## Original Article
# Short-term effects of radiation in glioblastoma spheroids

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**Abstract:** Glioblastoma is the most frequent and malignant primary brain tumor. The standard treatment includes surgery, radiation and chemotherapy. The limited efficacy of the current treatment has been explained by the existence of treatment-resistant stem-like tumor cells. The aim of this study was to investigate the short-term effects of radiation of spheroids containing tumor-initiating stem-like cells. We used a patient-derived glioblastoma stem cell enriched culture (T76) and the standard glioblastoma cell line U87. Primary spheroids were irradiated with doses between 2 and 50 Gy and assessed after two and five days. We found a small reduction in primary spheroid size after radiation and an associated small increase in uptake of the cell death marker propidium iodide. Using immunohistochemistry, P53 expression was found to be significantly increased, whereas the Ki-67 proliferation index was significantly reduced. Both number and size of secondary spheroids formed after radiation were significantly reduced. In a limiting dilution assay, the spheroid formation capacity upon irradiation was higher for T76 compared to U87, but for both T76 and U87 irradiation led to a reduced spheroid formation capacity. Gene expression analysis of nine stem cell- and two hypoxia-related genes did not reveal any upregulation after radiation. In conclusion, this study suggests that a major short-term effect of radiation is pronounced reduction of tumor cell proliferation. We found no upregulation of stem cell-related genes. This may suggest a limited effect of targeting these genes within the first days after radiation therapy.

**Keywords:** Glioblastoma, radiation, short-term, tumor stem cells, spheroids

## Introduction

Glioblastoma is the most frequent and malignant brain tumor with patients having a median survival of only 14.6 months [1]. Treatment consists of surgical resection, radiotherapy and concomitant as well as adjuvant temozolomide [1]. Radiotherapy is the most successful non-surgical treatment of brain tumors and has provided a significant survival benefit [2, 3]. As a part of the current treatment a total standard dose of 60 Gy in 30-33 fractions are given to glioblastoma patients [1, 4]. Radiation induces oxidative stress, which impacts several biological parameters including DNA damages [5, 6] and chromosomal aberrations [7].

