Plastic induction of CD133^{AC133}-positive cells in the microenvironment of glioblastoma spheroids

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Abstract. Recent studies showed that the stemness of cancer stem cells is maintained under a hypoxic microenvironment. However, the relationship of the hypoxic microenvironment in a three-dimensional cell mass and the induction of cancer stem cell-like phenotype is not well known. We examined the relationship between CD133 expression and the hypoxic microenvironment using glioblastoma spheroids formed with the T98G cell line. CD133^{AC133}- and HIF-1α-positive cells were observed in the marginal region of the central hypoxic area positive for HIF-1α 10 days after plating T98G cells. CD133^{AC133}-positive cells were positive for nestin. Quantitative PCR analysis showed that the *CD133* expression level is not different in spheroids during the tested period after spheroid formation, indicating that post-translational regulation of the CD133 protein mediates positivity to CD133^{AC133}. When spheroids were trypsinized and the dissociated cells were cultured under the adherent monolayer conditions, the CD133^{AC133}-positive cells gradually disappeared. These results show that CD133^{AC133}-positive cells, which may incline toward undifferentiated cells because of nestin positivity, are plastically induced under the different culture conditions, spheroid and monolayer. In this plasticity, HIF-1α is involved in the induction and maintenance of CD133^{AC133}-positive cells. Spheroids as an *in vitro* tumor model are useful to study the dynamic changes in the tumor cell phenotype in the different cell microenvironments.

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Introduction

Glioblastomas are highly radio- and chemotherapy-resistant cancers, because they contain cellular hierarchies with cancer stem cells (CSCs) that are radio- and chemoresistant (1-4). CSCs are maintained within the niche for stem cells, such as the hypoxic microenvironment (5), as previously shown in normal stem cells (6). Hypoxia contributes to the self-renewal, differentiation, and quiescence of CSCs (7). Hypoxia-inducible factors (HIFs) become stable under the hypoxic condition and transcriptionally upregulate genes that promote cell survival, motility, and tumor angiogenesis, leading to the promotion of cancer cells to a more malignant state. However, the role of HIFs in the maintenance of CSCs is not fully understood. Recent studies have shown that HIFs maintain glioma stem cells via overexpression of ZNF217 (8), promote expansion of glioma stem cells (9), and upregulate the expression of CD133 protein in glioblastoma-derived neurospheres (10). Furthermore, HIFs may mediate the expression of chemoresistance markers in CSCs (11). It has been shown that in glioblastoma-derived neurospheres, hypoxia induces increases in the expression of not only HIFs and CD133 but also chemoresistance-related markers (MGMT, TIMP-1, Lamp-1, MRP1 and MDR-1). Similarly, in cancer cell lines as well as in CSCs, HIFs contribute to the regulation of stemness-related cellular responses such as neurosphere formation (7) and the upregulation of CD133 (12) and the stem cell factors OCT4, NANOG, SOX2, KLF4 and c-MYC (7,13). These findings indicate that the hypoxic condition play an important role in the stemness state of cancer cells as well as CSCs.

CD133 has recently attracted much attention as a marker for identifying CSCs in glioblastoma, hepatic cancer, melanoma, osteosarcoma and colon cancer (14-19). However, the physiological function of the transmembrane protein CD133 remains unknown. On the basis of the assessment of graft survival rate in a xenograft model and ability of sphere formation under the routine culture condition, it was demonstrated that CD133-positive cells exhibit properties of CSCs. In contrast,

controversial results indicating that CD133-negative cells also exhibit properties of CSCs have been reported (20-23). A possible explanation for this discrepancy is the plasticity of CD133 expression, which is dependent on the cellular microenvironment. In glioblastoma neurosphere cells, the level of CD133 expression was reported to vary with oxygen concentration (24). In addition, hypoxia-induced CD133 expression has been reported in human lung cancer cell lines (12). It has been reported that in SW620 colorectal cancer cells, CD133 expression changes depending on the type of the microenvironment (25). Under the low-nutrient culture condition, CD133-negative SW620 colorectal cancer cells re-expressed CD133. These results indicate that CD133 expression is dynamic and modulated by the cellular microenvironment. It seems that CD133 is not a critical marker of glioblastoma CSCs. The plasticity of CD133 expression in the non-stem and stem-like glioblastoma populations provides careful consideration about effects of the hypoxic microenvironment on both cell populations.

