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

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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 (GICs), responsible for therapy resistance and tumor recurrence, suggesting that targeting GICs 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

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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.

Conflict of Interest: J.E.A. is an employee and shareholder of Oncoceutics and has ownership interest (including patents) in Oncoceutics. F.P. holds stock in Chimerix Inc.. T.F.C. is cofounder, major stock holder and board member of Katmai Pharmaceuticals, Member of the board for the 501c3 Global Coalition for Adaptive Research, holds stock option of Notable Labs, has provided consulting services to QED, Roche, Trizel, Medscape, Bayer, Amgen, Odonate Therapeutics, Pascal Biosciences, Bayer, Del Mar Pharmaceuticals, Tocagen, Karyopharm, GW Pharma, Kiyatec, Abbvie, Boehinger Ingelheim, VBI, Deciphera, VBL, Agios, Merck, Roche, Genocea, Celgene, Puma, Lilly, BMS, Cortice, Wellcome Trust, Novocure, Novogen, Boston Biomedical, Sunovion, Human Longevity, Insys, ProNai, Pfizer, Notable labs, Medqia and has contracts with UCLA for the Brain Tumor Program with Amgen, Abbvie, DNAtrix, Beigene, BMS, AstraZeneca, Kazia, Agios, Boston Biomedical, Deciphera, Tocagen, Orbus, AstraZenica, Karyopharm. No potential conflicts of interests are disclosed by the other authors.

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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

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 treatment [1]. 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 intratumoral heterogeneity and plasticity of GBM [2-6]. Past attempts to improve survival using classical chemotherapeutic drugs or immunotherapy have been hampered by the inability of many drugs or biologics to cross the blood brain barrier (BBB) [7-9].

ONC201 is the first member of a novel class of anti-cancer small molecules called imipridones, originally discovered by screening for p53-independent inducers of the immuno-surveillance cytokine TNF-related apoptosis-inducing ligand (TRAIL) and tumor cell death in human colorectal cancer cells [10, 11]. With p53-independency, low toxicity and excellent BBB penetration, ONC201 is currently being evaluated in multiple clinical trials for select advanced malignancies [12-19].

Emerging data suggest that a hierarchical organization within glioblastoma plays a crucial role in tumor development, therapy resistance and tumor recurrence [20-23] and interactions between tumor cells and the microenvironment are involved in tumor progression and the invasive nature of GBM [24]. Strategies to eradicate GICs 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 radio-therapy largely target only the proliferative cell population [25-28]. 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

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overall survival in mouse models of GBM *in vivo*. Transcriptome analyses revealed shifts in gene 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 Oncoceutics, Inc. (Philadelphia, PA, USA). A 10 mM stock solution was prepared with dimethylsulfoxide (DMSO) for all *in vitro* experiments and 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.

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.

A detailed description of the cell culture conditions, *in-vitro* limiting dilution assay, clonogenic and cell viability assays, *in-vivo* bioluminescent imaging, irradiation procedures, immunohistochemistry, immunofluorescent staining, western blot, quantitative reverse transcription-PCR and RNA-sequencing is available in Supplementary Material. Primers are listed in Supplementary Table1. The RNA-Seq data are available via GEO accession number GSE153982.

TCGA data mining and analysis

The TCGA Provisional dataset (captured June 1, 2020) was accessed via cBioPortal [29, 30]. Kaplan-Meier estimates were calculated for patients with upregulation (z-score >1.5) in one or more genes enriched in qGBM cells (gene list is available in Supplementary Material) and compared to patients with no alterations in the expression of those genes.

Statistics

All data were represented as means ± standard error mean for at least 3 independent biological samples. All analyses were performed in the GraphPad Prism 8.0 software. An unpaired two-sided Student's t-test or Two-way ANOVA with a post hoc Bonferroni adjustment were used for comparisons. A log-rank test was used for Kaplan-Meier estimates. A p-value≤0.05 was considered as statistically significant.

Results

ONC201 has been previously studied in diffuse intrinsic pontine gliomas (DIPG) carrying the H3K27 mutation and is a known inhibitor of DRD2/3 and an agonist of ClpP. In order to demonstrate target engagement after treatment of glioma cells that do not carry the H3K27 mutation we treated HK-374 and HK-382 glioma cells with ONC201 for 1 hour and performed Western blotting for Erk/p-Erk and Akt/p-Akt. ONC201 down-regulated

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p-Akt and -to a lesser extent- pErk indicating that ONC201 inhibited DRD2/3 in both lines [31]. Likewise, treatment with ONC201 for 24 hours increased protein levels of ATF4 and TRAIL, consistent with an activation of ClpP [31] (Supplementary Fig. 1).

A radiobiological gold-standard for assessing the radiosensitizing properties of drugs is the clonogenic survival assay. Treatment of both HK-374 and HK-382 cells with ONC201 led to a significant, dose-dependent reduction in plating efficiency. Normalized to 0 Gy controls, ONC201 (0.5 μM) decreased the surviving fraction from 33.1 ± 3% to 1.5 ± 0.7% (p=1.2x10^{-6}) in HK-374 cells (Fig. 1A), while ONC201 (1 μM) decreased the surviving fraction from 22.2 ± 1.5% to 0.7 ± 0.12% (p=3.9x10^{-3}) in HK-382 cells (Fig. 1B). Higher concentrations of ONC201 in combination with 2 Gy prevented colony formation completely (Fig. 1C/D, Supplementary Fig. 2).

In order to assess an effect of ONC201 on DNA repair, we irradiated HK374 GBM in the presence/absence of ONC201. Using γH2AX foci as surrogate markers for DNA double strand breaks (DSBs) we found that combined treatment of radiation and ONC201 significantly increased the number of initial DSBs at 1 hour when compared to the DMSO or radiation controls. Likewise, the combination treatment significantly increase the number of residual DSBs at 24 hours (Fig. 1 C/D), thus indicating interference of ONC201 with DNA DSB repair.

Gliomaspheres are known to enrich for GICs and gliomasphere formation from a single cell is a functional measure of self-renewal capacity [32, 33]. 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 (Table 1). 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 μM). Treatment with ONC201 led to a dose-dependent reduction in sphere-formation (Fig. 1E/G, Supplementary Fig. 3B/D/F). Curve fitting of the sphere-forming data using a linear-quadratic model indicated that ONC201 did not radiosensitize GICs (Fig. 1F/H, Supplementary Fig. 3C/E/G).

Radiotherapy prolongs the survival of GBM patients, 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 performed secondary and tertiary sphere-formation assays in the absence and presence of ONC201 (Supplementary Fig. 3H). Combined treatment reduced primary, secondary and tertiary sphere-formation in both, the HK-374 line (established from a primary GBM) (Fig. 1I) and the HK-308 line (established from a recurrent GBM) (Fig. 1J) when compared to spheres formed from cells treated with 4 Gy only.

In order to better understand the effects of ONC201 in combination with radiation, we performed RNA-Sequencing on HK-374 cells. Principal component analysis showed that biologically-independent replicates of each experimental group clustered closely, indicating reproducibility of the results (Supplementary Fig. 4A). Hierarchical clustering of differentially expressed genes revealed a distinct gene expression profile after combined treatment (Supplementary Fig. 4B). Radiation caused differential up-regulation of 1711

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genes and down-regulation of 1680 genes when compared to DMSO control, while combined treatment 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 (Supplementary Fig. 4C). The top 10 up- and down-regulated genes with their $\log_2$-fold changes are shown in Supplementary Fig. 4D. The top up- and down-regulated genes in cells after combined treatment compared to irradiated only were validated using qRT-PCR (Supplementary Fig. 4E).

Next, we performed a GO enrichment analysis of DEGs in irradiated versus control samples. Differentially up-regulated genes overlapped with gene sets containing ECM components (Supplementary Fig. 4F, **Top**), while down-regulated genes overlapped with gene sets involved in DNA replication, chromatin binding, and mitotic nuclear division (Supplementary Fig. 4F, **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 (Supplementary Fig. 4G, **Top**), while down-regulated DEGs overlapped with gene sets involved in cell adhesion, mitotic nuclear division, and DNA replication (Supplementary Fig. 4G, **Bottom**).

