A lignan from *Alnus japonica* inhibits glioblastoma tumorspheres by suppression of FOXM1

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Abstract

Introduction

Glioblastoma (GBM) is the most common and aggressive malignant brain tumor. Forkhead Box M1 (FOXM1) has been shown to regulate cell proliferation, apoptosis, angiogenesis, DNA damage repair and tumorigenesis. The lignin, (–)-(2R,3R)-1,4-O-diferuloylsecoisolariciresinol (DFS), from *Alnus japonica* (Betulaceae) has shown anti-cancer effects against colon cancer cells by suppressing FOXM1. However, the efficacy of DFS in GBM has not yet been determined. The present study hypothesized that DFS can have anti-cancer effects against GBM tumorspheres (TSs).

Methods

Immunoprecipitation and luciferase reporter assays were performed to evaluate the ability of DFS to suppress nuclear translocation of β -catenin through β -catenin/FOXM1 binding. GBM TSs were treated with DFS to assess the ability of DFS to inhibit GBM TSs and to evaluate their transcriptional profiles. The *in vivo* efficacy of DFS was examined in orthotopic xenograft models of GBM.

Results

Expression of FOXM1 was higher in GBM than in normal tissues. DFS-induced FOXM1 protein degradation blocked β -catenin translocation into the nucleus and consequently suppressed downstream target genes of FOXM1 pathways. DFS considerably inhibited cell viability and ATP levels in GBM TSs, while increasing the proportion of apoptotic cells. Treatment with DFS also reduced neurosphere formation and the invasive properties of GBM TSs. Transcriptome analyses showed that DFS reduced the activities of transcription factors related to tumorigenesis, stemness, and invasiveness. In addition, DFS significantly inhibited tumor growth and prolonged the survival rate of mice in orthotopic xenograft models of GBM.

Conclusions

DFS inhibits the proliferation of GBM TSs by suppressing FOXM1. These findings suggest that DFS may be a potential therapeutic agent to treat patients with GBM.

Introduction

Glioblastoma (GBM), the most common primary malignant tumor in the brain, is highly aggressive [1]. Despite intensive treatment with surgery, chemotherapy and radiotherapy, patients with GBM have a high mortality rate and poor prognosis [1–3]. To date, furthermore, relatively little is known about the cells from which GBM originates or the genetic mutations that accompany GBM tumorigenesis, so GBM still

remains a difficult disease to conquer [4, 5]. However, previous studies reported that GBM tumorsphere(TS), characterized by GBM resident cells in culture has a treatment refractory properties, as well as a stemness profile and an ability to form tumors, and it has been shown to have value for clinical use [6, 7].

Forkhead Box M1 (FOXM1) is a member of the Forkhead box transcription factor family that has been shown to regulate DNA damage repair, cellular proliferation, apoptosis, angiogenesis and tumorigenesis [8]. Expression of FOXM1 is significantly elevated in many human tumors, including non-small cell lung cancer, breast cancer, basal cell carcinoma, hepatocellular carcinoma, pancreatic cancer, prostate cancer, colon cancer, medulloblastoma and GBM [9–13]. High expression of FOXM1 in glioma cells enhances tumorigenicity, invasiveness and angiogenesis in GBM animal models [14–16]. Furthermore, FOXM1 was found to regulate the Wnt/ β -catenin signaling pathway by promoting the nuclear translocation of β -catenin [17, 18].

Alnus japonica (Betulaceae) is a plant used as a medicinal herb in east Asia. This plant contains pharmacologically active compounds, including the isolated lignan, (-)-(2R,3R)-1,4-*O*-diferuloylsecoisolariciresinol (DFS), which was found to reduce the viability of colon cancer cells and to interrupt the cell cycle. We previously reported that DFS interfere with β -catenin translocation into the nucleus, and to subsequently reduce the levels of expression of β -catenin mediated genes through suppression of FOXM1 protein expression in colon cancer [19]. DFS also induced autophagy and endoplasmic reticulum (ER) stress in prostate and colon cancer cells [20]. FOXM1/ β -catenin interactions can also regulate the stemness and tumorigenicity of glioma stem cells [18]. Because GBM TSs are resistant to conventional treatments such as radiotherapy and chemotherapy, they have been considered a good platform for testing the therapeutic efficacy of drug candidates. We have tried to find promising anti-GBM agent from medicinal plants such as *A. japonica*. Relatively little is known, however, about the effects of the DFS in GBM TSs.

