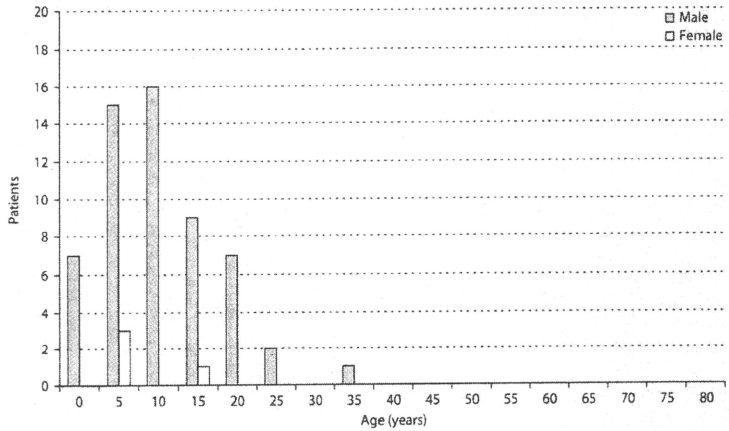

i

Fig. 1. **i** Malignant teratoma (n = 62; male:female ratio = 14.5:1).

Cumulative survival rates of each pineal tumor were calculated by Cutler's method. Five-year survival rate of germinoma was 89.4%, while those of embryonal carcinoma, yolk sac tumor and choriocarcinoma were 35.3, 37.5 and 58.1%, respectively (table 4).

Discussion

The incidence of the pineal tumors according to the centralized brain tumor registries varies from 0.4 to 1% among adult patients and from 3 to 5% among children [7]. Especially germ cell tumors are very frequent in Asian countries such as Japan and Korea [8]. The frequency in Japan is 5 times as high as in the western countries. In the registries of the United States, germ cell tumors were classified only into germinoma and mixed germ cell tumors, and the details of choriocarcinoma, embryonal carcinoma and yolk sac tumors were not reported. Even in Japan these tumors are rare, but 95 cases apart from 68 cases with mixed germ cell tumors were registered in BTRJ during 1984 and 2000 and survival rates were calculated. Compared with pure germinomas, survival rates of these tumors were very low, and especially those of embryonal carcinoma and yolk sac

Table 3. Frequencies of pineal tumors by histology (BTRJ 1984–2000)

	Male	Female	Total
Germinoma	542	43	585 (49.2%)
Pineoblastoma	51	50	101 (8.5%)
Pineoblastoma	41	24	65 (5.5%)
Teratoma	57	4	61 (5.1%)
Malignant teratoma	58	4	62 (5.2%)
Embryonal carcinoma	31	3	34 (2.9%)
Yolk sac tumor	31	3	34 (2.9%)
Choriocarcinoma	23	4	27 (2.3%)
Other germ cell tumor	60	8	68 (5.7%)
Glioma	42	35	77 (6.5%)
Dermoid	4	1	5 (0.4%)
Epidermoid	13	2	15 (1.3%)
Others	32	22	54 (4.5%)
Total	985	203	1,188 (100.0%)
Unknown	84	72	105

Table 4. Cumulative survival rates of pineal tumors (BTRJ 1984–2000)

	Patients	1 year	2 years	3 years	4 years	5 years
Germinoma	486	96.2	92.4	91.3	90.3	89.4
Pineocytoma	77	95.4	89	87.5	87.5	84.1
Pineoblastoma	30	74.8	57.1	50.7	48.5	46.1
Teratoma	50	96.4	96.4	92.2	92.2	89.6
Malignat teratoma	41	84.2	74.9	72.8	70.6	70.6
Embryonal carcinoma	12	64.7	38.2	35.3	35.3	35.3
Yolk sac tumor	13	55.6	44.8	37.3	37.3	37.3
Choriocarcinoma	15	62.3	62.3	62.3	62.3	58.1

Rates are expressed as percentages.

tumors were less than 40%. Although chemotherapies such as ifosfomide, cisplatin and etoposide and carboplatin + etoposide improved the survival, the latter is still unsatisfactory.

Nationwide registry is important. It is a retrospective study and the evidence level is not as high as it would be in a prospective study. But thanks to it we can understand the global aspects of the tumors, especially rare tumors such as pineal tumors. In 2008, BTRJ started to register tumors online. The registration rate is expected to be much higher, and most of the brain tumors in Japan will be registered.

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14. 転移性脳腫瘍の治療方針にエビデンスはあるのか？

1 序論

日本における転移性脳腫瘍の頻度についての正確な統計は存在しない．しかしながら，がん患者の剖検による脳転移の発見率は20～30％とも言われ，日本のがん死亡者数が年間30万人であることから，推定患者数は6～9万人に達し，原発性脳腫瘍を凌駕するとも言われている．一方，脳腫瘍全国統計調査の転移性脳腫瘍登録数は，過去10年にわたりほぼ1,000件に過ぎない．このように，これまで日本の脳神経外科医は，転移性脳腫瘍の治療に関して関心が低かったと言わざるを得ない．

転移性脳腫瘍の治療法には，摘出術，全脳照射，そしてガンマナイフやXナイフといった定位照射があるが，その有効性に関しては個別の評価が多く，ランダム化比較臨床試験は少ない．そのため多くの患者が発生しているにもかかわらず各施設でまちまちの治療が実施されてきたのが実状である．

2 指針

転移性脳腫瘍の治療法は，転移個数により異なるため，ここでは単発と多発の場合に分けて標準治療とされているものを記載する．

単発例

これまでに行われたPatchell（1990，1998）[1,4]，Vecht（1993）[2]，Mintz（1998）[3]の4つのランダム化比較試験の結果により，原疾患がコントロールされている場合，単発の脳腫瘍に対する欧米での標準治療は摘出術＋全脳照射と考えられている．

ただし，現実には最大径が3 cm未満の病変に対しては，定位照射が急速に普及しつつある．例えばO'Neillら[5]は，Mayo Clinicの35 mm以下，単発の転移性脳腫瘍に関して，摘出術＋全脳照射を行った74例と，定位照射＋全脳照射24例のretrospectiveな解析を行い，頭蓋内の再発（30％ vs 29％），1年生存割合に差がなかったとの報告がある．

