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DJ-1 Contributes to Self-renewal of Stem Cells in the U87-MG Glioblastoma Cell Line

YUKI TODA[1], RYOSUKE YOSHIMURA[1], MASAO ITAHARA[1], YURI IMAI[1], KANAE YAMADA[1], TOMOKO UNO[1], SUSUMU NAKATA[2], SHIGEKUNI HOSOGI[1], KAZUYUKI TAKATA[3] and EISHI ASHIHARA[1]

[1]*Department of Clinical and Translational Physiology, Kyoto Pharmaceutical University, Kyoto, Japan;*
[2]*Department of Clinical Oncology, Kyoto Pharmaceutical University, Kyoto Pharmaceutical University, Kyoto, Japan;*
[3]*Division of Integrated Pharmaceutical Sciences, Kyoto Pharmaceutical University, Kyoto, Japan*

Abstract. *Background/Aim: DJ-1, an oncogenic molecule, helps to maintain somatic stem cells by reducing the intracellular level of reactive oxygen species (ROS). This study investigated the role of DJ-1 in glioma stem cells (GSCs). Materials and Methods: U87-MG (U87) and U251-MG (U251) glioblastoma cell lines that express wild-type and mutant p53, respectively, were used. These were cultured with DJ-1-targeting siRNA and subjected to a variety of in vitro experiments or intracranial transplantation into nude mice. Results: Knockdown of DJ-1 reduced clonogenicity only in U87 cells, which was rescued by p53 depletion. ROS accumulated in DJ-1-depleted cells, although treatment with N-acetyl cysteine, which quenches ROS, did not affect exhaustion of CSCs among U87 cells by DJ-1 knockdown. In a serial transplantation study, DJ-1 knockdown prolonged the survival of mice in secondary transplantation. Conclusion: DJ-1 plays a pivotal role in maintenance of stem cell self-renewal in the U87 cell line.*

Glioblastoma (GBM), defined as a grade IV astrocytoma, is the most aggressive and commonly diagnosed brain cancer and accounts for 16% of all primary brain and central nervous system neoplasms (1, 2). The current standard treatment protocol for GBM is surgery, followed by temozolomide chemotherapy and radiotherapy. A variety of novel drugs have been developed; however, the median survival of patients with GBM who receive any therapy is only 15 months (3).

Among several factors responsible for these poor outcomes, traditional therapies are ineffective against cancer stem cells (CSCs). CSCs are postulated to be a small population of cells located at the top of the cellular hierarchy that supply other tumor cells by undergoing self-renewal and differentiation (CSC hypothesis) (4, 5). Glioma stem cells (GSCs) were first identified as CD133-expressing cells with high tumorigenicity *in vivo* and resistance to current therapy (6). This implies that GSCs can initiate cancer recurrence and metastasis. Consequently, molecular strategies targeting GSCs must be urgently developed to treat GBM.

Parkinsonism-associated deglycase (PARK7, DJ-1) functions in anti-apoptotic signaling and protein quality control in response to oxidative stress. Failure of these functions due to genetic mutations is associated with the early-onset and familial form of Parkinson's disease (7). *DJ-1* was originally cloned as an oncogene that enhances RAS-driven neoplastic transformation (8). Moreover, DJ-1 is overexpressed in many types of cancer and this contributes to survival, proliferation, and metastasis of cancer cells (9-11). However, the functional role of DJ-1 in maintenance of GSCs remains elusive.

CSCs and somatic stem cells share many characteristics that are established by common molecular mechanisms (12). The Mortalin/DJ-1 complex sustains the stemness of hematopoietic stem cells, one of the most characterized types of somatic stem cells, by modulating mitochondrial oxidative stress (13). We hypothesized that this mechanism also operates in GSCs. The present study examined the effect of DJ-1 knockdown on the self-renewal capability of human GBM cell lines.

