Biological characteristics comparison of glioblastoma tumorsphere obtained from the fresh and cryopreservation sample

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ABSTRACT

Biological characteristics comparison of glioblastoma tumorsphere obtained from the fresh and cryopreservation sample

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Background: Glioblastoma (GBM) is the most common and aggressive form of a primary tumor with poor prognosis. Tumorsphere (TS) isolated from human GBM have been shown to possess the potential for studying cancer biology, preclinical in vitro screening of anticancer drugs and use of patient-derived xenograft (PDX) models. Several studies have shown the high clinical significance of TS, but the storing of patient samples at an appropriate time point after surgical treatment is very limited. Recently some studies have demonstrated the benefits of cryopreservation, but to date, there have been no studies comparing the molecular comparison of Fresh and Cryo TS in detail. In this study, we tried to compare and analyze whether TS isolated by cryopreservation really maintains the same pattern as fresh sample devised from various aspects.

Methods: Surgically obtained primary GBM tissues were divided into a fresh

and cryopreserved group. Each tissue was chopped and further processed to cells isolation with or without cryopreservation media. Acquired TSs with two different culture method were analyzed for transcriptome analysis, stem cell characterization, neurosphere formation, invasive properties, Temozolomide (TMZ) response and mouse orthotopic models.

Results: A total of 40 diagnosed primary GBM patient samples were used in this study. TS isolation success rate were 65% in fresh and 60% in cryopreserved group, respectively. There were no significant differences in neurosphere formation, stemness, differentiation between fresh and cryopreserved groups. TSs in two different environments featured the similar invasiveness, TMZ responsiveness and animal results highly showed similar capacity of tumor generation. In the RNA sequencing, the cancer subtypes between different TSs may be different, but the similarity between fresh and cryopreserved TSs did not change.

Conclusions: These results showed that cryopreservation cells can be used for experiment as primary cultured cells, suggesting that cryopreservation is the alternative method when TS cannot be isolated immediately after tumor resection.

Key words: cryopreservation, glioblastoma, cell isolation, tumorsphere
orthotopic xenograft model

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I. INTRODUCTION

Despite advances in modern surgery and postoperative therapy, glioblastoma (GBM) is a devastating and incurable brain tumor with a median overall survival of 15 months.[1-4] One of the reasons GBM is particularly difficult to treat is the resistance and recurrence that occurs during treatment. GBM is composed of highly complex heterogenic cells and produces a high recurrence rates due to the repopulation of cancer stem cells (CSCs).[5,6] Tumorspheres (TSs) isolated from GBM tissue contain glioma CSCs (gCSCs) with characteristics such as ability to proliferate extensively, self-renewal properties, expression of neural stem cell markers, and ability to differentiate into neural lineages.[6] Primary tumor culture can serve as a selective model for TS isolation to study invasiveness, treatment resistance and tumorigenicity, which are key mechanisms of glioblastoma biology, including the stem cell layer. Previous studies have successfully isolated TS from primary tumors and have shown that GBM TS isolation is a prognostic indicator of clinical outcome.[7,8] The presence of CSCs also showed stronger resistance to current conventional treatments compared to other cancer cells that do not exhibit stem cell-like properties.[5] Therefore, an improved understanding of the cellular and

molecular mechanisms regulating the growth and invasion of GBM has been the focus of current research, and obtaining TSs in a timely manner immediately after surgery is important for understanding of the molecular and cellular basis of cancer biology and incorporating preclinical PDX models as potential drug testing.

In the case of brain tumors, tissues obtained immediately after surgery cannot be readily dissociated to obtain TSs, and its success rate is also known to be less than 50%.[9] Therefore, these limitations often impede conducting clinical applicability studies. As a way to cope with this, cryopreservation is the process of maintaining a biological sample frozen at cryogenic temperatures for a long time using a cryoprotectant that preserves the microstructure of cells.[10,11] Cryopreservation has been commonly used in the preservation of cells involved in human fertility[12], embryonic stem cell bodies,[13] and reproduction.[1] Researchers had successfully cryopreserved human and mouse embryonic stem cells through cryopreservation, demonstrating that cryopreserved cells retain pluripotency and viability upon thawing.[14-16] In addition to other previous study, it was reported that CSCs isolated by cryopreservation from patient-derived GBM samples maintain higher viability and stemness than CSCs isolated by conventional methods.[17] As such, Cryopreservation techniques can be particularly useful for obtaining TS.

TS obtained from primary culture is known to retain its properties even after serial transplantation in immune-compromised mice.[7,18] However, it is not always possible to isolate TS from fresh tumor tissue and apply to research at appropriate conditions and times in a clinical setting, and not all institutions have laboratory facilities capable of performing TS isolation. To pursue successful clinical applicability studies, isolated TSs should be free of genetic alterations and should be kept as close to the parental tumor as possible with as few passages as possible. Therefore, there is a need for an alternative preservation method of GBM tissue that allow TS isolation without changing

the properties of the CSCs in fresh tissue. Chong et al.[18] reported that GBM TS cryopreservation preserves biological phenotype and genetic characteristics. They analyzed the profile of parental tumor cells and performed comparative analysis with differentiated cells to demonstrate that the genetic profile was not changed. Mullins et al.[19] showed that cryopreservation of GBM tissue did not alter TS isolation rate, morphology, growth kinetics, and drug response.

Although many studies have been conducted on cryopreservation methods in different tumors, it is unknown whether cryopreservation of GBM tissues preserves the biological properties of gCSCs. In this study, we attempted to compare the biological properties of GBM TS obtained from fresh tissue and cryopreserved tissues such as self-renewal ability, neuroglial differentiation, invasiveness, and tumorigenesis. The cryopreservation method introduced by us is not only very simple, but also very useful because it allows cells to be isolated at any time after long-term storage of tissues for a certain period of time.

II. MATERIALS AND METHODS

1. CSC isolation from fresh GBM tumor tissue specimen

Patient-derived GBM tissue was transferred directly from the operating room to the laboratory. The process of isolating gCSC from GBM tissue was carried out according to the previous studies.[20,21] Briefly, the tissue obtained after surgery was separated into two pieces for Fresh TS and Cryo TS isolation. For Fresh TS isolation, the tissues were soaked in Dulbecco's modified Eagle medium: nutrient mixture F-12 (DMEM/F-12, Corning), minced with a surgical scalpel, and passed through a 70 μm nylon mesh cell strainer (Corning). Cells were then resuspended in DMEM/F-12 medium twice and grown for TS in DMEM/F-12 containing 1% (v/v) B27 (Thermo Fisher Scientific, 1x), 20ng/ml basic fibroblast growth factor (bFGF, BON-OPUS), 20ng/ml epidermal growth factor (EGF, BON-OPUS), 50U/ml penicillin and 50mg/ml streptomycin (Thermo Fisher Scientific).

2. Cryopreservation process

For cryopreservation, a cryopreservation solution of 90% (v/v) fetal bovine serum (FBS, Thermo Fisher Scientific) and 10% (v/v) Dimethyl Sulfoxide (DMSO, Applichem) was prepared and used. The tissue obtained after surgery was chopped with a surgical scalpel by adding about 1ml of chilled cryopreservation medium prepared earlier. The minced tissue was transferred to a cryo-vial and stored in liquid nitrogen (LN2) for one month. After 1 month, cryopreserved cells were post-thawed in a water bath at 37℃ and filtered through a 70-μm nylon cell strainer for TS cultivation.

3. Neuro-sphere formation assay

TS was collected by centrifugation at 200 g for 3 min, and the pellet was

gently resuspended in 500 μL of Accutase (Thermo Fisher Scientific) to dissociate into single cells. Cells were counted using Trypan Blue (NanoEntek) and plated at 10 cells/well in 96-well plate. After 3 weeks of incubation, plates were imaged in brightfield, and acquired images were analyzed using ToupView software (ToupView3.7). The radius of the TS was measured and compared to well-formatted spheres and empty wells. The measured values were statistically analyzed using Prism 7 software (GraphPad).