さらに，原発巣も含めた他臓器病変の治療法の向上により生存期間が延長し，全脳照射による高次脳機能障害が問題視されるようになってきた．このため，術後の全脳照射に代わる局所放射線療法に関する報告も増加している[7]．

多発例

多発例の場合，画像上明らかではない他の転移巣の存在も疑われるため，全脳照射が標準治療とされる．照射量に関しては，米国のRadiation Therapy Oncology Group（RTOG）で多くの検討がなされ，現在は30 Gy（3 Gy×10 fr）や37.5 Gy（2.5 Gy×15 fr）が多く用いられている．

ただし多発例であっても一部の腫瘍径が大きく放置すると致死的と考えられる場合や，症状

が高度である場合には，摘出術が行われることがある．

また，腫瘍径が3 cm未満で，転移個数が少なければ定位照射が用いられる[10]．Serizawaら[9]は，頭蓋内総線量が10 J以下で照射可能な多発症例1,386例をガンマナイフ単独で治療し，中央生存期間は9.4カ月，1年における神経死予防率88.5%，神経機能温存率81.5%，新規病変非出現率56.3%，追加照射回数平均0.69の成績を得て，多発病変におけるガンマナイフ単独治療の有用性を報告している．ただし，定位照射で治療可能な転移個数に関するエビデンスレベルの高い研究はいまだなされていない．

3 エビデンス

1 Patchell RA, et al. Postoperative radiotherapy in the treatment of single metastases to the brain: a randomized trial (JAMA. 1998; 280: 1485-9)[4]

目的：単発転移摘出術後の全脳照射に再発および新病変出現予防効果があるか否か，生存期間の延長をもたらすか否かの検討．

対象・方法：摘出術により腫瘍が全摘され，組織型が確定しており，KPS（Karnofsky performance scores）が70以上の患者に対して，①全脳照射群（術後28日以内に全脳照射50.4 Gy）と，②経過観察群（全脳照射を行わず，経過観察）の治療法を検討する多施設共同ランダム化比較試験．

一次エンドポイントは頭蓋内再発，二次エンドポイントは生存期間・死因・機能温存率．

結果：全脳照射群49名，経過観察群46名，計95名で評価．生存期間，機能温存期間に有意差はなかったが，摘出部位の再発予防効果が認められた（表1）．

表1 結果

Treatment arms	no. of cases	Median survival time (weeks)	Overall local control rate (%)	Median duration of functional independence (KPS≧70) (weeks)
Surgery＋WBRT (50.4 Gy)	49	48	90	35
Surgery alone	46	43	54	37
		n. s.	$p<0.001$	n. s.

結論：単発性転移性脳腫瘍摘出術後の全脳照射には，頭蓋内再発予防効果があり，中枢神経死を回避する効果が認められる．

2 Aoyama H, et al. Stereotactic radiosurgery plus whole-brain radiation therapy vs stereotactic radiosurgery alone for treatment of brain metastases. a randomized controlled trial (JAMA. 2006; 295: 2483-91)[8]

目的：1～4個の転移性脳腫瘍に対して定位照射単独と定位照射＋全脳照射の有効性を比較した前方視的ランダム化比較臨床試験．

対象・方法：対象は1～4個の転移性脳腫瘍患者で，転移巣の最大径が3 cm未満，KPSが70以上の症例．治療アームは，①定位放射線照射（最大径2 cm以下は，marginal dose 22-25 Gy/最大径2 cmを超える場合は，18-20 Gy）と，②定位照射＋全脳照射群（定位照射に先立ち30 Gyの全脳照射．定位照射の線量は30%減）．

結果：定位照射群67例，定位照射＋全脳照射群65例の合計132例で検討．生存期間中央値は，定位照射群8.0カ月，定位照射＋全脳照射群7.5カ月で有意差なし．一年後の再発率は，定位照射群76.4%，定位照射＋全脳照射群46.8%と有意差を認めた（$p<0.001$）．神経死の割合は，定位照射群19.3%，定位照射＋全脳照射群22.8%で有意差なし（$P=0.64$）．KPSおよびMMSEで評価した機能温存率にも有意差なし．

結論：定位照射＋全脳照射は，定位照射単独と比較して再発防止には有効であるが，生存期間延長の効果はなかった．

3] Chang EL, et al. Neurocognition in patients with brain metastases treated with radiosurgery or radiosurgery plus whole-brain irradiation: a randomised controlled trial（Lancet Oncol. 2009; 10: 1037-44）[6]

目的：定位照射に全脳照射を加えることにより学習および記銘力障害の発生率が上昇するかを検討するランダム化比較試験．

対象・方法：対象は1～3個，RPA classが1～2，KPS 70以上の患者．治療アームは，定位照射単独群（照射線量はRTOG90-05の基準）と定位照射に全脳照射（30 Gy）を加えた群の2アーム．治療後の1，2，4，6，9，12，15，18カ月，以後6カ月ごとに頭部MRIと高次脳機能検査（HVLT-R，WAIS-Ⅲ，COWAなど）で評価．

結果：定位照射群30例，定位照射＋全脳照射群28例の時点で，治療後4カ月のモニタリング結果から定位照射単独群では高次脳機能の低下が52%であったのに対して全脳照射併用群では96%の低下が認められたことから，この時点で試験は中止された．

結論：定位照射と全脳照射併用療法は，治療後4カ月でも高次脳機能障害のリスクが高いことが明らかとなった．この結果から，初回治療としては全脳照射を併用せず，定位照射のみを行い，頻回に経過観察を行うことが推奨される．

4] Rades D, et al. Whole brain radiotherapy plus stereotactic radiosurgery（WBRT＋SRS）versus surgery plus whole brain radiotherapy（OP＋WBRT）for 1-3 brain metastases: results of a matched pair analysis（Eur J Cancer. 2009; 45: 400-4）[11]

目的：転移個数1～3の症例に関して摘出術＋全脳照射と定位照射＋全脳照射の有効性を比較するmatched pair analysis．

対象・方法：対象は，転移個数1～3の症例で，全脳照射スケジュール，年齢，性別，Performance status，腫瘍組織，転移個数，RPSクラス，診断から全脳照射までの期間をすべてマッチさせた摘出術＋全脳照射群52例と定位照射＋全脳照射群52

例の比較．定位照射は，平均 20 Gy（15～25 Gy），全脳照射は，5×4 Gy，10×3 Gy，20×2 Gy のいずれか（両群で施行数は同じ）．Overall survival（OS），intracerebral control（IC），local control（LC）を評価．