Materials and Methods

Cell culture. The human GBM cell lines U251-MG (U251) and U87-MG (U87) were purchased from American Type Culture Collection (Manassas, VA, USA). p53 is mutated (R273H) in U251 cells (14)

Correspondence to: Yuki Toda, Department of Clinical and Translational Physiology, Misasagi-Nakauchi-cho 5, Yamashina-ku, Kyoto 607-8414, Japan. E-mail: tda@mb.kyoto-phu.ac.jp and Eishi Ashihara, Department of Clinical and Translational Physiology, Misasagi-Nakauchi-cho 5, Yamashina-ku, Kyoto 607-8414, Japan. E-mail: ash@mb.kyoto-phu.ac.jp

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and not mutated in U87 cells (15). Both cell lines were cultured in Dulbecco's modified Eagle's medium containing 1 g/l glucose (Wako, Osaka, Japan) at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

RNA interference. RNA interference was performed by transfecting siRNA oligonucleotides using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. To scavenge ROS, cells were transfected with siRNA in the presence of 1 mM *N*-acetyl cysteine (NAC) and the culture medium was additionally supplemented with 1 mM NAC at 24 h prior to harvest. The following sequences of siRNA oligonucleotides (Thermo Fisher Scientific) were used: *DJ-1*: s22304; sense: 5'-GGUUUUGGAAGUAAAGUUATT-3', antisense: 5'-UAACUUUACUUCCAAAACCTA-3'; *p53* (s605; sense: 5'-GUAAUCUACUGGGACGGAATT-3', antisense: 5'-UUCCGUCCC AGUAGAUUACCA-3'. The knockdown efficiency was determined by western blotting.

Sphere-formation assay. CSCs were prepared by culturing cells in Dulbecco's modified Eagle's medium/F12 (Thermo Fisher Scientific) containing 1% penicillin/streptomycin, 2% B-27 (Thermo Fisher Scientific), 20 ng/ml epidermal growth factor (Peprotech, Rocky Hill, NJ, USA), and 20 ng/ml basic fibroblast growth factor (Peprotech) in ultra-low attachment plates (Corning International, Corning, NY, USA). Epidermal growth factor and basic fibroblast growth factor were added at a concentration of 20 ng/ml on days 0 and 3. Cultures were incubated in 5% CO_2 at 37˚C. To analyze clonogenicity, cells were seeded at a density of 500-1,000 cells/well and cultured for 7 days. Sphere-forming efficiency (clonogenicity) was calculated as the number of spheres per well.

Immunoblot analysis. Total proteins from U87 or U251 cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membranes. Membranes were incubated with a primary antibody (Ab) followed by an HRP-linked secondary Ab (GE Healthcare, Uppsala, Sweden). Mouse anti-DJ-1 (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan), mouse anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-c-MYC (Cell Signaling Technology, Danvers, MA, USA) and mouse anti-actin (Merck, Darmstadt, Germany) antibodies were used. Protein bands were detected using enhanced chemiluminescence.

Aldehyde dehydrogenase (ALDH) assay. An ALDEFLUOR kit (STEM CELL Technologies, Vancouver, BC, Canada) was used to detect tumor cells with high or low ALDH activity. Briefly, cells were suspended in pre-warmed staining buffer containing the ALDH substrate and then incubated at 37˚C for 30 min. Diethylaminobenzaldehyde, an inhibitor of ALDH, was used as a negative control. Stained cells were analyzed on a FACS LSRFortessa™ X-20 instrument (BD Biosciences, Franklin Lakes, NJ, USA), and data were analyzed using FlowJo Software (BD Biosciences). The percentage of $ALDH^{bright}$ cells was calculated.

ROS analysis. To assess the intracellular level of ROS, cells were incubated with 10 μM 2',7'-di-chlorofluorescein diacetate, acetyl ester (CM-H2DCFDA; Thermo Fisher Scientific) at 37˚C for 1 h. Stained cells were harvested and analyzed on FACS.

Orthotopic tumor model. Male Balb/c nu/nu mice (7-8 weeks old) were obtained from Shimizu Laboratory Supplies (Kyoto, Japan). The mice were anesthetized and placed in a stereotaxic instrument (51730D; Stoelting Co., Wood Dale, IL, USA). U87 cells (200,000 cells suspended in 2 μl of phosphate-buffered saline) were injected into the right cerebral cortex using a Hamilton syringe driven by a digital syringe pump (Legato130; KD Scientific, Holliston, MA, USA) at a rate of 1 μl/min at coordinates 2.0 mm lateral, 0.5 mm posterior, and 3.0 mm ventral from bregma. The syringe was left in place for 2 min after each injection to allow cell diffusion and then slowly removed. The skin was sutured, and mice were allowed to recover in a warm box before being returned to their cages. For secondary transplantation, brain tumor cells were harvested from mice that had received the primary transplantation and were intracerebrally injected into secondary recipient mice. These studies were approved by the Committee on Animal Research of Kyoto Pharmaceutical University. Animal health was monitored daily (approval number DCTP-15-001). Mice were sacrificed and the existence of tumor xenograft in brain was identified at a humane endpoint when they showed signs of disease progression with a decrease in body weight of 20%.

