SUPPLEMENTARY MATERIALS AND METHODS

Cell culture and treatments

Cells were cultured in a level 2 cell culture facility according to the regulations of the RIMUHC Biohazard Safety Certificate. All cell lines were established by the co-author (I.N) as Glioblastoma stem cells (GSC) within less than 5 years of their initial publication in 2013[34]. The sphere cultures were derived from clinical GBM samples, as previously described, and were characterized as GSCs representing either mesenchymal (MES83, MES1123) or proneural subtype (PN157, PN528)[34-37]. GSCs were cultured as neurospheres in DMEM/F12 media supplemented with B27 (2%), Glutamax, EGF (20 ng/mL), bFGF (20 ng/mL), Penicillin and Streptomycin (Life Technologies, Burlington, ON, Canada), and Heparin 5µg/mL (Stem Cell Technologies, Vancouver, BC, Canada). In some experiments cells were treated with Temozolomide (Selleckchem, S1237, Burlington, ON, Canada) or O6BG (Sigma-Aldrich, B2292, Oakville, ON, Canada), dissolved in DMSO and used at indicated concentrations.

Mouse intracranial xenograft injection

To generate brain tumours in mouse brains, 1 x 10^4 GSCs were stereotactically injected into the brain of NSG mice (Charles River Labs, Senneville QC) in a total volume of 2µL, using a Stereotaxic Injector (PW, Stoelting, L. L. Kiel, WI, USA) at the coordinates (AP: +1.5 ML: +2.5 DV -3.0) from bregma as previously described [38]. Mice were treated intraperitoneally (i.p.) with TMZ at 120 mg/kg repeatedly at time points of overt weight loss over 3 consecutive days/measurements, in the presence of high luciferase signal and showing signs of neurobehavioural deterioration (circling) - a state that in our hands was indicative of the loss of

therapeutic response. Mice were monitored daily and euthanized at humane endpoints and after their therapeutic responses were no longer observed. All procedures involving animals were performed in accordance with the guidelines of the Canadian Council of Animal Care and the Animal Utilization Protocols, approved by the Institutional Animal Care Committee at McGill University Health Centre Research Institute and McGill University.

Tumor dissociation

Secondary GSC (SGSC) lines were isolated from intracranial GBM xenografts. Brain tumors were dissected, cut into small pieces, and subjected to collagenase/dispase digestion (Roche, Laval, QC, Canada), for 15 min on ice and then for 15 min at 37°C under agitation. After mechanical dissociation by pipetting, the preparation was centrifuged for 10 min at 1,200 rpm, re-suspended in stem cell media, filtered through a 100 μm strainer (BD Biosciences, Mississauga, ON, Canada) and single cells plated in neurosphere cultures.

Mouse subcutaneous xenograft injection

In some experiments the *in vivo* validation of TMZ-resistant phenotype was carried out through the use of a more rapid subcutaneous xenograft method. To accomplish this, 1 x 10^6 SGSCs were re-suspended in 200 μL of Matrigel (BD Biosciences) and injected subcutaneously (s.c.) into the flank of NSG mice. Tumor size was then measured several times a week using a digital Vernier's caliper, and tumor volume (TV) was calculated using the formula: $TV = (a^2 \times b) \times 0.52$, where a and b are the smallest and the largest perpendicular diameters of the tumor [38].

Bioluminescent imaging

Luciferase-expressing GSCs were obtained through transduction of GSCs with lentiviral particles containing luciferase (ABM, Applied Biochemical Materials Inc., Richmond, BC, Canada; 10^8 IU/ml). After intracranial injection, bioluminescence imaging was carried out under general Isofurane anaesthesia to monitor tumor growth, following the injection of mice with D-Luciferin (PerkinElmer, Waltham, MA, USA; 15µg/mL) and imaging with the IVIS 200 scanner (PerkinElmer) as described [38].

