Synergistic Effects of the DRD2/3 Antagonist ONC201 and Radiation in Glioblastoma

¹Ling He, ¹Kruttika Bhat, ¹Angeliki Ioannidis, ¹Le Zhang, ¹Nhan T. Nguyen,
²Joshua E. Allen, ^{3,4}Phioanh Leia Nghiempfu, ^{3,4}Timothy F. Cloughesy, ^{3,5}Linda
M. Liau, ^{3,6}Harley I. Kornblum and ^{1,3}Frank Pajonk*

¹Department of Radiation Oncology, David Geffen School of Medicine at UCLA

²Oncocyte Inc., Philadelphia, Pennsylvania

³Jonsson Comprehensive Cancer Center at UCLA

⁴Department of Neurology at UCLA

⁵Department of Neurosurgery at UCLA

⁶NPI-Semel Institute for Neuroscience & Human Behavior at UCLA

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Authorship: LH, KB, AI, LZ performed the experiments and collected the data. LML and HIK provided the patient-derived specimen. JEA provided ONC201, FP designed the experiments, FP conceived of the study, LH and FP analyzed the data and wrote the manuscript. All authors edited and approved of the final version of the manuscript.

***Correspondence address:** Frank Pajonk, MD, PhD, Department of Radiation Oncology, David Geffen School of Medicine at UCLA, 10833 Le Conte Ave, Los Angeles, CA 90095-1714, Phone: +1 310 206 8733, Fax: +1 310 206 1260, Email: pajonk@ucla.edu

Abstract

Background Glioblastoma (GBM) is the deadliest of all brain cancers in adults. The current standard-of-care is surgery followed by radiotherapy and temozolomide, leading to a median survival time of only 15 months. GBM are organized hierarchically with a small number of glioma-initiating cells, responsible for therapy resistance and tumor recurrence, suggesting that targeting glioma-initiating cells could improve treatment response. ONC201 is a first-in-class anti-tumor agent with clinical efficacy in some forms of high-grade gliomas. Here we test its efficacy against GBM in combination with radiation.

Methods Using patient-derived GBM lines and mouse models of GBM we test the effects of radiation and ONC201 on GBM self - renewal *in vitro* and survival *in vivo*. A possible resistance mechanism is investigated using RNA-Sequencing.

Results Treatment of GBM cells with ONC201 reduced self-renewal, clonogenicity and cell viability *in vitro*. ONC201 exhibited anti-tumor effects on radioresistant GBM cells indicated by reduced self-renewal in secondary and tertiary glioma spheres. Combined treatment with ONC201 and radiation prolonged survival in syngeneic and patient-derived orthotopic xenograft mouse models of GBM. Subsequent transcriptome analyses after combined treatment revealed shifts in gene expression signatures related to quiescent GBM populations, GBM plasticity, and GBM stem cells.

Conclusions Our findings suggest that combined treatment with the DRD2/3 antagonist ONC201 and radiation improves the efficacy of radiation against GBM *in vitro* and *in vivo* through suppression of GICs without increasing toxicity in mouse models of GBM. A clinical assessment of this novel combination therapy against GBM is further warranted.

Keywords: Glioblastoma, radiotherapy, dopamine receptor antagonist, transcriptome analyses, quiescent glioblastoma cells

Key points:

- Combined treatment of ONC201 and radiation exhibit anti-tumor effects on cells from primary and recurrent GBM
- Combined treatment significantly prolongs survival *in vivo*
- Combined treatment potentially targets the quiescent GBM cell population

Importance of the Study

The survival rates for patients with GBM are unacceptably low and novel treatment approaches are needed. This study provides evidence that a combination of radiation and the dopamine receptor antagonist ONC201 significantly prolongs survival in mouse models of glioma.

Introduction

GBM is the deadliest of all brain cancers in adults and all patients ultimately succumb to the tumor. The standard-of-care involves surgical removal of the bulk tumor followed by radiotherapy and temozolomide treatment¹⁻⁴. Reasons for treatment failure include the spread of tumor cells into the normal parenchyma, far beyond the detectable tumor, radio- and chemo-therapy resistance of the tumor cells and the intratumoral heterogeneity and plasticity of GBM⁵⁻⁹. Past attempts to improve the outcome of these patients using classical chemotherapeutic drugs or immunotherapy have been hampered by the inability of many drugs or biologics to cross the blood brain barrier (BBB)¹⁰⁻¹².

ONC201 is the first member of a novel class of anti-cancer small molecules called imipridones, originally discovered by screening with the luciferase-reporter system for p53-independent inducers of the immuno-surveillance cytokine TNF-related apoptosis-inducing ligand (TRAIL) and tumor cell death in human colorectal cancer cells^{13,14}. ONC201 has been found to specifically bind to and inhibit the dopamine receptor D2-like receptors DRD2 and DRD3 and acts as an allosteric agonist of the mitochondrial protease caseinolytic protease P (ClpP)¹⁵. This results in dual inactivation of Akt/ERK signaling that drives Foxo3a-mediated TRAIL gene induction to promote pro-apoptotic and anti-proliferative effects in tumor cells, but not in normal cells¹⁶⁻²⁰. With p53-independency, low toxicity and excellent penetration of the BBB, ONC201 is currently being evaluated in multiple clinical

trials for select advanced malignancies, including myeloma, leukemia, lymphoma, endometrial cancer, high grade glioma, and other solid tumors²¹⁻²⁸.

Emerging data suggest that a hierarchical organization within glioblastoma plays a crucial role in tumor development, therapy resistance and tumor recurrence²⁹⁻³² and interactions between tumor cells and the microenvironment are involved in tumor progression and the invasive nature of GBM^{33,34}. Strategies to eradicate glioma-initiating cells at the apex of this hierarchy could be a valid approach for developing novel therapeutic approaches or modifying existing treatment regimen. Furthermore, GBM comprises of both a fast-dividing population and a relatively quiescent population, while conventional chemo and radiation therapies largely only target the proliferative population³⁵⁻³⁸. Hence, mobilizing the quiescent cell population into proliferation to further sensitize them to existing therapies could be an approach to improve GBM treatment outcome.

In this study we demonstrate that the first-in-class compound of imipridones, ONC201, in combination with radiation has anti-tumor efficacy against GBM *in vitro* and prolongs overall survival in mouse models of GBM *in vivo*. Transcriptome analyses revealed shifts in gene expression signatures in response to radiation and ONC201, indicating effects of the combination treatment on quiescent GBM cells and their ability to interact with the extracellular matrix (ECM).

Material and Methods

Reagents

ONC201 was kindly provided by Oncocentrics, Inc. (Philadelphia, PA, USA). A 10 mM stock solution was prepared for ONC201 with dimethylsulfoxide (DMSO) for all *in vitro* experiments. All stock solutions were stored in aliquots at -20°C . For *in vivo* studies, ONC201 was freshly prepared with sterile saline at a concentration of 5.5 mg/mL before injection.

Cell culture

Primary human glioma cell lines were established as previously described ³⁹. A description of the cell lines can be found in **Table 1**. The GL261 murine glioma cell line was obtained from Charles River Laboratories, Inc., Frederick, MD. GL261 cells were cultured in log-growth phase in DMEM (Invitrogen, Carlsbad, CA) (supplemented with 10% fetal bovine serum, penicillin and streptomycin). Primary glioblastoma cells were grown under serum-free conditions in ultra-low adhesion plates in DMEM/F12, supplemented with B27, EGF, bFGF and heparin as described previously. All cells were grown in a humidified atmosphere at 37°C with 5% CO_2 . The unique identity of all patient-derived specimen was confirmed by DNA fingerprinting (Laragen, Culver City, CA). All lines were routinely tested for mycoplasma infection (MycoAlert, Lonza).