Analysis of PrognoScan data. The relationship between DJ-1 expression and overall survival of glioma patients was evaluated by analysis of the public database, PrognoScan (http://dna00.bio. kyutech.ac.jp/PrognoScan/index.html) (16, 17). Survival analysis in PrognoScan employs the minimum *p*-value approach to find the cut-off point in continuous gene expression measurement for grouping patients. The datasets of GSE4412-GPL97 and GSE7696 were the groups of patients with grade III/IV glioma and glioblastoma, respectively. These patients were ordered by expression of DJ-1 gene and divided into two groups at all possible cut-off points. The risk differences between the two groups were calculated using the log-rank test.

Statistical evaluation. Results of ROS quantification and sphere-formation assay are reported as mean±SD. Data from $ALDH^{bright}$ cell quantification is reported as mean±SEM. Means were compared using the two-tailed unpaired Student's *t*-test or a one-way ANOVA with the Bonferroni/Dunn test (Prism, GraphPad Software, Inc., San Diego, CA, USA). Survival curves were drawn using the Kaplan–Meier method and analyzed using the log-rank test. Values of $p<0.05$ was considered significant.

Results

DJ-1 is up-regulated in GSCs. CSCs can grow in an anchorage-independent manner. Upon culture in ultra-low attachment plates containing serum-free media, CSCs among U251 and U87 cells proliferated to form spheres (sphere-formation assay). Spheres were harvested on various days and analyzed by western blotting. DJ-1 protein expression was up-regulated in spheres harvested from both cell lines after more than 10 days of culture (Figure 1A). These data suggest that DJ-1 is up-regulated during sphere formation in these GBM cell lines.

Knockdown of DJ-1 suppresses maintenance of U87 GSCs. We depleted DJ-1 using siRNA to elucidate the significance