The present study hypothesized that DFS has the ability to block β -catenin translocation into the nucleus by suppressing the formation of FOXM1/ β -catenin complex in GBM TSs. In addition, the anti-cancer potential of DFS against GBM was evaluated using GBM TSs and an orthotopic xenograft mouse model.

Materials And Methods

Culture of GBM TSs and reagents

GBM TSs were derived from two individual patients with GBM. These GBM TSs, denoted TS15-88 and TS13-64, were established from fresh GBM tissue specimens, as approved by the institutional review board of the Yonsei University College of Medicine. TSs were cultured according to standard methods [21–23]. Briefly, cells were cultured in TS complete medium, consisting of Dulbecco's modified Eagle's medium/F12 (Mediatech, Manassas, VA, USA), 1x B27 (Invitrogen, Carlsbad, CA, USA), 20 ng/mL basic fibroblast growth factor (bFGF; Novoprotein, Summit, NJ, USA) and 20 ng/mL epidermal growth factor

(EGF; Novoprotein). All *in vitro* experiments were performed under the culture conditions for TSs. DFS, an anti-GBM lignan, was isolated from a chloroform soluble fraction of *A. japonica* extracts, as described [19].

Western blot

Lysates of GBM TSs were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on Tris-glycine gels. Cytosolic and nuclear fractions were prepared using cytoplasmic and nuclear extraction reagents (Thermo Scientific, Rockford, IL, USA), respectively, according to the manufacturer's instructions. Proteins were transferred to nitrocellulose membranes and probed with antibodies against cleaved capase-3, CD133, PDPN, β -catenin, CD44, snail, Cyclin D1 and cMYC (Cell Signaling Technology, Danvers, MA, USA); Sox2 (Merck Millipore, Darmstadt, Germany); Msi-1 (Abcam, Cambridge, UK); nestin (Novus Biologicals, Centennial, CO, USA); N-cadherin, Zeb1 (Sigma-Aldrich, St. Louis, MO, USA); Twist, Oct3/4, BAX, Bcl-2, phospho- β -catenin, PARP and GAPDH (Santa Cruz Biotechnology, Dallas, TX, USA); and FOXM1 (Bethyl Laboratories, Montgomery, TX, USA), along with Western Lightning Plus-enhanced chemiluminescence reagent (PerkinElmer, Lawrence, MA, USA). Images were captured using an ImageQuant LAS 4000 mini (GE Healthcare Life Sciences, Little Chalfont, UK).

Immunoprecipitation

To determine interactions between FOXM1 and β -catenin, GBM TSs were treated with DFS for 72 h, lysed with lysis buffer and incubated with anti-FOXM1 or anti- β -catenin antibody on a rotator overnight at 4°C. Protein-antibody-protein A/G agarose complexes were prepared using protein A/G sepharose beads (Santa Cruz Biotechnology) for 4 h at 4 °C. After extensive washing with RIPA lysis buffer, the immunoprecipitated complexes were resuspended in reducing sample buffer and boiled for 10 min. The precipitated proteins were subjected to immunoblotting with the indicated antibodies.

Luciferase reporter assay

Canonical TCF/LEF signaling activity was measured using TOPflash/FOPflash reporter assays. Briefly, 1×10^5 cells were seeded into each well of a 24-well plate for 1 day before transfection. The cells were transfected with 100 ng of firefly luciferase reporter plasmid and 10 ng of internal control plasmid pRL-TK (Promega, Madison, WI, USA) using Lipofectamine 3000 (Invitrogen) for 72 h according to the manufacturer's protocol. Samples were lysed with 1 $\times$ reporter lysis buffer for 15 min and centrifuged, and the supernatants were used for luciferase and β -galactosidase assays, according to the manufacturers' protocols. After confirming that the cells had been transfected using β -galactosidase assays, luciferase activity in 10 μ g of protein lysate was quantified using the Dual Luciferase Reporter Assay System (Promega). Each sample was analyzed in triplicate.

Evaluation of cell viability

The effects of DFS on the survival of GBM TSs were determined using WST assays (Promega). Briefly, 1×10^4 single dissociated GBM TSs were seeded into each well of a 96-well plate. The plates were incubated at 37°C for 24 h and treated with DFS for 72 h. WST reagent (10 μ l/well) was added and

absorbance was measured at 450 nm after incubating at 37°C for 1 h. Each experiment was repeated three times in triplicate, and the results are expressed as the percentage of viable cells relative to controls.