Assessing global gene expression changes using GSEA in combination-treated versus irradiated cells we found DEGs in combination-treated cells overlapping with the Hallmark gene sets of apoptosis and the p53 pathway (Fig. 2A), consistent with the pro-apoptotic properties of ONC201 and a p53 response to radiation. Furthermore, DEGs up-regulated in response to combined treatment overlapped with gene sets of the unfolded protein response, TNFα and IL-6/Stat3 and mTORC1 signaling, IFNγ response, xenobiotic and heme metabolism, genes up-regulated in response to UV and an inflammatory response (Supplementary Fig. 4H). Down-regulated DEGs in combination-treated cells overlapped with the gene sets of E2F targets, G2M checkpoint, mitotic spindle, apical surface, epithelial mesenchymal transition (EMT) and myogenesis (Fig. 2B, Supplementary Fig. 4J). Heat maps of genes contributing to the leading edge of each gene set are shown in Supplementary Fig. 4I/K.

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 signatures [25]. We had previously confirmed the presence of quiescent and proliferating cells with self-renewal capacity in GBM and also reported recruitment of qGICs cells into the cell cycle in response to radiation [34]. 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 (Supplementary Fig. 4L, **left**) consistent with the recruitment of qGICs 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,

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mTORC1 signaling, UV response, apoptosis, unfolded protein response, TNFα and IL2/Stat5 signaling (Supplementary Fig. 4L, **right**). Compared to radiation alone, combined treatment 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, TNFα, IL2/Stat5, TGFβ and inflammatory response (Fig. 2C). A subpopulation of GBM cells is thought to acquire and maintain quiescence through ECM organization and interaction with niche factors [25].

When compared to irradiated samples, the combined treatment 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. 2D). Western blotting confirmed this effect in a subset of proteins down- or up-regulated in qGICs (Fig. 2E). Lastly, when compared to 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. 2F, Supplementary Fig. 4M).

Based on the *in-vitro* effect of ONC201 on the self-renewal capacity, 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) and no toxicity (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 survival (Fig. 3C). Likewise, a single dose of 10 Gy led to a central necrosis of the tumors and ONC201 treatment alone had little effect, while the combined treatment caused an almost complete regression of the tumors (Fig. 3D). Ki67 staining of coronal brain sections of mice that reached euthanasia endpoints did not indicate a reduction in proliferation by radiation or ONC201 alone. However, the combined treatment decreased the number of proliferating cells at the injection site (Fig. 3E), which was in line with the observed reduction of the bioluminescence signal (Fig. 3C), the reduced tumor size (Fig. 3D) and the improved survival after combined treatment (Fig. 3A).

Furthermore, immunohistochemistry staining revealed induction of c-Myc expression by radiation, which agreed with our previous report on radiation-induced acquisition of a GIC phenotype of non-tumorigenic cells [34]. This was also seen after ONC201 treatment but was completely abolished after combined treatment (Fig. 3F). A single dose of 10 Gy or ONC201 treatment reduced the number of CD133-positive cells in GL261 tumors. Combined treatment with radiation and ONC201 further reduced the number of CD133-positive and reduced the number of Ki67-positive cells (Fig. 3G).

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Next, we verified these findings using patient-derived orthotopic xenografts. The same implantation and treatment procedures were performed with HK-374 glioblastoma cells in NSG mice. 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 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 combined treatment 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. 3H) and toxicity (Fig. 3I).

To further investigate whether qGBM-enriched ECM-related gene expression is indeed associated with clinical outcome in GBM, 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 PFS (5.4 *v.s.* 8.2 months, *p*-value=6.745e-3) and OS (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 GBM patients, OS for this disease still remain dismal. The current standard-of-care with postoperative radiotherapy and temozolomide treatment prolongs PFS by only 3 months and almost all tumors recur [35]. Reasons for treatment failure include the lack of BBB penetration of drugs, the infiltrative nature of GBMs, cellular plasticity and intratumoral heterogeneity [4, 36, 37] with a small number of therapy-resistant GICs able to re-grow a tumor [38-40].

Previous studies have shown expression of DRD2 in GBM, with elevated DRD2 expression in the GIC population [41, 42]. DRD2 signaling is involved in maintaining self-renewal [42], activating hypoxia response and functionally altering metabolism of GBM in a potentially autocrine manner [41], indicating that DRD2 could be a therapeutic target in GBM.

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 [13, 17, 18].

We have recently reported that radiation in combination with the first-generation dopamine receptor antagonist trifluoperazine prolonged survival in mouse GBM models [34]. Here we show that radiation in combination with ONC201 reduced the viability of bulk 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, combined treatment prevented the radiation-induced expression of gene sets correlated with GBM plasticity, glioma stem cells, Oct4 targets and interfered with cell

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growth, ECM interaction and EMT genes, thought to be involved in neural-to-mesenchymal transition, and the metabolism of GBM cells. This agreed with a previous study reporting effects of ONC201 on glioma stem cells [43].

In vivo, a single dose of radiation combined with continuous, weekly ONC201 treatment translated into significantly prolonged median survival in mouse models of GBM. 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 efficacy of ONC201 in combination with radiation. Most importantly, the combined treatment was well tolerated and did not lead to treatment-related toxicity.

Our data suggest that the combination of radiation with ONC201 counteracts defense mechanisms of GBM cells against radiation without acting as a classical radiosensitizer against GICs but only non-stem GBM cells. We conclude that ONC201 in combination with radiation could be a promising new strategy against GBM that should be tested in clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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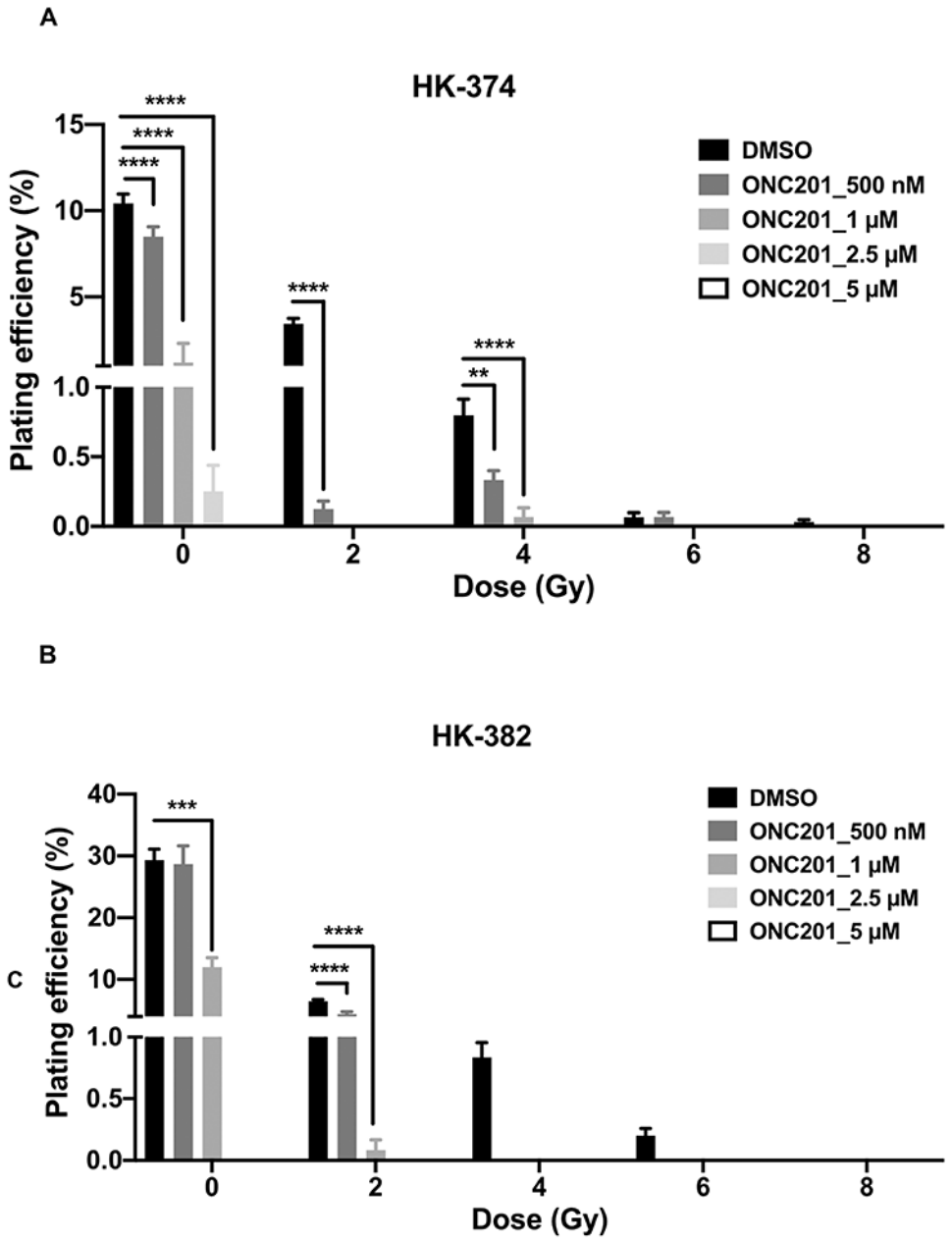