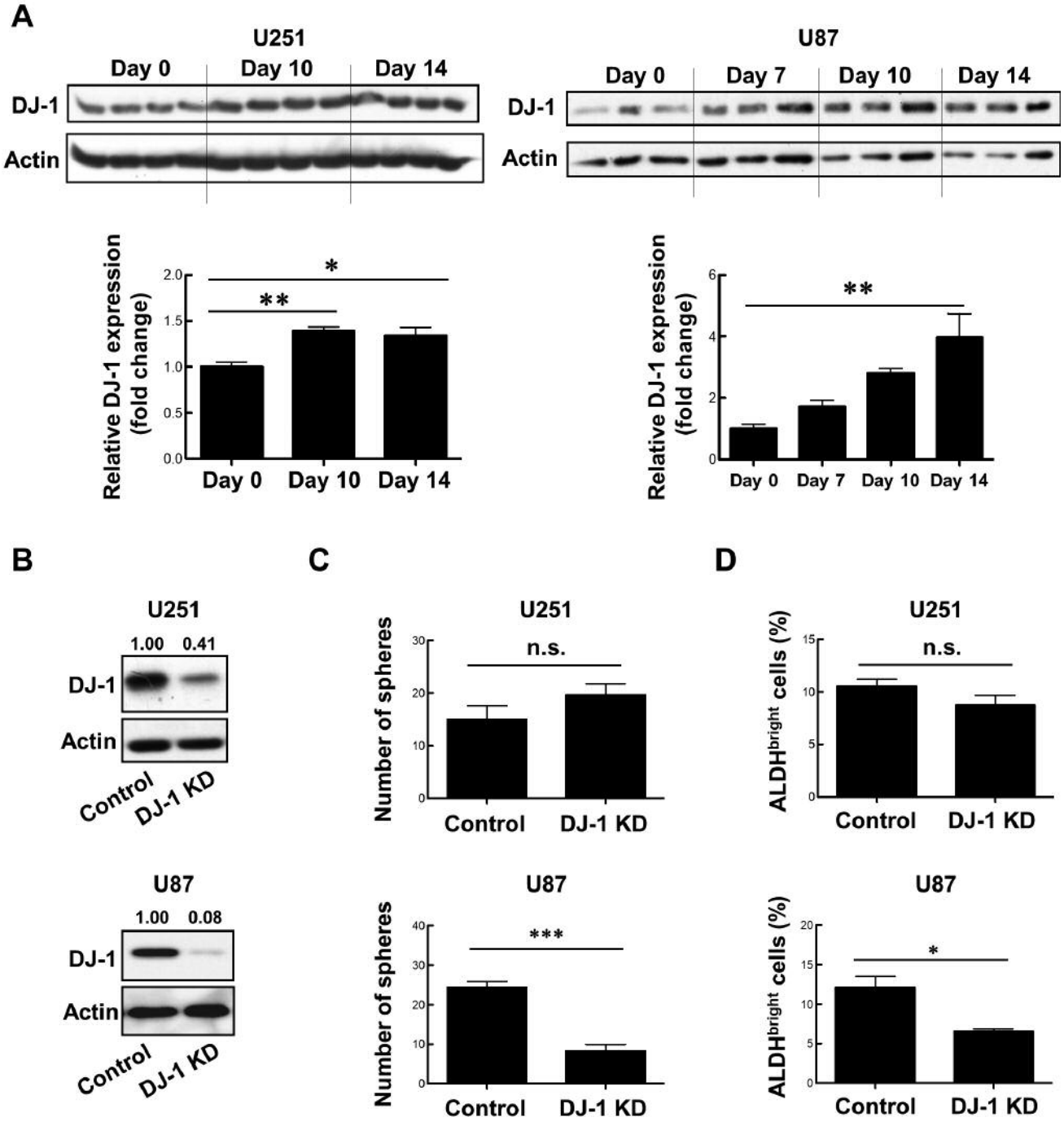

Figure 1. *Knockdown (KD) of Parkinsonism-associated deglycase (DJ-1) attenuates self-renewal of glioma stem cells (GSCs). A: DJ-1 protein expression was analyzed in total lysates of GSCs collected on specified days (n=3-4 samples per group). Bars indicate the mean±SEM. Significantly different at *p<0.05, **p<0.01 (one-way ANOVA with the Bonferroni/Dunn test). B: Knockdown efficiency of DJ-1 siRNA. U251 and U87 cells were transfected with control or DJ-1-targeting siRNA oligonucleotides for 72 h and then DJ-1 expression was analyzed by western blotting. C: Clonogenicity of DJ-1-depleted cells. U251 and U87 cells described in (B) were cultured under sphere formation conditions for 7 days. The number of spheres in each well was calculated. Bars indicate the mean±SD. Significantly different at ***p<0.001 (two-tailed unpaired Student's t-test). D: Aldehyde dehydrogenase (ALDH) activity in DJ-1-depleted cells. ALDH activity in U251 and U87 cells described in (B) was assessed using the ALDEFLUOR system. The percentage of ALDHbright cells was calculated. Bars indicate the mean±SEM. *Significantly different at p<0.05 (two-tailed unpaired Student's t-test). n.s.: Not significantly different.*