Spheroids, larger cell masses compared with spheres, can be used as an *in vitro* tumor model, which exhibits marked similarity to the three-dimensional growth and morphological characteristics of *in vivo* tumors (26). Cells in the outer region of spheroids are exposed to a normoxic, nutrient-rich, and neutral pH microenvironment, but cells in the central area of spheroids are exposed to a hypoxic, low-nutrient, and low pH microenvironment. Cellular oxygen consumption of V79 and EMT6 spheroid cells decreases ~3-fold with the increase in the size of spheroids (27,28). Such three-dimensional characteristics of spheroids are expected to provide useful information for the behavior of stemness-related markers under a hypoxic microenvironment. In this study, we examined the expression of CD133 using cryostat sections of glioblastoma spheroids formed using the T98G cell line.

Materials and methods

Cell culture. A human glioblastoma cell line (T98G) was maintained in α-minimum essential medium (α-MEM) supplemented with 20 mM 4-(2-hydroxyethyl) piperazine ethane sulfonic acid (HEPES), 8 mM $NaHCO_3$, 50 μg/ml streptomycin, 50 U/ml penicillin and 10% fetal calf serum. T98G cells were cultured in a humidified incubator at 37˚C with a mixture of 98% air and 2% CO_2.

Spheroid culture. T98G cells were seeded onto non-adherent U-shape bottom 96-well plates (PrimeSurface 96U, MS-9096U, Sumitomo Bakelite Co., Ltd., Tokyo, Japan) in α-MEM at a density of 5,000 or 10,000 cells/well and cultured for 3 days at 37˚C with a mixture of 98% air and 2% CO_2. Three days later, spheroids were transferred to non-adherent 30-mm dishes (PrimeSurface 35Φ, MS-9035X, Sumitomo Bakelite Co., Ltd.) at a density of 6-10 spheroids/dish to decrease the frequency of medium change during culturing for 7-10 days. The spheroids grew to a diameter of ~300 μm (Fig. 1a).

Preparation of frozen cryostat sections. Spheroids were rinsed with PBS and fixed with 10% formalin containing 10% sucrose for ≥1 h. After rinsing with PBS, spheroids were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and cut into 10-20-μm-thick frozen sections using a cryostat.

Stable transfection. To monitor tumor hypoxia, a stable reporter-transfectant, T98G/5HRE-EGFP, which expressed GFP in response to hypoxic stress, was isolated. A plasmid, p5HRE-EGFP, into which was inserted a GFP reporter gene under the regulation of an artificial HIF-1α-dependent promoter, 5HRE, was transfected into T98G cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Cells were trypsinized 24 h after the transfection and replated onto 100-mm dishes in α-MEM containing G418 (200 μg/ml, Sigma Chemical Co., St. Louis, MO, USA) and then incubated at 37˚C for 7-10 days to enable colony formation. Colonies were isolated using cloning cylinders. The hypoxic area was monitored by GFP fluorescence in spheroids formed using T98G/5HRE-EGFP transfectants, as shown in Fig. 1b.

Immunofluorescence staining. Cryostat sections were rinsed twice with PBS and incubated with anti-CD133/1 (AC133) monoclonal antibody (Miltenyi Biotechnology, Auburn, CA, USA), anti-HIF-1α polyclonal antibody (Novus Biologicals, Littleton, CO, USA), or anti-nestin polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at 37˚C. After the incubation, cells were washed with PBS three times and then incubated with Alexa Fluor 488 anti-rabbit and/or 546 anti-mouse secondary antibodies (Nacalai Tesque, Kyoto, Japan) for 1 h at 37˚C. The sections were then washed with PBS, treated with SlowFade Gold antifade regent with DAPI (Invitrogen), and covered with a glass cover slip.

Quantitative PCR analysis. Total RNA was extracted with a RNAiso Plus kit (Takara, Siga, Japan). RNA extracts (1 μg) were treated with DNase to degrade genomic DNA and then reverse transcribed according to the protocol of PrimeScript RT regent kit (Takara). Quantitative PCR was performed with SYBR Premix Ex Taq™ II (Takara) in 25-μl reactions using 1/25 of the cDNA. The following conditions were used for PCR by Thermal Cycler Dice Real Time System II (Takara): one cycle at 95˚C for 30 sec and 95˚C for 5 sec, 40 cycles at 60˚C for 30 sec, and one cycle at 95˚C for 30 sec, 60˚C for 30 sec, 95˚C for 15 sec for dissociation. The sequence-specific primers of CD133 and β-actin were as follows: CD133: 5'-GGA CCCATTGGCATTCTC-3' (sense) and 5'-CAGGACACA GCATAGAATAATC-3' (antisense); β-actin: 5'-GGCACCC AGCACAATGAAG-3' (sense) and 5'-TCATAGTCCGCCTA GAAGCA-3' (antisense).