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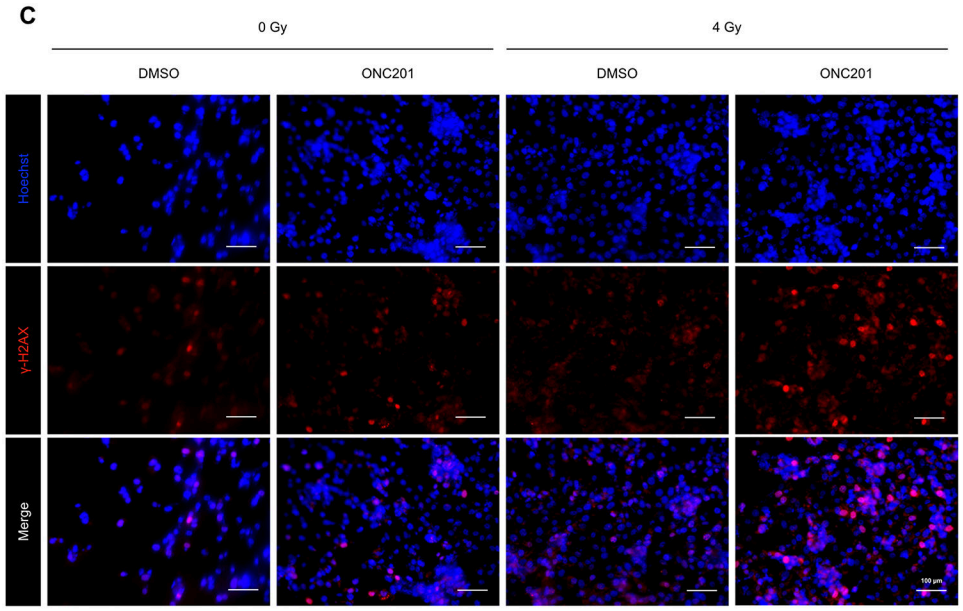

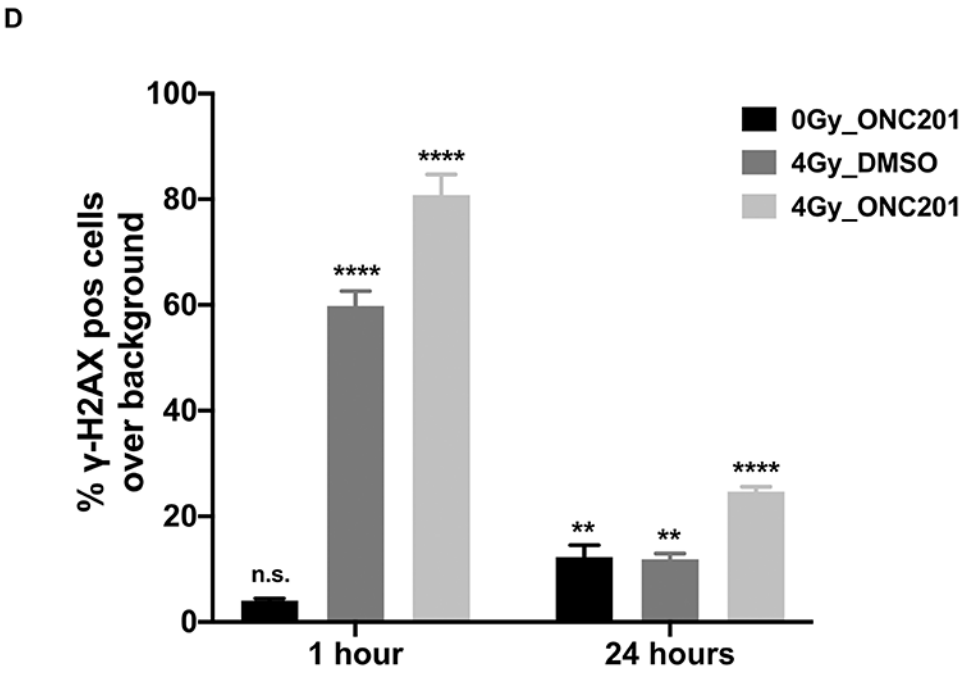

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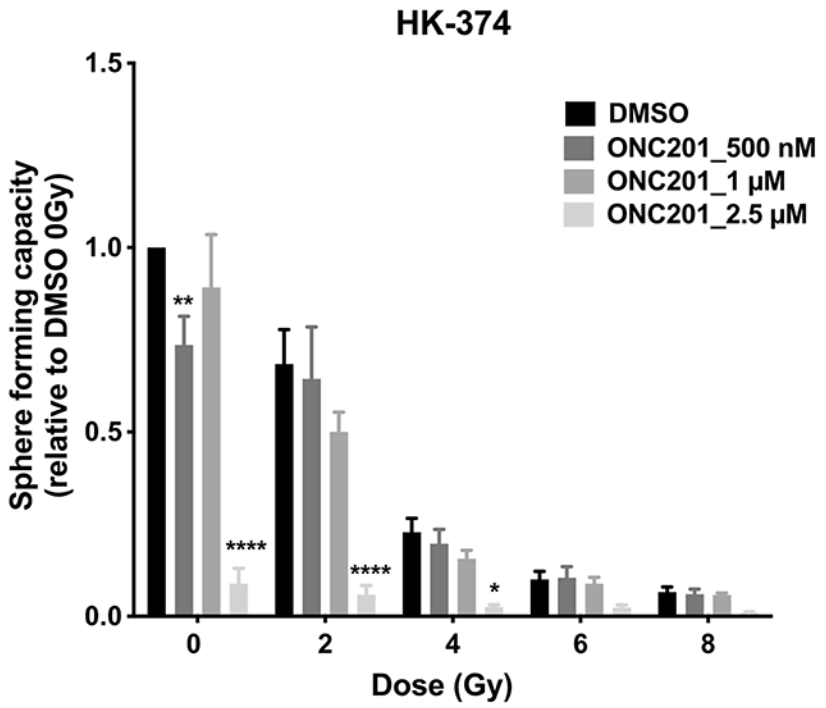