Animals

6–8-week-old C57BL/6 mice, or NOD-*scid* IL2Rgamma^{null} (NSG) originally obtained from The Jackson Laboratories (Bar Harbor, ME) were re-derived, bred and maintained in a pathogen-free environment in the American Association of Laboratory Animal Care-accredited Animal Facilities of Department of Radiation Oncology, University of California, Los Angeles, in accordance to all local and national guidelines for the care of animals. Weight of the animals was recorded every day. 2×10^5 GL261-Luc or 3×10^5 HK-374-Luc cells were implanted into the right striatum of the brains of mice using a stereotactic frame (Kopf Instruments, Tujunga, CA) and a nano-injector pump (Stoelting, Wood Dale, IL). Injection coordinates were 0.5 mm anterior and 2.25 mm lateral to the bregma, at a depth of 3.0 mm from the surface of the brain. Tumors were grown for 3 days with successful grafting confirmed by bioluminescence imaging. Mice that developed neurological deficits requiring euthanasia were sacrificed.

In-vitro sphere formation assay

For the assessment of self-renewal *in vitro*, primary GBM cells were irradiated with 0, 2, 4, 6 or 8 Gy and seeded under serum-free conditions into non-tissue-culture-treated 96-well plates in DMEM/F12 media, supplemented with 10 mL / 500 mL of B27 (Invitrogen), 0.145 U/mL recombinant insulin (Eli Lilly, Indiana), 0.68 U/mL heparin (Fresenius Kabi, Illinois), 20 ng/mL fibroblast growth factor 2 (bFGF, Sigma) and 20 ng/mL epidermal growth factor (EGF, Sigma). The number of spheres formed at each dose point was normalized against the non-irradiated control. The resulting data points were fitted using a linear-quadratic model.

Secondary and tertiary glioblastoma spheres

Primary HK-308 and HK-374 spheres were grown in 10-cm petri dishes and treated with 2.5 μ M ONC201 one hour before a single dose of 4 Gy radiation. The irradiated spheres were dissociated after 4-5 days and plated at clonal densities to generate secondary spheres. Re-plated secondary spheres were treated with a single dose of ONC201 (2.5 μ M) 24 hours later, dissociated, and plated at clonal densities after 4-5 days in suspension and cultured to form tertiary spheres.

Clonogenic assay

GBM HK-374 cells were trypsinized and plated at a density of 100 cells per well in a 6-well plate for 0 and 2 Gy, 200 cells per well for 4 and 6 Gy, and 400 cells per well for 8 Gy. ONC201 was applied one hour before irradiation at a concentration of 500 nM, 1 μ M, 2.5 μ M and 5 μ M. After 10 days, the colonies were fixed and stained with 0.1% crystal violet. Colonies containing at least 50 cells were counted in each group and presented as the percentage to the initial number of cells plated.

Cell viability assay

GBM HK-374 cells were trypsinized and plated at a density of 1×10^5 cells per well in a 6-well ultra-low adhesion plate. ONC201 was applied one hour before a single dose of irradiation (0 or 8 Gy) at a concentration of 500 nM, 1 μ M, 2.5 μ M and 5 μ M. The spheres were collected and dissociated after 24, 48 and 72 hours. Live, trypan blue-excluding cells were counted using a hemocytometer and normalized

to the cell counts of the 0 Gy DMSO-treated group.

In vivo bioluminescent imaging

The mice were injected intraperitoneally with 100 μ l of D-luciferin (15 mg/ml, Gold biotechnology). Five minutes later, animals were anesthetized (3% isoflurane gas in O₂) and luminescence was recorded (IVIS Spectrum, Perkin Elmer, Waltham, MA). Images were analyzed with Living Image Software (Caliper Life Sciences).

Drug treatment

After confirming tumor grafting via bioluminescent imaging, mice bearing GL261 tumors or HK-374 specimen were injected intraperitoneally (i.p.) on a weekly basis either with ONC201 (50 mg/kg) or saline until they reached the euthanasia endpoint. ONC201 was dissolved in sterile saline at a concentration of 5.5 mg/ml.

Irradiation

Cells were irradiated at room temperature using an experimental X-ray irradiator (Gulmay Medical Inc. Atlanta, GA) at a dose rate of 5.519 Gy/min for the time required to apply a prescribed dose. The X-ray beam was operated at 300 kV and hardened using a 4mm Be, a 3mm Al, and a 1.5mm Cu filter and calibrated using NIST-traceable dosimetry. Corresponding controls were sham irradiated.

All mice were irradiated using an image-guided small animal irradiator (X-RAD SmART, Precision X-Ray, North Branford, CT) with an integrated cone beam CT

(60 kVp, 1 mA) and a bioluminescence-imaging unit. The X-ray beam was operated at 225 KV and calibrated with a micro-ionization chamber using NIST-traceable dosimetry. Cone beam CT images were acquired with a 2 mm Al filter. For delivery of the radiation treatment the beam was hardened using a 0.3 mm Cu filter. During the entire procedure the interior of the irradiator cabinet was maintained at 35° C to prevent hypothermia of the animals. Anesthesia of the animals was initiated in an induction chamber. Once deeply anesthetized, the animals were then immobilized using custom 3D-printed mouse holder (MakerBot Replicator+, PLA filament) with ear pins and teeth bar, which slides onto the irradiator couch. Cone beam CT images were acquired for each individual animal. Individual treatment plans were calculated for each animal using the SmART-Plan treatment planning software (Precision X-Ray). Radiation treatment was applied using a square 1 cm x 1 cm collimator from a lateral field. For the assessment of the effect of ONC201 in combination with irradiation *in vivo*, mice were treated with ONC201 (50 mg/kg, i.p.) or saline one hour prior to irradiation. Animals received a single dose of 10 Gy on day 3 after tumor implantation.

Immunohistochemistry

Brains were explanted, fixed in formalin for twenty-four hours and embedded in paraffin. 4 µm sections were stained with hematoxylin and eosin (H&E) using standard protocols. Additional sections were baked for 30 minutes in an oven at 65 °C, dewaxed in two successive Xylene baths for 5 minutes each and then hydrated for 5 minutes each using an alcohol gradient (ethanol 100%, 90%, 70%,

50%, 25%). Antigen retrieval was performed using Heat Induced Epitope Retrieval in a citrate buffer (10 mM sodium citrate, 0.05% tween20, pH 6) with heating to 95 °C in a steamer for 20 minutes. After cooling down, the slides were blocked with 10% goat serum plus 1% BSA at room temperature for 30 minutes and then incubated with the primary antibody against Ki67 (Cell signaling, Cat #12202S, 1:800) or c-Myc (Cell signaling, Cat #5605S, 1:800) overnight at 4 °C. The next day, the slides were rinsed with PBS and then incubated with ready-to-use IHC detection reagent (Cell signaling, Danvers, MA; 10 µl) at room temperature for one hour, rinsed, and then incubated with DAB (Cell Signaling) for 3-5 minutes. Tissues were counterstained with Harris modified Hematoxylin (Fisher scientific, Waltham, MA) for 30 seconds, dehydrated via an alcohol gradient (ethanol 25%, 50%, 70%, 90%, 100%) and soaked twice into Xylene. A drop of Premount mounting media (Fisher Scientific) was added on the top of each section before covering up with a coverslip.

Quantitative Reverse Transcription-PCR

Total RNA was isolated using TRIZOL Reagent (Invitrogen). cDNA synthesis was carried out using the SuperScript Reverse Transcription IV (Invitrogen). Quantitative PCR was performed in the QuantStudio™ 3 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) using the PowerUp™ SYBR™ Green Master Mix (Applied Biosystems). C_t for each gene was determined after normalization to IPO8, TBP, PPIA and $\Delta\Delta C_t$ was calculated relative to the designated reference sample. Gene expression values were then set equal to

$2^{-\Delta\Delta Ct}$ as described by the manufacturer of the kit (Applied Biosystems). All PCR primers were synthesized by Invitrogen with PPIA, TBP and IPO8 as housekeeping genes (for primer sequences see **Supplementary Table1**).

