 4A, B).

The grafted 253G1-NS/PCs differentiated into β-III tubulin-positive neurons, GFAP-positive astrocytes, and Oligo-1-positive oligodendrocyte progenitor cells. In addition, we also observed cells that stained positively for Nestin, which is a neural progenitor marker, and octamer-binding transcription factor 4 (Oct4), which is a marker of undifferentiated pluripotent stem cells [30] (Fig. 4C–G). Cells also stained positively for the Ki-67 antigen, a marker of actively diving cells (Fig. 4H). The ratio of cells that stained positively for Ki-67 to cells that stained positively for Hoechst was 6.60 ± 0.22%.

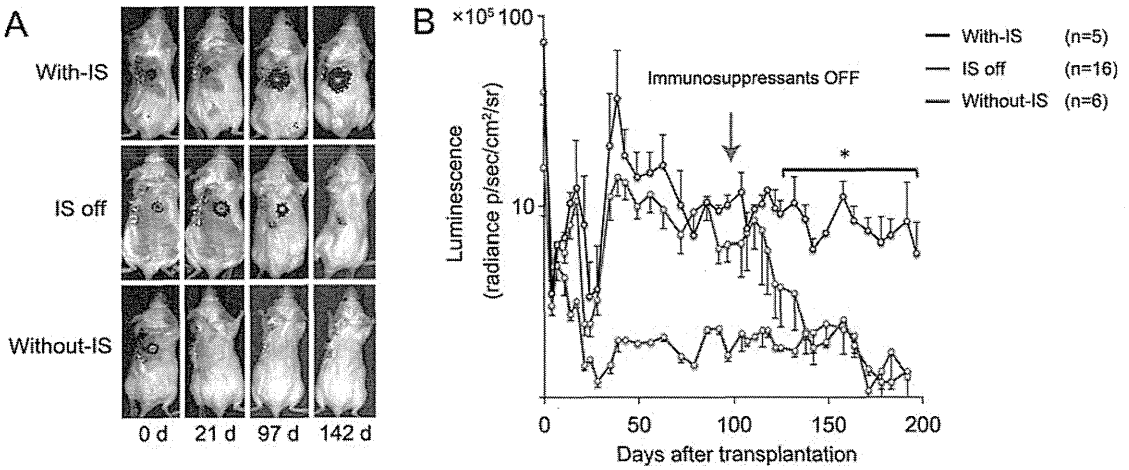

Fig 2. Representative *in vivo* images and quantitative analysis of photon counts derived from grafted hiPSC-NS/PCs. (A) Bioluminescence images of representative mice at 0, 21, 97, and 142 days after human induced pluripotent stem cell-derived neural stem/progenitor cell (hiPSC-NS/PC) transplantation. Upper panel: BALB/cA mouse with immunosuppressant treatment (FK506 plus anti-cluster of differentiation (CD) 4 monoclonal antibody (mAb); With-IS group); middle panel: BALB/cA mouse with immunosuppressant treatment, followed by discontinuation of immunosuppressants 100 days later (IS off group); lower panel: BALB/cA mouse without immunosuppressant treatment (Without-IS group). (B) Quantitative analysis of photon counts derived from grafted hiPSC-NS/PCs. Graft survival rate was 100% (n = 17/21, three animals died and one animal was sacrificed by day 21) in BALB/cA mice with immunosuppressant treatment (FK506 plus anti-CD4 mAb), versus 0% (n = 0/6) in BALB/cA mice without immunosuppressant treatment. After discontinuing the administration of FK506 and anti-CD4 mAb, all the grafted cells were rejected by day 164 and drastic reductions in signal intensity were observed. Data represent the mean value ± the standard error of the mean. (*$p<0.05$; Friedman's test followed by Dunn's post-hoc test.)