Evaluation of ATP levels

Dispersed GBM TSs were plated in 96-well plates at a density of $1 \cdot 10^4$ cells per well. After incubating for 24 h, TSs were treated with DFS for 3 days, and then ATP levels were compared using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer's protocol. Briefly, a volume of CellTiter-Glo Reagent equal to the volume of cell culture medium was added to each well, after which cells were incubated at room temperature for 10 min, and luminescence was measured.

Cell cycle and apoptosis analysis

To analyze the cell cycle, GBM TSs treated with DFS for 72 h were collected, fixed with 70% ethanol, dissociated with accutase (Sigma), and stained with 100 µg/ml of propidium iodide (PI, Sigma) at 4°C for 15 min in the dark. To evaluate DFS-induced apoptosis in GBM TSs, GBM TSs were treated with the indicated concentration of DFS. Apoptotic cell death was detected by double staining with fluorescein-isothiocyanate (FITC)-conjugated annexin V (BioLegend, San Diego, CA, USA) and PI. The stained cells were analyzed using an LSR II flow cytometer (BD Biosciences, Bedford MA, USA).

Neurosphere formation assays

Ten single dissociated GBM TSs were seeded in each well of a 96-well plate and treated with DFS after 24h. The plates were incubated for 3 weeks in TS complete medium, with medium changed every week. The number of sphere-positive wells was counted, and the ratio of sphere-positive wells in the treated relative to the control group was calculated. The radius of spheres in each group was also measured. Images were captured and analyzed using ToupView software (ToupTek Photonics, Zhejiang, China).

Three-dimensional (3D) invasion assays

Each well of a 96-well plate was filled with mixed matrix composed of matrigel (Corning Incorporated, Tewsbury, MA, USA), collagen type I (Corning Incorporated) and TS complete medium. Single spheroids had been seeded inside the matrix prior to gelation, followed by the addition of TS complete medium over the gelled matrix to prevent drying. The invaded area was quantified as occupied area at (72 h - 0 h)/0 h.

Processing of transcriptome data

Total RNA was extracted from GBM TSs and used for RNA sequencing. The quality of the reads was checked using FastQC (v.0.10.1) and the sequencing adapters were removed using Trimmomatic (v.0.38). Low quality reads were filtered according to the following criteria: reads containing more than 10% of skipped bases (marked as 'N's), reads containing more than 40% of bases with quality scores less than 20, and reads whose average quality scores of each read were less than 20. Filtered reads were mapped to the human reference genome using the aligner Tophat [24]. Gene expression levels were measured with Cufflinks v2.1.1 [25], using the gene annotation database of Ensembl release 72 [26]. Non-coding regions were removed using the -mask option. Using GENE-E software, the average linkage of hierarchical

clustering was determined with Pearson's correction as a distance metric and expression levels depicted as heat maps.

Mouse orthotopic xenograft model

Male athymic nude mice aged 4–8 weeks (Central Lab. Animal Inc., Seoul, Korea) were housed in micro-isolator cages under sterile conditions and monitored for at least one week before study initiation to ensure proper health. Lighting, temperature, and humidity were controlled centrally. Dissociated Luc-TS13-64 cells were pretreated with DFS for 72 h using the pretreatment protocol [27, 28]. Cells were selected by trypan blue staining, and 5×10^5 viable cells were implanted into the right frontal lobe of each mouse at a depth of 4.5 mm using a guide-screw system [21–23, 29]. Mice showing a >15% reduction in body weight compared with the maximum were euthanized according to the approved protocol.

Statistical analysis

Levels of significance for comparisons among treatment groups were determined using one-way ANOVA with Tukey's post hoc test for multiple comparisons. Survival was analyzed using the Kaplan-Meier method and compared by log-rank tests. All the graphs and statistical analyses were performed using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, USA) software, with P-values < 0.05 considered statistically significant.

Results

Expression of FOXM1 and β -catenin mRNAs in GBM tissues and TSs

Tissue specimens were obtained from 49 patients who underwent surgery for GBM from May 2009 to December 2016, with both GBM and normal brain tissue specimens obtained from eight of these patients. Similar to previous findings, the levels of expression of FOXM1 and β -catenin mRNAs were significantly higher in GBM than in normal tissues (Fig. 1a and b).