RNA-Sequencing

Primary HK-374 cells were treated with 2.5 μ M ONC201 one hour before a single dose of radiation (4 Gy), RNA was extracted at 48 hours using Trizol followed with RNeasy kit (Qiagen) isolation. The next-generation sequencing was performed by Novogene (Chula Vista, CA). Quality and integrity of total RNA was quantified on the Agilent Technologies 2100 Bioanalyzer (Agilent Technologies; Waldbronn, Germany). The sequencing and analysis process was performed as previously reported⁴⁰.

Briefly, differential expression analysis between irradiated and control samples (three biological replicates per condition) was performed using the DESeq2 R package (2_1.6.3). The resulting *p*-values were adjusted using the Benjamin and Hochberg's approach for controlling the False Discovery Rate (FDR). Genes with an adjusted *p*-value of <0.05 found by DESeq2 were assigned as differentially expressed. To identify the correlations between differentially expressed genes (DEGs), we generated heatmaps using the hierarchical clustering distance method with the function of heatmap, SOM (Self-organization mapping) and k-means using the silhouette coefficient to adapt the optimal classification with default parameters in R software.

Gene Ontology (GO) enrichment analysis of differentially expressed genes was generated using the **GO**rilla (**G**ene **O**ntology **e**n**R**ichment **a**na**L**ysis and **v**izua**L**iz**A**tion) tool. GO terms with corrected p -values less than 0.05 were considered significantly enriched by differentially expressed genes.

Gene set enrichment analysis (GSEA) using all genes ranked by their differential expression as input was performed for *Hallmark* and *Curated* gene sets (<http://software.broadinstitute.org/gsea/index.jsp>; accessed 05/2020). GSEA with gene sets related to quiescent glioblastoma signatures were manually searched by the gene sets list and ECM-related genes were sorted out for analysis³⁵.

TCGA data mining and analysis

The TCGA Provisional dataset (captured June 1, 2020) was accessed via cBioPortal^{41,42}. Kaplan-Meier estimates were calculated for patients with upregulation (z-score >1.5) in one or more genes enriched in qGBM cells (FN1, CAV1, COL12A1, EFNB2, AKAP12, TNC, CDH13, SPARC, COL6A2, ITGA3, COL4A1, AHNK, CD44, CTSB, LAMA4, CD59, SVIL, VCL, LAMC1, COL15A1, ACTN1, SYNE2) and compared to patients with no alterations in the expression of those genes.

Statistics

Unless stated otherwise all data shown are represented as mean $\pm$ standard error mean (SEM) for at least 3 biologically independent experiments. A p -value of ≤ 0.05 in an unpaired two-sided Student's t -test or Two-way ANOVA test indicated a statistically significant difference. Kaplan-Meier estimates were calculated using the GraphPad Prism Software package. For Kaplan-Meier estimates a p -value ≤ 0.05 in a log-rank (Mantel-Cox) test indicated a statistically significant difference.

Data sharing

All data and methods are included in the manuscript. Patient-derived cell lines will be made available upon reasonable request. The RNA-Seq data are available via GEO accession number GSE153982.

Results

ONC201 decreases cell viability, clonogenicity and self-renewal in GBM.

ONC201 was originally discovered by screening drug libraries for compounds that induce TRAIL to initiate death receptor-mediated apoptosis. To study the effects of ONC201 on GBM, exponentially growing HK-374 cells were treated with ONC201 at 0.5, 1, 2.5 or 5 μ M, concentrations known to be reached in the brain lesions of patients with GBM²¹, and irradiated with 0 or 8 Gy. At 24, 48 and 72 hours after the start of the treatment, we observed a significant, dose-dependent reduction in the number of viable cells, thus suggesting cytostatic or cytotoxic activity of ONC201 on the bulk population of HK-374 GBM cells (**Fig. 1A**).

A radiobiological gold-standard for assessing the radiosensitizing properties of drugs is the clonogenic survival assay, which measures the formation of adherent colonies of more than 50 cells from an individual clonogenic cell. Treatment of HK-374 cells with ONC201 led to a significant, dose-dependent reduction in the plating efficiency of HK-374 cells. Normalizing to the plating efficiency at 0 Gy, 0.5 μ M concentrations of ONC201 caused a drop in the surviving fraction of the cells from $33.1 \pm 3\%$ to $1.5 \pm 0.7\%$ ($p=1.2 \times 10^{-6}$, Student's t-test). Higher concentrations of ONC201 in combination with 2 Gy prevented colony formation completely (**Fig. 1B**).

Gliomaspheres are known to enrich for glioma-initiating cells and gliosphere formation from a single cell is a functional measure of self-renewal capacity^{43,44}. To assess the effect of ONC201 on self-renewal capacity, we first

performed an *in vitro* limiting dilution assay using the patient-derived GBM lines HK-382, HK-374, HK-157, HK-345 and HK-308. Cells were treated with a single dose of radiation (0, 2, 4, 6 or 8 Gy) in the presence or absence of ONC201 (0, 0.5, 1 or 2.5 μ M). Treatment with ONC201 led to a dose-dependent reduction in sphere-formation (**Fig. 1C/E/G/I/K**). Curve fitting of the sphere-forming data using a linear-quadratic model indicated that ONC201 did not act as a classical radiosensitizer for glioma-initiating cells (**Fig. 1D/F/H/J/L**).

Radiation therapy prolongs the survival of patients with GBM, but the tumors almost always recur. In order to test if ONC201 inhibits self-renewal of GBM cells surviving combined treatment of a sublethal dose of radiation and ONC201 we next performed secondary and tertiary sphere formation assays in the absence and presence of ONC201 using cells that survived a combination of 4 Gy and ONC201 (2.5 μ M) (**Fig. 1M**). Irradiated, ONC201-treated cells displayed reduced primary, secondary and tertiary sphere formation in both, the HK-374 line (established from a primary GBM) (**Fig. 1N**) and the HK-308 line (established from a recurrent GBM) (**Fig. 1O**) when compared to primary spheres formed from cells treated with 4 Gy only.

When surviving HK-374 cells from primary spheres from the combined treatment with radiation and ONC201 were further treated with ONC201, the line showed exhaustion of sphere-forming cells in secondary and tertiary spheres (**Fig. 1N**). Primary sphere-formation in the HK-308 line was more than an order of magnitude lower than in the HK-374 line. However, sphere-formation after

additional rounds of ONC201 treatment did not indicate further loss of sphere-forming capacity in secondary or tertiary spheres (**Fig. 1O**).

Gene signature profiles of GBM cells in response to radiation and ONC201

In order to better understand the diverse effects of ONC201 on different populations of GBM cells, including the reduction in viability of bulk cell populations, the loss in clonogenicity and radiosensitization of clonogenic cells as well as the diminished self-renewal of glioma-initiating cells, we performed RNA-Sequencing (RNA-Seq) on HK-374 cells, 48 hours after irradiation and/or ONC201 treatment. Principal component analysis (PCA) showed that biologically-independent replicates of each experimental group clustered closely, indicating reproducibility of the results (**Fig. 2A**). Hierarchical clustering of differentially expressed genes revealed a distinct gene expression profile after combined treatment with radiation and ONC201 (**Fig. 2B**). Radiation caused differential up-regulation of 1711 genes and down-regulation of 1680 genes when compared to DMSO control, while the combination of ONC201 and radiation led to 3048 differentially up-regulated and 3156 down-regulated genes when compared to radiation only. We also compared combination-treated samples and ONC201-treated samples to DMSO-treated controls, and found 3190 differentially up-regulated and 3158 down-regulated genes in combination-treated, and 2989 differentially up-regulated and 3223 down-regulated genes in ONC201-treated cells (**Fig. 2C**). The top 10 up- and down-regulated genes with their log2 fold changes for all four comparisons are shown in **Fig. 2D**.

The top up- and down-regulated genes in cells after combined treatment with radiation and ONC201 compared to irradiated cells were validated using qRT-PCR (**Supplementary Fig. 1**).