結果：1 年目の OS は，定位照射群 56%，摘出術群 47%（p＝0.034），同じく IC は定位照射群 66%，摘出術群 50%（p＝0.003），LC は，定位照射群 82%，摘出術群 66%（p＝0.006）といずれも定位照射群が有意に高値であった．

結論：定位照射＋全脳照射は，少なくとも摘出術＋全脳照射と同等に有効である．

4 根拠となった臨床研究の問題点と限界

転移性脳腫瘍患者の中枢神経死の割合は，約 30%と考えられる．このため，治療効果の比較試験では，生存期間で有意差を得ることが難しいという問題がある．さらに複数のランダム化試験を比較する場合にも，患者の全身状態や原発巣の組織型の割合が異なる場合には，比較自体が意味をなさない場合もあり，メタアナリシスを行うにも限界がある．

5 （本邦の）患者に適応する際の注意点

最新の脳腫瘍全国集計調査報告（1984～2000 年）[12]において，転移性脳腫瘍に対して選択された治療法の割合を見ると，肺がんでは，摘出術＋放射線治療が 16.2%，摘出術のみが 61.4%，放射線治療のみが 22.4%，乳がんでは，摘出術＋放射線治療が 22.6%，摘出術のみが 75%，放射線治療のみが 2.4%，胃がんでは，摘出術＋放射線治療が 2.7%，摘出術のみが 96.2%，放射線治療のみが 1.1%であった（図 1 参照）．前述のごとく，この統計調査に登録される症例数は年間わずか 1,000 例であり，悉皆性が高くはないが，いずれにしても転移性脳腫瘍に関して我が国では，国際的な標準治療が長年にわたり標準治療とはなっていなかったと言える．また，我が国では，他国と比較して定位照射が多用されている点も特徴的である．

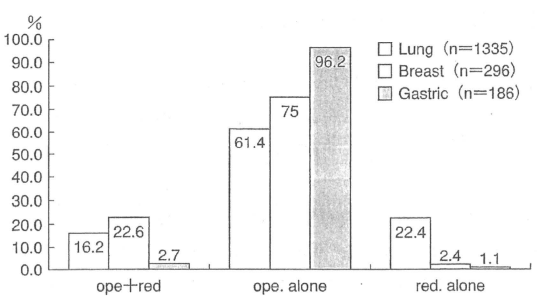

図 1 治療法（1984-2000）[12]
Ratio of Cases who lived more than 30 days.

6 コメント

日本においては転移性脳腫瘍の治療法としてすでに多くの施設で定位照射が行われていた2003年においても，米国のNational Comprehensive Cancer Networkの治療ガイドラインでは，1～3個の少数転移例の治療として定位照射のアームは存在していなかったが，2006年度版，最新の2009年度版と版を重ねるたびに定位照射の比重が高くなっている．今後の転移性脳腫瘍の治療は，いかにうまく定位照射を利用するかが世界共通のテーマではないだろうか．

定位照射の活用のように，転移性脳腫瘍の治療に関して我が国は，決して遅れをとっていたわけではない．しかしながら新たな治療法を導入する際に常に標準的治療を意識して，国際的評価に耐えうる臨床試験を行ってこなかったために，せっかくの先進性が国際的に評価されてこなかった．現在，この反省に立ち，術後全脳照射による高次脳機能障害を定位照射を活用して回避する新たな治療法の有効性を問うJapan Clinical Oncology Group（JCOG）の臨床試験JCOG0504が進行中である．

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ORIGINAL ARTICLE

Association of stem cell marker CD133 expression with dissemination of glioblastomas

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Abstract Dissemination of glioblastoma was once considered rare but is now increasingly encountered with longer survival of glioblastoma patients. Despite the potential negative impact of dissemination on clinical outcome, however, molecular markers useful for prediction of dissemination risk still remains ill defined. We tested in this study for an association between the expression of stem cell marker CD133 and the risk of dissemination in 26 cases of glioblastoma (16 with dissemination and 10 without dissemination). The protein expression of CD133 was examined by western blot analysis of tumor specimens, and the CD133 expression levels were quantified by densitometry and normalized to β-actin. The results indicated that CD133 expression levels are significantly higher in glioblastomas with dissemination (mean 10.3, range 0.20-27.8) than in those without (mean 1.18, range 0.07-3.58). The results suggest that CD133 could be a molecular predictor of glioblastoma dissemination, and also give rise to an intriguing idea that CD133-positive cancer stem cells may be implicated in the initiation of disseminated lesions.

Keywords Glioblastoma · Dissemination · Cancer stem cell · CD133

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Introduction

Malignant gliomas are the most common and intractable primary neoplasms of the central nervous system. Despite aggressive treatments, recurrence is inevitable and fatal in most cases of malignant glioma. Recurrent tumors usually arise locally close to the primary tumor site, but they occasionally appear as disseminated lesions at sites distant from the primary tumor [1, 3, 9, 12, 19, 23, 24, 35, 37, 44]. The incidence of disseminating malignant gliomas, which was once considered rare [44], ranges from 8% to 27% [1, 9, 23, 24] and reaches as high as 44% in one recent report, which could be ascribed to longer survival of the patients

analyzed [17]. Given the apparent negative impact of dissemination on survival of patients with malignant gliomas [1, 24], control of dissemination will have increasingly greater clinical significance as progress is made in local control of the tumors. Understanding the biology that underlies dissemination would help improve diagnosis and treatment of disseminated tumors, yet information on genetic and/or molecular aspects of dissemination is still very limited [2, 15, 17, 18, 21].

The cancer stem cell hypothesis holds that tumors are composed of a rare subpopulation of cancer stem cells having the ability to self-renew indefinitely and initiate tumor formation and of the other majority of tumor cells having limited ability to divide and therefore incapable of initiating tumor formation [25]. Recent studies have documented existence of such cancer stem cells in several tumor types including gliomas [10, 27, 34, 38], and the cell surface antigen CD133 has been established as a useful marker molecule for identifying cancer stem cells of glioma (glioma stem cells) [31]. CD133 is expressed preferentially in glioma stem cells, and the $CD133^+$ but not the $CD133^-$ population of glioma has been shown to retain the ability to self-renew and, upon orthotopic transplantation into immunodeficient mice, initiate formation of tumor that recapitulates the characteristics of the original tumor from which it is derived [4, 28–30, 43]. According to this cancer stem cell hypothesis, the cells that give rise to disseminated lesions should be "cancer stem cells" capable of initiating tumors, since those cells are presumed to be individual (single) tumor cells migrating away from the primary tumor to the distant sites of dissemination. If this is actually the case, then, it is expected that the proportion of the cancer stem cell population within primary tumors would be a critical factor determining the chance of developing dissemination.