4. Cell proliferation assay

TS was collected by centrifugation at 200 g for 3 min, and the pellet was gently resuspended in 500 μL of Accutase (Thermo Fisher Scientific) to dissociate into single cells. Cells were seeded at 2×10^3 cells/well in 96 well plate, and incubated in DMEM/F-12 containing 1% (v/v) B27 (Thermo Fisher Scientific, 1x), 20ng/ml bFGF (BON-OPUS), 20ng/ml EGF (BON-OPUS), 50U/ml penicillin and 50mg/ml streptomycin (Thermo Fisher Scientific) for 9 days. Cell proliferation and viability were assessed after incubation for 90 min with Cell Counting Kit-8 (WST-8/CCK8, Donginls, 10 μl/well), and absorbance was measured at a wavelength of 450 nm with VERSA max (Molecular Devices, Softmax pro 6.2.2). The measurements were made every day for 8 days, and the measured values were statistically analyzed using Prism 7 software (GraphPad).

5. Immunocytochemistry (ICC)

The cultured TS were fixed in 2% paraformaldehyde (PFA) (Sigma-Aldrich) for 7 min at 4℃. The immobilized TS was transferred to a cover slide and placed in methanol (Merck Millipore) and acetic acid (Merck Millipore) in a 3:1 ratio (v/v) for 3 min. Permeabilization was performed for 10 min using 0.1% (v/v) Triton X-100 (Bio-RAD) and blocking was performed in 1% (v/v) bovine

serum albumin (BSA, RDtech) for 60 min. The primary antibodies, rabbit anti-CD133 (1:250, Abcam Dawinbio, ab19898), rabbit anti-Nestin (1:250, Abcam, ab5968), rabbit anti-Musashi (1:250, Abcam, ab52865) and rabbit anti-podoplanin (PDPN) (1:250, Abcam, ab10274) were separately incubated for 120 min in antibody diluent reagent solution (Invitrogen). The secondary antibody goat anti-rabbit IgG conjugated with Alexa Fluor 555 (1:2000, Invitrogen, A21428) for CD133 and Nestin, Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:2000, Invitrogen, A11034) for Musashi and PDPN were diluted with antibody diluent reagent solution (Invitrogen) and incubated for an additional 60 min. For nuclear staining, Vectashield h-1200 mounting medium containing 4'6-diamidino-2-phenylindole (DAPI; Vector Laboratories) was used. Washing steps were performed with Phosphate-buffered saline (PBS, Enzynomics) between all processes, and images were acquired through a fluorescence microscope (OLYMPUS Korea) and DP Controller software (OLYMPUS Cell Sens Standard 3.1).

6. Neural differentiation

Neuronal differentiation of GBM TS was confirmed by ICC via neuronal and glial marker genes.[22] TSs were seeded onto 4-chamber culture slides (Lab-Tek II; Nalge Nunc International), and cultured in DMEM/F-12 containing 1% (v/v) B27 (Thermo Fisher Scientific, 1x) and 10% (v/v) FBS for 13 days. On day 13, the neural differentiation medium was removed and the differentiated cells were fixed in 2% PFA for 7 min at 4℃. Permeabilization was performed with 0.1% (v/v) Triton X-100 for 10 min, and serial blocking was performed with 1% (v/v) BSA for 1 h. Primary antibodies were diluted rabbit anti-GFAP (1:200, Merck Millipore, AB5804), mouse anti-MBP (1:200, Abcam, ab62631), mouse anti-NeuN (1:100, Abcam, EPR12763) and mouse Add anti-TUBB3 (1:200, biolegend, 801201) to the antibody diluent reagent solution and incubated for 60 min. Goat anti-rabbit IgG conjugated with Alexa Fluor 555

(1:2000, Invitrogen, A21428) for GFAP, goat anti-mouse Alexa Fluor 546 (1:200, Invitrogen, A11003) for MBP, NeuN and TUBB3 were used as secondary antibody for 60 min. For nuclear staining, Vectashield H1200 mounting medium containing 4'6-diamidino-2-phenylindole (DAPI; Vector Laboratories) was used, and the image were taken using a fluorescence microscope (OLYMPUS Korea) and DP Controller software (OLYMPUS Cell Sens Standard 3.1).

7. Three-dimensional (3D) invasion assay.

The TS were cultivated for 7 days to form spheroids. We coated 96 wells plate with a collagen I/Matrigel matrix by adding highly concentrated rat tail collagen type I (Corning Life Sciences, 2.4 mg/ml), Matrigel (Corning Life Sciences, 2.1 mg/ml), 10% NahCO3, and 2x DMEM/F-12 culture medium. Collagen I/Matrigel matrix was stored at 4℃ prior to use and single TS was seeded into 96-well plates/well in 100 µl collagen I/Matrigel matrix. In the 96-well coated gel matrix, 50 µl of TS culture medium was included and stored in an incubator at 37.4℃, 5% CO2 for 30 min.[23] Invasion images were taken with an inverted microscope (Intron Biotechnology) and the values of the invaded area were measured and analyzed using ToupView image analysis software (ToupView3.7). Each measurement value was analyzed statistically using Prism 7 software (GraphPad).

8. Western blot analysis

TSs were harvested and pelleted by centrifugation at 1,300 rpm for 5 min. Cell pellets were lysed with cell extraction buffer (Invitrogen) containing 1% (v/v) protease inhibitor and phosphatase inhibitor (Thermo Fisher Scientific) on ice for 30 min. The samples were vortexed 3 times every 10 min, centrifuged at 14,000 rpm for 20 min, and then supernatants were collected. Protein

quantification was quantified by Bradford and mixed with 5x sample dye (ELPIS BIOTECH). The sample was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on Tris-glycine gels and proteins were transferred to nitrocellulose membrane (GE Healthcare Life Sience). The blots were blocked with 3% BSA in 1x Tris-saline buffer with 0.1% Tween-20 (TBS-T) for 60 min at room temperature. The primary antibody was diluted with 1x TBS-T and incubated at 4° C. for 24 h, followed by incubation of the secondary antibody for 60 min. The probed protein detection was done with Western Lightning Plus-ECL, Enhanced Chemiluminescence Substrate (Thermo Fisher Scientific), and the band quantity was checked using ImageQuant LAS 4000 mini (GE Healthcare Life Sciences).

9. TMZ treatment response

TS was collected by centrifugation at 200 g for 3 min, and the pellet was gently resuspended in 500 μL of Accutase (Thermo Fisher Scientific) to dissociate into single cells. Cells were seeded at 1×10^4 cells/well in 96 well plate, and incubated in DMEM/F-12 containing 1% (v/v) B27 (Thermo Fisher Scientific, 1x), 20ng/ml bFGF (BON-OPUS), 20ng/ml EGF (BON-OPUS), 50U/ml penicillin and 50mg/ml streptomycin (Thermo Fisher Scientific) for 24 h. After 24 h of incubation, TMZ (Merck Millipore) was treated with 0, 250, 500, 750, and 1000 μM and incubated for 3 days. After 3 days, 10 μL of WST-8/CCK8 per well was added, and absorbance was measured at 450 nm wavelength after 90 min. ATP levels were also measured using the CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega, 70 μl/well). Each measurement value was analyzed statistically using Prism 7 software (GraphPad).

10. RNA QC, library construction, and sequencing

The quality and quantity of total RNA were assessed by Agilent 2100 Bioanalyzer with a Eukaryotic Total RNA Pico chip (Agilent Technologies). Using this instrument, the 18s/28s ratio and RNA Integrity Number (RIN) values were determined from the Bioanalyzer traces. 100ng of non-FFPE RNA from each sample was chemically fragmented to a size appropriate for library preparation. The RNA fragments were bound to random primers and synthesized to strand specific cDNA with Actinomycin D solution. 3' end A tailing and adaptor-ligated cDNA were amplified for 14 cycles. The samples were concentrated to 200 ng in 3.4 μL DW using a Speedvac machine (Thermo Scientific) and hybridized with All Human V6+UTR baits at 65℃ for 16 h. After hybridization, the biotinylated libraries were captured by M-270 streptavidin beads (Thermo Fisher Scientific). PCR was performed for 12 cycles to amplify the captured libraries and add the unique index tags. Libraries were quantified using the Agilent TapeStation 4200 HSD1000 screen tapes (Agilent Technologies) and KAPA Library Quantification Kit (KK4824, Kapa Biosystems). The individual samples were pooled and sequenced on the Illumina NovaSeq6000 with 150 bp paired-end by following the manufacturer's protocols. Image analyses were performed using the NovaSeq6000 control Software version 1.3.1 and the output data was demultiplexed with bcl2fastq v2.2 generating fastqc files.