Next, we performed a GO enrichment analysis of DEGs in irradiated versus control samples. Differentially up-regulated genes overlapped with gene sets containing extracellular matrix (ECM) components (**Fig. 2E, Top**), while down-regulated genes overlapped with gene sets involved in DNA replication, chromatin binding, and mitotic nuclear division (**Fig. 2E, Bottom**). DEGs in combination-treated versus irradiated cells, overlapped with gene sets involved in catabolism, cellular response to extracellular stimuli, and DNA-binding transcription repressor activity (**Fig. 2F, Top**), while down-regulated DEGs overlapped with gene sets involved in cell adhesion, mitotic nuclear division, and DNA replication (**Fig. 2F, Bottom**).

Thereafter, we assessed global gene expression changes in combination-treated cells relative to irradiated cells using GSEA. DEGs in cells treated with radiation and ONC201 revealed an overlap with the Hallmark gene sets of apoptosis and the p53 pathway gene sets (**Fig. 2G**), consistent with the pro-apoptotic properties of ONC201 and a p53 response to radiation. Furthermore, DEGs up-regulated in response to a combination of radiation and ONC201 overlapped with gene sets of the unfolded protein response, TNF α and IL-6/Stat3 and mTORC1 signaling, IFN γ response, xenobiotic and heme metabolism, genes up-regulated in response to UV and an inflammatory response (**Fig. 2G**). Down-regulated DEGs in cells treated

with radiation and ONC201 overlapped with the gene sets of E2F target, G2M checkpoint, mitotic spindle, apical surface, epithelial mesenchymal transition (EMT) and myogenesis (**Fig. 2H**). Heat maps of genes contributing to the leading edge of each gene set are shown in **Supplementary Fig. 2**.

GBM are known for their intratumoral heterogeneity. A recent study reported the presence of a relatively quiescent cell population in GBM (qGBM) that exhibited self-renewal capacity comparable to its proliferative counterparts (pGBM) but showed higher therapeutic resistance and distinct gene expression signatures³⁵. We had previously confirmed the presence of quiescent and proliferating cells with self-renewal capacity in GBM but also reported recruitment of qGBM cells into the cell cycle in response to radiation⁴⁰. Analyzing our transcriptome data, we observed pattern changes in gene sets associated with cell cycle growth, EMT, metabolism, mitochondria, and stress pathway in response to radiation (**Fig. 2I, left**) consistent with the recruitment of quiescent GBM into the cell cycle. Treatment of the cells with ONC201 led to a down-regulation of genes involved in the G2/M checkpoint, EMT-related genes and genes involved in oxidative phosphorylation, consistent with ClpP agonism, while up-regulating angiogenesis, mTORC1 signaling, UV response, apoptosis, unfolded protein response, TNF α and IL2/Stat5 signaling (**Fig. 2I, middle**). Compared to radiation alone, the combination of radiation and ONC201 reduced the expression of genes involved in G2/M checkpoint control, protein secretion, EMT, oxidative phosphorylation and DNA repair, while increasing the expression of Myc target genes, hypoxia-related

genes, genes involved in xenobiotic metabolism, UV response, $TNF\alpha$, IL2/Stat5, $TGF\beta$ and genes of a general inflammatory response (**Fig. 2I, right**). A subpopulation of GBM cells is thought to acquire and maintain quiescence through ECM organization and interaction with niche factors³⁵.

When compared to irradiated samples, the combined treatment of radiation and ONC201 significantly down-regulated genes involved in ECM interaction, while irradiation or treatment with ONC201 alone did not significantly affect the expression of these genes (**Fig. 2J**). Lastly, when compared to samples treated with radiation alone, the combined treatment reduced the expression of genes overlapping with gene sets of GBM plasticity, glioma stem cells and targets of the developmental transcription factor Oct4 (**Fig. 2K/L**).

ONC201 prolongs survival in mouse models of GBM.

Based on the *in vitro* effect of ONC201 on the self-renewal capacity of patient-derived glioma specimens, we tested whether ONC201 also had anti-tumor activity in a murine model of GBM *in vivo*. In C57BL/6 mice, bearing orthotopic GL261 tumors, a single radiation dose of 10 Gy three days after implantation led to a significant increase in survival (10 Gy + Saline v.s. Saline: 120.5 v.s. 24 days, $p < 0.0001$). Weekly treatment with ONC201 alone had a smaller but significant effect on median survival (ONC201 v.s. Saline: 42 v.s. 24 days, $p < 0.0001$; ONC201 v.s. 10 Gy + Saline: 42 v.s. 120.5 days, $p < 0.0001$). However, a single dose of 10 Gy combined with weekly ONC201 treatments led to a significant increase in survival compared to irradiation or ONC201 alone (10 Gy + ONC201

v.s. 10 Gy + Saline: $p=0.0173$, median survival not reached in 10 Gy + ONC201 group) with 80 % of the animals showing no signs of tumor growth 240 days after the start of the experiment (**Fig. 3A**). Importantly, ONC201 did not show any toxicity and irradiated, ONC201-treated animals showed no weight loss over the course of 240 days (**Fig. 3B**). Bioluminescence imaging of the tumors at day 3 (pre-treatment) and at day 28 (days post-implantation) showed a reduction of signal intensity after combined treatment, consistent with the observed increase in median survival (**Fig. 3C**). Likewise, while a single dose of 10 Gy led to a central necrosis of the tumors and ONC201 treatment alone had little effects, the combined treatment with radiation and ONC201 caused an almost complete regression of the tumors. (**Fig. 3D**). Staining of coronal brain sections of mice that reached euthanasia endpoints against Ki67 did not indicate a reduction in proliferation by radiation or ONC201 alone. However, the combined treatment with radiation and ONC201 decreased the number of proliferating cells at the injection site in animals euthanized at day 227 after tumor implantation (**Fig. 3E**), which was in line with the observed reduction of the bioluminescence signal (**Fig. 3C**), the reduced tumor size in H&E staining (**Fig. 3D**) and the improved median survival after combined treatment (**Fig. 3A**).

Furthermore, immunohistochemistry staining revealed induction of c-Myc expression by radiation, which was in agreement with our previous report on radiation-induced acquisition of a glioma-initiating cell phenotype of previously non-tumorigenic cells⁴⁰. This was also seen after ONC201 treatment but was

completely abolished after combined treatment with radiation and ONC201 (**Fig. 3F**).

Next, we verified these findings using patient-derived orthotopic xenografts. Three days after implantation of HK-374 glioblastoma cells into the striatum of NSG mice, animals were treated with a single dose of 0 or 10 Gy followed by weekly injection of ONC201. Again, ONC201 treatment led to a small increase in median survival (ONC201 v.s. Saline: 60.5 v.s. 34 days, $p < 0.0001$), while a single dose of 10 Gy alone did not significantly prolong median survival (10 Gy + Saline v.s. Saline: 100 v.s. 34 days, $p = 0.1155$; 10 Gy + Saline v.s. ONC201: 100 v.s. 60.5 days, $p = 0.1472$). However, the combination of a single dose of 10 Gy followed by weekly treatments with ONC201 increased overall survival when compared to 10 Gy group (10 Gy + ONC201 v.s. 10 Gy + Saline: $p = 0.0059$, median survival not reached in 10 Gy + ONC201 group) with 70% of the animals showing no signs of tumors growth 250 days after start of the experiment (**Fig. 3G**). Similarly to what was seen in immune-competent animals, ONC201 did not show any toxicity and did not lead to weight loss in NSG mice (**Fig. 3H**).

Altered qGBM-enriched ECM-related gene expression is associated with decreased overall survival and progression-free survival in GBM patients.