DFS reduces β -catenin nuclear translocation by suppressing FOXM1 expression

To test the inhibitory effects of DFS on FOXM1/ β -catenin interactions, proteins encoded by β -catenin pathway-related genes were quantified by western blotting. DFS significantly reduced the levels of FOXM1, active- β -catenin and total β -catenin expression in whole-cell lysate of GBM TSs (Fig. 1c) and in their nuclei (Fig. 1d). Immunoprecipitation (IP) analyses showed that DFS suppressed the interaction between FOXM1 and β -catenin in GBM TSs (Fig. 1e). In addition, DFS markedly reduced TCF/LEF signaling activity in GBM TSs in a dose-dependent manner (Fig. 1f), as well as reducing the expression of cyclin D1 and cMyc, which are regulated by FOXM1/ β -catenin forming a complex with the TCF/LEF

transcription factor (Fig. 1g). Taken together, these results indicate that DFS inhibited the binding of FOXM1 and β -catenin, thereby inhibiting the formation of complexes with TCF/LEF and sub-steps.

DFS inhibits GBM TSs viability and ATP levels in vitro

To assess the mechanism of action of DFS against GBM TSs, we evaluated the effects of DFS on cell viability and ATP levels. WST assays confirmed that DFS dose-dependently reduced cell viability in GBM TSs (**Fig. 2a**), as well as reducing ATP levels in a concentration dependent manner (**Fig. 2b**). Based on these results, DFS concentrations of 5 μ M and 10 μ M were selected for further studies. DFS treatment significantly increased the percentage of GBM TSs in sub-G1 phases (more than 55%) compared with control (about 10%) (**Fig. 2c**), as well as markedly increasing the percentage of cells in early stages of apoptosis (**Fig. 2d**). DFS also altered the levels of apoptotic markers, such as Bcl-2, BAX and cleaved caspase-3, indicating that DFS induced cell apoptosis (**Fig. 2e**). Taken together, these results indicated that DFS decreased cell proliferation while inducing cell death of GBM TSs at dose of higher than 5 μ M.

DFS treatment suppresses stemness and invasiveness of GBM TSs

Stemness and invasiveness are critical aspects of neural stem cells. We therefore measured the effects of DFS on stemness and invasiveness of GBM TSs. Neurosphere formation assays evaluate the stemness of GBM TSs, a characteristic essential for the self-renewal, proliferation and multipotency of cells. Treatment of GBM TSs with 10 μ M DFS for 3 weeks significantly reduced the percentage of sphere-positive wells and sphere radii (**Fig. 3a**), as well as reducing the levels of expression of stemness-related marker proteins, including CD133, Nestin, Sox2, CD44, Msi-1 and PDPN (**Fig. 3b**). Treatment with 10 μ M DFS also significantly inhibited the invasive capacity of GBM TSs, as determined by 3D matrigel invasion assays (**Fig. 3c**), as well as markedly reducing the expression of invasiveness-associated marker proteins, including Zeb1, N-cadherin, Snail and Twist (**Fig. 3d**). Taken together, these results suggest that DFS significantly suppressed the stemness and invasiveness of patient-derived GBM TSs.

Transcriptional profiles following DFS treatment

RNA sequencing was performed to assess the effects of DFS treatment on the transcriptional reprogramming of GBM TSs. Samples were subjected to single sample gene set enrichment analysis (ssGSEA) using transcription factor target gene sets [30]. DFS significantly reduced the activities of several transcription factors associated with tumorigenesis, stemness, invasiveness, and mesenchymal transition, while significantly increasing the activities of tumor suppressor transcription factors (Fig. 4a). DFS consistently downregulated the levels of expression of mRNAs associated with the Wnt signaling pathway and with the activation of β -catenin/TCF complex-associated genes (Fig. 4b), as well as stemness- and invasiveness-related genes (Fig. 4c). Analysis of differentially expressed genes (DEGs) by DFS-treated and control GBM TSs showed that the DEGs significantly downregulated in DFS treated cells included several cell cycle-associated gene sets (Fig. 4d), whereas the DEGs specifically upregulated included most genes involved in sub-G1, S, and G2 phase transitions (Fig. 4e).

Therapeutic responses in a mouse orthotopic xenograft model

The effects of DFS pretreatment of GBM TSs on tumor growth were evaluated in orthotopic xenograft models of GBM using TS13-64. Magnetic resonance imaging (MRI) showed that tumors in the control group were larger than those in the DFS-pretreated group, indicating that pretreatment with DFS reduced the ability of GBM TSs to promote tumor growth in a mouse orthotopic xenograft model (Fig. 5a **and b**). Kaplan-Meier survival analysis showed that DFS pretreatment significantly prolonged mouse survival compared with the control group ($p=0.0018$) (Fig. 5c). Collectively, these results clearly demonstrate that DFS was able to reduce the tumorigenesis capacity of TS13-64.