In this study, in an attempt to identify molecular predictors of dissemination, we investigated whether the expression level of CD133, which is expected to reflect the proportion of glioma stem cells within a tumor, is associated with development of dissemination in glioblastoma cases.

Materials and methods

Patient population

The subjects of this study were patients with newly diagnosed glioblastoma multiforme (GBM) who were treated at the neurosurgical departments of the participating institutions and for whom snap-frozen samples of the primary tumor available for the following expression analysis have been obtained upon informed consent. The subject patients consisted of two groups: "patients with dissemination" and "patients without dissemination". "Patients with dissemination" were those who had evidence of dissemination documented on magnetic resonance (MR) imaging during the clinical course. "Patients without dissemination" were those who survived 12 months or longer without any evidence of dissemination on MR images taken during the course. Dissemination was defined as the appearance of an enhanced nodule(s) and/or diffuse enhancement of the leptomeningeal space at sites distant from (i.e., not contiguous to) the primary tumor location on T_1-weighted MR images with contrast enhancement. Patients were treated according to each institution's protocol, and outpatient follow-up was done at 1-2 month intervals. MR examinations were conducted at least every 2-3 months.

Clinical data acquisition

Clinical records including the MR images were reviewed for each patient. We recorded patient age at diagnosis, sex, pathological diagnosis, location of the primary tumor, extent of surgical resection, and history of radiation therapy and chemotherapy. The extent of resection was described as total (100% resected), subtotal (95% ≤, <100% resected), partial (5% ≤, <95% resected), and biopsy. The date of primary diagnosis, the date dissemination was detected, final outcome, the date of death (the date of the latest clinical follow-up for living patients) were recorded to calculate time interval between primary diagnosis and dissemination (= timing of dissemination) and overall survival.

Western blot analysis of CD133 expression

Samples from the main body of the primary tumors, which were snap frozen in liquid nitrogen at the time of tumor resection and stored at −80°C until use, were lysed in the lysis buffer (62.5 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol) and sonicated. After determination of protein concentration using the BCA protein assay kit (Pierce), cell lysates were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride membrane. The membrane was, after being blocked in Tris-buffered saline with 5% nonfat dry milk, probed with anti-CD133 primary antibody (W6B3C1, Milteny Biotechnology, 1:3,000) and then with an anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, 1:2,000) using Can Get Signal (Toyobo). The membrane was also probed with anti-β-actin (Sigma, 1:6,000 in phosphate-buffered saline-0.1% Tween 20 [PBS-T] with 5% nonfat dry milk) and then with an anti-mouse HRP-conjugated secondary antibody (Santa Cruz Biotechnology, 1:3,000 in PBS-T with 5% nonfat milk). Blots were visualized on X-ray films using Immobilon Western Chemiluminescent HRP Substrate (Millipore).

Developed films were scanned and band densitometry was done using Image J (National Institutes of Health).

CD133 densitometry results were normalized to β-actin. A positive control sample (patient ID: D4) was always run on each gel and used as a standard for comparison of results from different gels.

Statistical analysis

Mann-Whitney *U* test was used to test for statistically significant difference of CD133 expression and age between two independent groups. Chi-square test and Fisher's exact probability test were used to examine the difference of sex, tumor location, and initial therapies (the extent of surgical resection, with or without radiation and chemotherapy). Correlation of CD133 expression with timing of dissemination and overall survival was assessed using the nonparametric Spearman rank correlation assay (correlation coefficient: r_s). Two-sided values of $P<0.05$ were considered statistically significant.

Results

Patient characteristics

A total of 26 GBM patients were available for analysis in this study, and their baseline characteristics are summarized in Table 1. Of the 26 patients analyzed, 16 were patients with dissemination, and 10 were without dissemination. Of the 16 patients, with dissemination, 14 were adult (>17 years old) and two were pediatric patients, with the median age of 58.5 years (range 8–74 years). The location of the primary tumor was spinal in one patient and supratentorial in the other 15. Of the 10 patients without dissemination, all were adult, with the median age of 54 years (range 44-74 years). The location of the primary tumor was supratentorial in all patients without dissemination. In principle, both patients with and without dissemination received initial therapies consisting of surgical resection, radiation therapy, and chemotherapy. There were no statistically significant differences in age, sex, tumor location, and the initial therapies (the extent of surgical resection, whether or not accompanied by radiation and chemotherapy) between the two groups. The median overall survival was 14 months (range 5-129 months) for patients with dissemination and 29.5 months (range 12-88 months) for those without.

CD133 expression in GBMs with and without dissemination

To investigate CD133 expression, we subjected samples of the primary tumors from patients with and without dissemination to western blot analysis. Varying levels of CD133 expression was observed in tumors from patients with dissemination, ranging from very high levels to nearly undetectable. In contrast, the expression levels of CD133 in patients without dissemination were uniformly low: CD133 was almost undetectable in the majority of patients, and was at most barely detectable even in patients with highest expression (Fig. 1). We then quantified CD133 expression by densitometry. Since inevitable inclusion of red blood cells within the tumor samples made determination of protein concentration somewhat inaccurate, CD133 expression level was normalized to β-actin level, an internal control for protein loading. The levels of CD133 expression after normalization to β-actin (CD133/β-actin ratio) were remarkably higher in patients with dissemination (mean 10.3, range 0.20-27.8, $n=16$) than in those without dissemination (mean 1.18, range 0.07-3.58, $n=10$). This difference of CD133/β-actin levels was statistically significant ($P<0.05$, Fig. 2a), and the difference remained significant even when the analysis was limited to adult GBM patients with (mean 9.48, range 0.20–22.7, $n=14$) and without (mean 1.18, range 0.07–3.58, $n=10$) dissemination ($P<0.05$, Fig. 2b).