Immunohistochemistry

Brain tissues were fixed in 4% paraformaldehyde, processed overnight, embedded in paraffin, sectioned (5µm) and mounted on glass slides. Sections were stained with haematoxylin and eosin (H&E) to reveal the morphology or with specific antibodies against indicated molecular markers: rabbit anti-Nestin (BioVision Inc. Milpitas, CA, USA; 3208-100), rabbit anti-TGM2 (Cell Signalling Technology Inc., Danvers, MA, USA, 3557); rabbit anti-CD44 (Abcam, Toronto, ON, Canada, ab51037), rabbit anti-OLIG2 (EMD Millipore, Darmstadt, Germany, AB9610), goat anti-SOX2 (Santa Cruz Biotech, Santa Cruz, CA, USA, sc-17320), rabbit anti-MAP2 (Abcam, ab5622), as described earlier [38]. Briefly, slides were dewaxed and processed through ethanol series with subsequent washing with PBS (pH 7.4). Endogenous peroxidases were quenched by placing the slides in a mixture of 49% methanol:49% PBS:2% H_2O_2. Brain sections were blocked with normal serum of the secondary antibody species and then incubated with indicated primary antibodies (1:100) overnight at 4^{o}C in a humidified chamber. The following day slides

were incubated the appropriate HRP-conjugated secondary antibodies at 1:200 concentration (Vector rabbit ABC kit, Vector Laboratories, Burlingame, CA, USA) for 30 minutes at room temperature (RT). Signals were detected using DAB substrate kit (Vector). Tissues were counter stained with hematoxylin and viewed as indicated. In some cases, changes in the invasive morphology of the tumour mass were quantified by calculating the percentage of the total tumour perimeter with detectable infiltrative features such as satellite nests of cancer cells, projection of tumour tissue into the surrounding parenchyma or irregular tumour margin. Briefly, the tumour circumference within each H&E stained tissue section was defined by precise free-hand draw tool separating tumour parenchyma and surrounding host tissues. This length was measured morphometrically using image analysis software (below), including infiltrative projections and nests of cancer cells. Subsequently, segments the tumour infiltrative margins (as above) were visually identified and their lengths measured and expressed as percentage of the overall length of the tumour margin. Measurements of infiltration (%) of individual xenografts were plotted as open or black diamonds (Fig. S5). Slides were analysed and microphotographed using Axiomager Z1 microscope (Carl Zeiss, Gottingen, Germany). Image analysis was performed using Zen software (Carl Zeiss).

Cell viability and growth assays

Cells were seeded into 96-wells plates at the density of 2,500 cells/well and exposed to indicated treatments followed by addition of MTS reagents (Promega, San Louis Obispo, CA, USA, G3580) and absorbance at 490nm was measured. For the viability assay on irradiated cells following indicated doses of radiation in culture the cells were harvested and counted in the

presence of trypan blue. Briefly, to assess the changes in GSC viability and numbers following treatment with TMZ, O6BG, or radiation, cells were either directly counted using haemocytometer, or evaluated using MTS Cell proliferation assay (Promega, San Louis Obispo, CA, USA, G3580), according to the manufacturer's instructions [38]. Briefly, for MTS assay cells were seeded into 96-wells plates at the density of 2,500 cells/well and 24h later TMZ with or without O6BG were added at indicated concentrations. The cultures were further incubated for 4 days at 37C in 5 % CO2 at which point 30μL of MTS reagent/well were applied for 2 h. Following this incubation the absorbance was read at 490nm using Epoche plate reader equipped with Gen5 software (BioTek, Winooski, VT, USA).

Western blotting

As described earlier[39] indicated protein lysates were resolved and transferred to nitrocellulose membranes which were probed with the following primary antibodies: rabbit anti-NOTCH1 (Cell Signaling 4380), rabbit anti-NESTIN (Abcam, ab105389), goat anti-SOX2 (Santa Cruz Biotechnology, sc17320), rabbit anti-MAP2 (Millipore, AB5622), rabbit anti-TGM2 (Cell Signaling, 3557), rabbit anti-RAD50 (Cell Signaling, 3427), rabbit anti-EGFR (Cell Signaling, 4267S), mouse anti-Keratin18 (Cell Signaling, 4548), mouse anti-MGMT (Millipore, AB16200); and mouse anti-β-actin (Sigma). Appropriate horseradish peroxidise (HRP) – conjugated secondary antibodies (Dako – Agilent Technologies, Mississauga, ON, Canada) were used to visualise the protein bands following enhanced chemiluminescence detection (GE Healthcare, Mississauga, ON, Canada).

