discontinuing immunosuppressants at 7 days after transplantation (Fig. 4A), resulting in motor functional recovery of the hind limbs (Fig. 4B). On the other hand, all of the mice (N=20/20) died before tumor rejection when immunosuppressant treatment was discontinued at 14 days after transplantation.

Lymphocyte subsets in the peripheral blood were then examined by using FACS with anti-CD4 and anti-CD8 Abs. CD4+ T cells were immediately depleted with administration of immunosuppressants, but recovered after immunosuppressant treatment was discontinued (Fig. 4C).

Histological analysis of U251 cells in C57BL/6J mice with immunosuppressant treatment

Histological analysis revealed that xenografted human GBM U251 cells survived in C57BL/6J mice with immunosuppressive therapy. In some mice, the tumor protruded to the outside from the posterior median sulcus. However, the grown tumors were rejected after discontinuation of immunosuppressants (Fig. 5). The graft rejection status is shown at various post-transplantation time points in Figure 6A. Infiltration of inflammatory cells into the tumor, such as CD11b-, NKp46-, and CD3-positive cells, as well as an increase in the number of TdT-positive cells detected by the TUNEL assay, were observed during the process of tumor rejection. These cells became prominent by 39 days after discontinuation of immunosuppressants (Fig. 6B, C).

Immunorejection of hiPS-NS/PCs grafted into BALB/cA mice after

discontinuation of immunosuppressant treatment

We also performed xenotransplantation of hiPS-NS/PCs into the intact spinal cord of BALB/cA mice (N = 5) and observed tumor formation. This tumor showed biphasic pattern with areas of high and low cell density. The high cell density areas contained compact bipolar cells with Rosenthal fibers, whereas the low cell density areas contained loose-textured multipolar cells with microcysts. Histologically, these tumors resembled low-grade gliomas rather than teratomas. After confirming the tumor growth, we discontinued the immune suppression at 100 d after transplantation. Thereafter, we confirmed complete tumor rejection by BLI and histopathology (Fig. 7).

DISCUSSION

In terms of the clinical application of iPSC-based cell therapy, the most serious issue is the prospective tumorigenicity of the transplanted cells (21,30,44). Indeed, there is no report of the formation of tumors other than teratomas after transplantation of iPS derivatives. However, here, we observed formation of a glioma-like tumor after the transplantation of NS/PCs derived from an inappropriate iPS cell line that was established using a retroviral vector (Nori et al., in submission). Based on this result, in regard to potential tumorigenesis after iPS-NS transplantation, we have to consider not only teratoma formation, but also tumors derived from cells of the neural lineage. In fact, we plan allogeneic iPS-NSC transplantation into the injured spinal cord in a first-in human study from the viewpoints of the timing of transplantation after injury, safety and cost performance. To minimize the risk of the therapy using iPS-NSCs, it will be

important to examine whether the allo-grafted iPS-NSC-derived tumors could be eliminated without any adverse events by inducing immunorejection. As a first step, to confirm the feasibility of this concept, we used human GBM, because GBM is the most aggressive and toughest growing tumor against immunorejection. To this end, we established a human GBM-grafted mouse model with or without immunosuppressant therapy. This system allowed the course of the tumor fate to be observed in real time using BLI under immunodeficient and immunocompetent conditions. We also utilized the same approach against the glioma-like tumor that developed after the transplantation of NS/PCs derived from inappropriate iPS cells. We propose that our model may serve as a valuable tool to perform translational research of iPSC-derived NS/PC transplantation for SCI.

Human glioma-grafted model

Many brain tumor models using rat glioma cell lines have been reported (5,15,16,19,45). However, there are several limitations to the use of these rodent tumor models, such as the different response of rodent vs. human glioma cell lines to treatment with chemotherapy agents (34,40,41). Therefore, xenotransplantation models of human glioma cell lines into animal models have begun to be investigated (31-33). In the present study, we developed a novel mouse model of an intramedullary spinal cord tumor by using the human GBM U251 MG cell line. We also refined the human GBM xenograft model by using BLI, which is a reliable method for live monitoring of tumor growth *in vivo* (24,26,29,39). The advantage of BLI is that tumor cells are tagged with

the luciferase gene, and only living tumor cells are visualized, while GFP-based imaging can also detect dead cells. Furthermore, in contrast to magnetic resonance imaging, BLI detects only implanted cells without the inclusion of inflammatory regions (11,29,36). By measuring this luminescence, we can quantitatively evaluate the survival, growth, and rejection of grafted tumor cells over time.