CD133 expression and timing of dissemination

The median time interval between primary diagnosis and dissemination (timing of dissemination) for 16 patients with dissemination was 8 months (range 0–107 months) (Table 1). Dissemination occurred within 1 year after primary diagnosis in 10 patients (62.5%) and later than 1 year in six (37.5%). We examined whether there is any correlation between timing of dissemination and CD133 expression in primary tumors, and found positive correlation between the two parameters ($r_s=0.67$, $n=16$), which was statistically significant ($P<0.05$, Fig. 3).

CD133 expression and overall survival in adult GBMs with dissemination

To test whether CD133 expression in the primary tumor is associated with overall survival of patients independent of dissemination, we examined if any correlation exists between the two parameters among patients with dissemination. To exclude confounding factors and conduct analysis on a uniform group of patients, analysis was limited to adult GBMs, and patients who were alive at the time of latest follow-up were censored. The result indicated that there is no significant correlation between the level of CD133/β-actin ratio and overall survival in patients with dissemination ($r_s=0.55$, $n=10$; Fig. 4a). When the patients with dissemination were grouped into those who survived 12 months or longer and those who survived less than 12 months, the mean CD133/β-actin ratio was higher in the former group than in

Table 1 Baseline clinical characteristics of study subject

Patient ID	Age	Sex	Histopathology	Location	Initial therapies			Dissemination	TOD (months)	Outcome	OS (months)
					Surgery	Radiation (Gy)	Chemotherapy				
D1	13	F	GBM	Lt. F	Total	Local 60	CDDP, VP-16	+	47	Alive	56
D2	69	F	GBM	Rt. P-O	Total	Local 60	ACNU iv	+	18	Dead	25
D3	59	M	GBM	Lt. O	Total	Local 60	ACNU iv	+	8	Dead	12
D4	67	F	GBM	Rt. T	Total	Local 60	ACNU iv	+	14	Dead	16
D5	64	M	GBM	Lt. T	Total	Local 60	CDDP, VP-16, INF-β	+	8	Dead	10
D6	45	F	GBM	Rt. F	Total	Local 60	ACNU iv	+	8	Dead	10
D7	71	M	GBM	Rt.P-O	Partial	Local 60	–	+	4	Dead	6
D8	58	F	GBM	Lt. T	Total	Local 60	–	+	107	Dead	129
D9	17	M	GBM	Lt. O	Total	Local 60	ACNU iv	+	18	Alive	46
D10	55	F	GBM	Rt. F-T	Partial	Local 60	ACNU iv	+	12	Alive	29
D11	8	F	GBM	Spinal	Partial	Local 44	CDDP,VP-16,IFM	+	0	Dead	5
D12	69	F	GBM	Lt. T	Partial	Local 60	ACNU iv	+	0	Alive	26
D13	74	M	GBM	Blt. F	Partial	Local 60	ACNU iv	+	8	Alive	9
D14	61	M	GBM	Rt. T	Total	Local 60	MTX it	+	6	Dead	10
D15	57	F	GBM	Lt. F	Subtotal	Local 60	ACNU iv	+	5	Dead	7
D16	49	M	GBM	Rt. T	Total	Local 60+24	ACNU ia, MTX it	+	5	Dead	17
ND1	52	F	GBM	Rt. P	Total	Local 60	ACNU iv	–	–	Dead	15
ND2	52	M	GBM	Lt. F	Total	Local 60	ACNU iv	–	–	Dead	12
ND3	74	M	GBM	Rt. F	Total	Local 60	ACNU iv	–	–	Dead	21
ND4	62	F	GBM	Lt. F	Total	–	–	–	–	Dead	40
ND5	56	F	GBM	Lt. T	Subtotal	Local 60	ACNU iv,INF-β	–	–	Dead	45
ND6	72	F	GBM	Rt. F	Partial	Local 60	ACNU iv	–	–	Dead	19
ND7	50	M	GBM	Lt. insular	Partial	Local 60	ACNU iv	–	–	Alive	24
ND8	51	M	GBM	Rt. F	Total	Local 60	ACNU iv	–	–	Alive	88
ND9	44	M	GBM	Rt. insulo-operculum	Subtotal	Whole 30+Local 30	ACNU iv	–	–	Alive	70
ND10	64	M	GBM	Rt. hippocampus	Total	Local 60	–	–	–	Alive	35

GBM glioblastoma, *TOD* timing of dissemination, *OS* overall survival, *F* denotes frontal, *P-O* parieto-occipital, *O* occipital, *T* temporal, *F-T* fronto-temporal, *T-O* temporo-occipital, *P* parietal, *Lt* left, *Rt* right, *Blt* bilateral, *iv* intravenous injection, *ia* intraarterial injection, *it* intrathecal injection

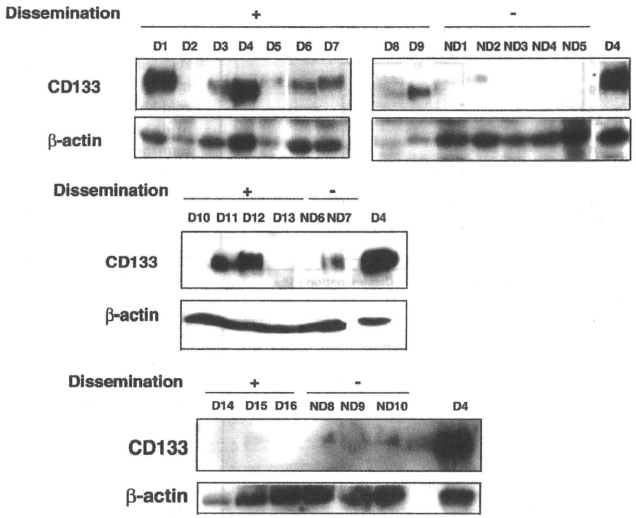

Fig. 1 Western blot analysis of CD133 expression in primary tumor samples from glioblastoma patients with and without dissemination. Primary tumor samples from patients with (D1–D16) and without (ND1–ND10) dissemination was analyzed by western blotting for CD133 expression. Actin blots show the relative amount of protein loaded onto each lane

the latter (13.9 for those who survived ≥12 months and 7.1 for those who survived <12 months), although the difference was not statistically significant (Fig. 4b).