For each sample, nonspecific PCR products were checked using dissociation curves. The threshold cycle (Ct) values for gene expression in each sample were normalized by the relative expression of β-actin.

Results

Observation of CD133^{AC133}- and HIF-1α-positive cells in adherent monolayer cells and spheroids. Double immunofluorescence staining with CD133^{AC133} and HIF-1α antibodies showed that T98G cells under the adherent monolayer culture condition were not positive for either CD133^{AC133} or HIF-1α

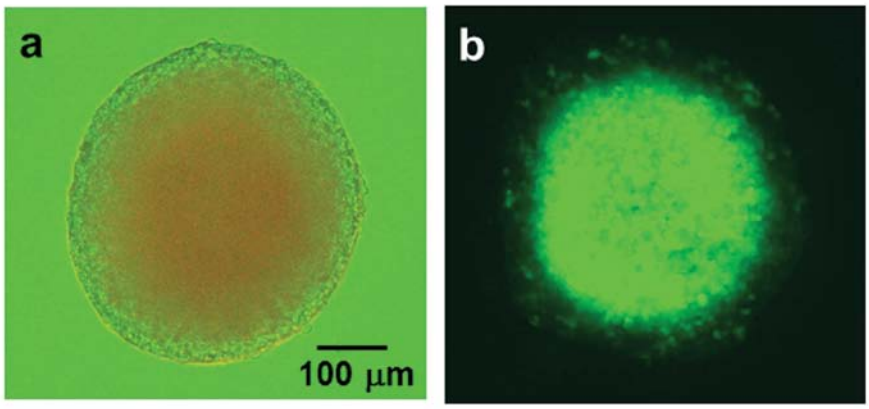

Figure 1. Spheroids formed using T98G (a) and T98G/5HRE-EGFP (b) transfectants. Images taken with white light (a) and peak excitation wavelength of 488 nm (b) 10 days after seeding of 5,000 cells. Area exhibiting green fluorescence shows the hypoxic area (b).