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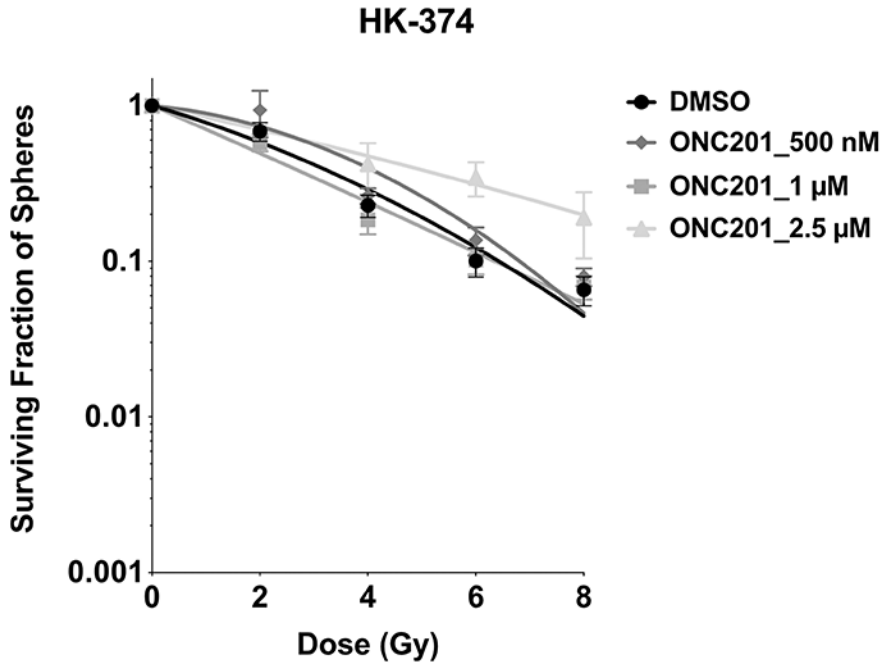

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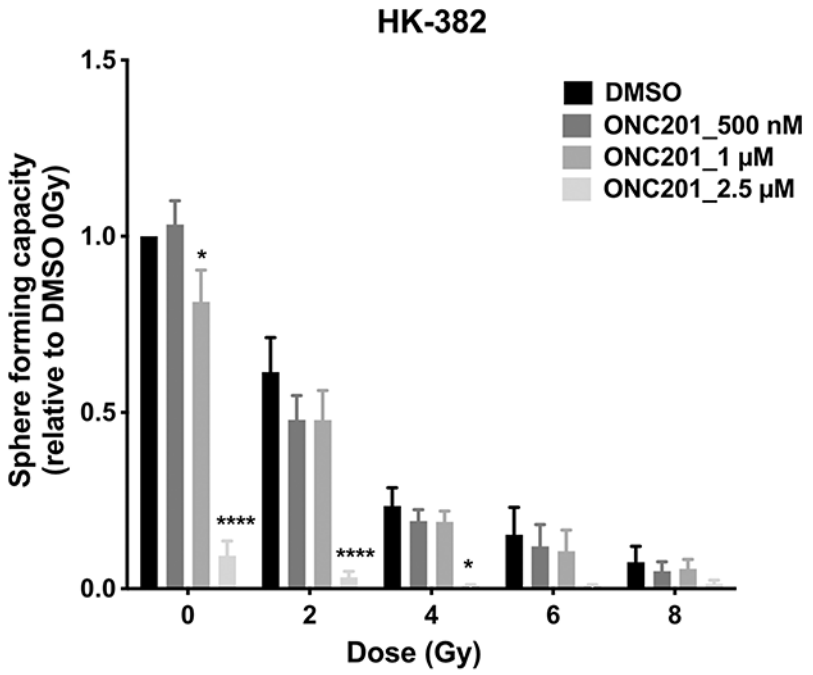

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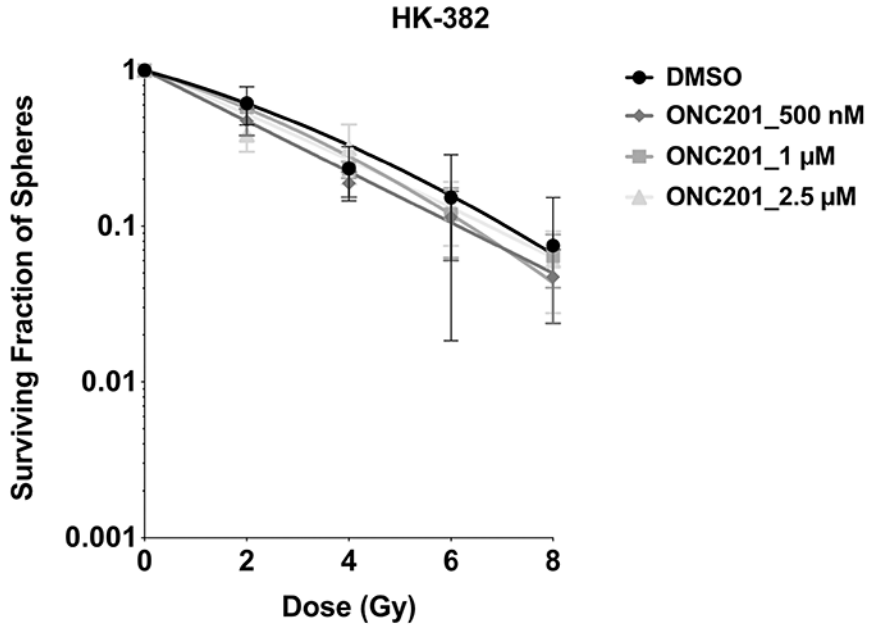

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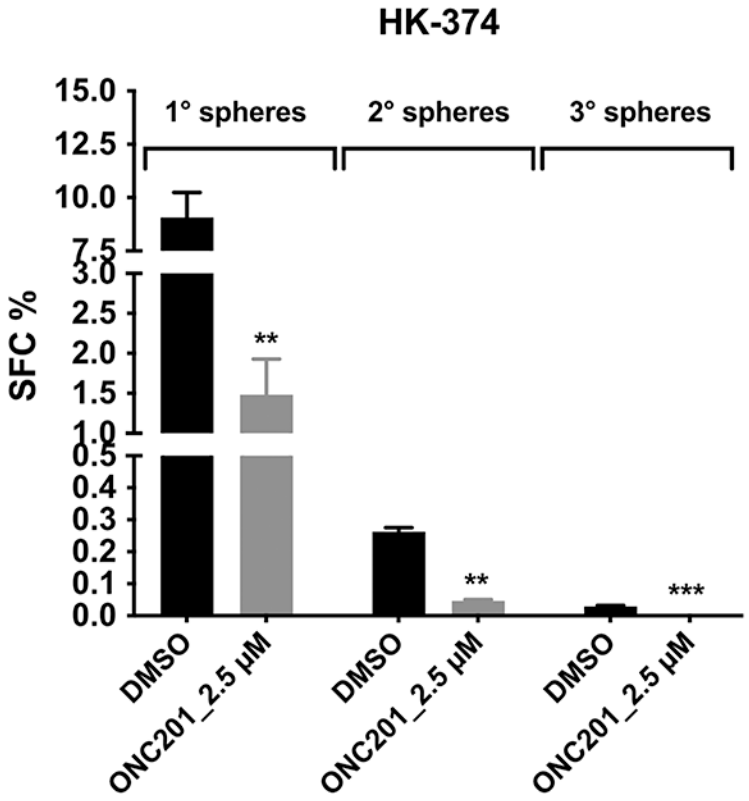

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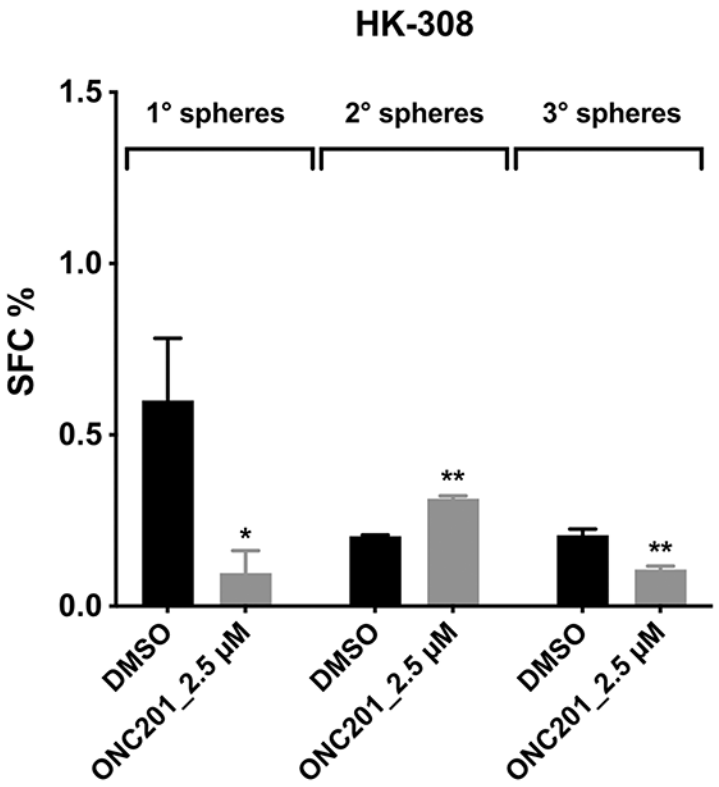

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Figure 1. Effects of ONC201 on GBM cells *in vitro*.