Discussion

In this study, to explore the possibility that CD133, a glioma stem cell marker, could be a molecular predictor of dissemination, we investigated whether there is significant association between CD133 expression in the primary tumors from GBM patients and development of dissemination. The results clearly indicated that CD133 expression levels assessed by western blot analysis are significantly higher in patients with dissemination than in those without, suggesting that CD133 protein expression may be a useful indicator of dissemination risk in GBM cases. The findings also prompt us to put forward a novel hypothesis that CD133-positive glioma stem cells could be a potential source/seed of dissemination.

We analyzed in this study, for patients without dissemination, only those who survived 12 months or longer without the evidence of dissemination. This inclusion criterion was added for the purpose of preventing "potentially disseminating cases (cases in which dissemination would have occurred if the patient had survived longer)" from being included in the group of patients without dissemination. The median or mean time interval between primary diagnosis and dissemination falls within 6-12 months in the majority of previous studies [17, 24]; summarized in Ref. [39], which was also the case in this study. We therefore expect that, with this inclusion criterion, we could have substantially reduced, albeit not totally eliminated, the risk of including potentially disseminating cases in the group of patients without dissemination. As one potential drawback of this inclusion criterion, it is possible that CD133 expression levels are low in patients without dissemination in this study just because we have selected patients with favorable prognosis, which may be associated with low CD133 expression. However, this is unlikely to be the case for two reasons. First, no significant correlation was observed between CD133 expression level and overall survival when the analysis was conducted on patients (adult GBMs) with dissemination, suggesting that CD133 expression may not be a prognostic factor of overall survival independent of dissemination (Fig. 4a). Of note, when we divided the patients with dissemination into two groups—those surviving 12 months or longer and those surviving less than 12 months—the CD133 expression levels tended to be even higher in the former group than in the latter (Fig. 4b). Thus, it seems unlikely that we have artificially selected patients with low CD133 expression by limiting the analysis of patients without dissemination to those who survived 12 months or longer. Second, in line with our finding, a recent study failed to show significant association between CD133 expression and overall survival

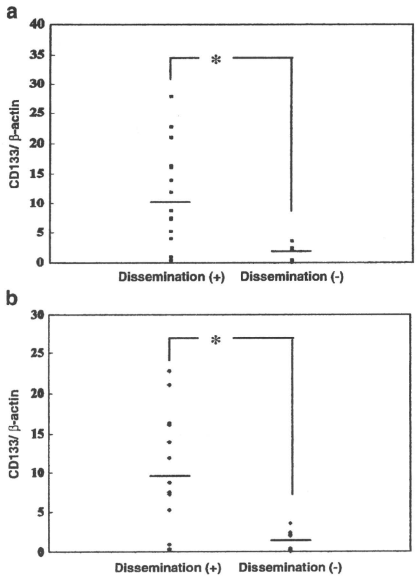

Fig. 2 Association between dissemination and CD133 expression in glioblastoma patients. CD133/β-actin ratio of primary tumors from patients with (dissemination +) and without (dissemination −) dissemination. *Bars* indicate mean values. **a** All subjects (16 patients with dissemination and 10 without dissemination) were included in the analysis. **b** Analysis was limited to adult patients (14 with dissemination and 10 without dissemination); $*P<0.05$

of 72 adult GBM patients [7]. Although another report, in which a series of grade 2-4 astrocytoma patients were analyzed, suggested that CD133 expression may be associated with overall survival of astrocytoma patients, it remains to be shown in that study whether it holds true if the analysis is limited to grade 4 (i.e., GBM) patients [45]. Together, these observations are in support of the idea that CD133 expression may be associated with dissemination rather than with overall survival of GBM patients. It may, however, deserve emphasizing here that association of CD133 expression with dissemination does not necessarily preclude its association with overall survival. Given that CD133 is a marker of glioma stem cells that represent only a small fraction within the entire tumor, the association between CD133 expression and overall survival would become more apparent as the remaining majority of tumor cells, i.e., the non-stem cell population, becomes better controlled in the future.

Whereas higher CD133 expression is thus associated with increased risk of dissemination, the results of this study also suggested that it is not associated with "shorter" interval between primary diagnosis and dissemination. This could be naturally understood if we assume that the timing of dissemination mainly depends on the time required for formation of tumor mass, for which the intrinsic growth properties of tumor cells, irrespective of whether they are stem cells or not, are considered to be key determinants. In line with this idea, higher MIB-1 labeling index has been associated with shorter time interval before development of dissemination in patients with disseminating GBM [17].

Recently, we have reported that nestin expression may be associated with dissemination of central nervous system (CNS) germ cell tumors [26]. Although it remains to be shown whether cancer stem cells exist in CNS germ cell tumors and whether nestin expression, presumed to be a marker for multi-lineage progenitor cells [41], could also be a marker for such cancer stem cells of CNS germ cell tumor, the association of nestin expression and dissemination suggests the possibility that cancer stem cells are the potential source of dissemination in CNS germ cell tumors. Thus, the results of the present study, in conjunction with this earlier report of ours [26], support the emerging hypothesis that brain tumor stem cells may play an important role in brain tumor dissemination within the CNS. Although, in the present study, the exact identity of the cells expressing CD133 remains to be determined, demonstrating the expression of stem cell markers including CD133 by tumor cells in future immunohistochemical studies would help further validate this hypothesis. In this respect, nestin has been classically regarded as a marker for glioma stem cell, and nestin immunohistochemistry has

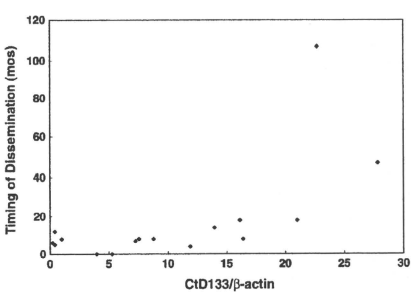

Fig. 3 Relationship between CD133 expression level and timing of dissemination in glioblastoma patients. A scatter plot of time interval between primary diagnosis and dissemination (timing of dissemination) in relation to CD133/β-actin ratio in 16 glioblastoma patients with dissemination. Correlation coefficient, $r_s=0.67$, $n=16$, $P<0.05$