Immunodeficient mouse model

The work described herein made use of the NOD/SCID mouse, which is an immunodeficient mouse lacking both T and B lymphocytes. The transplantation of luciferase-transfected U251 cells into this mouse strain produces enormous tumors with a consistent rate of growth, permitting the use of BLI to quantitatively measure the extent and duration of the tumor response to immunosuppressive therapy. For animal subjects receiving an injection of U251 cells, histopathological analysis revealed that tumor growth had progressed to the medulla, and our approach allowed anatomically accurate modeling of this type of brain tumor. Furthermore, histological assessment showed that this spinal cord tumor had cells characterized by nuclear atypia, microvascular proliferation, necrosis, and a high Ki-67 index (18%), similar to the findings of human grade IV GBM. Our model is simple and reproducible, and may be useful to evaluate the effectiveness of new treatments for spinal cord GBM.

Immunocompetent mouse model

The CNS, comprising the brain and the spinal cord, has long been assumed to exhibit higher immunological tolerance than other organs due to the following anatomical and physiological characteristics: 1) The CNS is in a state of immunological neglect because the brain and spinal cord are isolated from the rest of the body by the blood-brain barrier; 2) the brain and spinal cord contain a paucity of lymphocytes; 3) expression of major histocompatibility complex is low in the CNS; 4) antigen-presenting cells do not function well in the CNS; and 5) T cell apoptosis is induced by Fas/FasL in the CNS (23,35). We also transplanted U251 cells into the spinal cord of immunocompetent C57BL6/J mice without immunosuppressant administration. Consequently, immunorejection of the grafted cells was precipitated, even though the CNS is a so-called immune-privileged site. These observations may be due to the invasion of activated T cells and natural killer cells into the CNS beyond the blood-brain barrier (12). To avoid this immunorejection, we administered immunosuppressants (FK506 and anti-CD4 Ab) to the immunocompetent mice. FK506 treatment prevented immunorejection in 80% of mice, whereas the combination of FK506 and anti-CD4 Ab treatment resulted in the complete engraftment of xenografted cells in all mice. These findings suggest that our human GBM model has great merit for the study of the immune dynamics of xenotransplantation.

Immunorejection of the grafted tumor

To obtain a proof of concept regarding the immunorejection of graft-derived tumor cells by immunomodulation, we first confirmed their growth in the presence of

immunosuppressants via BLI. Next, we discontinued the administration of FK506 and anti-CD4 Ab to induce rejection of the tumor cells. Our GBM model showed hind limb paralysis and eventual death at 4 weeks after transplantation under immunosuppressant administration. Given that the half-life of FK506 in the blood is 12 hours and that of mouse anti-CD4 Ab is 9 days, the recovery of CD4-positive cells in the blood took about 14 days after the discontinuation of the immunosuppressants. At the beginning of this study, we discontinued administration of the immunosuppressants at 2 weeks after transplantation. Although CD4-positive cells in the peripheral blood were recovered at this time-point, all the mice died due to tumor growth. Therefore, we discontinued administration of the immunosuppressants on day 7 after U251 MG cell transplantation, when the tumors were rejected in all the mice. Based on this result, we considered that we may take advantage of immunorejection to control the survival of grafted highly malignant tumors such as GBM. In general, in the immunorejection of a xenograft, the problem is hyperacute rejection by NK cells to α-gal (13). However, this mechanism does not occur in xenografts of human cells in mice. In the present study, it is likely that the immunorejection occurred mainly through T cells as well as by an interaction with APCs, rather than via NK cells (Fig. 6), which is supported by several previous reports (1,2,8,38). Although it is well known that GBM can avoid immune attack through tumor immunity (43), transplantation immunity caused rejection through T cells, resulting in complete eradication of the grafted GBM without any adverse events such as neurological deterioration in this study. We also performed xenotransplantation of NS/PCs derived from an inappropriate hiPS cell line into the intact spinal cord of

BALB/cA mice and observed tumor formation. After discontinuation of immune suppression, we observed complete tumor rejection by BLI and histopathology. Finally, we confirmed the effectiveness of this paradigm against the tumor derived from the iPS-NS/PCs. These findings suggest that it is possible to induce immunorejection of any type of xenografted tumor cells by immunomodulation. However, the tumorigenic mechanisms of iPS-NS/PCs are still to be elucidated. Therefore there may be various differences between iPS-NS/PCs derived tumor and glioblastoma which are thought to arise from intrinsic glial cells with genetic mutations, abnormal epigenetic modifications and altered cellular metabolisms (7). These issues should be addressed in the future study.