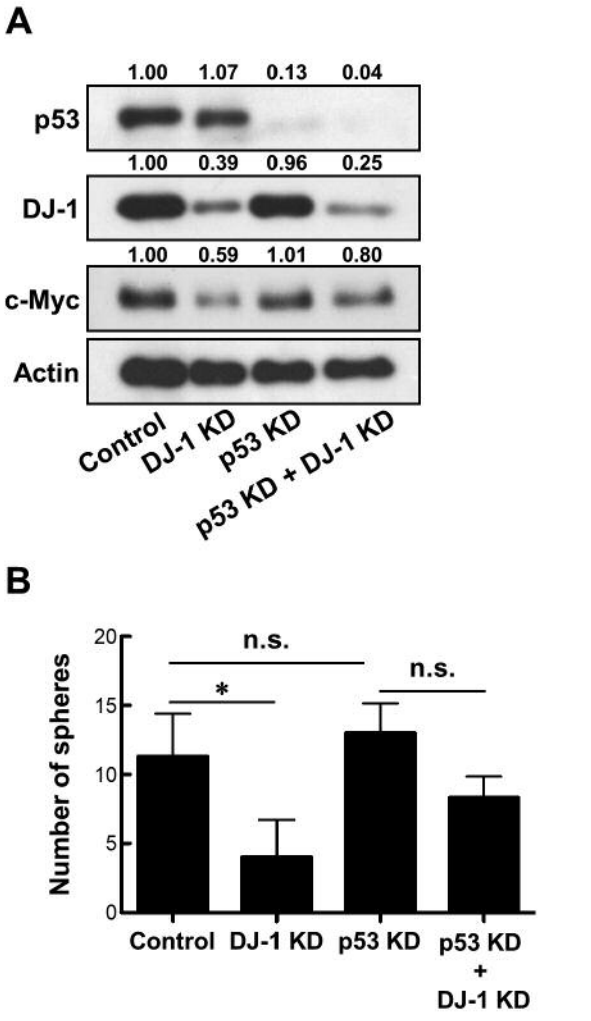

Figure 2. *p53 Mediates Parkinsonism-associated deglycase (DJ-1)-regulated glioma stem cell (GSC) clonogenicity in U87 cells. A: The effect of DJ-1/p53 double-knockdown (KD) on protein expression of c-MYC. U251 and U87 cells were transfected with control, DJ-1-targeting, or p53-targeting siRNA oligonucleotides for 72 h and then expression of DJ-1, p53 and c-MYC was analyzed by western blotting. B: U87 cells described in (A) were subjected to the sphere-formation assay. Bars indicate the mean±SD. *Significantly different at p<0.05 (one-way ANOVA with the Bonferroni/Dunn test). n.s.: Not significantly different.*

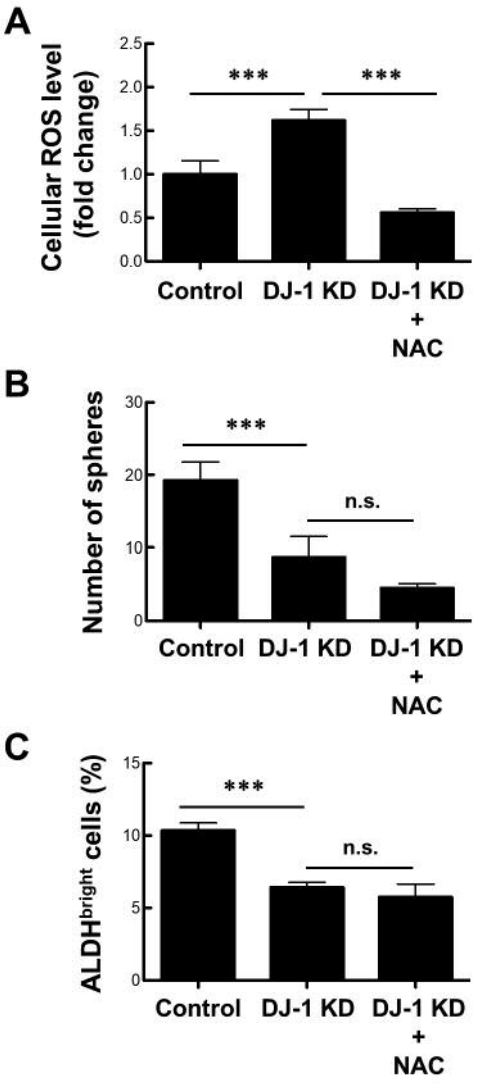

Figure 3. *Accumulation of reactive oxygen species (ROS) is unrelated to the reduced clonogenicity of cells after Parkinsonism-associated deglycase (DJ-1) knockdown (KD). A: Level of ROS in DJ-1-depleted cells and its down-regulation upon N-acetyl cysteine (NAC) treatment. U87 cells were transfected with siRNA oligonucleotides in the presence or absence of 1 mM NAC for 72 h. Fluorescence of 2',7'-di-chlorofluorescein diacetate acetyl ester was analyzed by flow cytometry. B: Effect of NAC on the clonogenicity of DJ-1-depleted cells. U87 cells described in (A) were subjected to the sphere formation assay. n.s.: Not significant. C: Effect of NAC on aldehyde dehydrogenase (ALDH) activity of DJ-1-depleted cells. U87 cells described in (A) were subjected to the ALDH assay. Bars indicate the mean±SEM. Significantly different at ***p<0.001 (one-way ANOVA with the Bonferroni/Dunn test). n.s.: Not significantly different.*