11. RNAseq

The three GBM tissue samples and six samples of TSs were hybridized with All Human V6+UTR baits (Three GBM cases of 18-110, 18-117, and 19-65; Triple samples from each case: one sample of the origin tissue, one directly isolated TS, and one TS of cryo-preserved). All the GTF files in this analysis were merged and labeled after same alignment and counting process. Gene

expression level data were calculated by summing up the transcripts in the gene location (GRCh38.p5). Controversial transcripts were reconfirmed in the sequence level that is extracted from gffread (-w option)[24,25]. An unsupervised selection of the expressed genes (Coefficient of variation > 10 and mean FPKM more than one, n=2559) were included for the t-SNE analysis (Dimension = 2, Perplexity = 2, Max iteration = 5000)[24,26]

12. Gene set variation analysis

A gene set variation analysis (GSVA) was conducted on the gene level merged FPKM data of the RNAseq using three gene sets of GBM subtypes (GSVA package in R, version 1.36.3)[25-27]. The raw output of GSVA was displayed as a heatmap with column split option by the original cases (Complexheatmap in R, version 2.4.3).

13. Mouse orthotopic xenograft model

Male thymic nude mice aged 4 to 8 weeks (n=5 for each group, Central Lab. Animal) were used, and the experiment was performed after an adaptation period in which temperature and humidity were adjusted for about 1 week. All animal experiments were processed under the approval of Yonsei University College of Medicine Institutional Animal Care and Use Committee. Mice were anesthetized by injection peritoneal using Zoletil (Virbac Korea, 30mg/kg) and xylazine (Bayer Korea, 10mg/kg). 5×10^5 cells were injected into the right frontal lobe of the nude mouse at a depth of 4.5 mm using a guide-screw system.[28] Mouse weight was measured every week and euthanized if they lost more than 15% of their initial weight. Brain was fixed with formalin to make paraffin-embedded tissue block. Tissue slide was made from paraffin-embedded tissue block and conducted histologic observation through

hematoxylin and eosin (H&E) staining. When all animals died, survival curve was evaluated using the Kaplan–Meier method.

14. Immunohistochemistry (IHC)

Paraffin-embedded tissue block was sectioned to 5-μm-thick using microtome and transferred to adhesive slide glass. Antigen retrieval and antibody attachment were performed using an automated instrument (Discovery XT, Ventana Medical Systems) with anti-Nestin (1:400, Cell Signaling Technology, 33475S) and anti-Zeb1 (1:100, Abcam, ab203829). Peroxidase/3,3′-diaminobenzidine staining system was performed to detect Nestin and Zeb1, and edge detection the boundaries of the cell nuclei were counted and statistically plotted using Prism 7 software (GraphPad).

III. RESULTS

1. Isolation of TS from fresh and cryopreserved tissue.

A total of 40 surgically resected tumor samples were used in this study. All tumor samples were primary glioblastomas with 97.5% of IDH1 wild-type. Tissues from GBM patients who underwent surgical resection were divided into two groups. For the fresh TS group, TS was immediately isolated from fresh tissue. In the case of the Cryo TS group, the TS was isolated after cryopreserving the tissue for 1 month. Each group of isolated TS was calculated for the success rate of TS isolation. Of the 40 cases, 22 cases (55%) succeeded in TS isolation from both fresh and cryopreserved tissues, 4 cases (10%) succeeded only in fresh tissue, and 2 cases (5%) succeeded only in cryopreserved tissues. In total, the TS isolation failure rate was 12 cases (30%) (**Figure. 1B**). TSs are closely clustered together, regardless of the preparation method (See details in Method of TS culture) (**Figure. 1C**). GBM subtypes are preserved regardless of the TS in this cohort. Two cases (18-110 and 18-117) are classified under the proneural dominant type without the invasive signature.[27,29] A GBM 19-65 case co-expressed the mesenchymal subtype genes as well as the invasive subtype genes (**Figure. 1D**). We found two GBM samples are classified into proneural type and one GBM sample as neural type. PTPRZ1, the outer radial glia marker, is overexpressed in the tissue and preserved in the TS and Cryo TSs of proneural type. In contrast, neural type sample showed partially positive dopaminergic pathway-related genes (PPP1CB, CALM1, GNB2; $P = 0.0194$, adjusted $P = 0.483$) and ECM-receptor interaction-related genes (COL6A1, COL6A2, ITGA7, SPP1, CD44; $P < 0.0001$, adjusted $P < 0.01$) in the gene enrichment analysis with KEGG database (**Figure. 1E**). Further descriptive clinical data are shown in Table 1.

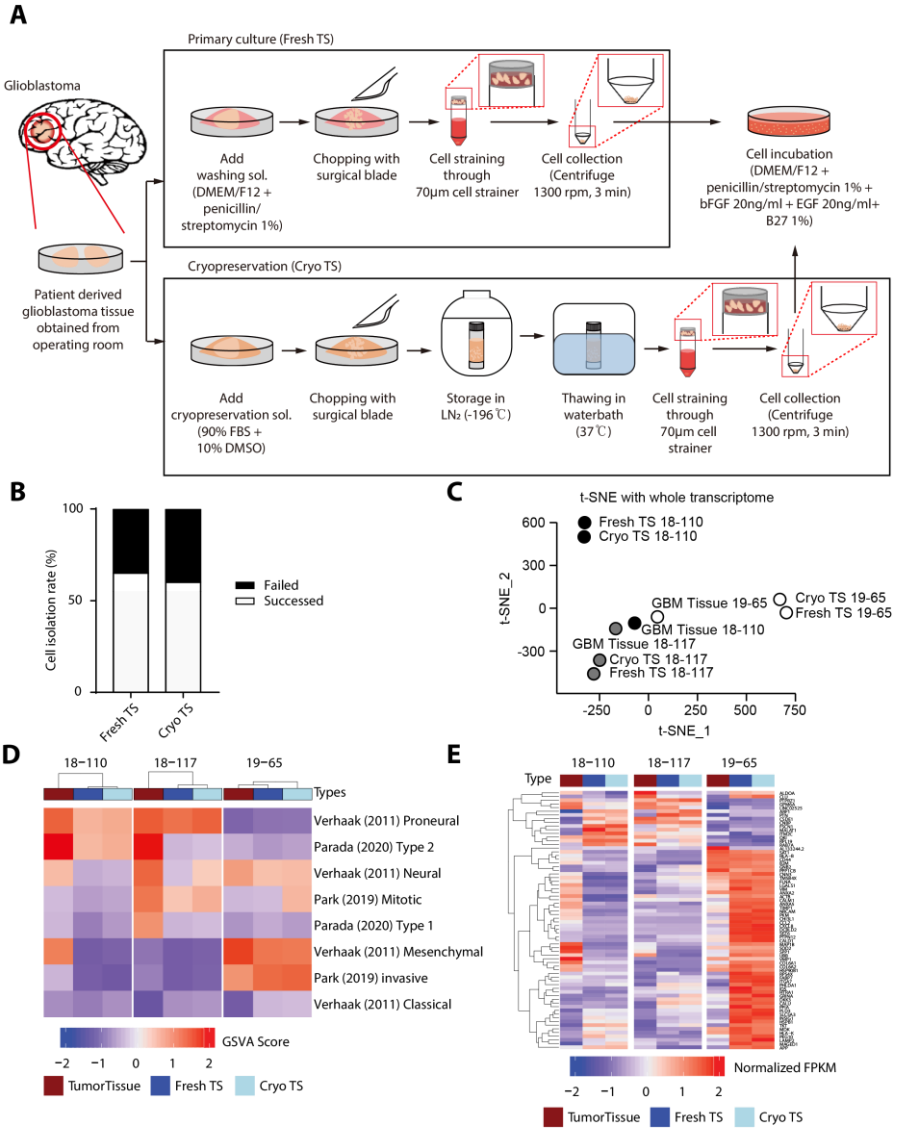

Figure. 1 Cell isolation in primary culture and cryopreservation.