CONCLUSIONS

In conclusion, the present study demonstrated that a mouse xenograft model of U251 glioma might be a reliable tool to target human spinal cord tumors in preclinical studies. Using this model, we were able to manipulate the survival of the grafted cells, even high-grade glioma cells, by immunorejection. This model may also be useful for the study of the therapeutic effect of anticancer drugs against malignant tumors. We are currently developing an iPSC-derived NS/PC transplantation therapy for SCI in animal models (18,27). The issue of the tumorigenicity of iPSC-derived NS/PCs remains a major concern towards clinical application of cell therapy in the near future. By applying the results of this study, we can evaluate whether the allografted cells can be

eliminated by immunorejection in the case of tumors developing after transplantation of iPSC-derived NS/PCs for SCI.

Acknowledgments and Financial Disclosures

We appreciate the help of Dr. K. Fujiyoshi, Dr. O. Tsuji, Dr. Y. Takahashi, Dr. S. Kaneko, Dr. A. Yasuda, Dr. S. Nori, Dr. T. Konomi, Dr. S. Shibata, Dr. M. Shinozaki, Dr. R. Zhang, Dr. S. Tashiro, Dr. S. Kawabata, Dr. Y. Nishiyama, Dr. K. Hori, Dr. M. Ozaki, Dr. T. Iida, Dr. K. Matsubayashi, and Dr. S. Suzuki, who are all members of the spinal cord research team at the Department of Physiology, Orthopaedic Surgery and Rehabilitation Medicine, Keio University School of Medicine. We also thank Ms. T. Harada, Ms. S. Miyao, Ms. M. Mizutani, Ms. Y. Fukaya, Ms. R. Yoshida, and Ms. H. Shimada for their assistance with experiments and animal care.

This work was supported by grants from the Japan Science and Technology (JST)-California Institute for Regenerative Medicine (CIRM) collaborative program to M.N.; a grant-in-Aid for Research Center Network for Realization of Regenerative Medicine, Centers for Clinical Application Research on Specific Disease/Organ, and the Ministry of Education, Culture, Sports, Science and Technology (MEXT) from the JST to H.O.; Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) and the MEXT; Keio Gijuku Academic Development Funds; a Grant-in-Aid for the Global Center of Excellence (COE) program from the MEXT to Keio University; and by a Grant-in-Aid for Scientific Research on Innovative Areas (Comprehensive Brain Science Network) from the MEXT. H.O. is a scientific

consultant for San Bio, Inc., Eisai Co., Ltd., and Daiichi Sankyo Co., Ltd. The other authors declare they have no competing financial interests.

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FIGURE LEGENDS

Figure 1. Luminescence and fluorescence in lentivirally-transfected U251 cells

A-B) Microscopic images of well-dispersed transfected U251 MG cells.

C) BLI was detected luminescence in a different cell numbers (0, 2×10^5, 7.5×10^5, 1.5×10^6, 3×10^6 cells).

D) Correlation between photon counts and cell numbers.

E) One thousand cells were sufficient to elicit a BLI signal *in vitro*.

Scale bars in B and C: 200 μm.

Figure 2. BLI images and photon counts over time after transplantation of U251 cells into NOD/SCID mice, as well as C57BL/6J mice with and without immunosuppressant treatment

A) BLI images of representative mice (upper: NOD/SCID mouse, middle: C57BL/6J mouse with immunosuppressant treatment, lower: C57BL/6J mouse without immunosuppressant treatment) at 0, 18, and 28 days after GBM U251 cell transplantation. Even if it was xenogeneic transplantation, transplanted tumor engrafted and grew as same as in the NOD/SCID mice group, by using two immunosuppressants.

B) Quantitative analysis of the photon counts derived from grafted cells. Graft survival rate was 100% (N=9/9, 2/9 animals were dead at day 14) in NOD/SCID mice, 100% (N=10/10) in C57BL/6J mice with immunosuppressant treatment, and 0% (N=0/6) in C57BL/6J mice without immunosuppressant treatment. Data represent the mean ± SEM. (*$p<0.05$; Repeated measures ANOVA followed by Bonferroni post hoc test.)