A subset of tumor cells in glioblastomas is believed to have stem cell properties and is most likely responsible for tumor recurrence. This subpopulation has specific characteristics including self-renewal, unlimited proliferative capacity and differentiation into non-stem progenies [8-10]. Furthermore, they have been reported to be less sensitive to radiation-induced cell death through activation of the DNA damage checkpoint and increased DNA damage repair [11]. It has been suggested by several authors that tumor stem cells have increased radioresistance compared to more differentiated cells [12-14]. Previously, irradiated glioblastoma cells expressing the suggested stem cell marker CD133 were shown to be more resistant than CD133^{-} cells. Moreover, the fraction of CD133^{+} cells was enriched in gliomas after radiotherapy, suggesting that the more stem-like cells contribute to radioresistance [12].
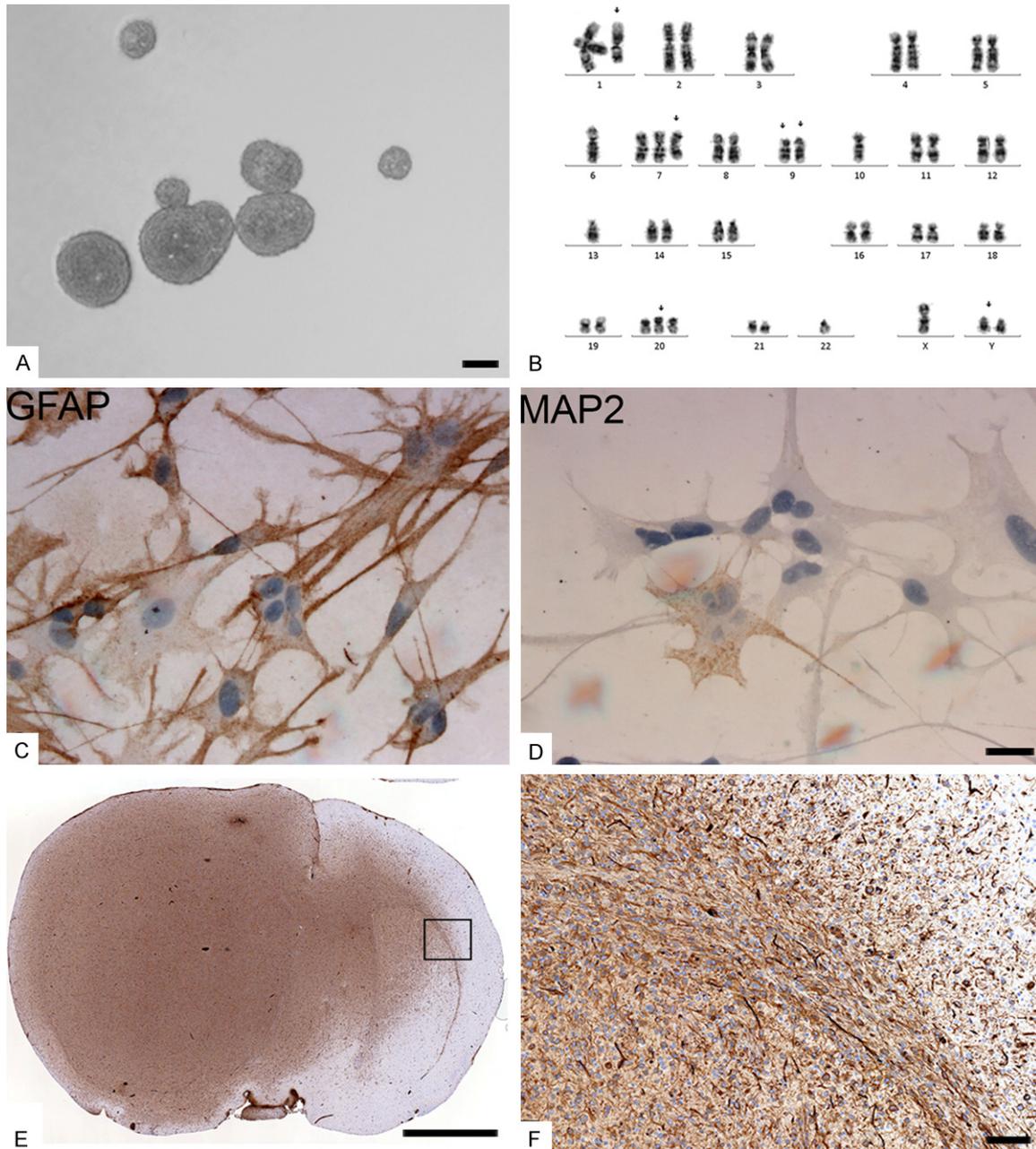
**Figure 1.** Characterization of the patient-derived glioblastoma culture T76. Cells from this culture formed spheroids upon culturing in serum-free medium (A), and had a karyotype with characteristic gain of chromosome 7 and loss of 10 (B). Serum deprivation resulted in differentiation of tumor cells, which expressed the astrocytic marker GFAP (C) and the neuronal marker MAP2 (D). T76 were able to induce tumor formation when implanted into the brain of immunodeficient mice. The brains were fixed, paraffin embedded, sectioned (3 μm) and immunohistochemically stained with anti-human specific CD56 in order to identify tumor cells. The CD56 staining showed that T76 formed invasive tumors (E, F). Scalebar: A=100 μm, C-D=30 μm, E=2 mm, F=100 μm.

Previous experimental studies have focused on long-term effects of radiation up to 30 days after exposure [15, 16], using both standard and patient-derived glioblastoma cell cultures. These studies suggested dose-dependent growth inhibition of U87 spheroids [15] as well as dedifferentiation and reacquisition of stem cell properties 30 days after exposure of patient-derived cell cultures [16]. However, to elucidate the potential of future concomitant anti-tumor stem cell therapy and radiation, a short-term perspective on effects of radiation

response is required. Several models have been used to study radiosensitivity in glioblastoma cells. Among these are monolayer cultures of cell lines, although, it is assumed that spheroid cultures better reflect the actual in situ environment [17, 18]. Culturing glioblastoma tissue as spheroids in serum-free medium supplemented with growth factors has been shown to enrich for more immature tumor cells with stem-cell properties [19-21] making this approach clinically relevant.