(**A**) Primary culture and cryopreservation method were schematically described. For primary culture, tissues were added DMEM/ F12 including 1% penicillin/ streptomycin and chopped. Chopped tissues were pass through a cell strainer and cells were collected. These cells were incubated in DMEM/ F12 including

1x B27, 20ng/ml bFGF, 20ng/ml of EGF and 1% penicillin/ streptomycin. For cryopreservation, tissues were added cryopreservation solution (90% FBS + 10% DMSO) and chopped. Chopped tissues were transferred to the cryo-vial and stored in LN2. When using cryopreserved tissues, thawing in 37℃ water bath. These tissues were pass through cell strainer and cells were collected. Collected cells were incubated in complete media. (**B**) Cell isolation rate of primary culture (Fresh TS) and cryopreservation (Cryo TS). Total 40 samples were cultured. 21 samples were isolated in both groups and 5 samples were isolated from only Fresh TS group and 2 samples were isolated from only Cryo TS group. (**C**) Selected genes by the coefficient of variables and mean expression of FPKM across the six samples. (**D**) Three GBM cases and its derived Fresh TS and Cryo TS are illustrated after GSVA analysis. Unnormalized GSVA data from unnormalized FPKM data. (**E**) Gene-level normalized heatmap that compared proneural type samples (18-110, 18-117) from neural type samples (19-65). Relatively variable genes with relatively high expression (Mean FPKM more than 100) were scaled, centered, and visualized after differential gene expression with student T-test ($P<0.05$, minimal absolute difference of FPKM 1).

2. Fresh TS and Cryo TS maintain the same characterization and differentiation.

Three different types of GBM-TS (TS18-110, TS18-117, and TS19-65) were isolated from fresh and cryopreserved tissues respectively, and their morphological changes were analyzed. It was confirmed whether the stemness characteristics and the differentiated phenotype were changed by cryopreservation. The three types of GBM-TS were shown to have different morphologies, but no morphological changes were found in TS isolated by fresh or cryopreservation using bright field microscopy (**Figure. 2A**). The proliferation ability was confirmed that Fresh TS and Cryo TS grew at the same

rate in the subsequent survival assay results (**Figure. 1B**). The self-renewal ability was confirmed by staining four stem cell markers (CD133, Nestin, Musashi and PDPN by immunocytochemical staining in three types of GBM-TS. Of the four markers, TS18-100 was all positive, Nestin was negative in TS18-117, and Musashi was negative in TS19-65 (**Figure. 2C**). In neuroglial differentiation, glial fibrillary acidic protein (GFAP), myelin basic protein (MBP), neuronal nuclei (NeuN), and tubulin beta 3 (TUBB3) were identified by immunocytochemistry, respectively. MBP and NeuN were not observed in TS18-110, whereas MBP and TUBB3 were not detectable in TS18-117. In the case of TS19-65, MBP, NeuN, and TUBB3 were all not expressed. However, both fresh TS and Cryo TS showed the same expression pattern (**Figure. 2D**). We found that the expression patterns in GBM-TSs may be different, but no change in stemness characteristics or differentiation within TS isolated either by fresh or cryopreserved method were confirmed. For further information regarding molecular characteristics including molecular subtypes, the presence of codeletions on chromosomes 1p and 19q, the methylation status of the O6-DNA methylguanine-methyltransferase promoter, and the presence of isocitrate dehydrogenase mutations are summarized in **Table S1** and **S2**.

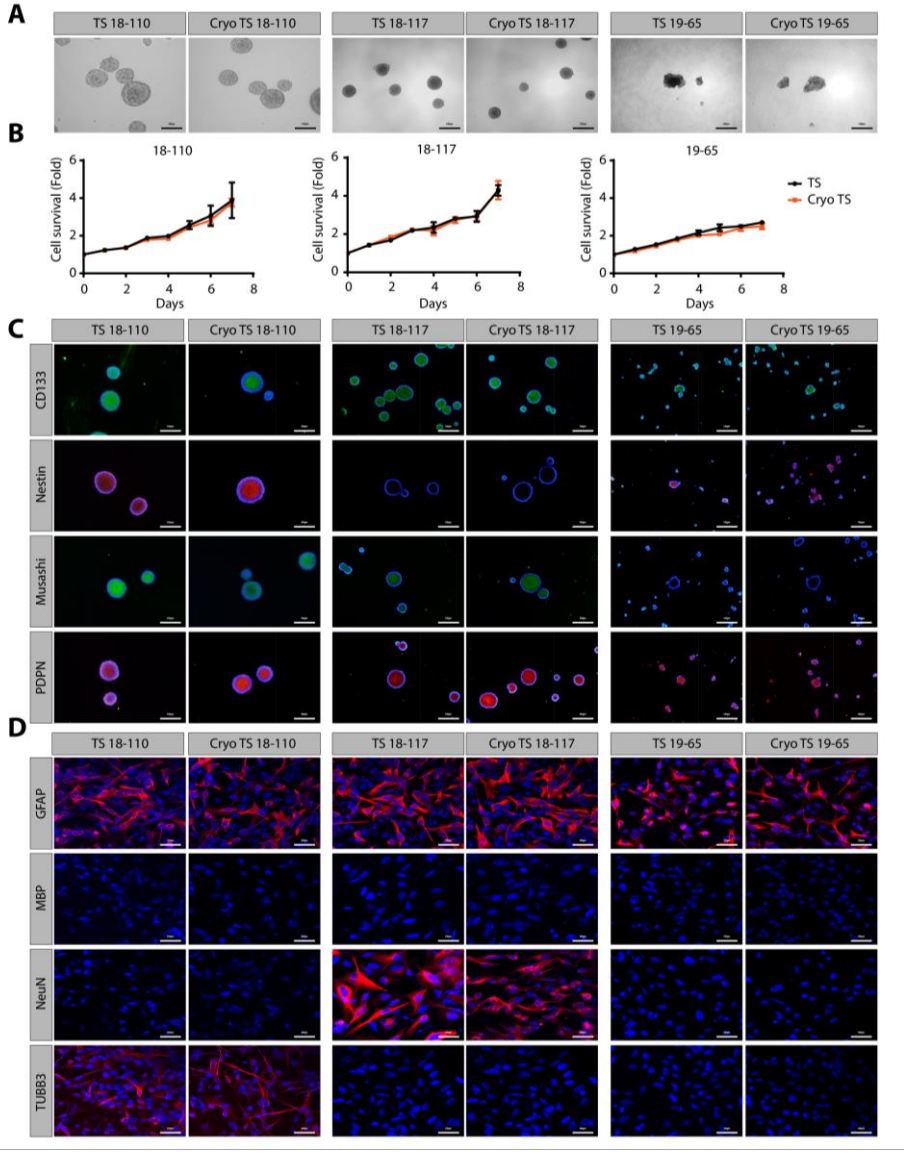

Figure. 2 Cell characterization of Fresh TS and Cryo TS.