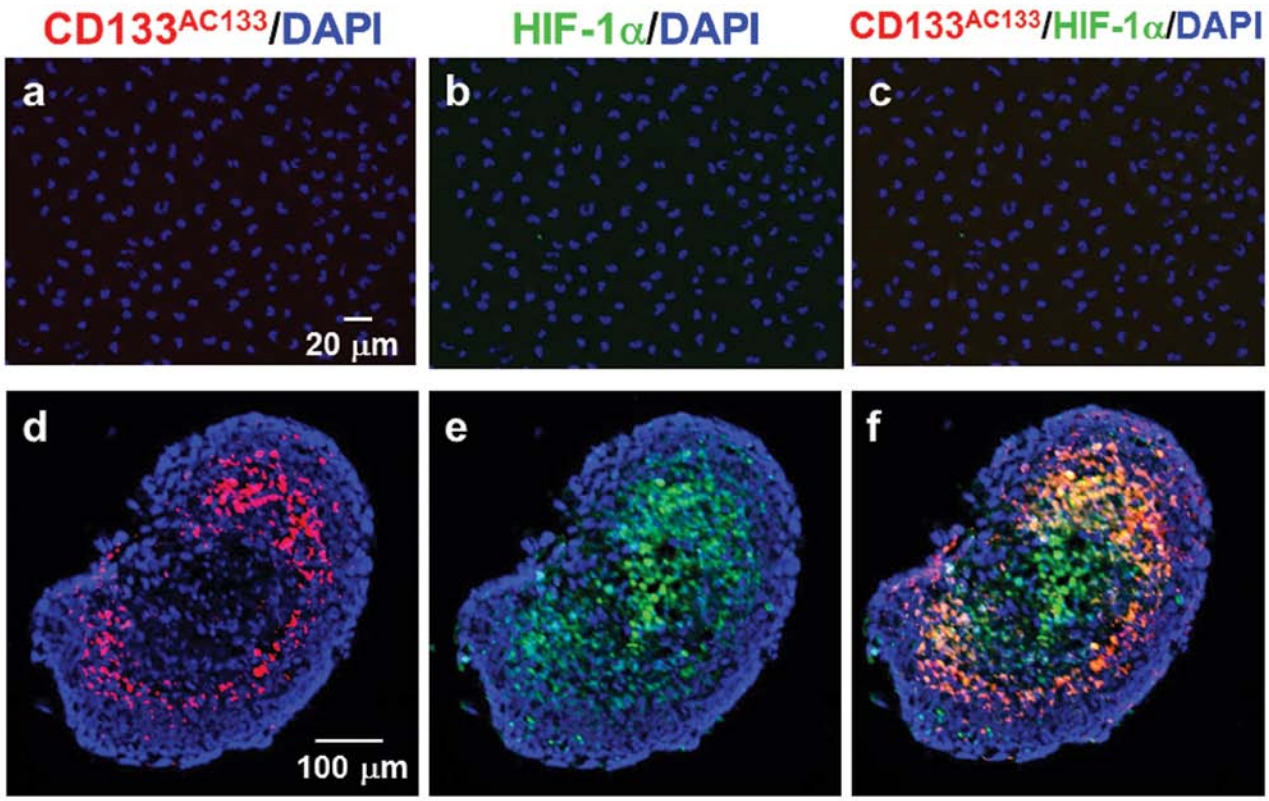

Figure 2. Images of T98G cells cultured under the adherent monolayer condition (a-c), and images of a cryostat section of T98G spheroid 10 days after spheroid formation (d-f). $CD133^{AC133}$/HIF-1α/DAPI triple staining was performed.

(Fig. 2a-c). However, immunofluorescence staining of cryostat sections showed that 10 days after spheroid formation, many $CD133^{AC133}$- and HIF-1α-positive cells were located mainly in the marginal region of the central area of T98G spheroids positive for HIF-1α (Fig. 2d-f). In addition, a few $CD133^{AC133}$- and HIF-1α-positive cells were observed in the outer region near the surface of spheroids. Three days after spheroid formation, $CD133^{AC133}$- and HIF-1α-positive cells were sparsely distributed in the outer region of spheroids (Fig. 3a-c) and were more abundant 5 days after spheroid formation (Fig. 3d-f). At this early stage after spheroid formation, a few $CD133^{AC133}$- and HIF-1α-positive cells were observed in the central areas of spheroids positive for HIF-1α.

Observation of $CD133^{AC133}$- and nestin-positive cells in adherent monolayer cells and spheroids. T98G cells under the adherent monolayer culture condition were not positive for $CD133^{AC133}$ or nestin after double immunofluorescence staining with $CD133^{AC133}$ and nestin antibodies (Fig. 4a-c). However, immunofluorescence staining of cryostat sections showed that $CD133^{AC133}$- and nestin-positive cells were located mainly in the marginal region of the central area of spheroids positive