The aim of this study was to investigate the short-term effects of irradiation of spheroids with to tumor stem-like cells. We used a patient-derived spheroid culture established in our laboratory [22, 23] and the standard glioblastoma cell line U87 for comparison [24]. Both the patient-derived spheroid culture and U87 were kept in serum-free medium. In order to evaluate the short-term response of radiation we investigated the subsequent cell death and proliferative potential of these spheroids. Moreover, the spheroid formation-capacity and gene expression after two and five days was investigated using a panel of nine stem cell- and two hypoxia-related genes.

## Materials and methods

### *Establishment and characterization of glioblastoma short term culture*

The Regional Scientific Ethics Committee approved the use of human glioma tissue in the present study (approval number S-20110022). The glioblastoma stem cell-like containing spheroid culture (T76) was established in our laboratory. Glioblastoma tumor tissue was collected and the tissue was processed and characterized as previously described [22, 25], by a spheroid formation assay (**Figure 1A**), karyotyping (**Figure 1B**), a differentiation assay (**Figure 1C**, **1D**), and in vivo xenografting (**Figure 1E**, **1F**). The use of animals for glioblastoma mice xenografts were approved by The Animal Experiments Inspectorate in Denmark (permission J. No. 2008/561-1572). The experimental procedure was performed as previously described [22]. Female Balb/c nude (BALBNU-F, Taconic) mice were anesthetized subcutaneously and placed in a stereotactic instrument. Through a burr hole a 2-µL suspension of 300,000 single cells was injected into the striatum. The mice were euthanized in a carbon

dioxide chamber upon symptoms such as weight loss (20 % loss of body weight) and general poor state including lethargy, hunched posture and failure to groom. The standard glioblastoma cell line U87 (from European Collection of Cell Cultures (ECACC)) was also included. Both T76 and U87 were cultured in serum-free medium as previously described [22].

### *Cell culturing*

U87 and T76 cells were seeded as single cells in 24-well plates and cultured in 900 µl serum free medium/well allowing them to form free floating spheroids. U87 cells were seeded at a density of 5000 cells/well eight days before radiation. T76 cells were seeded at a density of 8000 cells/well 28 days before radiation. These protocols were used to obtain spheroids of similar size. Images were taken prior to irradiation with a digital camera connected to the microscope (Leica DFC 300 FX) using a 5× lens. The diameter of the spheroids was measured using the software program ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA).

### *Radiation of spheroids*

Radiation was performed after formation of primary spheroid as described above. Spheroids received radiation doses of 0, 2, 5, 10 or 50 Gy, respectively, given in one fraction. Radiation was carried out at the Laboratory of Radiation Physics at the Department of Oncology, Odense University Hospital. The samples were irradiated on an Elekta Synergy accelerator (Elekta Ltd.) using 6MV photons at gantry angle 180 and field-size 30×30 cm$^2$, with the samples centered in the field. Two cm perspex provided build-up. The treatment setup was CT-scanned on a Somatom scanner (Siemens AG) and dose calculation performed in Pinnacle (Philips).

### *Propidium iodide uptake in primary spheroids*

Two and five days after radiotherapy, cell death was evaluated using a concentration of 2 µM propidium iodide (PI, Sigma), a dye which enters dead and dying cells with a compromised cell membrane [26, 27]. PI uptake was visualized using fluorescence microscopy with a standard rhodamine filter and a digital camera (Leica DFC 300 FX). The captured images were ana-

**Table 1.** Quantitative PCR: Genes and endogenous control

| Gene symbol | Gene | Assay ID (Applied Biosystems) |
|---|---|---|
| PDPN | Podoplanin | Hs00366766_m1 |
| POU5F1 | Oct 4 | Hs00999632_g1 |
| PROM1 | CD133 | Hs01009257_m1 |
| PROM1 | CD133 | Hs01009250_m1 |
| NES | Nestin | Hs00707120_s1 |
| MSI1 | Musashi | Hs01045894_m1 |
| NANOG | Nanog | Hs02387400_g1 |
| SOX2 | Sox2 | Hs01053049_s1 |
| BMI1 | Bmi1 | Hs00180411_m1 |
| HIF1A | Hif-1α | Hs00936370_m1 |
| FUT4 PIWIL4 | CD15 | Hs00275643_s1 |
| EPAS1 | Hif-2α | Hs01026142_m1 |
| MYC | C-Myc | Hs99999003_m1 |
| 18S rRNA | Endogenous control | Hs99999901_s1 |
| GAPDH | Endogenous control | Hs99999905_m1 |
| ACTB | Endogenous control | Hs00357333_g1 |

lyzed using ImageJ. Mean PI intensities, which corresponded to cell death levels, were measured. In addition, number and diameter of spheroids were measured and counted. Subsequently, the spheroids were fixated, paraffin embedded, sectioned and investigated with immunohistochemistry.