(A/B) Clonogenic assay of patient-derived primary HK-374 and HK-382 GBM cells treated with ONC201 (500 nM, 1 μM, 2.5 μM and 5 μM) or solvent control (DMSO) in combination with a single dose of radiation (0, 2, 4, 6 or 8 Gy). The colony number was counted and presented as the percentage relative to the initial number of cells plated. **(C)** γH2AX immunofluorescent staining of HK-374 GBM cells treated with 2.5 μM ONC201 or DMSO in combination with a single dose of radiation (4 Gy) for 24 hours. **(D)** Quantification of the percentage of γH2AX positive cells over background at 1 hour and 24 hours after radiation, respectively. **(E-H)** Patient-derived HK-374 and HK-382 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 μM, 2.5 μM). 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. **(I/J)** 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 μM 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-H; multiple Student's t-tests for J and K. * *p*-value<0.05, ** *p*-value<0.01, *** *p*-value<0.001 and **** *p*-value<0.0001.

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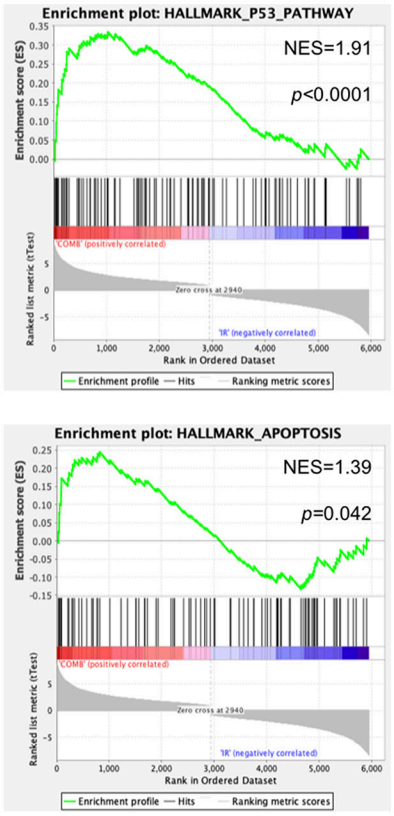

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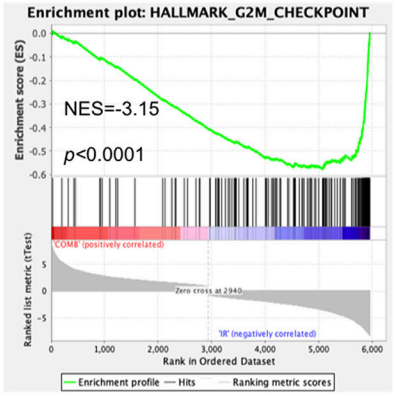

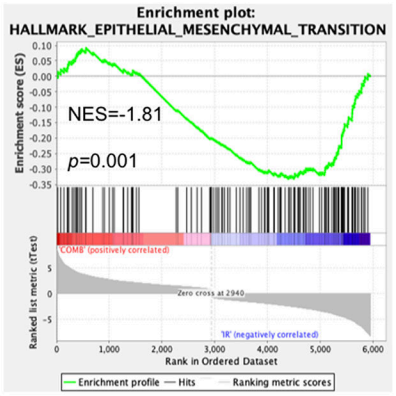

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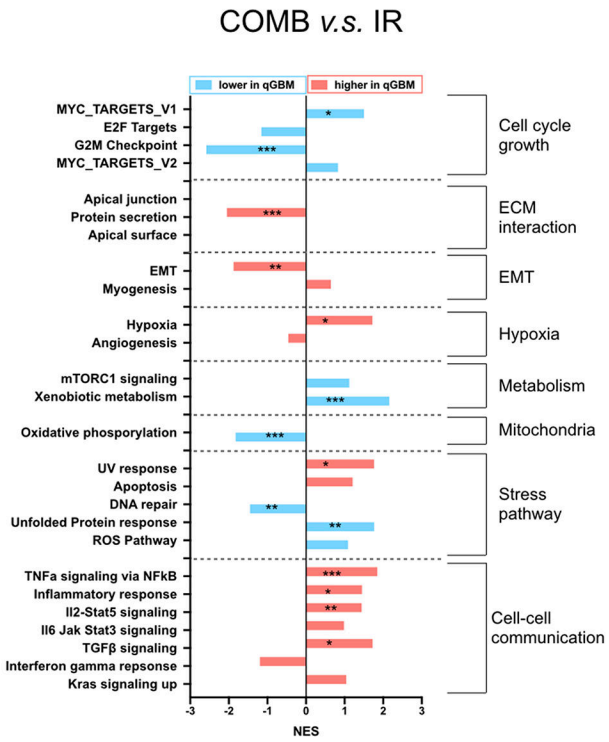

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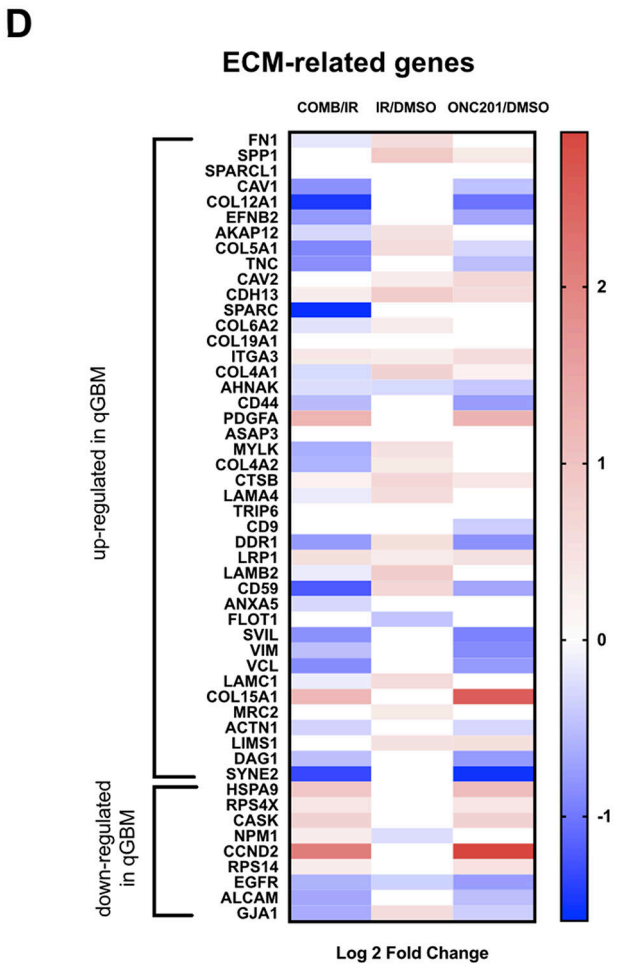