(**A**) The morphology of Fresh TS and Cryo TS images were captured in a bright field. TS from two groups showed identical morphology (Scale bar= 100μm). (**B**) The cell proliferation rate of Fresh TS and Cryo TS was compared for 8 days and measured using WST-8/CCK8. One-way ANOVA with Tukey's post

hoc test was performed for analysis (n=4, *P < 0.05, **P < 0.01, ***P < 0.001). The rate of proliferation between two different culture groups were statistically not significant. (**C**) We conducted immunocytochemistry (ICC) staining for GBM stem cell surface markers that CD133, Nestin, Musashi and PDPN. Surface markers of Fresh TS and Cryo TS confirmed the same expression (Scale bar= 100μm). (**D**) Fresh TS and Cryo TS were incubated in differentiation media (DMEM/F-12 containing 10% FBS and 1x B27 supplement) for 13 days. After the following day, ICC staining for differentiation markers as GFAP, MBP, NeuN, TUBB3 was performed. Differentiation markers of Fresh TS and Cryo TS showed identical expressions (Scale bar= 100μm).

3. Fresh TS and Cryo TS exhibit the same neurosphere-forming ability.

We hypothesized that cryopreservation would induce delayed cell growth or loss of stemness, an intrinsic property of TS. Therefore, stemness formation of Fresh TS and Cryo TS in each of the three different GBM-TS (TS18-110, TS18-117, and TS19-65) was confirmed by neurosphere formation assay. As a result, it was found that all three types of Fresh TS maintain the same stemness as Cryo TS (**Figure. 3A**). The majority of sphere cells were positive and the sphere radius also showed almost no difference between Fresh TS and Cryo TS (**Figure. 3B**). In the subsequent western results, no changes in stemness-related proteins were found in Fresh TS and Cryo TS (**Figure. 3C**). There was no significant difference of stemness related gene (PROM1, PDPN, POU5F1, MSI1, NES) between Fresh TS and Cryo TS, except that SOX2 did not show same pattern in two cases (18-110 and 18-117). These data are expressed in the bar plots (**Figure 3D**). These findings suggest that Fresh TS and Cryo TS maintains the same properties for both stemness and proliferation.

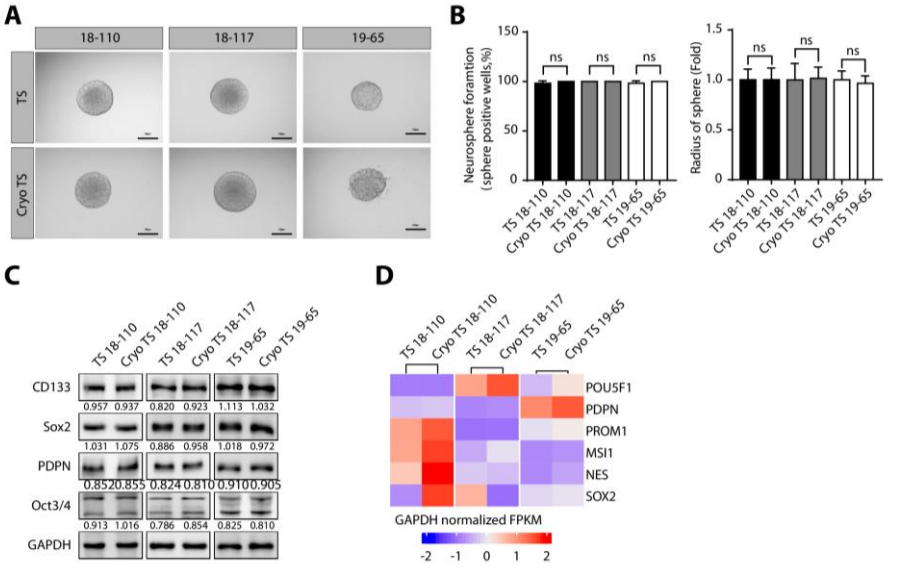

Figure. 3 Comparison of stemness between Fresh TS and Cryo TS.

(**A**) Fresh TS and Cryo TS were seeded into 10 cells each for neurosphere formation. After 3 weeks of incubation, Fresh TS and Cryo TS were formed neurosphere and images were captured in a bright field. Fresh TS and Cryo TS neurosphere showed identical morphology (Scale bar= 100μm). (**B**) The positive wells were counted according to the formation of tumor spheres in the two groups and the radius of the tumor spheres were measured and compared. Statistical analysis was done using one-way ANOVA with Tukey's post hip test (n=60 each group). (**C**) Protein expression of stemness including CD133, Sox2, PDPN and Oct3/ 4 was confirmed using lysate samples of Fresh TS and Cryo TS. GAPDH was used as a control. (**D**) An unsupervised clustering of a row normalized FPKM data. Two types of cells were expressed similar gene expression and clustered. There was no significant difference between stemness capacity of Fresh TS and Cryo TS.

4. Fresh TS and Cryo TS showed the same pattern in terms of invasive and metastatic potential.

TS exhibits the characteristics of cancer and is often highly invasive, which can be correlated with their metastatic potential. We thought that cryopreservation could cause highly invasive TS less aggressive or less invasive. Therefore, each Fresh TS and Cryo TS derived from three types of GBM-TS (TS18-110, TS18-117, and TS19-65) grown with 3D multicellular spheroids were embedded in collagen matrix for quantitative analysis of the directional invasive area. The invasiveness of each spheroid was evaluated through the infiltration area normalized to the initial area of the spheroid (invasion area at a specific time (72 h)/spheroid area at the initial time (0 h) × 100. Comparing the invaded area of Fresh TS and Cryo TS in counterparts, it was confirmed that the invasiveness increased when all three types of Fresh TS and Cryo TS reached 72 h (**Figure. 4A**). Also, there was no difference between Fresh TS and Cryo TS in the measured invaded area (**Figure. 4B**). Similarly, it was confirmed that the expression of several invasive markers maintained the same expression patterns in the western blot results (**Figure. 4C**). Invasiveness related genes (CDH2, CD44, ZEB1, CTNNB1) were expressed same patterns in Fresh TS and Cryo TS. As a result, it was determined that Fresh TS and Cryo TS maintained the same invasive properties. These data are expressed in the bar plot (**Figure 4D**). As a result, it was determined that Fresh TS and Cryo TS maintained the same invasive properties. A clustering of the two cases (18-110 and 18-117) was reminiscent of the subtypes (**Figure. 1D**).

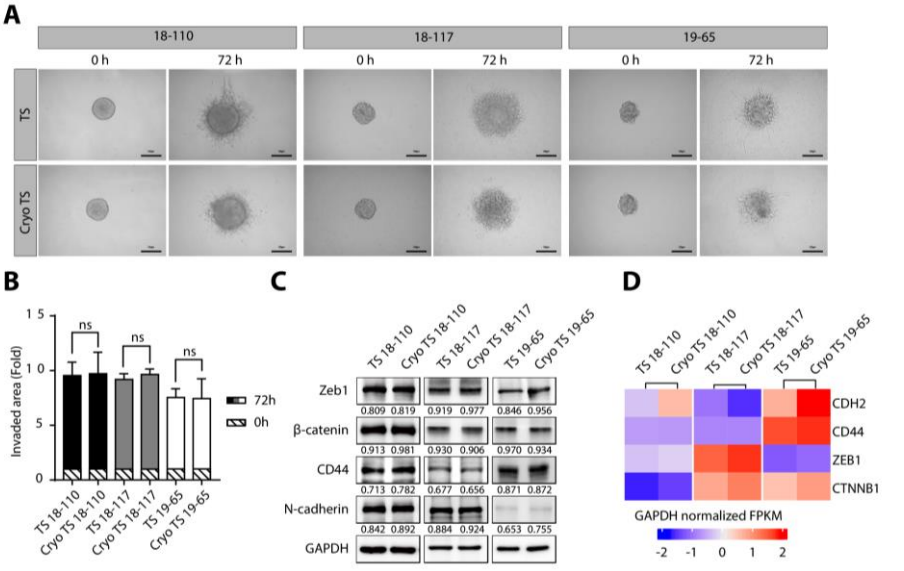

Figure. 4 Comparison of invasiveness between Fresh TS and Cryo TS.