To further investigate whether qGBM-enriched ECM-related gene expression is indeed associated with the clinical outcome of GBM patients, we analyzed overall survival (OS) and progression-free survival (PFS) data from 206 GBM patients in

the TCGA Provisional dataset, stratified into subgroups with altered or unaltered expression of one or more ECM-related genes over-expressed in qGBM cells. Patients with increased expression of ECM-related genes showed an inferior median progression-free (5.4 v.s. 8.2 months, p -value=6.745e-3) and overall survival (11.9 v.s. 14.3 months, p -value=0.0357) compared to those with unaltered expression of these genes (**Fig. 4A/B**).

Discussion

Despite decades of research aimed at improving the treatment outcome for patients suffering from GBM, overall survival rates for this disease still remain dismal. The current standard-of-care with postoperative radiation therapy and temozolomide treatment only prolongs progression-free survival by approximately 3 months and almost all tumors recur³. Reasons for the failure of current therapies to control GBM are multiple and include the lack of BBB penetration of drugs used, the infiltrative nature of GBMs, intratumoral heterogeneity, and cellular plasticity^{7,33,45,46}. A large number of studies support that GBM are organized hierarchically with a small number of radio- and chemotherapy-resistant glioma-initiating cells at the apex of that hierarchy, able to re-grow a tumor and give rise to differentiated progeny⁴⁷⁻⁴⁹.

Previous studies have shown expression of dopamine receptor 2 in GBM cells, with elevated DRD2 expression in the GIC population^{50,51}. DRD2 signaling is involved in maintaining self-renewal⁵¹, as well as activating hypoxia response⁵⁰ and functionally altering metabolism of GBM in a potentially autocrine manner⁵⁰, indicating that DRD2 could be a therapeutic target in GBM.

Recent preclinical and clinical data have hinted at an efficacy of ONC201 in several solid cancers including GBM^{21-23,25,27}. Originally identified as an inducer of TRAIL, ONC201 inhibits D2-like dopamine receptors DRD2 and DRD3 and activates the mitochondrial protease ClpP, a key player in the mitochondrial unfolded protein response^{52,53}. So far, clinical trials using ONC201 against GBM

have shown pharmacodynamic activity in biomarker-defined recurrent GBM patients, as well as in pediatric and adult H3 K27M-mutant glioma^{22,26,27}.

We have recently reported that radiation in combination with the first-generation dopamine receptor antagonist trifluoperazine prolonged survival in mouse models of GBM⁴⁰. Here we show that radiation in combination with ONC201 reduced the viability of bulk GBM cells, radiosensitized clonogenic GBM cells and reduced the self-renewal capacity of GICs *in vitro*. Combined treatment led to distinct changes in gene expression, consistent with an induction of cell death, downregulation of DNA repair and elimination of GICs. Furthermore, the combination of ONC201 and radiation prevented the radiation-induced expression of gene sets correlated with GBM plasticity, glioma stem cells, and upregulation of Oct4 targets (a developmental transcription factor⁵⁴) and interfered with cell growth, ECM interaction and EMT genes thought to be involved in neural-to-mesenchymal transition, and the metabolism of GBM cells. This was in agreement with a previous study reporting effects of ONC201 on glioma stem cells⁵⁵.

In vivo, a single dose of radiation combined with ONC201 followed by continuous, weekly ONC201 treatment translated into significantly prolonged median survival in mouse models of GBM when compared to radiation or ONC201 treatment alone. This was accompanied by tumor regression and loss of Ki67 and c-Myc staining, which was consistent with our *in vitro* data and indicated a previously unknown synergism of ONC201 and radiation. At the same time the treatment combination was well tolerated and did not lead to treatment-related toxicity.

Taken together, our data suggests that the combination of radiation with ONC201 severely affects defense mechanisms of GBM cells against radiation without acting as a classical radiosensitizer. We conclude that ONC201 in combination with radiation could be a promising new strategy against GBM that should be tested in clinical trials.

Figure legends

Figure 1

Effects of ONC201 on GBM cells *in vitro*.

(A) Live cell counts of HK-374 cells treated with ONC201 (500 nM, 1 μ M, 2.5 μ M and 5 μ M) or solvent control (DMSO) for 24, 48 or 72 hours. **(B)** Clonogenic assay of patient-derived primary HK-374 GBM cells treated with ONC201 (500 nM, 1 μ M, 2.5 μ M and 5 μ M) or solvent control (DMSO) in combination with a single dose of radiation (0 or 2 Gy). The colony number was counted and presented as the percentage relative to the initial number of cells plated. **(C-L)** Patient-derived HK-374, HK-345, HK-157, HK-382 and HK-308 GBM cells were used to perform sphere-forming assays with sham-irradiated or irradiated cells in the presence or absence of ONC201 (500 nM, 1 μ M, 2.5 μ M). The spheres were cultured in suspension for 7-10 days. The number of spheres formed under each condition were counted and presented as percentage spheres formed and normalized against the sham-irradiated control. **(M)** Schematic flow chart of secondary and tertiary spheres from patient-derived HK-308 and HK-374 GBM cells. **(N/O)** Sphere-forming capacity (SFC%) was evaluated using HK-374 and HK-308 derived secondary and tertiary spheres in the presence or absence of 2.5 μ M ONC201 using a limiting dilution assay. All experiments have been performed with at least 3 biological independent repeats. *p*-values were calculated using two-way ANOVA for A-L; multiple Student's *t*-tests for N and O. * *p*-value<0.05, ** *p*-value<0.01, *** *p*-value<0.001 and **** *p*-value<0.0001.

Figure 2

RNA-Seq analysis of glioma cells treated with ONC201 and radiation.

(A) Principal Component Analysis (PCA) plot displaying all 12 samples along PC1 and PC2, which describe 50.42% and 14.59% of the inter- and intra-group variability respectively, within the expression dataset. PCA was applied to normalized (reads per kilobase of transcript per 1 million mapped reads) and log-transformed count data. **(B)** Heatmap of differentially expressed genes in HK-374 cells, 48 hours after treatment with radiation (4 Gy), ONC201, COMB (ONC201 in combination with radiation) or DMSO control. **(C)** Volcano diagrams of differentially expressed genes in HK-374 cells, 48 hours after treatment with radiation, ONC201 or COMB compared to DMSO-treated control cells. **(D)** Top 10 up- and down-regulated genes with their Log2 fold change in the comparisons, e.g., IR v.s. DMSO, COMB v.s. IR, COMB v.s. DMSO, ONC201 v.s. DMSO. **(E)** The top 20 significantly enriched signaling pathways in the Gene Ontology (GO) database between IR and DMSO. Up-regulated in IR v.s. DMSO (top) and down-regulated in IR v.s. DMSO (bottom). **(F)** The top 20 significantly enriched signaling pathways in the Gene Ontology (GO) database between COMB and IR. Up-regulated in COMB v.s. IR (top) and down-regulated in COMB v.s. IR (bottom). **(G/H)** GSEA results of the gene sets that are positively or negatively enriched in the combination of ONC201 and radiation as compared to radiation alone. NES, normalized enrichment score. **(I)** GSEA of quiescent GBM (qGBM) gene signatures in the comparisons IR v.s. DMSO, COMB v.s. IR, and ONC201 v.s. DMSO, displayed with normalized enrichment scores. Red indicates the gene sets highly expressed

in qGBM, while the blue represents gene sets with low expression in qGBM. The nominal p -value was labeled with * <0.05 ; ** <0.01 ; *** <0.001 . **(J)** Heatmap of ECM-related gene expressions in the comparisons, e.g., IR v.s. DMSO, COMB v.s. IR, ONC201 v.s. DMSO, stratified by whether these genes are up- and down-regulated in qGBM. **(K/L)** GSEA results of the gene sets of glioblastoma plasticity, glioma stem cells and targets of the developmental transcription factor Oct4 in the combined treatment of ONC201 and radiation (COMB) compared to radiation alone (IR). Enrichment plots and corresponding heatmaps displayed.

Figure 3

Combination of ONC201 and radiation prolongs survival in mouse models of glioblastoma.