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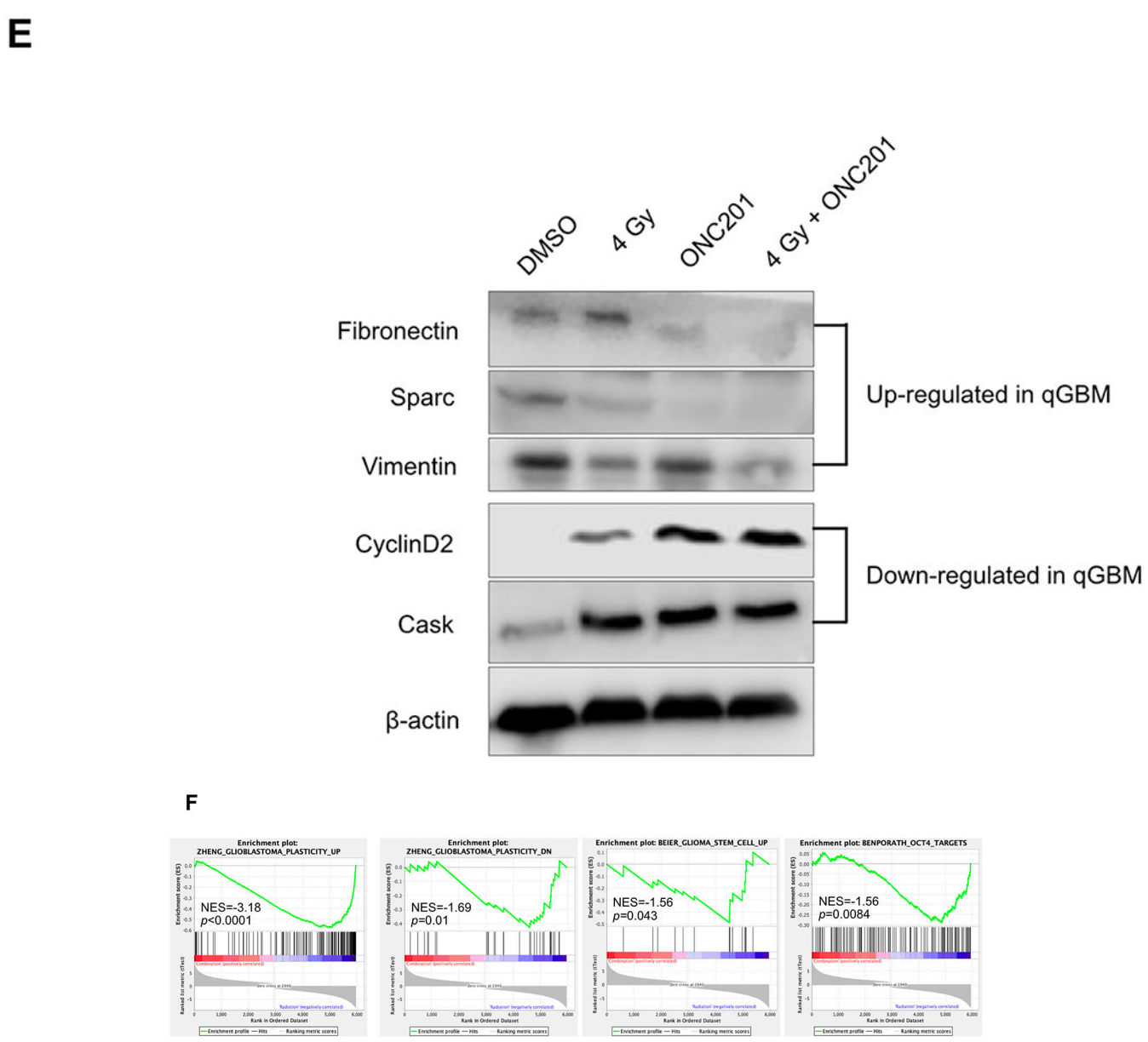

Figure 2. RNA-Seq analysis of glioma cells treated with ONC201 and radiation.

(A/B) 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. **(C)** GSEA of quiescent GBM (qGBM) gene signatures in the comparisons COMB *v.s.* IR, 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. **(D)** 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. **(E)** Western blot verification of selected up-regulated ECM-related proteins (Fibronectin, Sparc and Vimentin) and down-regulated ECM-related proteins (CyclinD2 and Cask) in qGICs, with β-actin as a loading control. Proteins were extracted from HK374

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spheres treated with 2.5 μM ONC201 or DMSO in combination with a single dose of radiation (4 Gy) for 48 hours. **(F)** 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), displayed with Enrichment plots.

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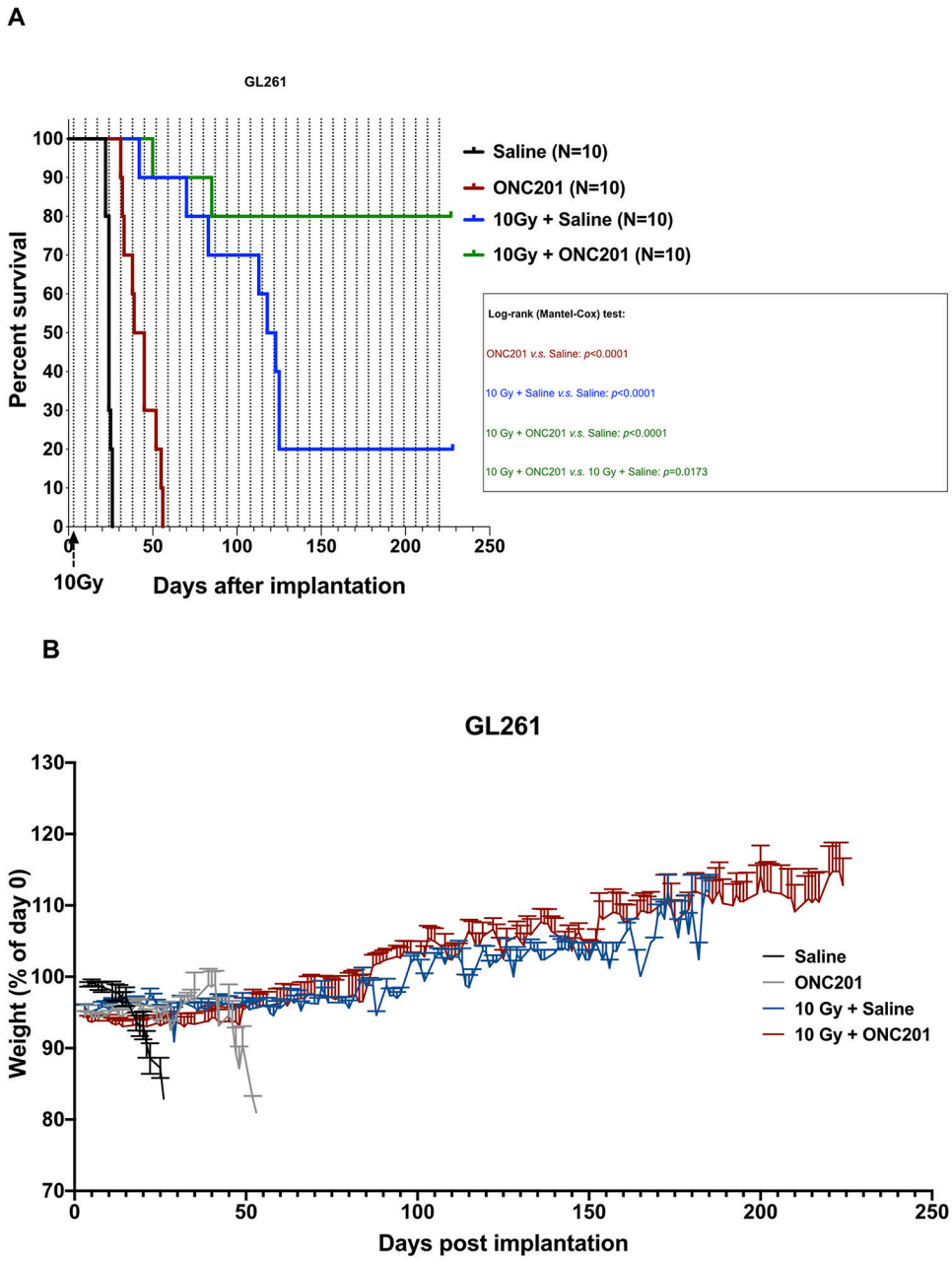