Mass spectrometry (MS)

Sample preparation and proteomic analyses were conducted as previously described [40]. Cell pellets were subjected to denaturation, reduction, alkylation, followed by trypsin digestion as described elsewhere [41]. Briefly, cell pellets were re-constituted in 150 µL of 50% (v/v) 2,2,2-trifluoroethanol (TFE) in PBS buffer. The suspension was incubated at 60°C for two hours with brief agitation every 30 min. Proteins were reduced with 5 mM of DTT (Sigma, Canada) at 60°C for 30 min. Carbamidomethylation of reduced cysteines was performed with 25 mM of IAA (Sigma) for 30 min at room temperature in the dark. Subsequently, samples were diluted five times using freshly prepared 100 mM ammonium bicarbonate buffer at pH 8.0. Proteins were digested using 5 µg of mass spectrometry grade trypsin (Promega, USA) at 37°C overnight. Digested peptide mixtures were desalted and purified using OMIX C18 pipette tips (Agilent, USA) and concentrated by vacuum centrifugation. The semi-dry pellet of peptides was reconstituted using LC–MS grade water/0.1% formic acid. Peptide concentrations were determined using a NanoDrop spectrophotometer (Thermo Scientific). Mass spectrometry was carried out using a QExactive tandem mass spectrometer (Thermo Scientific) equipped with an Easy-Spray nano-electrospray ionization source (Thermo Scientific) coupled to an EASY-nLC 1000 nano flow ultra-performance liquid chromatography system (Thermo Scientific). Analytical chromatography was performed using a 50 cm EASY-Spray column (PepMap C18, 2 µm particles, 100 Å pore size; Thermo Scientific) heated to 40°C. For each analysis 2 µg of peptides were loaded on the analytical column. Elution buffers used for reverse phase chromatography consisted of 0.1% (v/v) formic acid as solvent A and 100% acetonitrile with 0.1% (v/v) formic acid as solvent B. Peptides were separated over an analytical gradient of 5–30% solvent B in 230 min at a flow rate of 250 nL/minute. Data was acquired on a QExactive mass spectrometer

running a top 10 data dependent acquisition method. All MS1 spectra were acquired at resolution of 70,000 with scan range of 400–1600 m/z, while MS/MS spectra were acquired at resolution of 17,500. The acquired data was searched by MaxQuant version 1.3.0.3 [42] against the UniProt complete human proteome protein sequence database (version: 2012-07-19, number of sequences: 20,232). Searches were performed with fragment ion mass tolerance of 20 ppm, maximum missed cleavage of 2 and carbamidomethylation of cysteine was specified as a fixed modification and oxidation of methionine as variable modification. False discovery was controlled using a target/decoy approach with false discovery level set to 1% (for peptides and proteins). Only protein groups identified with at least two or more peptides (sum of razor and unique) were carried forward in the analysis.

Quantitative Real-Time PCR

The first strand of complementary DNA was synthesized by using 500 ng of total RNA and the RT^2 First Strand Kit (Qiagen, Mississauga, ON, Canada, 330404). Equal amounts of complementary DNA were distributed to each well of customized 96 wells (24 drug resistance genes) or DNA Repair specific RT2 Profiler PCR Arrays (SABiosciences/Qiagen, Mississauga, ON, Canada, PAHS-042Z), and amplified with Quantitect SYBR Green PCR Kit (Qiagen, 330503) on a Roche LightCycler 480 (Roche). PCR was performed according to the manufacturer's instructions, including 4 housekeeping genes (ACTB, GAPDH, Hsp90AB1, B2M) for data normalization. A genomic DNA control, a reverse-transcription control and a positive PCR control were also used for PCR quality control, to verify the efficiency of the reverse transcription, the efficiency of the PCR and the absence of DNA contamination. Raw

data were collected with LightCycler® 480 Software, and analyzed using the ΔΔCt method with the RT^2 Profiler PCR Array Data Analysis software, version 3.5 (Qiagen, SA Biosciences).

Preparation of extracellular vesicles

EVs were obtained by ultracentrifugation as described earlier[39,43]. Briefly, cell culture supernatants were centrifuged for 10min at 400g, and 30 min at 10,000g to remove cell debris. The liquid fraction was then centrifuged for 1h at 100,000g to pellet EVs, and then washed extensively in phosphate buffered saline (PBS).

Nanoparticle tracking analysis (NTA)

Measurement of the size and number of particles released by cells in the cell culture was performed using the NS500 NTA system (Nanosight, Amesbury, UK)[44].