Figure 3. Histological analysis of grafted U251 cells in NOD/SCID mice

A) Low magnification HE staining (top) and GFP staining (bottom). Scale bar: 500 μm.

B) High magnification HE staining (top), and Hoechst, GFP, and Ki-67 (bottom) staining.

Grafted GBM cells showed good survival and migrated into the host spinal cord. The tumor showed nuclear atypia, microvascular proliferation, and necrosis. Ki-67 index was 18%.

Scale bars: 500 μm in A and 25 μm in B.

Figure 4. Regulation of the fate of grafted U251 cells by immunomodulation

A) Representative BLI images and quantitative analysis of the photon counts over time after transplantation of U251 cells into C57BL/6J mice with immunosuppressant treatment. Graft survival rate was 82% (N=9/11) in the FK506 treatment group and 100% (N=37/37) in the FK506/anti-CD4 Ab treatment group. Immunorejection of all the grafted U251 cells was achieved by discontinuing immunosuppressants. In the FK506 group, the immunosuppressive therapy was discontinued at 28 days after cell transplantation, By contrast, in the FK506/anti-CD4 Ab group, immunosuppressive therapy was discontinued at 7 days after cell transplantation.

B) Hind limb motor function was assessed by the Basso mouse scale (BMS). Hind limb motor function was lost to the level of complete paralysis with increasing tumor in some mice. After discontinuation of immunosuppressants, grafted mice showed functional

recoveries with disappearance of the tumor in the survived group (N=14/48).

C) Lymphocyte monitoring in C57BL/6J mice with immunosuppressant treatment. Lymphocyte subsets were observed by FACS with anti-CD4 and anti-CD8 Abs. $CD4^+$ T cells were immediately depleted following administration of immunosuppressants, and recovered after immunosuppressant treatment was discontinued.

Figure 5. Histological analysis of grafted U251 cells in C57BL/6J mice with and without immunosuppressant treatment

Consistent with the BLI results, grafted U251 cells survived in C57BL/6J mice with immunosuppressant treatment. The tumors were rejected after discontinuation of immunosuppressant treatment.

Scale bar: 500 μm in A and 100 μm in B.

Figure 6. Immunohistochemistry analysis during the immunorejection of grafted U251 cells

A) Rejection graft status.

B) Quantitative analyses of the numbers of HNA-, CD3-, CD11b-, NKp46-, and TdT-positive cells (detected by the TUNEL assay) cells after discontinuing immunosuppressant treatment.

C) HNA staining clearly shows the gradual reduction of tumors.

D) CD11b, CD3, NKp46, and TdT staining in inflammatory cells. The numbers of CD11b-, CD3-, and NKp46-positive cells increased up to 32 days after transplantation

and then gradually decreased. TdT-positive cells also became more prominent following transplantation.

Scale bars in C and D: 100 μm.

Figure 7. Representative quantitative analysis of photon counts derived from grafted hiPS-NS/PC and pathological analysis of iPS-NS/PC derived tumor.

(A) Quantitative analysis of photon counts derived from grafted hiPS-NS/PCs. The graft survival rate was 100% (n = 5/5) in the BALB/cA mice given immunosuppressant treatment (FK506 plus anti-CD4 mAb). After discontinuing the administration of FK506 and anti-CD4 mAb, all the grafted cells were rejected and drastic reductions in the signal intensity were observed.

(B, C) Representative HE-stained sagittal images of the mouse spinal cord after cell transplantation. HE staining revealed a biphasic tumor pattern with areas of high and low cell density. The high cell density areas contained compact bipolar cells with Rosenthal fibers, whereas the low cell density areas contained loose-textured multipolar cells with microcysts. The image within the square box is shown at higher magnification. After discontinuing the administration of immunosuppressants, the tumor was rejected completely. Scale bars: 500 μm in B-1, C-1, 50 μm in B-2, C-2 and 25 μm in B-3, C-3.

Table 1: Graft survival for the different treatment groups

		Graft survival					
Host	immunosuppressant	0 d	7 d	14 d	21 d	28 d	35 d
NOD/SCID	none	9/9	9/9	9/9	7/7	7/7 sacrifice	
C57BL6/J	none	6/6	6/6	0/6	0/4	0/3	0/3
C57BL6/J	FK506	11/11	11/11	11/11	9/11	9/11	0/11
C57BL6/J	FK506 + anti-CD4	37/37	37/37	37/37	22/22	8/8	0/5