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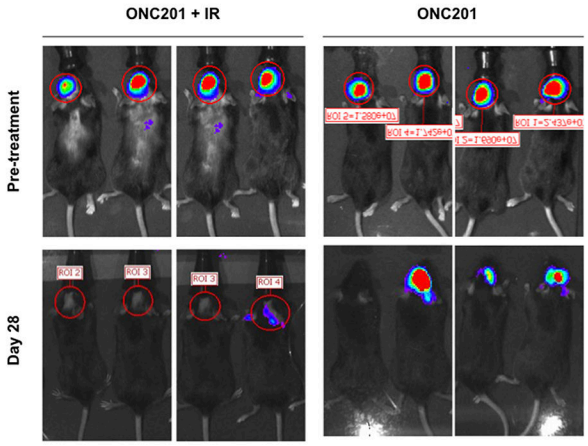

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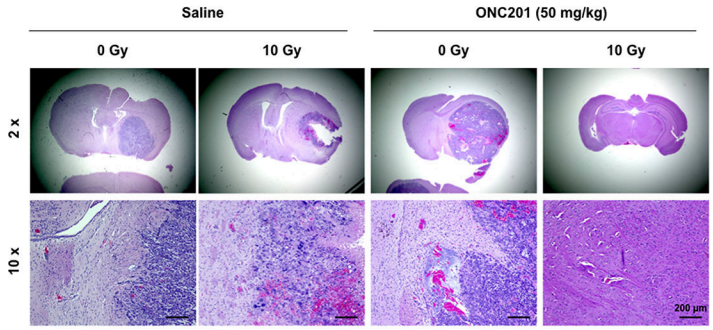

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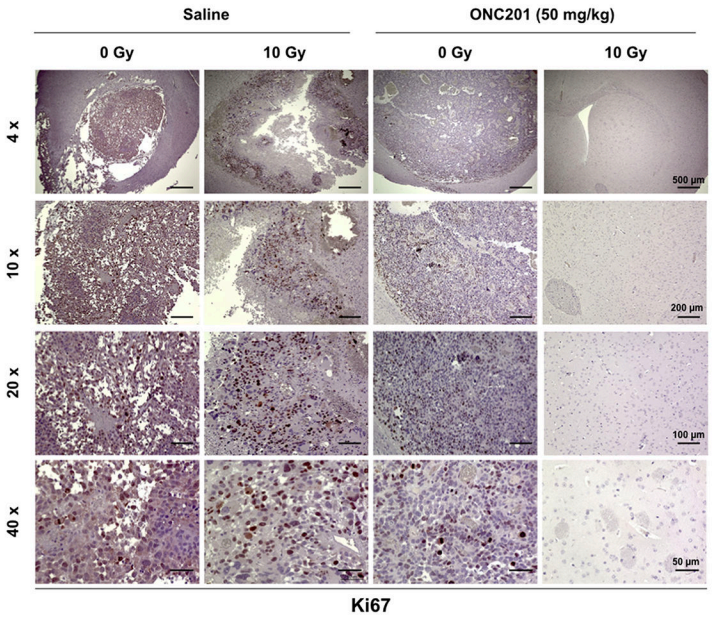

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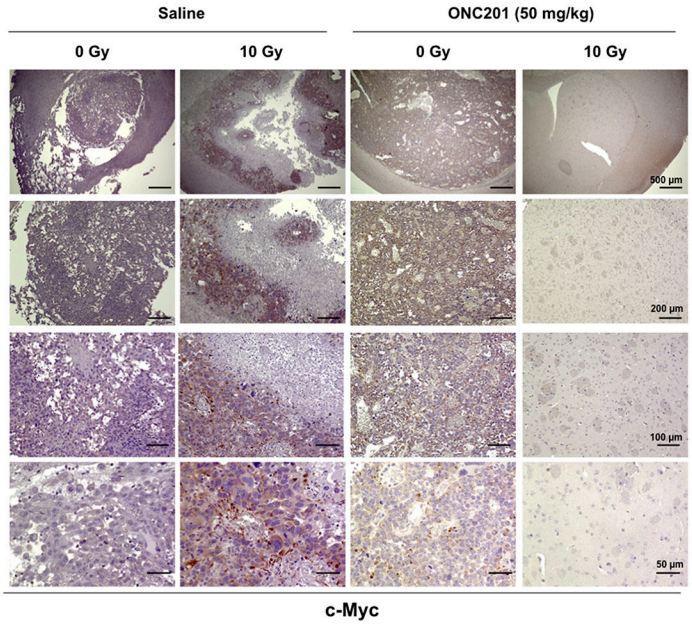

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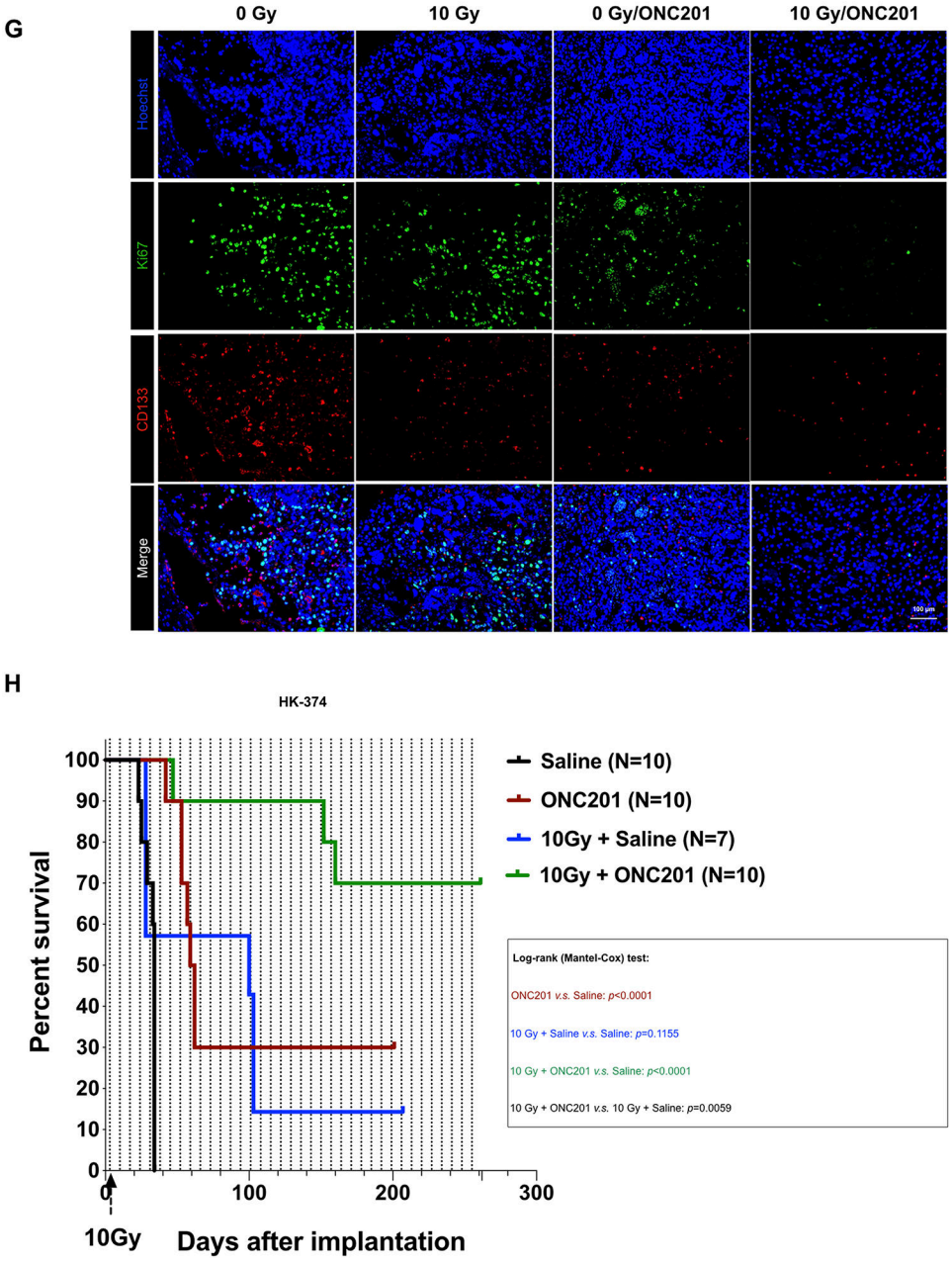