Discussion

The Wnt/ β -catenin signaling pathway is aberrantly activated during the development and progression of various human cancers. This pathway plays important roles in the tumorigenesis and progression of malignant astrocytic gliomas [31–34], including in the promotion of stem cell genes [35–38], in tumor migration and invasion, and in the epithelial-to-mesenchymal transition (EMT) [39–41]. FOXM1, a key mediator of the Wnt/ β -catenin pathway, is overexpressed in many human cancers, including glioma. The finding in the present study, that the level of FOXM1 mRNA was significantly higher in GBM than in normal brain tissue, suggests that targeting FOXM1 in GBM can suppress tumor progression.

A. japonica is a plant widely used as both a food and a medicinal herb in east Asia [19, 42]. It is effective in various medical conditions, such as fever, diarrhea and hemorrhage, because it contains diverse pharmacologically active components. DFS, a lignan isolated from a methanol extract of *A. japonica* stems [42], has shown anticancer activity in colon cancer by suppressing the nuclear translocation of β -catenin through lysosomal-dependent degradation of FOXM1 protein [19].

The interaction between FOXM1 and β -catenin has been shown to promote the nuclear translocation of β -catenin and to increase the expression of β -catenin target genes, thereby promoting self-renewal and tumorigenesis of GBM initiating cells (GICs) [18]. The present study found that DFS reduced interactions between FOXM1 and β -catenin in the nucleus, subsequently reducing the expression of proteins such as cyclin D1 and cMyc by suppressing formation of the complex with TCF4. Moreover, the levels of expression of mRNA associated with Wnt signaling pathway and the activation of β -catenin/TCF complex-related genes were downregulated in DFS treated GBM TSs.

The present study investigated the mechanisms of action of DFS in GBM TSs through the FOXM1/ β -catenin signaling pathways. Inhibition of FOXM1 was found to reduce cell proliferation and induce apoptosis [43, 44], whereas overexpression of FOXM1 was found to promote cell cycle progression [45, 46] and cell proliferation by directly activating the transcription of genes encoding cyclin D1 and cyclin B1 [47, 48]. The present study found that DFS inhibited the viability of GBM TSs and significantly increased the number of cells in sub-G1 phase of the cell cycle, indicating that DFS cytotoxicity is mediated by

apoptosis. In addition, DFS decreased the protein expression of cleaved caspase-3 and Bcl-2 and increased the expression of BAX. Analysis of DEGs showed that DFS upregulated genes involving the sub-G1, S and G2 phase transitions of the cell cycle. Collectively, these results demonstrated that DFS has anticancer potential in human GBM cells through the induction of cell cycle arrest and apoptosis.

FOXM1 has been shown to promote the stemness of GBMs by regulating the master stem cell regulator SOX2 [49]. Furthermore, FOXM1 promotes EMT and is deeply involved in the acquisition in pancreatic cancer cells of a cancer stem cell (CSC) phenotype [50]. The present study investigated the prominent phenotypes of GBM TSs, which have stemness and invasiveness. Stem-like cells that induce GBM TS in tumors are known to affect treatment resistance and recurrence, making the inhibition of stemness and invasiveness a positive indicator for evaluating the potential efficacy of DFS.

In summary, DFS can interfere with signaling steps of stemness, invasiveness and proliferation by suppression of FOXM1. DFS consequently inhibited the proliferation of GBM TSs *in vivo*, prolonging the survival time of mice with orthotopic GBM xenografts. These results were obtained from GBM patient-derived cells and a corresponding mouse model, highlighting the clinical applicability of DFS as a new treatment for GBM. DFS may be a potential therapeutic agent to inhibit GBM progression by increasing apoptosis and reducing cell viability, stemness and invasiveness in patients with refractory GBM.

Declarations

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Conflict of Interest

The authors declare that they have no conflicts of interest.

Availability of data and material

The datasets generated and/or analyzed during the current study are not publicly available due to the risk of compromising individual privacy but are available from the corresponding author on reasonable request and with an appropriate agreed upon collaboration.

Code availability

Not applicable.