(**A**) Invasion morphology of Fresh TS and Cryo TS were captured at 0 and 72 h in a bright field (Scale bar= 100μm). (**B**) The invaded area was measured and compared between Fresh TS and Cryo TS. Statistical comparison was done using one-way ANOVA with Tukey's post hoc test (n=4, *P < 0.05, **P < 0.01, ***P < 0.001). (**C**) Invasion markers that Zeb1, β-catenin, CD44 and N-cadherin were identified by western blot and compared. GAPDH was used as a control. (**D**) An unsupervised clustering of a row normalized FPKM data. Two types of cells were expressed similar gene expression and clustered. There was no significant difference between invasiveness of Fresh TS and Cryo TS.

5. TMZ drug reactivity was not different between Fresh TS and Cryo TS.

We decided to test whether the reactivity of the drug could produce varying responses different from that of conventional TS due to the cryopreservation

process. TMZ has been the current standard drug in GBM chemotherapy for more than a decade.[30,31] Therefore, TMZ was treated at a concentration of 0 to 1000 μM in three different Fresh TS and Cryo TS (TS18-110, TS18-117, TS19-65), respectively, and WST assay and intracellular ATP levels were measured for drug reactivity evaluation. According to the **Figure. 5A** result, the cellular viability decreased in a concentration-dependent manner when both Fresh TS and Cryo TS were treated with TMZ. However, regardless of whether it was Fresh TS or Cryo TS, there were TS with significantly reduced viability depending on the characteristics of GBM-TS. The relative TMZ IC50 value of TS18-117 was 713 μM and Cryo TS18-117 was 733 μM, which showed a lower TMZ IC50 value than other TS (TMZ IC50 of TS18-110, Cryo TS18-110, TS19-65, and Cryo TS19-65 was over 1000 μM). At subsequent ATP levels, TS18-117 was found to be more sensitive to the TMZ response as the ATP level was lower when TMZ was treated than that of other TSs (**Figure. 5B**). Bax and Bcl2 were checked in western blot to confirm whether apoptotic proteins initiate the apoptosis pathway in Fresh TS or Cryo TS when TMZ was treated. When 500 μM of TMZ was treated, Bax, a pro-apoptotic factor, was observed in three types of Fresh TS or Cryo TS. On the contrary, it was confirmed that the anti-apoptotic factor, bcl-2, decreased (**Figure. 5C**). From these results, it was confirmed that TMZ induces apoptosis in TS and the drug reactivity of Fresh TS or Cryo TS does not change significantly.

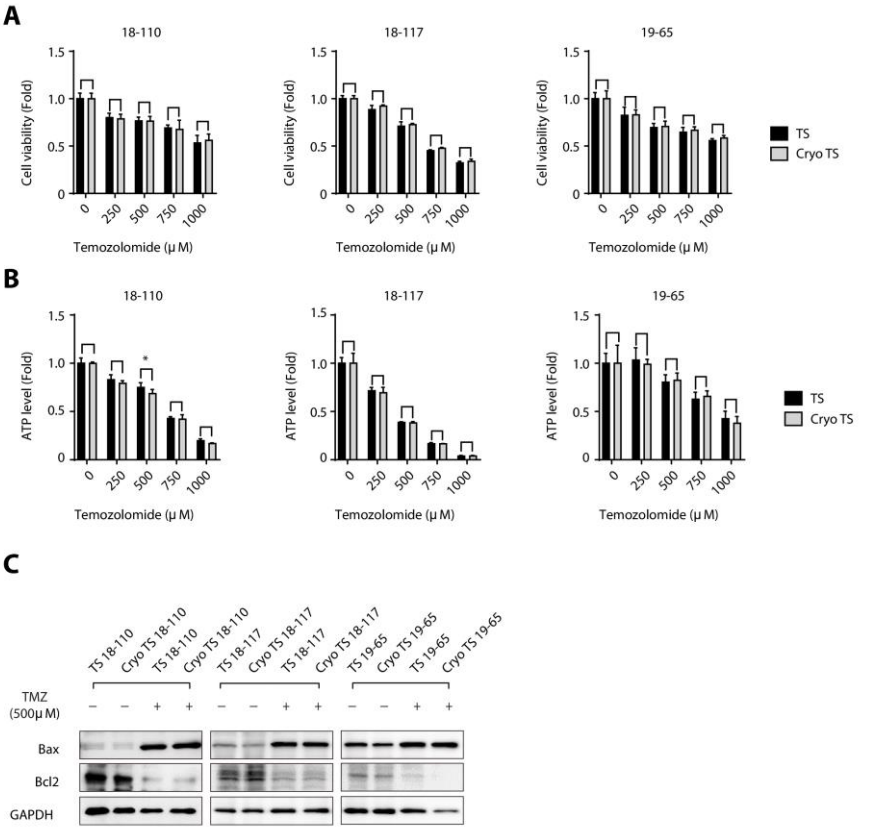

Figure. 5 TMZ response between Fresh TS and Cryo TS.

(**A** to **B**) Cell viability and ATP level was measured 72 h after treatment with TMZ with 5 different concentrations. WST-8/CCK8 was used to measure the cell viability, and the ATP level was measured as CellTiter-Glo® Luminescent Cell Viability Assay Kit. One-way ANOVA with Tukey's post hoc test was performed for analysis (n=4, *P < 0.05, **P < 0.01, ***P < 0.001). (**C**) Changes of apoptotic protein markers include Bax, Bcl-2, Cleaved caspase-3 due to TMZ were verified and compared through western blot. There was no significant difference with Fresh TS and Cryo TS.

6. Fresh TS and Cryo TS displayed no difference in tumorigenesis in the orthotopic xenograft model.

Finally, we decided to check whether cryopreservation maintains the properties of TS and does not differ from Fresh TS in tumorigenesis. Therefore, approximately 5×10^5 of 3 different Fresh TS and Cryo TS were implanted into the right frontal lobes of 6-8-week-old of nude mice brain, respectively, to create a xenograft model, and then to checked whether tumor formation was successful (n=5). The mouse survival curve was evaluated using the Kaplan–Meier method, and the difference in the median survival of all three types of Fresh TS and Cryo TS was not significant (**Figure. 6A**). When mice were euthanized, each brain was removed and sectioned, and hematoxylin and eosin (H&E) staining for final evaluation of tumorigenesis, Nestin immunostaining to determine stemness and Zeb1 immunostaining to analyze invasiveness was performed. In the H&E results, Fresh TS and Cryo TS formed tumors with similar size and shape (**Figure. 6B**), and the same expression pattern was also shown for Nestin and Zeb1 expression in tumors. Nestin and Zeb1-positive cells outside the total tumor boundaries separated by H&E staining were counted and identical expression levels were confirmed (**Figure. 6C**). All experimental procedures were approved by the Yonsei University College of Medicine Institutional Animal Care and Use Committee.