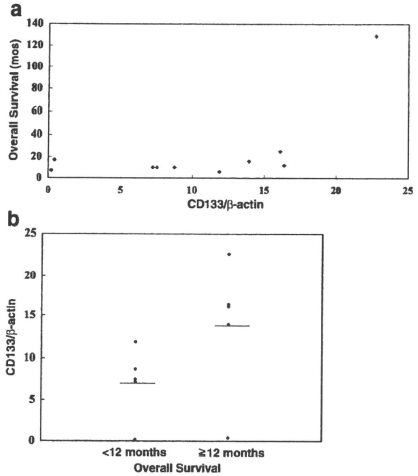

Fig. 4 Relationship between CD133 expression level and overall survival of adult glioblastoma patients with dissemination. **a** A scatter plot of overall survival in relation to CD133/β-actin ratio in adult glioblastoma patients with dissemination. Correlation coefficient, r_s= 0.55, n=10, P>0.05. **b** CD133/β-actin ratio in adult glioblastoma patients who survived 12 months or longer (≥12 months) and less than 12 months (<12 months). *Bars* indicate mean values. In **a** and **b**, live patients at the time of latest follow-up were censored from the analysis. Consequently, a total of 10 patients were analyzed

been well established [8]. We therefore conducted a pilot immunohistochemical analysis of our GBM samples for nestin expression and found that the level of nestin expression in tumor cells is indeed higher in primary tumors from patients with dissemination than in those from patients without dissemination (unpublished data, the Tohoku Brain Tumor Study Group). However, the difference of nestin expression was much less pronounced compared with that of CD133 demonstrated in this study, with substantial overlap of nestin expression levels in the two groups. This could be explained by the idea that nestin is a less specific marker for stem cells than CD133 is, given the recent reports suggesting that nestin expression may not be restricted to stem cells [6, 20].

The results of the present study suggest that stem cell marker CD133 may be a novel molecular marker for dissemination of GBM. Our data clearly indicated that there is little overlap of CD133 expression between the two groups of patients with and without dissemination (Fig. 2). Importantly, whereas the expression levels of CD133 in patients with dissemination somewhat varied, those in patients without dissemination were uniformly low. This finding implies that, although low CD133 expression may not exclude the possibility of dissemination, high levels of CD133 expression may be associated with a high risk of dissemination. Thus, CD133 expression could become a useful predictor for selective identification of patients at high risk of dissemination. On the other hand, some tumors in this study disseminated despite low CD133 expression. This could be explained by the recent observations that there may exist a distinct class of glioma stem cells that do not express CD133 [5, 16, 22, 40]. To date, several clinical parameters such as young age, male sex, incomplete tumor removal, multiple resections, ventricular entry, and proximity of the tumor to the ventricular system, have been suggested as possible risk factors of dissemination [1, 3, 12, 19], but their significance as predictors of dissemination still remains to be shown. As for genetic and molecular markers of dissemination, gain at the *1p36* chromosomal region [18], *PTEN* mutation [15, 17], and tissue inhibitor of metalloproteinase 2 (TIMP-2) expression [21] have been associated with dissemination. Although the significance of these genetic/molecular markers as risk factors of dissemination also remains to be established, they could become a useful predictor, because the incidence of these genetic abnormalities (*1p36* gain, *PTEN* mutation) and the expression level of TIMP-2 in patients with dissemination were markedly higher than in control patients. Both increased TIMP-2 expression and inactivation of PTEN by mutation are presumed to contribute to dissemination via promotion of glioma cell migration/invasion away from the primary tumor, an essential step in the process of dissemination [17, 21]. However, it would be intriguing to speculate that *PTEN* mutation also contributes to dissemination by increasing the population of glioma stem cells within the primary tumors, given the observations that PTEN negatively regulates the population size of neural stem cells which are considered to share characteristic stem cell properties with glioma stem cells [13, 14]. Aside from whether and how these genetic and molecular markers are involved in dissemination, these markers are expected to help predict the risk of dissemination and their significance as predictors of dissemination will be tested and verified in future studies.

Currently, radiation therapy and intrathecal chemotherapy are the major treatment options for disseminated lesions in malignant glioma cases, yet their effects against established tumors are nonetheless limited [32, 33, 36, 42]. Given the principle idea that therapy resistance of tumor cells develop in a time-dependent manner [11], those treatments would be more effective if delivered prophylactically before disseminated lesions form discrete masses. Thus, patients would benefit from prophylactic radiation therapy and/or intrathecal chemotherapy against dissemina-

tion if we knew in advance that disseminated tumors would eventually develop during the course. In this respect, although the results of this present study need to be confirmed in large-scale studies in the future, our results suggest that CD133 could become a useful molecular predictor to prospectively identify such patients at high risk of dissemination, alone or in combination with other dissemination markers reported to date.

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Comments

Karl Frei, Zurich, Switzerland

In this study, the authors tested for an association between the expression of the stem cell marker CD133 and the risk of dissemination in 26 cases of glioblastomas (10 without and 16 with dissemination). The tumor specimens were examined by western blot analysis and the CD133 expression levels were quantified by densitometry and normalized to β-actin.

The authors suggest from their data that CD133 might be a molecular predictor of glioblastoma dissemination and that CD133-positive cancer stem cells may be implicated in the initiation of disseminated lesions.

This intriguing idea has to be taken with caution due to the following reasons:

1. The study is only based on western blot analysis and no immunohistochemistry has been performed to confirm the biochemical data.

2. The western blot analysis was not done in a quantitative manner. The number of 26 cases is low and the observed variability (five negative cases in each group) high.

Oliver Heese, Hamburg, Germany

In this study, the authors investigated the association between the expression of CD133 and the risk of tumor dissemination in 26 cases of glioblastoma multiforme in order to identify new potential predictors of an unvaivorable outcome of this tumor. The CD133 expression was measured on protein level by western blot. Dissemination was defined as appearance of enhanced nodules and/or diffuse enhancement of the leptomeningeal space at distance sites of the primary tumor location on contrast enhanced MRI.

The results indicated that significant higher CD133 level were found in GMB patients with a disseminated tumor progression. These findings imply that high levels of CD133 expression may be associated with a high risk of dissemination whereas low CD133 expression may not exclude the possibility of dissemination.

Despite the recent surge of interest in CD133+ brain tumor stem cells, the clinical significance of this cell population remains unclear and with this study an interesting hypothesis is made. Striking evidence suggests a dynamic process of expression of CD133 positive cells.