(A) Survival curves for C57BL/6 mice implanted intra-cranially with 2×10^5 GL261-GFP-Luciferase mouse glioma cells. Tumors were grown for 3 days for successful grafting. Mice were irradiated with a single fraction of 0 or 10 Gy and weekly treated with Saline or ONC201 (50 mg/kg, i.p.) continuously until they reached the study endpoint. Log-rank (Mantel-Cox) test for comparison of Kaplan-Meier survival curves indicated significant differences in the ONC201 treated mice compared to their respective controls. ONC201 v.s. Saline (**** p -value <0.0001), 10 Gy + Saline v.s. Saline (**** p -value <0.0001), 10 Gy + ONC201 v.s. Saline (**** p -value <0.0001), 10 Gy + ONC201 v.s. 10 Gy + Saline (* p -value=0.0173). **(B)** Weight curves for the C57BL/6 mice in the different treatment groups. **(C)** Bioluminescence images of mice bearing tumors obtained at day 3 (pre-treatment)

and at day 28 (days post implantation). **(D)** H&E stained coronal sections of the C57BL/6 mice brains implanted with GL261-GFP-Luc cells which were treated continuously with either ONC201 or saline until they met the criteria for study endpoint. **(E/F)** 4x, 10x, 20x and 40x images of Ki67 and c-Myc stained coronal sections of brains from C57BL/6 mice implanted with GL261-GFP-Luc cells and treated with either ONC201 (50 mg/kg) or saline in the presence or absence of a single dose of radiation (10 Gy) at the study endpoint. The brain samples for ONC201 in combination with radiation were from the mice at post-op day 227 (the mice did not reach the study endpoint). **(G)** Survival curves for NSG mice implanted intra-cranially with 3×10^5 HK374-GFP-Luciferase patient-derived GBM cells. Tumors were grown for 3 days for successful grafting. Mice were irradiated with a single fraction of 0 or 10 Gy and weekly treated with Saline or ONC201 (50 mg/kg, i.p.) continuously until they reached the study endpoint. Log-rank (Mantel-Cox) test for comparison of Kaplan-Meier survival curves indicated significant differences in the ONC201 treated mice compared to their respective controls. ONC201 v.s. Saline (**** p -value<0.0001), 10 Gy + Saline v.s. Saline (n.s. p -value=0.1155), 10 Gy + ONC201 v.s. Saline (**** p -value<0.0001), 10 Gy + ONC201 v.s. 10 Gy + Saline (** p -value=0.0059). **(H)** Weight curves for the NSG mice in the different treatment groups.

Figure 4

Evaluation of qGBM-enriched ECM-related gene signatures in TCGA glioblastoma patients.

(A) The overall survival (OS) in glioblastoma patients from TCGA stratified by qGBM-enriched ECM-related gene expression. OS in qGBM-enriched ECM-related gene altered group (higher expression) was significantly worse than that of the unaltered group (log rank test, p -value=0.0357). **(B)** The progression-free survival (PFS) in glioblastoma patients from TCGA stratified by qGBM-enriched ECM-related gene expression. PFS in qGBM-enriched ECM-related gene altered group (higher expression) was significantly worse than that of the unaltered group (log rank test, p -value=6.745e-3).

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Figure 1.

Figure 1.

Figure 1.

Figure 1.

K

L

Figure 1.

M

N

O

Figure 2.

E

IR v.s. DMSO_Upregulated

Significantly enriched GO terms (Top 20)

IR v.s. DMSO_Downregulated

Cellular Component Biological Process Molecular Function

F

COMB v.s. IR_Upregulated

COMB v.s. IR_Downregulated

Cellular Component Biological Process Molecular Function

G

H

I

IR v.s. DMSO

ONC201 v.s. DMSO

COMB v.s. IR

J

ECM-related genes

K

L

ZHENG_GLIOMASTOMA_PLASTICITY_UP

ZHENG_GLIOMASTOMA_PLASTICITY_DN

BEIER_GLIOMA_STEM_CELL_UP

BENPORATH_OCT4_TARGETS

Figure 3.

Figure 3.

Figure 3.

Figure 4.

A

B

Supplementary Table 1

Gene name	Primer sequence (5'–3')
PAPPA2	Forward: AGAAGTGTCTCTGTTTCCTTGG Reverse: CTGTTCACTCCTGTCATCCTG
ADM2	Forward: AGGGAGGGGAACTCAGCAGTTCAGGAG Reverse: GTTCTTGTTCTTGCTGTCACTTGGGCCT
KLHDC7B	Forward: GTCCTCAGTCCATTTCATCCTG Reverse: AGCTTTGGTTCTCTGCCTG
INHBE	Forward: AAGACGGATGTGCCAGATATG Reverse: GATGCCTCCAGTCACAGATG
GTSE1	Forward: AAGTACTGCCACAGAAGTAGC Reverse: CGATGAGAGGAAGGTCAATGAG
BUB1B	Forward: CCGAGTCTCAGAAAATACCAGG Reverse: AAATGGAGAAAGGTACACTGGG
UBE2C	Forward: TGATGTCAGGACCATTCTGC Reverse: CTGGTGACCTGCTTTGAGTAG
KIFC1	Forward: TGGTAGTGCTAAGATGCTCATG Reverse: ATCCGTCTTCACTTCCTGTTG
ASF1B	Forward: CCTTTCCACAGCCCCTTC Reverse: AAATTCCTCACTCTCAGCCG
IPO8	Forward: CGAAGTTGCGGATTGCAG Reverse: GAATTCCACATGGTCAGAGACT
TBP	Forward: TGCACAGGAGCCAAGAGTGAA Reverse: CACATCACAGCTCCCCACCA
PPIA	Forward: ATGCTGGACCCAACACAAAT Reverse: TCTTTCACCTTGCCAAACACC

Supplementary Figure S1

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Top four up- and five down-regulated genes in cells after combined treatment with radiation and ONC201 compared to irradiated cells and their validation by qRT-PCR. All experiments have been performed with 3 biological independent repeats.

Supplementary Figure s2

A

Hallmark_Unfolded_Protein_Response

Hallmark_TNFa_Signaling_Via NFkB

Hallmark_IL6_JAK_STAT3_Signaling

Hallmark_P53_Pathway

Hallmark_Interferon_Gamma_Response

Gene name	COMB_1	COMB_2	COMB_3	IR_1	IR_2	IR_3
MTHFD2						
APOL6						
WARS1						
PTGS2						
UPP1						
EIF4E3						
IL6						
NAMPT						
RBCK1						
PIM1						
IRF1						
SOD2						
TNFAIP6						
TRIM25						
PLSCR1						
IFI44L						
BTG1						
CASP4						
IRF7						
JAK2						
ICAM1						
SECTM1						
C1S						
CDKN1A						
DHX58						
NCOA3						
NFKB1A						
UBE2L6						
RIPK2						
MT2A						
HELZ2						
PTPN2						
TAP1						
TNFAIP2						
PARP14						
TOR1B						
RNF213						
HLA-B						
ISOC1						
CD74						
IL4R						
CIITA						
EIF2AK2						
LGALS3BP						
TAPBP						
PML						
TNFAIP3						
LAP3						
NLRCS						
PFKP						
LY6E						
STAT3						
AUTS2						
NUP93						
TRIM21						
CASP8						
SP110						
HIF1A						
PDE4B						
TRAFD1						
MVP						
SAMHD1						
IFIIM2						
USP18						
PSME2						
PNP						
CMKLR1						
CFH						
SSPN						
IFIIM3						
IFI44						
TRIM14						
VAMP5						
HLA-DMA						
DDX58						
OASL						
ST3GAL5						
OAS2						
ST8SIA4						
ISG20						
BST2						
OAS3						