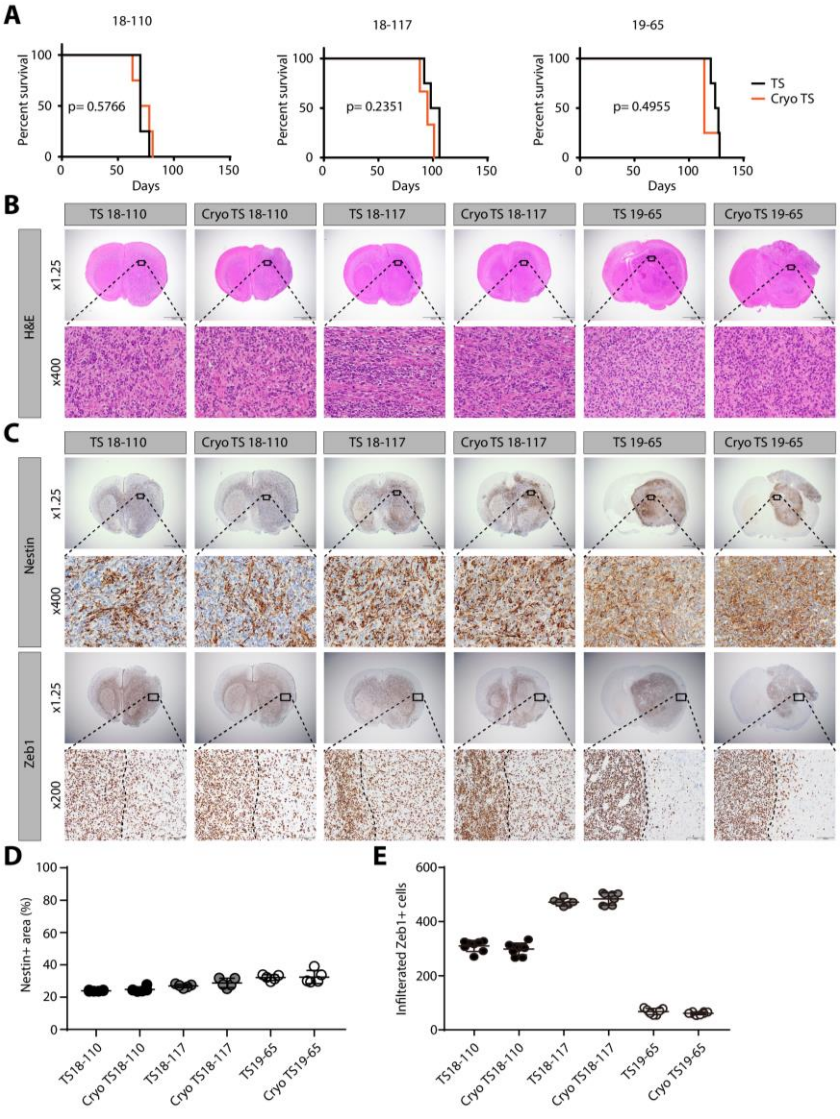

Figure. 6 Fresh TS and Cryo TS mouse orthotopic xenograft model.

(**A**) After implantation using a guide-screw system, the survival curve was estimated based on Kaplan–Meier curves. P value was analyzed using a log-rank test (n=5). There was no significant difference between Fresh TS and Cryo TS. (**B**) Hematoxylin and eosin (H&E) staining was done to sections of the brain tissue obtained from euthanized mice. Histological tumor

morphology was similar between Fresh TS and Cryo TS. (**C**) Nestin and Zeb1, proliferation and invasion markers, were IHC stained to compare Fresh TS and Cryo TS in in vivo conditions. (**D**) In the 10-fold magnification image, Zeb1 + cells infiltrating beyond the margin of the tumor (left of the black line) were counted and graphed. (**E**) The expression ratio of Nestin was measured and graphed in 10 high power field images. 2-way ANOVA with Tukey's post hoc test was performed for analysis (*P < 0.05, **P < 0.01, ***P < 0.001).

Table 1. Clinical information of 40 samples used in this study.

Characteristics	Success of TS isolation					P value
	Fresh (+) Cryo (+) (n=22)	Fresh (+) Cryo (-) (n=4)	Fresh (-) Cryo (+) (n=2)	Fresh (-) Cryo (-) (n=12)	Total (n=40)	
Pathological diagnosis						
Glioblastoma	22 (100%)	4 (100%)	2 (100%)	12 (100%)	40 (100%)	
Type						
Primary	22 (100%)	4 (100%)	2 (100%)	12 (100%)	40 (100%)	
Recurrent	0	0	0	0	0	
Sex						0.296
M, n (%)	8 (36.4%)	3 (75%)	0	4 (33.3%)	15 (37.5%)	
F, n (%)	14 (63.6%)	1 (25%)	2 (100%)	8 (66.7%)	25 (62.5%)	
Age (years)	65.6 ± 9.8	62 ± 3.2	64 ± 1.4	58.2 ± 12.5	62.9 ± 10.4	0.051
IDH1 mutation						0.840
Wild, n (%)	21 (95.5%)	4 (100%)	2 (100%)	12 (100%)	39 (97.5%)	
Mutation, n (%)	1 (4.5%)	0	0	0	1 (2.5%)	
MGMT promotor						0.341
Unmethylated, n (%)	14 (63.6%)	1 (25%)	1 (50%)	9 (75.0%)	25 (62.5%)	
Methylated,n (%)	8 (36.4%)	3 (75%)	1 (50%)	3 (25.0%)	15 (37.5%)	
1p19q						
No deletion	22 (100%)	4 (100%)	2 (100%)	12 (100%)	40 (100%)	
Co-deletion	0	0	0	0	0	

GBM, Glioblastoma; TS, Tumorsphere; RNA, Ribonucleic acid; DMSO, Dimethyl sulfoxide; FBS, fetal bovine serum; FCS, fetal calf serum; IDH, isocitrate dehydrogenase; MGMT, O-6-methylguanine-DNA methyltransferase; M, male; F, female; M, methylated; UM, unmethylated

A total of 40 surgically resected tumor samples were used in this study. All tumor samples were primary glioblastomas with 97.5% of IDH1 wild type. TS isolation success rate were 65% in fresh and 60% in cryopreserved group, respectively. Further descriptive clinical data are shown in **Table 1**.

Table 2. Clinical and mutational profiles of three tumor tissues selected for detailed comparative experiments

Case Number	18-110	18-117	19-65
Diagnosis	Glioblastoma	Glioblastoma	Glioblastoma
IDH type	wildtype	wildtype	wildtype
MGMT promoter methylation	Methylated	Unmethylated	Methylated
Age	65	75	66
Sex	Male	Male	Female
1p/19q status	Intact/intact	Intact/intact	Intact/intact
KI67 (%)	30%	15%	65%
Mutation status*			
TERT promoter mutation	C250T	C228T	C228T
EGFR	c.2132A>G c.2185-2A>G c.2582T>A c.2591C>A	c.2987A>G	-
TP53	-	c.742C>T	c.646 G>A
PTEN	-	-	c.598T>C
Nestin	c.19delG	c.*5520A>G (Moderate)	c.4227C>T (Moderate)
Musashi-1	-	-	-
CD133	-	-	-
SOX2	-	-	-
N-cadherin	c.62T>A	-	-
CD44	c.457A>G	c.454+2T>C	-

Mutation call was calculated based on the Mutect2 algorithm with RNAseq-based protocol (GATK version 4.1.7).

* All the mutation are high impact alterations of genes otherwise specified as moderate impact.

Cinical and mutational profiles of paired triple tumor samples selected for detailed comparative experiments are shown in **Table 2**.

Table 3. Methodological comparison between the present study and the previous two studies.

	Chong et al.	Mullin et al.	Present study
Number of patients	5	27	40
Diagnosis (n, %)			
Primary GBM	4 (80%)	18 (67%)	40 (100%)
Recurrent GBM	1 (20%)	8 (29%)	
Recurrent astrocytoma		1 (4%)	
Status at cryopreservation	Tumorsphere	Tumor tissue	Tumor tissue
Cryopreservation solution	10% DMSO 90% FBS	10% DMSO 90% FCS	10% DMSO 90% FBS
TS isolation (n, %)			
Fresh	5 (100%)	17 (63%)	26 (65%)
Cryopreservation	5 (100%)	16 (59%)	24 (60%)
Result			
Morphology	✓	✓	✓
Proliferation	✓	✓	✓
Molecular data		✓	
Drug response		✓	✓
Radiation response			✓
Stemness	✓		✓
Self-renewal	✓		✓
Karyotype	✓		
Xenograft	✓		✓
RNA sequencing	✓		✓

GBM, Glioblastoma; TS, Tumorsphere; RNA, Ribonucleic acid; DMSO, Dimethyl sulfoxide; FBS, fetal bovine serum; FCS, fetal calf serum;

Compared to previous studies, we tried to reduce heterogeneity by selecting a large number of all primary GBM patients. Chong et al. cryopreserved TS, Mullin et al. and we cryopreserved tumor tissues before isolate TS. There was no significant difference in cryopreservation solution concentration. The three

studies analyzed in various ways on the effectiveness of cryopreservation, and are presented in **Table 3**.