of its up-regulation during sphere formation. Western blotting analysis demonstrated that endogenous DJ-1 expression in U87 and U251 cells was greatly suppressed at 72 h after transfection of *DJ-1*-targeting siRNA (Figure 1B). These cells were seeded and subjected to the sphere-formation assay. After 7 days, the sphere-forming efficiency of DJ-1-depleted U87 cells was 2-fold lower than that of control U87 cells (Figure 1C). By contrast, clonogenicity did not significantly differ between DJ-1-depleted and control U251 cells. We next quantified $ALDH^{bright}$ cells as an indicator of CSCs (18-20). In agreement with the results of the sphere-formation assay, DJ-1 knockdown reduced the percentage of ALDHbright cells among U87 cells, but not among U251 cells (Figure 1D). These data demonstrate that DJ-1 maintains CSCs, and this is linked to the efficiency of sphere formation by U87 cells.

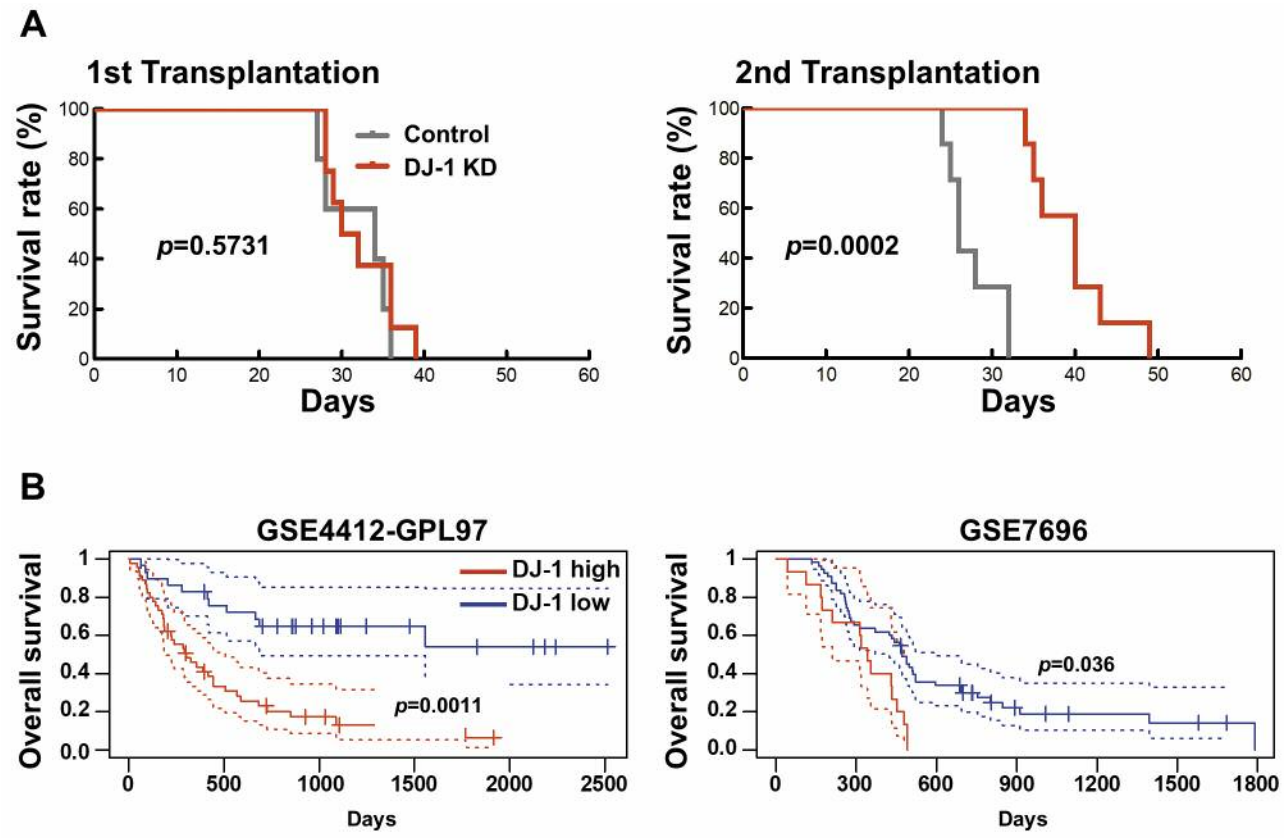

Figure 4. *Effect of Parkinsonism-associated deglycase (DJ-1) knockdown (KD) on survival of tumor-bearing mice, and its clinical implication. A: The survival curves of BALB/c nu/nu mice following transplantation of control or DJ-1-depleted U87 cells into the brain (first transplantation; left). Brain tumors derived from these mice were dissociated, and tumor cells were transplanted into secondary mice (second transplantation; right: Survival curves were generated using the Kaplan–Meier method and analyzed using the log-rank test. B: Kaplan–Meier analysis of overall survival of 74 patients with grade III and IV glioma (GSE4412-GPL97 dataset; left panel) and 70 patients with glioblastoma (GSE7696 dataset; right panel) stratified by high (red) and low (blue) DJ-1 expression (GSE4412-GPL97 dataset: high: n=45, low: n=29; GSE7696 dataset: high: n=55, low: n=15) using the PrognoScan database. The dotted lines represent 95% confidence intervals.*

Taken together, we conclude that DJ-1 supports the self-renewal of CSCs in the U87 cell line.

p53 affects GSC regulation by DJ-1 expression. Our results demonstrated that the impact of DJ-1 depletion on stemness differed between U87 cells and U251 cells. The mutational status of *p53* differs between these two cell lines: U87 and U251 cells express wild-type (WT) and mutant (R273H) *p53*, respectively. DJ-1 binds to *p53* and negatively controls its transcriptional activity (21). The suppressive function of *p53* on CSC maintenance might be intact and regulated by DJ-1 in U87 cell line. To test this, we conducted double knockdown