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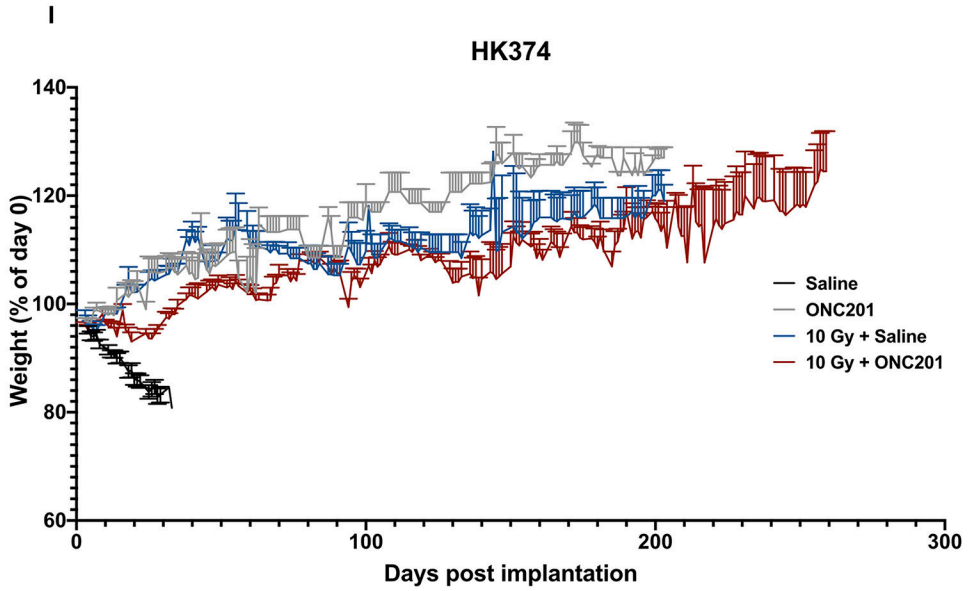

Figure 3. Combination of ONC201 and radiation prolongs survival in mouse models of glioblastoma.

(A) Survival curves for C57BL/6 mice implanted intra-cranially with $2x10^5$ 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)** Bio-luminescence 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)** Double immunofluorescent staining of GBM stem cell marker CD133 (red) and proliferating marker Ki67 (green) in coronal sections of brains from C57BL/6 mice implanted with GL261-GFP-Luc cells, 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. **(H)** Survival curves for NSG mice implanted intra-cranially with $3x10^5$ 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

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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). **(I)** Weight curves for the NSG mice in the different treatment groups.

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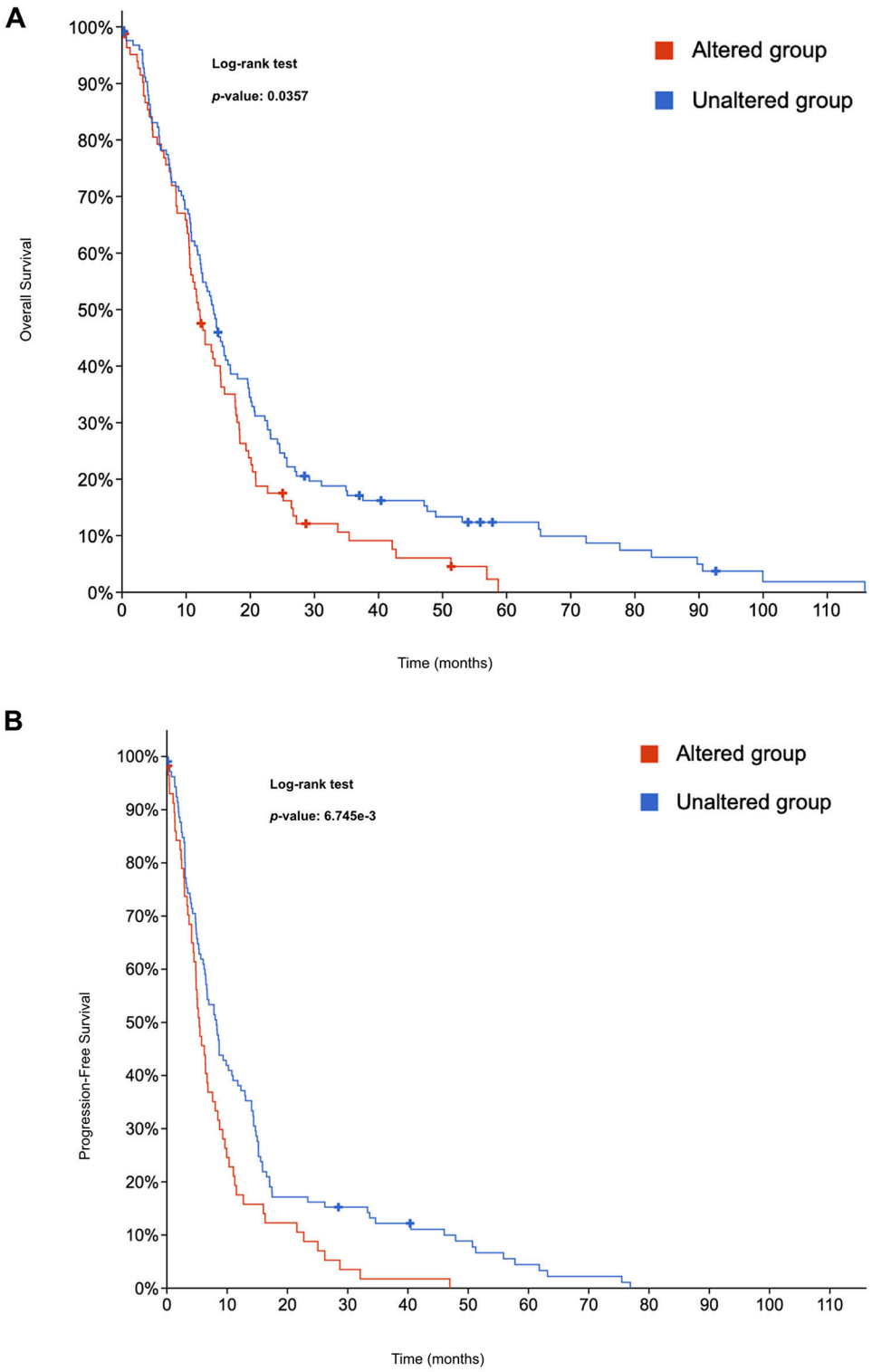

Figure 4. Evaluation of qGBM-enriched ECM-related gene signatures in TCGA glioblastoma patients.

(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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Table 1.

Patient demographics and TCGA-classification of GBM subtypes.

Line	Origin	Age	Gender	TCGA subtype	Culture P53 CN	EGFRvIII	PTEN	MGMT
HK-374	Primary GBM	45	Male	classical	Loss "mosaic"	Positive	Positive	not methylated
HK-345	Recurrent GBM	26	Female	mesenchymal	wt	Negative	Positive	not methylated
HK-157	Primary GBM	54	Female	proneural	wt	Negative	Positive	Unknown
HK-382	Primary GBM	66	Male	classical	Loss "mosaic"	Negative	Positive	methylated
HK-308	Recurrent GBM	50	Female	mesenchymal	Unknown	Positive	Positive	not methylated