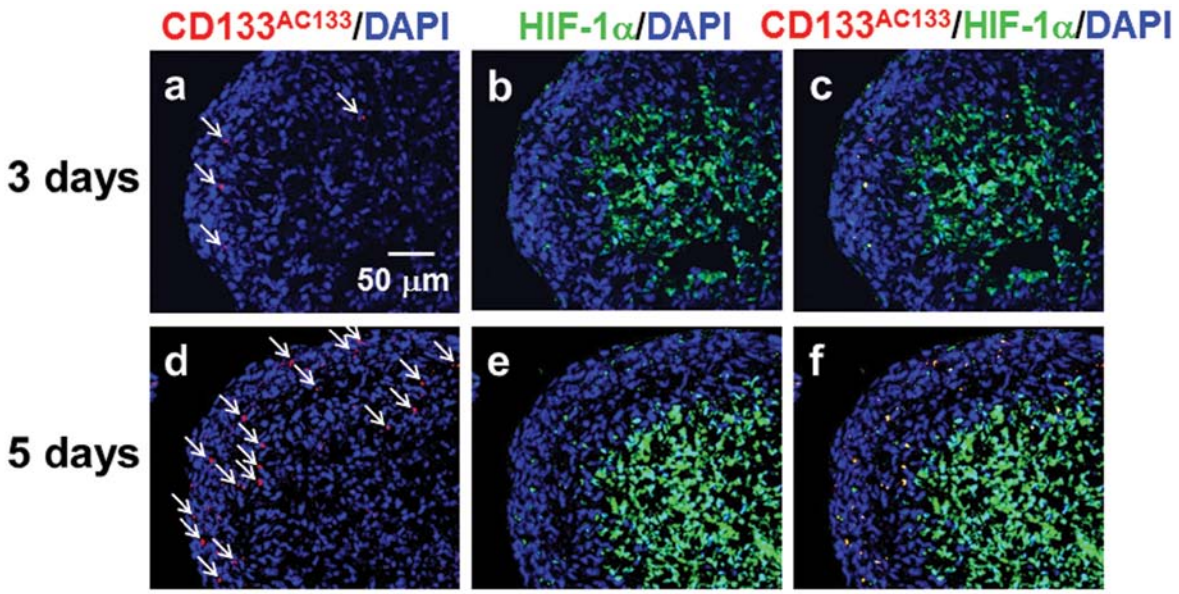

Figure 3. Images of CD133^{AC133}/HIF-1α/DAPI-stained cryostat sections of T98G spheroid 3 days (a-c) and 5 days after spheroid formation (d-f).

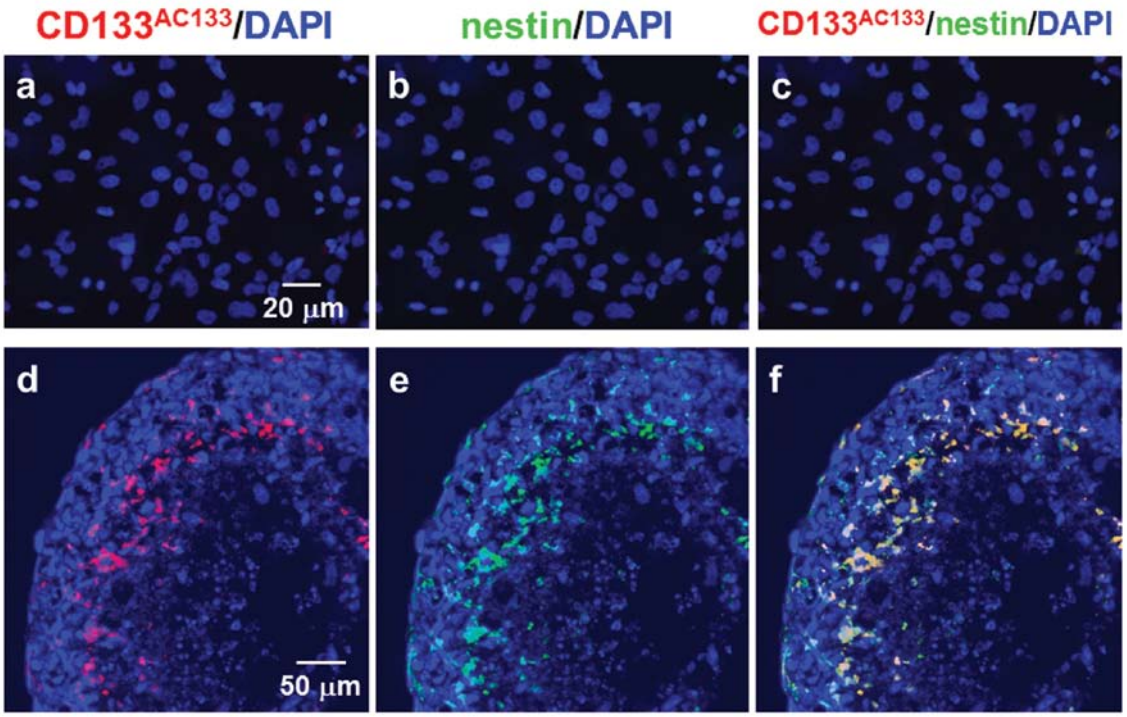

Figure 4. Images of T98G cells cultured under the adherent monolayer condition (a-c), and images of a cryostat section of T98G spheroid 10 days after spheroid formation (d-f). CD133^{AC133}/nestin/DAPI triple staining was performed.

for HIF-1α 10 days after spheroid formation (Fig. 4d-f). A few CD133^{AC133}- and nestin-positive cells were also observed in the outer region of spheroids.