doi:10.1371/journal.pone.0116413.g002

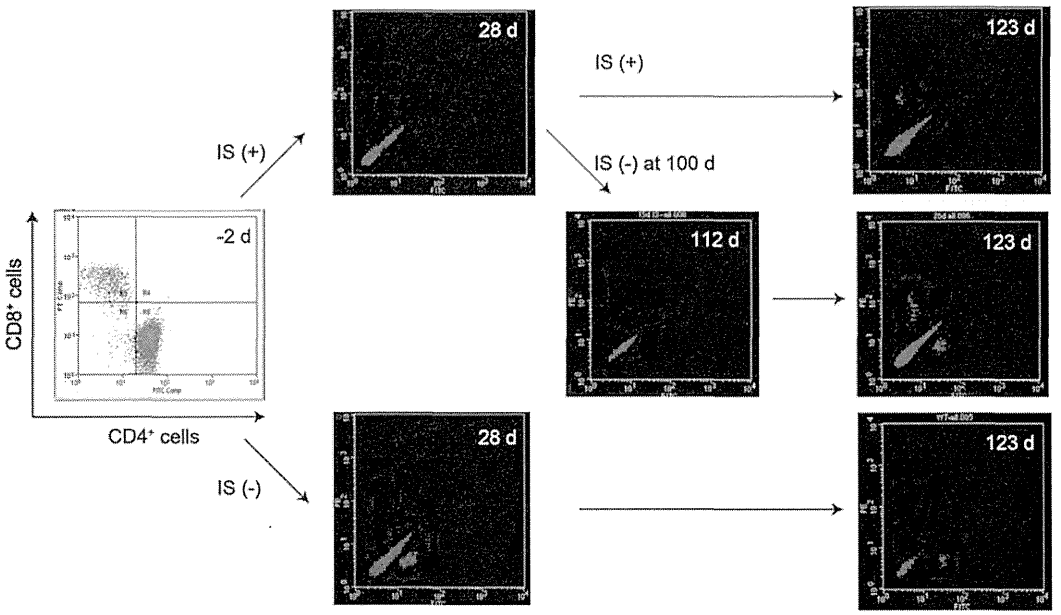

Fig 3. Lymphoid population in peripheral blood. Peripheral blood cells were analyzed using fluorescence-activated cell sorting-based flow cytometry. Data were gated on cluster of differentiation (CD) 4-positive or CD8-positive T-cell subsets. CD4-positive T-cells were depleted immediately after administration of FK506 plus anti-CD4 monoclonal antibody, but recovered after discontinuing immunosuppressant treatment. IS(+), with immunosuppressants; IS(–), without immunosuppressants.

doi:10.1371/journal.pone.0116413.g003

Recovery of CD4-positive T-cells, reconstitution of the host immune system, and immune rejection of hiPSC-NS/PC-derived tumors

To induce the ablation of the xenografted 253G1-NS/PC-derived tumors, we discontinued the immunosuppressant treatment ("IS off") at 100 days after cell transplantation. Consistent with the recovery of CD4-positive T-cells (described above in Fig. 3), the rejection of the hiPSC-NS/PCs was initiated with the cessation of FK506 and anti-CD4 mAb administration. The graft survival rate gradually decreased to 62.5% (n = 5/8 surviving animals) at 123 days and 25% (n = 2/8 surviving animals) at 138 days (Table 1). At 164 days, the transplanted hiPSC-NS/PCs were completely ablated in all of the surviving mice (Fig. 2B). No tumor recurrence was detected for at least 200 days in the absence of immunosuppressants (Fig. 2B).

Consistent with the BLI results, grafted hiPSC-NS/PC-derived tumors persisted in the immunocompetent BALB/cA mice with immunosuppressant treatment, but regressed after the cessation of FK506 and anti-CD4 mAb administration. During the course of immune rejection, CD11b-positive microglia, CD3-positive lymphocytes, and NKp46-positive natural killer (NK) T-cells infiltrated the tumors. TdT-positive cells were also observed within the tumors. Quantitative analyses of CD11b-positive, CD3-positive, NKp46-positive, and TdT-positive cells revealed that the numbers of all of these cells peaked at 122 days after iPSC-NS/PC transplantation (25 days after IS off), and then decreased at 164 days (64 days after IS off) to levels similar to those observed before immunosuppressant discontinuation (Fig. 5A–C).