*Immunohistochemistry*

Three-micrometer sections of paraffin-embedded spheroids were de-paraffinized and heat-induced epitope retrieval was performed in a TEG buffer solution (10 mmol/L Trisbase and 0.5 mmol/L EGTA). After blocking of endogenous peroxidase activity by incubation in 1.5% hydrogen peroxide ($H_2O_2$), the sections were incubated for 60 minutes with primary antibodies against P53 (1+200, clone: DO-7, Dako) and Ki-67 (1+200, clone: MIB-1, Dako). Detection of antigen-antibody complex was carried out using the detection system EnVision (Dako). The visualization was performed using diaminobenzidine as chromogen (Dako). Finally, the sections were counterstained with Mayer's hematoxylin and coverslips were mounted with Aquatex. Primary antibody omission was used as negative control. P53 staining of spheroids was evaluated using the software NIS-Elements AR 3.0 (Nikon). The KI-67 labeling index (KI-67 LI) was estimated as the fraction of Ki-67 positive cells.

*Spheroid proliferation assay*

Irradiated primary spheroids were dissociated two and five days after treatment and re-plated in 96-well plates at a density of 8000 cells/well in 900 μl serum free medium. At day 15 and 21, respectively, images were taken with a microscope connected to a digital camera (Leica DFC 300 FX). The number of secondary spheroids per well was counted and the diameter measured using the program ImageJ.

*Limiting dilution assay*

Irradiated primary spheroids were dissociated two and five days after treatment and re-plated in 96-well plates in serial dilutions ranging from 1000 cells per well to 1 cell/well in 200 μl serum free medium/well. Every week, 25 μl fresh medium was added to each well. Secondary spheroid formation was assessed after 8 (U87) and 22 (T76) days, respectively. The percentage of wells without spheroids for each cell plating density was calculated and plotted against the numbers of cells per well. Data was interpreted in ELDA: Extreme Limiting Dilution Analysis software [28]. Spheres with a diameter of at least 25 μm were included.

*Quantitative PCR (qPCR)*

Using the MagNA Pure System (Roche), RNA was purified from the spheroids two and five days after radiation using Isolation Kit-High Performance (Roche). Subsequently, cDNA was prepared, mixed with 2X TaqMan Universal Master PCR Mix (Applied Biosystems) and loaded on a Custom TaqMan® Low Density Array (Applied Biosystems). In the present study 8 samples were investigated per card investigating 12 stem cell-related genes of interest and three endogenous controls (for overview, see **Table 1**). All genes were analyzed in triplicates.