Exome sequencing and IDH1 gene analysis

Samples were prepared using the Agilend SureSelect exome capgture kit to generate 100bp reads and sequenced as paired end reads on an Illumina HiSeq 2000. Reads were aligned to the hg38 reference genome using BWA [45]. The Genome Analysis Toolkit (GATK) best practices were then applied [46]. Briefly, picard was used to mark duplicate reads then quality score was recalibrated with GATK followed by indel realignment and duplicate removal [47] [48]. Finally, MuTect2 was utilised to identify somatic mutations across all samples using COSMIC, 1000

genomes and dbSNP databases as inputs. Hard filters were then applied such that only variants with an allele frequency of >5% and covered by at least 10 reads were reported. All samples possessed wild type IDH1 except for the TMZ resistant cell line 1123-8. A single IDH1 variant was found within the 6^{th} intron of IDH1 (c.6775+1228G>A) and found at an allele frequency of 31% in this cell line.

Radiation treatment on GSC cultures

Cells from TMZ-sensitive and -resistant cell lines were seeded at 1 x 10^3 cells per well in a 6 well plate, left overnight and then irradiated using a Faxitron irradiator (Faxitron Bioptics, Tuckson, AZ, USA) at doses of 0, 2, 4, 6 and 8 Grey. After 11 days the cells and media were collected and centrifuged at 1,200 rpm for 5 min. The cell number was counted in the presence of trypan blue using the automated cell counter (Nexcelom Bioscience, Lawrence, MA, USA) to establish the final numbers of viable cells under different irradiation conditions, as indicated.

GSC's from resistant and sensitive cell lines were counted and suspended in a T-75 non-adherent flask (Sarstedt, Montreal, Canada) at a concentration of 10 - 15 x 10^6 cells in DMEM/F12 media. The flask was placed in the Faxitron irradiator and given doses of 0, 2 or 6 Gy, respectively. Cells were then collected immediately, recounted and suspended at a concentration of 5 X 10^6 cells per ml of DPBS. Cells were then injected into the flank of NSG mice at a concentration of 1 X 10^6 cells in 200 uls of DPBS. Tumors were measured every 2 days until the tumors reached the experimental endpoint.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Repeated exposure to TMZ *in vivo* leads to rapid acquisition of drug resistance by glioblastoma xenografts initiated through intracranial inoculation of GSC. (A) Plotting of Progression-free survival (PFS) for mice bearing MES (N=5) or PN tumors (N=4) and treated with TMZ suggests a slight delay in death for PN subtype. (B- C) Heterogeneity of the natural history of the disease in individual mice harbouring MES (123) xenografts. (B) Bioluminescent imaging of intracranial tumors was conducted using the IVIS 200 system over time and signal quantification was expressed as photons/sec, as computed by the Living Image 4.3.1 Software under identical conditions for individual tumour. (C) Luciferase signal in mice injected with MES GSCs confirms the acquisition of TMZ resistance after several cycles of treatment (red dots).

Figure S2. **Derivation of secondary GSC lines *in vivo***. The original MES (1123) or PN (528) GSC cell lines, isolated from clinical samples, were cultured *in vitro* as neurospheres and injected intracranially into NSG mice to form control (S) or TMZ-resistant (R) primary tumors (PT). Secondary GSCs (SGSCs) were cultured *in vitro* after isolation from those xenografts, and then injected again into NSG mice, subcutaneously to confirm the preservation of their resistant phenotype, or intracranially to investigate invasion and expression of MES/PN markers by immunohistochemistry (secondary tumors, ST).

Figure S3. TMZ resistance of secondary GSC (SGSCs) isolated from xenografts that escape drug toxicity is maintained in secondary tumor recipients. SGSCs were inoculated (s.c.) into

secondary NSG mice, and secondary tumors – ST (ST1123IC12S, ST1123IC15S – control xenografts; ST1123IC7R, ST1123IC8R – TMZ resistant (derived from GSC1123)) were left untreated or given a single dose of TMZ (120mg/kg;; See Fig. S1-2). (N=3)

Figure S4. Immunostaining for stem cell markers in SGSC initiated xenografts with and without TMZ therapy. Tissues were prepared from xenografts of MES-like SGSC lines, processed as indicated and stained for mesenchymal (TGM2) and proneural (Nestin, Sox2, MAP2, Olig2) markers. Control (ST1123ICxS) and TMZ-resistant (ST1123ICxR) tumors exhibit different staining profiles and reduced mesenchymal characteristics upon injection to secondary recipients. Objective 20x.