Hallmark_Xenobiotic_Metabolism

Gene name	COMB_1	COMB_2	COMB_3	IR_1	IR_2	IR_3
RAP1GAP						
SLC1A5						
IGFBP1						
UPP1						
HMOX1						
IDH1						
SMOX						
AKR1C2						
CES1						
PTGES						
ID2						
ETS2						
NINJ1						
ALDH3A1						
NPC1						
LONP1						
CYP1A1						
BLVRB						
KYNU						
ABCC3						
SHMT2						
ABCC2						
POR						
GCLC						
BCAT1						
N001						
ALDH2						
GABARAPL1						
AKR1C3						
AOP9						
MT2A						
IGFBP4						
PGD						
CYP27A1						
PMT						
PDK4						
TKFC						
COMT						
ALAS1						
ECH1						
TMBIM6						
IL1R1						
PPARD						
UGDH						
FBLN1						
PTGR1						
GSR						
ENTPD5						
ADH5						
KARS1						
EPHX1						
MTHFD1						
EPHA2						
MCCC2						
TPST1						
MAN1A1						
GSTO1						
ACP2						
CYB5A						
SLC35D1						
ATP2A2						
HPRT1						
XDH						
CROT						
BCAR1						
PGRM1						
ASL						
AP4B1						
SSR3						
GAD1						
PC						
GCNT2						
SLC6A6						
DHRS1						
PAPSS2						
SERPINE1						
TMEM97						
CA2						
CD36						
ACOX2						
FM03						
JUP						

Hallmark_Heme_Metabolism

Gene name	COMB_1	COMB_2	COMB_3	IR_1	IR_2	IR_3
ASNS						
SLC7A11						
RAP1GAP						
SLC6A9						
MOCOS						
TCEA1						
KDM7A						
SMOX						
MXI1						
SLC30A1						
FN3K						
BLVRB						
KLF3						
SLC25A37						
EZH1						
OPTN						
BTG2						
PGLS						
NFE2L1						
H1-0						
ERMAP						
C3						
GCLM						
GCLC						
CCDC28A						
SDCBP						
CTNS						
ELL2						
RIOK3						
RAD23A						
GYPC						
HEBP1						
DCUN1D1						
NR3C1						
FOXJ2						
DCAF10						
KHNYN						
YPEL5						
TERC						
MEFAS1						
MOSPD1						
MKRN1						
TRAK2						
BACH1						
VEZF1						
GMP5						
MARK3						
CLCN3						
CIR1						
MB0AT2						
REMS						
DCAF11						
FBX07						
XP07						
BCAM						
CTSB						
SLC6A8						
EIF2AK1						
PAHA2						
CP0X						
UR05						
NARF						
TFDP2						
SIDT2						
NEK7						
NNT						
FBX09						
TOP1						
CDC27						
PICALM						
SEC14L1						
PRDX2						
TMCC2						
ALAD						
UCP2						
TSPAN5						
ATG4A						
E2F2						
PC						
MPP1						
SELENBP1						
LM02						
ENDOD1						
ANK1						
CA2						
SLC2A1						
CND3						
MAP2K3						
KAT2B						
SLC30A10						

Hallmark_MTORC1_Signaling

Gene name	COMB_1	COMB_2	COMB_3	IR_1	IR_2	IR_3
	COMB_1	COMB_2	COMB_3	IR_1	IR_2	IR_3
	COMB_1	COMB_2	COMB_3	IR_1	IR_2	IR_3
	COMB_1	COMB_2	COMB_3	IR_1	IR_2	IR_3
	COMB_1	COMB_2	COMB_3	IR_1	IR_2	IR_3
SLC1A4						
ASNS						
PSAT1						
TRIB3						
SLC7A11						
SLC7A5						
CTH						
PHGDH						
VLDLR						
MTHFD2						
DDIT3						
NUPR1						
SLC3A5						
WARS1						
IFED1						
TCEA1						
PSPH						
NEFL3						
DDIT4						
PPP1R15A						
IDH1						
NAMPT						
EIF2S2						
TXNRD1						
HSPA9						
EPRS1						
ME1						
SOSTM1						
BTG2						
SHMT2						
CDKN1A						
GCLC						
GOT1						
PSMG1						
BCAT1						
XBP1						
NIBAN1						
FADS1						
TFRC						
G6PD						
INSIG1						
SLC2A3						
GTF2H1						
GMP5						
GSR						
RTT1						
LTA4H						
CACYP						
ETEF1						
CONG1						
CCT6A						
PSMC4						
PSME3						
PSMD13						
CANX						
USO1						
ACTR3						
PSMC2						
PROX1						
PPIA						
RAB1A						
GGA2						
GLRX						
SSR1						
CYB5B						
ADIPOR2						
SERP1						
YKT6						
STIP1						
TUBG1						
PSMB5						
SC5D						
PDAP1						
SERPINH1						
POLR3G						
UCHL5						
BHLHE40						
GPI						
ACACA						
TOMM40						
ATP2A2						
ODPR						
ENO1						
HPRT1						
HSPA5						
MLLT11						
PAHA1						
RPN1						
CTSC						
EKBP2						
HMGR						
ELOVL6						
ITGB2						
LDLR						
PNP						
SORD						
TM7SF2						
PFKF						
SCD						
HK2						
GIA						
ADD3						
DHCR7						
CNF						
TP11						
PPA1						
MCW4						
FADS2						
SLC6A6						
DDX39A						
TUBA4A						
STC1						
PDK1						
MCW2						
PGK1						
ATPSMC1						
HMGCS1						
SOLE						
PIOD2						
EBP						
AK4						
TMEM97						
SLC2A1						
LDHA						
STARD4						
PIK3R3						
MAP2K3						
DHCR24						
CAR						
EGLN3						
HSP90B1						
DHER						
SYTL2						
SDF2L1						
CORO1A						
AURKA						
CDC25A						
PLK1						
IGFBP5						
BUB1						
RRM2						

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Hallmark_Inflammatory_Response

Gene name	COMB_1	COMB_2	COMB_3	IR_1	IR_2	IR_3
	COMB_1	COMB_2	COMB_3	IR_1	IR_2	IR_3
	COMB_1	COMB_2	COMB_3	IR_1	IR_2	IR_3
	COMB_1	COMB_2	COMB_3	IR_1	IR_2	IR_3
	COMB_1	COMB_2	COMB_3	IR_1	IR_2	IR_3
P2RX7						
EREG						
CSAR1						
IL1A						
IL1B						
TNFRSF9						
CD55						
P2RX4						
AHR						
IL6						
NAMPT						
IRF1						
TNFAIP6						
CXCL8						
ITGB3						
IRF7						
ABCA1						
SLC31A1						
ICAM1						
BTG2						
FZD5						
MSR1						
CDKN1A						
TNHA						
KCNJ2						
SLC7A1						
NEKB1A						
ACVR2A						
AOP9						
TPBG						
RIPK2						
ATP2B1						
MMP14						
ADORA2B						
F3						
PSEN1						
TRAK2						
IL4R						
IL1R1						
HAS2						
GABBR1						
EIF2AK2						
RELA						
ACVR1B						
TIMP1						
ABI1						
PVR						
TAPBP						
ADM						
SPHK1						
ITGA5						
LIF						
LY6E						
ITGB8						
EMP3						
SELENOS						
ATP2A2						
KLF6						
HIF1A						
PDE4B						
KIF1B						
MET						
LDLR						
CMKLR1						
IFITM1						
ADGRE1						
DCBLD2						
SGMS2						
CCL24						
SERPINE1						
MYC						
CD70						
PTGER2						
SLCAA4						
RG516						
CXCL6						
BST2						
NDP						
CD82						
HBEGF						
EDN1						
IL7R						
CX3CL1						