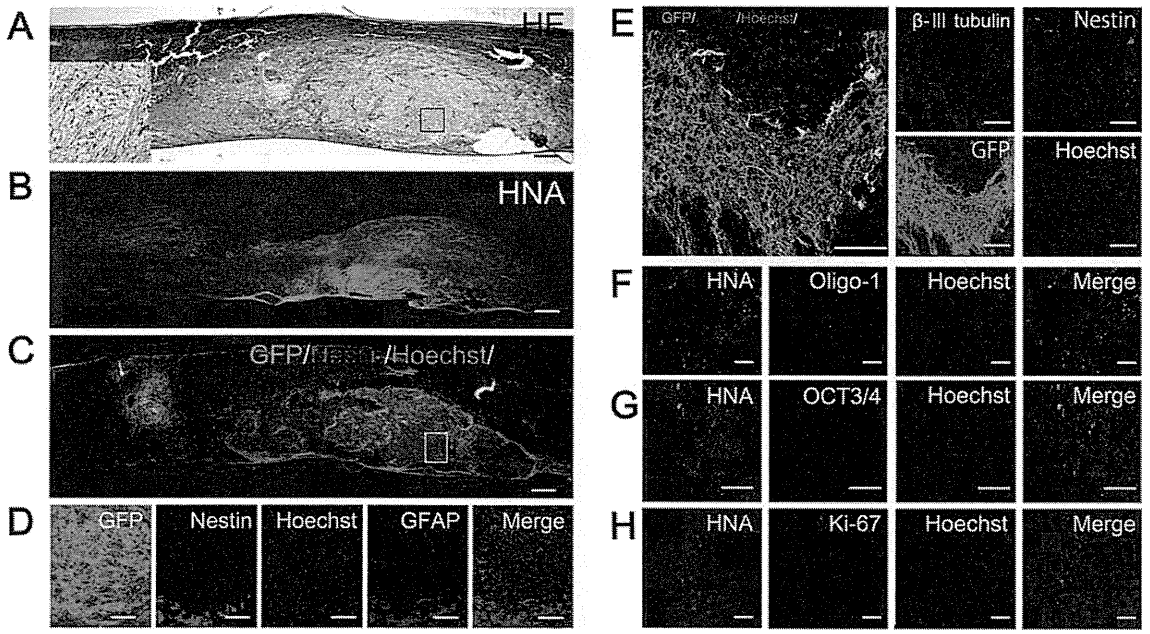

Fig 4. Tumor formation by grafted hiPSC-NS/PCs in the mouse spinal cord. Representative hematoxylin-eosin (HE)-stained (A) and human nuclear antigen (HNA)-stained (B) images of sagittal sections of spinal cord at 79 days after cell transplantation. HE staining revealed a biphasic tumor pattern with high and low cell density areas. The high cell density area contained compact bipolar cells with rosenthal fibers, whereas the low cell density area contained loose-textured multipolar cells with microcysts. The low cell density area surrounded by the square box is shown at higher magnification to the left of the image. Immunostaining for glial fibrillary acidic protein (GFAP), HNA, GFP, nestin, β-III tubulin (C, D, E), and Oligo-1 (F). The human induced pluripotent stem cell-derived neural stem/progenitor cell (hiPSC-NSC)-derived tumors mainly consisted of undifferentiated cells that stained positively for nestin, with low numbers of differentiated cells (e.g., β-III tubulin-positive neurons, GFAP-positive astrocytes, and Oligo-1-positive oligodendrocyte precursor cells). Nestin-positive cells were located in the center of the tumor, whereas differentiated cells were localized to the tumor margin (D, E, F). The boxed area in (C) corresponds to the higher magnification images in (D). Tumors contained a paucity of octamer-binding transcription factor (Oct) 4-positive (G) and Ki-67-positive (H) cells. The Ki-67 index was 7.0%. Scale bars in A–C, 500 μm; D–H, 100 μm.

doi:10.1371/journal.pone.0116413.g004

Discussion

Rejection of hiPSC-NS/PC-derived tumors in a xenogeneic setting

Following the transplantation of 253G1-NS/PCs into the spinal cord of BALB/cA mice, essentially all of the grafted human cells were rejected within 3 weeks, despite the immune-privileged status of the spinal cord. The spinal cord in particular and the CNS in general are thought to be immunologically tolerant based on the following criteria: 1) the brain and the spinal cord are isolated from the rest of the body by the surrounding blood-brain barrier (BBB); 2) the CNS does not contain lymphatic vessels; 3) microglia and dendritic cells do not function normally in the CNS; and 4) low expression levels of MHC molecules are found in the CNS [12].

The expression levels of MHC class I molecules are also low on the surface of human ESC-derived NS/PCs and fetus-derived NS/PCs *in vitro*, whereas the expression levels of MHC class II molecules and co-stimulatory molecules are not detectable. Therefore, the immunogenicity of these NS/PCs is considered negligible. However, NS/PCs exert diverse immunomodulatory actions that increase the host CD4-/CD25-/Forkhead box P3-positive T-cell population, augment the levels of secreted immunomodulatory cytokines (e.g., interleukin (IL)-6), inhibit the presentation of antigens to host T-cells, and stimulate the activation and proliferation of host T-cells [18,31–36].