experiment on U87 cells. The protein expression of c-MYC, a transcriptional target of p53, decreased in DJ-1-depleted cells (Figure 2A). The down-regulation of c-MYC by DJ-1 depletion was attenuated by the simultaneous knockdown of *p53* (Figure 2A). Consistently, while clonogenicity was not significantly affected by *p53* knockdown alone, the reduction of clonogenicity by DJ-1 depletion was attenuated by the simultaneous knockdown of *p53* (Figure 2B). These data suggest that *p53* is involved in DJ-1-regulated clonogenicity of U87 cells.

Attenuation of stemness upon DJ-1 depletion is not due to an increase in ROS. DJ-1 acts as an antioxidant by directly scavenging ROS (22). Depletion of DJ-1 might lead to accumulation of intracellular ROS, which resulted in exhaustion of CSCs. To substantiate this idea, the ROS level in DJ-1-depleted cells was measured by FACS using CM-H2DCFDA (Figure 3A). The ROS level was increased in DJ-1-depleted cells; however, the decrease in the number of spheres and in the percentage of $ALDH^{bright}$ cells upon *DJ-1* knockdown was not rescued by treatment with the ROS scavenger NAC (Figure 3B and C). Thus, DJ-1 regulates the

CSC population and the ROS level in the U87 cell line; however, these two effects were not related.

Aggressiveness of tumors generated by DJ-1-depleted cells decreases upon serial transplantation. To examine the effects of DJ-1 on tumorigenesis *in vivo*, we employed an orthotopically transplanted tumor xenograft model in BALB/c nu/nu mice using U87 cells (Figure 4A, left). Tumor size and the survival of mice were similar upon primary transplantation of control or DJ-1-depleted cells. To further assess the self-renewal of CSCs, the tumors were digested, and *in vitro*-expanded cells were serially transplanted into secondary nude mice (Figure 4, right). The survival duration of secondary mice transplanted with DJ-1-depleted cells was significantly longer than that of secondary mice transplanted with control cells. These data indicate that DJ-1 contributes to the aggressiveness of GBM tumors, possibly by helping to maintain CSCs.

Prognostic value of the DJ-1 expression in patients with brain cancer. To confirm our results in a clinical setting, we analyzed the relationship between the expression level of DJ-1 and the prognosis of patients with brain cancer using the PrognoScan database. A high expression level of DJ-1 positively correlated with poor overall survival rates in two datasets of patients with brain cancer (Figure 4C).