Figure S5. **Acquisition of TMZ resistance impacts the multicellular architecture and invasive tumour growth *in vivo***. (A) Morphological characterization of invasive phenotypes in intracranial secondary tumors after reinjection of control and TMZ-resistant SGSC lines. Objective 20x. (B) Quantification of invasive patterns in control and TMZ-resistant mesenchymal secondary intracranial tumors (see Supplementary Methods).

Figure S6. Differential expression and function of MGMT among TMZ resistant SGSC sublines derived from a single parental MES-like GSC line. SGSCs lines were isolated from TMZ naïve and refractory xenografts and analysed by western blotting for the expression of MGMT (A). (B) MTS assay was employed to assess the viability of indicated cell lines after

treatment with TMZ alone or in combination with O6BG, an MGMT inhibitor, at different concentrations. The results confirmed the involvement of MGMT in TMZ resistance for 1123IC9R cell line but not in cells that became TMZ resistant without MGMT upregulation (**** P< 0,0001; N=3).

Figure S7. Extended profiling of putative TMZ resistance markers using qPCR array. TMZ resistance associated genes were tested in established SGSC lines. These cultures were isolated from either mesenchymal (1123ICxS and 1123ICxR) or proneural (528ICxS or 528ICxR) GSC-initiated intracranial xenografts following TMZ exposure. The gene expression levels were compared between treatment naïve and TMZ-resistant cells (1 = average mRNA expression in TMZ-sensitive cell lines).

Figure S8. **Graphical representation of qPCR analysis for major differences observed in the expression of different drug resistance-associated genes**. The summary of changes observed for mesenchymal GSCs. (A) Volcano plot representation of statistical significance (P value)(y axis) against fold change (FC)(x axis) gene expression in MES control 1123ICxS cell lines versus TMZ-resistant 1123IC7R, 1123IC8R and 1123IC9R cell lines. N=3. Cut-off was set at Log2 (FC of 1123ICxR/Controls)=4. (B) Scatter plot representation of differentially expressed markers of drug resistance in MES control and TMZ-resistant SGSCs cell lines. Comparison between the normalized expression of every gene on the array between control 1123ICxS cell lines and TMZ-resistant 1123IC7R, 1123IC8R or 1123IC9R cell lines. The central line

corresponds to absence of variation in expression between the two groups. Fold regulation cut-off was set at Log10 (gene expression) =4.

Figure S9. Heterogeneous expression of genes associated with DNA repair among secondary GSCs. SGSCs isolated from MES (1123) GSC-initiated brain tumours either grown under control conditions (S) or exposed to TMZ (R) were profiled by qPCR for the expression of transcripts with reported association to DNA repair. (1 = average mRNA expression in TMZ-sensitive cell lines) . **** P< 0,0001; *** P< 0,001; ** P< 0,01; * P< 0,05 (N=minimum 2 independent experiments).

Figure S10. Extended analysis of the expression of genes associated with DNA repair among secondary GSCs. As in Fig. S9 SGSCs isolated from MES (1123) GSC-initiated brain tumours either grown under control conditions (S) or exposed to TMZ (R) were profiled by qPCR for the expression of transcripts with reported association to DNA repair. (1 = average mRNA expression in TMZ-sensitive cell lines) . **** P< 0,0001; *** P< 0,001; ** P< 0,01; * P< 0,05 (N=minimum 2 independent experiments).

Figure S11. Analysis of the GBM subtype-related expression of TMZ resistance markers in the human glioblastoma (GBM) gene expression database. Data were extracted from TCGA database and represented as a heatmap according to Verhaak GBM subtype classification (Proneural, Neural, Classical, Mesenchymal) [49].

Figure S12. Mesenchymal glioma stem cell evolution as a putative target of alternating chemo-radiation therapy. Our study shows that Nestin negative (NES-) and TGM2 positive (TGM2+) MES GSCs when selected for TMZ resistance in vivo acquire NES+/TGM2- phenotype coupled with increased responsiveness to radiation. This model may explain recent positive results of the clinical trial with neoadjuvant TMZ followed by radiation [50]

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