Author Contributions

Conceptualization: Jin-Kyoung Shim, Seung Hoon Lim and Seok-Gu Kang; Methodology: Jin-Kyoung Shim, Seung Hoon Lim, Ji Hye Jeong, Ran Joo Choi, Yoojung Oh, Junseong Park, Sunghee Choi, Junpyo Hong and Seo Jin Kim; Formal analysis and investigation: Jin-Kyoung Shim; Resources: Ji Hye Jeong, Sunghee Choi, Ju Hyung Moon, Eui Hyun Kim and Jong Hee Chang; Writing - original draft preparation: Jin-Kyoung Shim and Seung Hoon Lim; Writing - review and editing: Jin-Kyoung Shim, Seung Hoon Lim, Ran Joo Choi, Jae-Ha Ryu and Seok-Gu Kang.; Funding acquisition, Seok-Gu Kang. All authors have read and agreed to the published version of the manuscript.

Ethical approval and consent to participate

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures involving animals followed the ethical standards of the institution or practice at which the studies were conducted. This article does not contain any studies involving human participants performed by any of the authors.

Consent for publication

The work described here has not been published before and is not under consideration for publication elsewhere. All authors have approved the manuscript.

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Figures

Figure 1

Expression of FOXM1 and β -catenin mRNAs and proteins in GBM tissues and inhibition of FOXM1/ β -catenin interaction by DFS. a and b Levels of expression of (a) FOXM1 and (b) β -catenin mRNAs evaluated in previously described datasets. c GBM TSs were treated with DFS for 72 h and the protein levels of FOXM1, active- β -catenin and total β -catenin were determined by western blotting. d Levels of FOXM1 and β -catenin proteins in the cytosolic and nuclear fractions of GBM TSs determined by western

blotting. e DFS-treated GBM TSs were immunoprecipitated with anti-FOXM1 and β -catenin antibody, and endogenous expression of FOXM1 and β -catenin proteins were determined by western blotting to determine interactions between them. f Measurements of β -catenin/TCF signaling activity in GBM TSs transfected with TOPflash or FOPflash luciferase vector. g Expression of proteins encoded by β -catenin downstream genes (cyclin D1, cMYC) measured by western blotting. Differences among groups were compared by one-way ANOVA with Tukey's post hoc test; means $\pm$ SD; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, between indicated groups or compared with controls.

Figure 2

Inhibitory effects of DFS on cell survival. a cell viability and b ATP levels measured after 72 h of treatment with the indicated concentrations of DFS. Results are expressed as the means $\pm$ SD (n=5). c and d GBM TSs were treated with DFS for 72 h, and (c)cell cycle fractions and (d)apoptotic cell populations analyzed by FACS. e Expression of apoptosis-associated proteins measured by western blotting after treatment with DFS for 72 h. Differences among groups were compared by one-way ANOVA with Tukey's post hoc test for multiple comparisons; *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 3

DFS inhibition of GBM TS stemness and invasiveness. a Stemness was determined by neurosphere formation assay. Cells were treated with DFS for 3 weeks and the percentages of sphere-positive wells and sphere radii were calculated. b GBM TSs were treated with DFS for 72 h and stemness-associated protein levels were determined by western blotting. c GBM TSs were cultured on matrigel/collagen matrix with DFS for 72 h, and invasiveness was quantified by measuring areas of migration. d Western blotting assays of the expression of EMT-related proteins. All Images are x100 original magnification. (scale bar = 200 μ m) Differences among groups were compared by one-way ANOVA with Tukey's post hoc test; means $\pm$ SD; *P < 0.05, **P < 0.01, ***P < 0.001, between indicated groups or compared with controls.

Figure 4

Effects of DFS on transcriptional profile, as determined by RNA-sequencing. a Oncogenic and tumor suppressor transcription factors measured by single gene set enrichment analysis (ssGSEA). b Expression of Wnt signaling genes and β-catenin/TCF complex related genes evaluated by RNA-sequencing. c Heat map showing levels of expression of stemness- and invasiveness-associated genes. d

Set of genes downregulated after treatment with DFS for 72 hrs. e Heat map showing levels of expression of cell cycle-related genes.

Figure 5

Therapeutic effects of DFS in orthotopic xenograft models. a and b Tumor volumes of orthotopic xenograft models measured by magnetic resonance imaging (MRI). c Kaplan-Meier analysis of survival

probability of each group of mice, with comparisons by log-rank tests ($P < 0.05$) with Bonferroni adjustment. d Schematic summary of the study