Hallmark_UV_Response_UP

Gene name	COMB_1	COMB_2	COMB_3	IR_1	IR_2	IR_3
	COMB_1	COMB_2	COMB_3	IR_1	IR_2	IR_3
	COMB_1	COMB_2	COMB_3	IR_1	IR_2	IR_3
	COMB_1	COMB_2	COMB_3	IR_1	IR_2	IR_3
	COMB_1	COMB_2	COMB_3	IR_1	IR_2	IR_3
ASNS						
ATF3						
CEBPg						
CXCL2						
HM0X1						
IL6						
MAPK8IP2						
IRF1						
CDKN1C						
SOD2						
TARS1						
CYP1A1						
BTG1						
HSPA13						
ICAM1						
SOSTM1						
EIF5						
BTG2						
NEKB1A						
CHKA						
E2F5						
EIF2S3						
NRAA1						
MMP14						
TAP1						
TFRC						
JUNB						
GRINA						
CDC34						
PRKCD						
ALAS1						
TMBIM6						
IL6ST						
DLG4						
ATP6V1C1						
EPHX1						
DDX21						
SLC6A8						
HNRNPJ						
ENO2						
DNAJB1						
PSMC3						
NXF1						
CYB5B						
YKT6						
STIP1						
CNP						
PI1F						
PRPF3						
PDAP1						
KLHDC3						
DNAJA1						
POLR2H						
ACAA1						
FURIN						
SIGMAR1						
TYRO3						
CYB5R1						
MRPL23						
BID						
PRKACA						
MSX1						
GLS						
C1CN2						
POLR3						
RPN1						
CLTB						
RHOB						
SLC25A4						
AMD1						
GGH						
SELENOV						
HYAL2						
GPX3						
H2AX						
IGFBP2						
TUBA4A						
CDK2						
NPTX2						
CA2						
CND3						
FEN1						

Hallmark_Apopt

Hallmark_E2F_Targets

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Hallmark_G2M_Checkpoint

Hallmark_Mitotic_Spindle

Hallmark_Epithelial_Mesenchymal_Transition

Gene name	COMB_1	COMB_2	COMB_3	IR_1	IR_2	IR_3
ECM2						
CADM1						
MMP1						
FZD8						
ESTL3						
IL6						
BGN						
FGF2						
CXCL8						
SNAT2						
ID2						
MMP3						
PTX3						
GADD45A						
ITGB3						
NID2						
LAMA1						
VEGFA						
TNFR						
SLIT2						
TFPI2						
PMP22						
IGFBP4						
GADD45B						
TGFB1						
ANPEP						
LRP1						
MMP14						
TGFB3						
IGFBP3						
LRC15						
FBLN1						
FBN2						
TIMP1						
COL6A3						
PVR						
PRRX1						
SLC6A8						
MMP2						
HTRA1						
LAMC1						
FN1						
ENO2						
COL6A2						
ITGA5						
TNFAIP3						
LOX						
NTM						
CALU						
COL4A1						
LGALS1						
FSTL1						
COLGALT1						
SCG2						
SERPINH1						
PEN2						
MCW7						
PCOLCE						
EMP3						
DST						
LOXL2						
SDC1						
EUCAL						
EFEMP2						
VIM						
MSX1						
CD44						
CDH11						
TPM2						
JUN						
CALD1						
COL4A2						
ECM1						
RHOB						
ACTA2						
GEM						
MYLK						
GJA1						
PCOLCE2						
MYL9						
BDNF						
ITGAV						
FAP						
BMP1						
PLD1						
P3H1						
SAT1						
ITGB5						
TNC						
ELN						
COL5A1						
IGFBP2						
CDH2						
POLM4						
SERPINE1						
LOXL1						
DKK1						
TAGLN						
SERP1						
MFAP5						
PLD1						
PMEPA1						
TIMP3						
THBS1						
THY1						
TGM2						
PTIB						
PTH1H						
DYSL3						
APLP1						
CD59						
CHRC1						
TPM1						
CNN1						
COL1A2						
MEST						
ADAM12						
MATN2						
GLPR1						
COL12A1						
COL5A2						
SPARC						
CXCL6						
COL11A1						
MGP						
COL1A1						
RG54						
COL3A1						
CNN2						
MXRA5						

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Hallmark_Myogenesis

Gene name	COMB_1	COMB_2	COMB_3	IR_1	IR_2	IR_3
STC2						
COL15A1						
IFRD1						
KLF5						
PTPA43						
FGF2						
SGCA						
CDKN1A						
FST						
NOO1						
GAA						
TNNT1						
BIN1						
DMPK						
GADD45B						
FHL1						
AGRN						
IGFBP3						
TPD52L1						
ITGB4						
NOTCH1						
PRNP						
NAV2						
CRAT						
TEAD4						
CDH13						
PLXNB2						
COL6A3						
MEF2A						
RIT1						
PDE4DIP						
FLI1						
MRAS						
MEF2D						
SLC6A8						
SPHK1						
COL6A2						
PDLIM7						
PEKM						
TPM3						
MAPRE3						
BHLHE40						
AKT2						
CLU						
TPM2						
PSEN2						
SOD3						
COL4A2						
MYLK						
HSPB8						
SPTAN1						
PKIA						
ITGB5						
GPX3						
FDPS						
PC						
DTNA						
SMTN						
SVIL						
CHRNA1						
GSN						
PPP1R3C						
LSP1						
VIPR1						
PPIA4						
FABP3						
TAGLN						
LPIN1						
MEF2C						
CNN3						
WMT1						
PTGIS						
CD36						
CRYAB						
CKB						
ADAM12						
SPARC						
DMD						
COL1A1						
HBEF						
MYBP						
COL3A1						
CSRP3						

Hallmark_Myc_Target_V2

Gene name	COMB_1	COMB_2	COMB_3	IR_1	IR_2	IR_3
ATMP2						
SUPV3L1						
NPM1						
NOLC1						
PEST1						
PPRC1						
MYBBP1A						
GRND1						
PA2G4						
PBB						
LAS1L						
BYSL						
TBRG4						
UTP20						
RABEPK						
SLC19A1						
GNL3						
SORD						
SRM						
HK2						
DCTPP1						
MCM4						
SLC29A2						
MYC						
TMEM97						
MCM5						
PLK1						
PLK4						

Hallmark_Apical_Junction

Gene name	COMB_1	COMB_2	COMB_3	TR_1	TR_2	TR_3
ADAMTS5						
CLDN14						
PCDH1						
PKD1						
ICAM1						
MDK						
CLDN15						
SLIT2						
AMIGO2						
PARD6G						
AKT3						
PTEN						
TGFB1						
ITGB4						
TSC1						
NF1						
INSIG1						
RAS1						
GNAI1						
BAGALT1						
ITGA3						
CRAT						
TMEM8B						
CDH4						
EXOC4						
CDK8						
TJP1						
RRAS						
SRC						
BATAP2						
MMP2						
MPZL1						
RSU1						
ZYX						
ADAM15						
FLNC						
GTF2F1						
STRPA						
LIMA1						
YWHAH						
PARVA						
VASP						
PLCG1						
ACTN1						
GNAI2						
TUBG1						
ACTG1						
SDC3						
CAP1						
EVL						
AKT2						
TRO						
TAOK2						
TSPAN4						
SHC1						
CTNND1						
CNN2						
CDH11						
CADM3						
MYH10						
EPB41L2						
MVD						
EGFR						
ACTB						
CD99						
NEFASC						
LAYN						
FSCN1						
MYL9						
NEXN						
DLG1						
ADAM9						
BMP1						
ACTN4						
NECTIN3						
CERCAM						
VCL						
MSN						
NF2						
CLDN11						
THY1						
NEGR1						
ITGA10						
PIK3R3						
MNK4						
JUP						
SHROOM2						
ACTG2						
COL17A1						
CX3CL1						

Heat maps of genes contributing to the leading edge of the up-regulated DEGs overlapped gene sets of the unfolded protein response, TNFa/NFkB, IL-6/JAK/Stat3, P53 and mTORC1 signaling, IFN gamma response, xenobiotic and heme metabolism, inflammatory, UV response and apoptosis **(A)**. Heat maps of genes contributing to the leading edge of the down-regulated DEGs overlapped gene sets of E2F targets, G2/M checkpoint, Mitotic spindle, apical surface, epithelial-mesenchymal transition, myogenesis, Myc target and apical junction **(B)**.