Since the amount of CD133 positive cells may vary in a process of tumor progression the measured western blot data of the initial tumor specimen may not represent the amount of CD33 positive cells during tumor dissemination or during local tumor progression. In addition for future analysis, in order to correlate the relationship between CD133 expression and clinical prognosis not only the quantity of CD133 positive cells should be taken into account but also the quality of CD133+ cells in in vitro and in vivo model systems have to be evaluated.

Since various regimens target local tumor control of glioblastomas such as radiosurgery or local chemotherapy, disseminated tumor growth is difficult to treat and predictors are important in order to indentify this subpopulation of patients and CD133 expression may be one molecular parameter for prediction of the clinical course of a glioma patient group.

Michel Mittelbronn, Frankfurt, Germany

In their current manuscript, Sato et al. present CD133 as a molecular predictor for glioblastoma dissemination and suggest that CD133-positive stem cells may be implicated in the initiation of disseminated lesions. This very interesting finding might be—if constantly reproducible—a useful tool for the prediction of glioma growth and could impinge on treatment strategies. However, what is not proven in the present study and probably unprovable to date, is the open question if CD133 upregulation in glioblastoma is the cause of dissemination or rather a bystander effect or consequence of other conditions within gliomas. The authors strongly favor the hypothesis that the source of CD133 expression might be related to tumor stem cells (although not proven by means of immunohistochemistry or FACS analysis). Subsequently, I would like to provide some additional interpretation of the western blot data. CD133 is frequently considered as a marker for neural, hematopoietic, and brain tumor initiating stem or progenitor cells, however, the distribution of CD133 expression in brain tumors has remained controversial. CD133-positive cells not closely related to tumor vessels have been reported to reside in pseudopalisading areas of necrosis (1). These areas are mainly subjected to low oxygen concentrations. From cell experiments, it is known that cultured glioma cells are capable to express CD133 when kept under hypoxic conditions without immediately being considered as stem cells (2, 3). Furthermore, emerging studies point out that anti-angiogenic approaches in high grade gliomas either lead to (1) reactivating angiogenesis through upregulation of other proangiogenic factors, (1) invading normal CNS tissue via upregulation of matrix metalloproteinases-2, 9, 12, and sparc (secreted protein,

acidic, cysteine-rich) or even drive expression of critical genes associated with aggressiveness, invasiveness and poor survival in glioma patients (4, 5). Taking into account the fact that glioma cells upregulate CD133 under hypoxic conditions and that hypoxia strongly leads to a more migratory phenotype, one could assume that the findings of Sato et al. could more likely reflect a secondary CD133 upregulation in more migratory or disseminating glioblastomas.

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Regulation of neural stem/progenitor cell maintenance by PI3K and mTOR

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ABSTRACT

Control of stem cell state and differentiation of neural stem/progenitor cells is essential for proper development of the nervous system. EGF and FGF2 play important roles in the control of neural stem/progenitor cells, but the underlying mechanism still remains unclear. Here we show, using in vitro primary cultures of mouse neural stem/progenitor cells, that both PI3K and mTOR are activated by EGF/FGF2 but that inhibiting the activation of either PI3K or mTOR alone results in only reduced proliferation of neural stem/progenitor cells without affecting their stem cell state, namely, the capacity to self-renew. However, significantly, concurrent inhibition of PI3K and mTOR promoted exit from the stem cell state together with astrocytic differentiation of neural stem/progenitor cells. These findings suggest that PI3K and mTOR are involved in the EGF/FGF2-mediated maintenance of neural stem/progenitor cells and that they may act in parallel and independent pathways, complementing and backing up each other to maintain the stem cell state.

Neural stem/progenitor cells are the self-renewing, multipotent cells that generate progenies such as neurons and glia and serve as the common source of these fundamental components of the nervous system [17,18]. In the early stage of development, neural stem/progenitor cells first expand their population and then give rise to neurons and subsequently glia [11]. Thus, the maintenance and expansion of the neural stem/progenitor cell pool by self-renewal, as well as the timing and mechanism by which neural stem/progenitor cells become committed to differentiation into neurons and glia, are tightly regulated and critical to proper development of the nervous system. The maintenance of the neural stem/progenitor cell pool is governed by a variety of cell-intrinsic and extrinsic factors such as Notch ligands and secreted growth factors, and a large body of evidence now indicates that epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2) are critically involved in the control of neural stem/progenitor cells both in vitro and in vivo [2,3,7,12,19]. However, in contrast to their well-established role in the control of neural stem/progenitor cell, much still remains unknown about the mechanism by which these growth factors maintain neural stem/progenitor cells. For instance, it remains largely unclear *which* intracellular signaling molecule(s) activated by these growth factors contributes to neural stem/progenitor cell maintenance and *how*. Here in this study, we investigated the role of phosphatidylinositol 3-kinase (PI3K) and mTOR in the control of neural stem/progenitor cell.

LY294002 and rapamycin were purchased from Calbiochem. EGF and FGF2 were from Peprotech. Anti-Sox2 (MAB2018), anti-GFAP (AF2594), and anti-βIII-tubulin (MAB1199) were from R&D. Anti-phospho-Akt (Ser473) (#4098), anti-Akt (#9272), anti-phospho-p70 S6 kinase (Thr389) (#9209), anti-p70 S6 kinase (#2708), anti-phospho-4E-BP1 (Thr37/46) (#2855), anti-4E-BP1 (#9452) were from Cell Signaling Technology. Anti-β-actin (A5441) was from Sigma. Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Santa Cruz Biotechnology and Upstate.

The mouse strain used in this study for isolation of neural stem/progenitor cells was ICR. All animal procedures were done in accordance with the Declaration of Helsinki under the protocol approved by the Animal Research Committee of Yamagata University. Forebrain tissues from E14.5 mouse embryos were washed in chilled sterile Hanks' balanced salt solution (HBSS) with 0.6% glucose and penicillin/streptomycin (PS), minced with scissors, and incubated in TrypLE Express (Invitrogen) and 0.02% DNase type II (Sigma) in HBSS/PS for 30 min at 37 °C. After being washed with HBSS/PS, the tissues were suspended in a basal medium (a 1:1 mixture of Dulbecco's modified Eagle's medium and F-12 medium [DMEM/F12, GIBCO]) and filtered through a 70-μm strainer. The dissociated cells were subsequently cultured in the neural stem/progenitor cell culture medium (DMEM/F12 supplemented with the N2 supplement and PS) in the presence of EGF

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