IV. DISCUSSION

In past studies, cryopreservation approaches have often not been the best way to preserve cells. It has been considered to be applied only to a specific cell type or to be complicated in methodology[32,33]. This is because, when freezing cells, ice crystals are formed inside and outside the cells, changing the chemical settings of cells and causing cell damage[34,35]. Additionally, research has shown that storing cells in liquid nitrogen can halt their metabolism and lead to dramatic biological mutations and genetic drift[36]. Some common cryoprotectants used for cryopreservation, such as DMSO, induces cytotoxicity in cells[37,38], alters chromosomal stability[39], and induces epigenetic changes to histones[40]. There is a possibility of infection or contamination of cells that may occur during cryopreservation. In particular, freshly isolated CSCs from fresh GBM tissues have been preferred to obtain gCSCs.[41,42]

Even today, there is a debate about the negative and positive aspects of cryopreservation. In GBM research, there is a study showing that cryopreservation does not alter cell phenotype and genetic characteristics[18], and conserves EGFR and MGMT amplification[19]. In the construction of the PDX model, it has been reported that CSCs isolated by cryopreservation are not different from CSCs isolated from fresh tissue[43]. However, there are case studies suggesting that enzymatic digestion during cryopreservation should be used in conjunction with cryopreservation to increase cell viability after thawing, especially for GBM tissues[44], and cryopreserved CSC is more suitable for 2D culture than 3D culture[44]. In our case, the cryopreservation method we implemented found no difference in genetic profile, neurosphere formation, invasiveness property, TMZ treatment and radiation reactivity, tumorigenesis in the PDX model, and GBM transcriptomic subtype classification when compared to TSs isolated from fresh tissue regardless of any GBM tumor tissue.

We compared and analyzed various biological and molecular aspects of freshly isolated TS or TS isolated after cryopreservation from 40 patients

identified with GBM. Our TS isolation success rates are 65% for freshly isolated TS and 60% for frozen isolated TS, which is consistent with the results of other studies[19,45], and is convincing in that the separation success rates of fresh TS and Cryo TS are not significantly different (**Figure. 1B**). In the case of our study, it is very meaningful in that it compared and analyzed three TSs simultaneously isolated from Fresh TS and Cryo TS in parallel. These three TSs differ slightly from the original tumor tissue when looking at the transcript subtypes of the GBM after isolation (**Figure. 1D**). The reason is that the original tumor is thought to be due to an aggregation of multiple heterogeneities due to the tumor microenvironment[46]. However, the fact that cell surface markers remain the same between Fresh TS and Cryo TS (**Figure. 2C, D**) and that self-renewal and invasive abilities remain the same in proliferation, neurosphere formation and invasive properties (**Figure. 2A, Figure. 3, Figure. 4**) is convincing in that tumor properties remain intact. In terms of targeted therapy, Fresh TS and Cryo TS respond equally to TMZ drugs and radiation reactivity (**Figure. 5**), making them applicable for anticancer drug discovery, and the fact that tumorigenesis and animal survival are identical in PDX animal models may raise potential clinical studies.

Many basic and clinical studies such as tumorigenesis to progression, invasion, drug response, patient-derived xenograft models, and immune profiling still require primary cultured TS. Due to the limitations of the clinical environment, the majority of institutions do not have sufficient laboratory facilities and financial resources to perform primary cultures on the day of surgical collection. In particular, GBM tissue is difficult to obtain and may not always be successful in isolation. Our study is of high significance in that we isolated TS using patient-derived live tissue. In this study, we succeeded in preserving high-quality, viable GBM tissue specimens through cryopreservation. The data we studied showed that cryopreservation method could have high reliability, and it can be applied to various studies in that the

Fresh TS and Cryo TS gene profiles, proliferation, stem, invasiveness, xenografts of tumorigenesis, and GBM subtypes of tumors all remain the same. In this study, cryopreserved tissues were thawed and dissociated after 1 month, but recently, single cells were isolated from cryopreserved tissues after about 1 year and 2 months (*Data not shown*). Therefore, our protocol considers that cryopreserved tumor tissue can be used for studies months to years after preservation, and can be suggested as a step towards time-independent GBM studies.

V. CONCLUSION

Cryopreserved tissue can be used as freshly isolated TS in GBM studies. According to our results, Cryo TS showed no changes in biological phenotype and molecular properties such as cell surface markers and differentiation markers and in stemness, invasiveness and TMZ drug reactivity compared with Fresh TS. Our study is significant in that a parallel comparison analysis was performed between TSs simultaneously isolated from one tissue and it was verified once again through transcriptome analysis. Therefore, we can suggest that cryopreservation is an alternative when it is not immediately possible to isolate TS from GBM samples.

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ABSTRACT (IN KOREAN)

프레쉬 샘플과 동결 보존 샘플에서 획득된 교모세포종 종양구의 생물학적 특성 비교

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이 동 규

배경: 교모세포종은 가장 흔하고 공격적인 형태의 원발성 종양으로 환자의 예후가 매우 좋지 않은 종양에 속한다. GBM에서 분리된 종양구 (TS)는 종양의 공격적인 성향과 미세 환경을 잘 반영하여 종양 생물학, 항암제의 전임상 in vitro 스크리닝을 환자 유래 이종이식 (PDX) 모델을 통해 연구할 수 있는 잠재력이 있다. 임상적으로 여러 연구에서 TS의 높은 중요성을 제시하였으나 수술 후 적출된 환자 조직으로부터 바로 세포 분리를 하는 것은 매우 제한적이다. 최근 일부 연구에서 동결 보존의 이점이 입증되었으나 현재까지 프레쉬 샘플과 동결 보존 샘플의 생물학적 표현형과 분자생물학적 차이를 중점적으로 비교 한 연구는 없었다. 본 연구에서는 동결 보존에 의해 분리된 TS가 생물학적 표현형과 분자생물학적 차이가 Fresh TS와 나타나는지를 확인하고자 하였으며 동결 보존에 의해 분리된 TS가 향후 Fresh TS와 동일하게 실험에 사용 될 수 있는지를 비교 분석하고자 실시되었다.

방법: 교모세포종 진단을 받은 환자로부터 적출된 GBM 조직을 프레쉬군과 동결 보존군으로 나누었다. 각 조직을 수술용 칼로 잘게 자른 후 동결 보존 배지가 있거나 없는 상황에서 세포 분리를 실행하였다. 두 가지 다른 세포 분리 방법으로 획득한 TS를 줄기능, 신경구 형성, 침윤성, 테모졸로마이드 (TMZ) 반응, 전사체 분석 및 마우스 이종이식 모델에 대해 분석하였다.

결과: 총 40개의 GBM 환자 샘플이 본 연구에서 사용되었다. TS 분리 성공률은 프레쉬군에서 65%, 동결보존군에서 60%였다. 프레쉬군과 동결 보존군의 줄기능, 신경구 형성에는 유의한 차이가 없었다. 두 가지 다른 세포 분리 환경에서 획득한 TS는 동일한 침윤성, TMZ 반응성을 보였다. 서로 다른 TS 간의 암 아형은 다를 수 있지만 Fresh TS와 Cryo TS의 전사체 유사성은 변하지 않았다. 동물 실험 결과에서 프레쉬군과 동결 보존군의 종양 생성 능력을 동일하게 나타냈다.

결론: 이러한 결과는 Cryo TS가 Fresh TS와 비교하였을 때 생물학적 특성과 전사체의 변화가 없었다. 이종이식 동물 실험에서 종양의 형태와 분자생물학적 표지 인자의 발현 또한 동일한 것으로 보아 동결 보존으로부터 획득한 세포는 일차 배양 세포로써 임상적용성이 높은 실험에 사용될 수 있음을 보여주었다. 이는 동결 보존이 수술 후 획득된 GBM 조직으로부터 종양 세포를 분리할 수 없는 경우 대체할 수 있는 방법임을 실험을 통해 검증하여 시사한다.

핵심되는 말: 동결 보존, 교모세포종, 세포 분리, 종양구, 환자 유래 이종이식 모델