Observation of CD133^{AC133}- and nestin-positive cells in adherent monolayer cells dissociated from spheroids. T98G spheroids were trypsinized 10 days after spheroid formation, and the dissociated cells were then cultured under the adherent monolayer condition for scheduled periods (2, 4 and 10 days). The monolayer-cultured cells were immunofluorescently stained with CD133^{AC133} and nestin antibodies. Many CD133^{AC133}- and nestin-positive cells were observed 2 days after culturing under the adherent monolayer culture condition (Fig. 5a-c), but these cells were slightly decreased after 4 days (Fig. 5d-f). After further culture for 6 days, hardly any CD133^{AC133}- or nestin-positive cells were observed in the adherent monolayer cells (Fig. 5g-i).

CD133 expression in spheroids. Real-time quantitative PCR analysis was performed to evaluate CD133 expression in

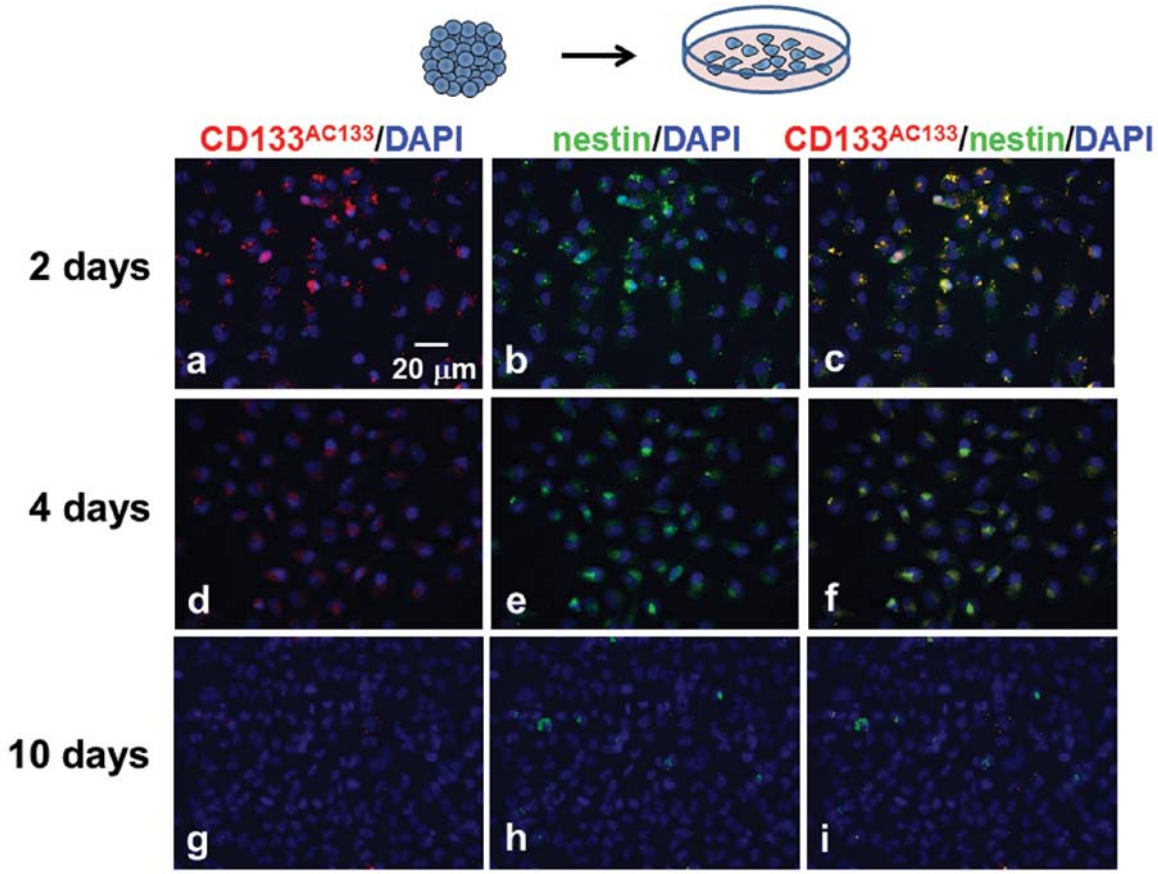

Figure 5. Images of T98G cells that were dissociated from spheroids and cultured under the monolayer condition. The cells were stained 2 days (a-c), 4 days (d-f), and 10 days (g-i) after monolayer culture. CD133^{AC133}/nestin/DAPI triple staining was performed.