Discussion

We revealed the significance of DJ-1 in maintenance of CSCs by performing gene knockdown experiments. *DJ-1* knockdown reduced the protein expression of c-MYC and clonogenicity in the U87 cell line. Absence of WT *p53* abolished these phenomena caused by *DJ-1* knockdown. p53 and phosphatase and tensin homolog deleted from chromosome 10 (PTEN) are the downstream signaling molecules of DJ-1 (21, 23). These target molecules cooperatively suppress *c-MYC* transcription, leading to the reduction of the GSC population (24). *PTEN* is mutated in both U87 and U251 cell lines (25). Additionally, U251 cells express R273H mutant *p53*. This is a hotspot mutation that disrupts binding of p53 to DNA (contact mutation) (26). Therefore, these major players in downstream signaling of DJ-1 might originally be inactivated in the U251 cell line. p53-dependent tumor-suppressive function is active in U87 cells. DJ-1 may regulate c-MYC expression through *p53* transcriptional activity, thereby helping to CSC maintenance. Consistent with this hypothesis, knockdown of *DJ-1* only affected CSCs in the U87 cell line with WT *p53*.

The function of DJ-1 as an antioxidant has been well-studied. DJ-1 quenches ROS *via* self-oxidation of cysteine 106, which can be converted into cysteine-sulfenic, -sulfinic, and -sulfonic acids (22). In addition, DJ-1 induces expression of redox- and detoxification-related genes *via* stabilization of nuclear factor erythroid-2-related factor 2 (27). Substitution of cysteine 106 with another amino acid or siRNA-mediated knockdown of DJ-1 increases the intracellular ROS level, which causes lethal damage. Tai-Nagara *et al*. reported that hematopoietic stem cells are exhausted in *DJ-1*-knockout mice due to intracellular accumulation of ROS (13). The ROS-related DJ-1 pathway is a key regulator of the differentiation of mesenchymal stem cells into smooth muscle cells upon sphingosylphosphorylcholine stimulation (28). These indications are inconsistent with the finding of the current study that accumulation of ROS was not involved in the exhaustion of CSCs upon DJ-1 depletion. The mechanism *via* which DJ-1 regulates stem cell function might differ between CSCs and normal stem cells.

Based on the CSC hypothesis, CSCs should be able to perpetuate xenograft tumors for multiple generations. Maintenance of tumorigenic potency can be evaluated by a serial transplantation assay, which has been used to identify and characterize CSCs in many types of cancer. In our study, the percentage of mice in which tumors formed did not significantly differ between the groups injected with control and DJ-1-depleted cells in the primary and secondary transplantation experiments (data not shown). However, the survival of mice upon secondary transplantation was markedly longer in the group injected with DJ-1-depleted cells than in the group injected with control cells. Expression of stem cell markers, such as podoplanin, CD133, and nestin, is a prognostic marker of clinical outcomes in GBM (29), indicating that there is a direct correlation between the frequency of CSCs and tumor aggressiveness. Survival duration is a surrogate endpoint for tumor aggressiveness. Thus, deregulation of CSC function upon *DJ-1* knockdown affected tumor aggressiveness, rather than tumorigenesis. siRNA-mediated depletion of DJ-1 prior to the primary transplantation affected self-renewal of CSCs. The effects of DJ-1 depletion may be enhanced *via* constitutive knockdown using shRNA.

Accumulating evidence from basic science studies indicates that DJ-1 is an emerging therapeutic target for cancer because it modulates various cellular pathways that support the survival, growth, and invasion of cancer cells. The results of the current study confirm this and demonstrate that DJ-1 is critical for the function of CSCs. A DJ-1-targeting drug has not been tested in clinical trials for cancer. Tashiro *et al*. discovered small chemical compounds that inhibit the function of DJ-1 (30). Drug delivery systems are critical for targeting central nervous system-related symptoms such as those observed in patients with brain cancer. We believe that our previous work showing that GBM cells effectively take up exosomes will assist the development of drug delivery systems for DJ-1 inhibitors (31). In conclusion, DJ-1 regulates the stemness of GSCs, and accumulation of ROS is not involved in exhaustion of CSCs upon depletion of DJ-1.

Conflicts of Interest

The Authors have no conflicts of interest to disclose in regard to this study.

Author's Contributions

Y.T., R.Y., M.I., Y.I., K.Y., T.U., and S.N. performed experiments; Y.T., R.Y., S.N., and E.A. analyzed data; S.N., S.H., K.T., and E.A. provided intellectual guidance; Y.T. and E.A. wrote the article; and Y.T. and E.A. conceptualized the study.

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