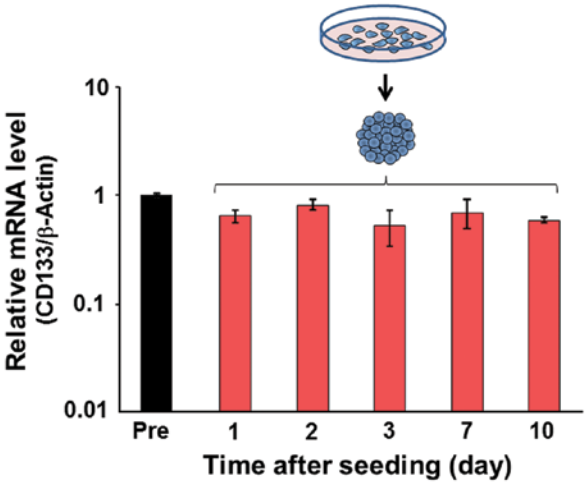

Figure 6. Real-time PCR analysis of T98G spheroids. Total RNA was extracted from 12 spheroids 1, 2, 3, 7 and 10 days after spheroid formation. Pre indicates the result obtained from monolayer-cultured T98G cells. No significant differences were observed in the relative mRNA level from 1 to 10 days after spheroid formation. Columns and bars indicate mean values and SD, respectively. Significant differences were examined using Student's t-test.

spheroids. No significant differences were observed in the relative mRNA level from 1 to 10 days after spheroid formation (Fig. 6).

Discussion

CD133^{AC133}-positive cells were observed in the outer region of spheroids 3 days after spheroid formation. The oxygenic condition of this region was considered to be normoxic, given that the region was HIF-1α-negative. At this early stage of spheroid formation, CD133^{AC133}-positive cells appear to be oxygen-independently induced. At this stage, most CD133^{AC133}-positive cells were HIF-1α-positive. Oxygen-independent stabilization of HIF-1α is likely to mediate the induction of CD133^{AC133}-positive cells. In contrast to this stage, at a late stage ~10 days after spheroid formation, most CD133^{AC133}-positive cells were observed in the marginal region of the central area of spheroids positive for HIF-1α indicating a hypoxic microenvironment. These results suggest that the different microenvironments within spheroids (the normoxic microenvironment in the outer region and the hypoxic microenvironment in the central region) contribute to the induction and maintenance of CD133^{AC133}-positive cells. However, it is unknown whether CD133^{AC133}-positive cells translocate from the normoxic to the hypoxic microenvironment in the spheroid.

The number of CD133^{AC133}-positive cells dramatically varied in accordance with the change in the culture condition between spheroid and monolayer culture. This finding suggests that the positivity to CD133^{AC133} may be plastic according to the cellular microenvironments such as low oxygen, low nutrients, and low pH. Given that *CD133* expres-

sion was not affected by the cell culture condition (Fig. 6), the positivity to CD133^{AC133} may result from a post-translational modification that enables the CD133^{AC133} antibody to bind to the AC133 epitope. CD133 is probably differentially folded in CD133^{AC133}-positive and CD133^{AC133}-negative cells as a result of differential glycosylation to mask specific epitopes. Such a post-translational modification of the AC133 epitope has been reported elsewhere in association with the differentiation of CD133^{AC133}-positive cells (29). However, it should be kept in mind that the change in *CD133* expression might not be detected by the present quantitative PCR analysis because of low number of CD133^{AC133}-positive cells.

CD133^{AC133}-positive cells in spheroids were also nestin-positive. Because nestin is known to be a marker for undifferentiated neural cells, it has been suggested that the CD133^{AC133}-positive cells may be undifferentiated neural cell-like cells. This finding proposes the possibility that CD133 is not only restricted to CSCs but is also expressed on cancer cells that began to express an undifferentiated phenotype. Tumor cells are exposed to a wide range of microenvironments (low oxygen, low nutrients, and low pH), and these microenvironments may strongly affect the phenotypic expression of their malignant properties.

The present study demonstrated that CD133^{AC133}-positive cells are plastically induced under the different culture conditions, spheroid and monolayer, in relation to stability of HIF-1α. Spheroids as an *in vitro* tumor model are useful to study the dynamic changes in the tumor cell phenotype in the different cell microenvironments.

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