Ct-values were calculated with SDS 2.1.1 (Applied Biosystems) using automatic threshold and baseline detection. The analysis was based on Relative Quantification using the comparative Ct method. Data was illustrated as a heat-map, with a median Ct-value for each gene across the samples. Red corresponds to high expression and green corresponds to low expression.
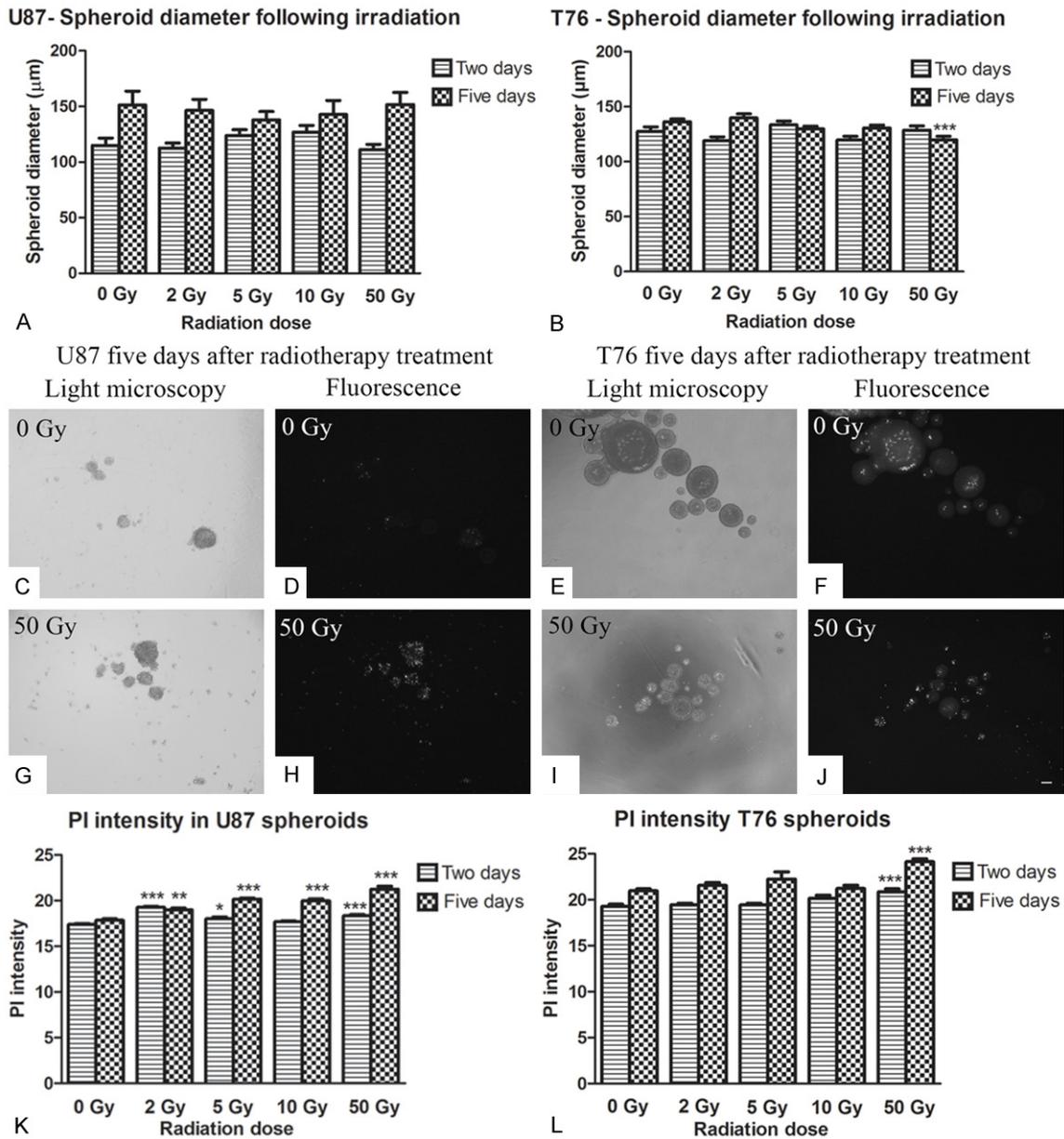
**Figure 2.** Effects of radiation on primary spheroids. Two and five days after radiation, spheroid diameter and propidium iodide (PI) uptake were measured. No effect on spheroid size was found for U87 (A), whereas a small but significant reduction in size was found for T76 five days after radiation with 50 Gy (B). Uptake of the fluorescent dye PI (C-J) showed a small to modest significant increase in uptake in U87 two and five days after radiation (2-50 Gy) (K), whereas T76 showed a significant uptake both two and five days after radiation with 50 Gy (L). Scalebar: 100 μm.

*Statistics*

All data were expressed as mean ± standard error of mean (SEM). Statistical significance was analyzed in Graph Pad Prism 5.0 (Graphpad Software, San Diego California USA), using one-way ANOVA test with Bonferroni correction for comparison of several groups. Statistical significance was defined as *$P<0.05$, **$P<0.01$,

***$P<0.001$. The PCR data was analyzed by hierarchical cluster analysis.

**Results**

*Effect of radiation on spheroids*

Radiation did not significantly affect the size of U87 spheroids two or five days post treatment
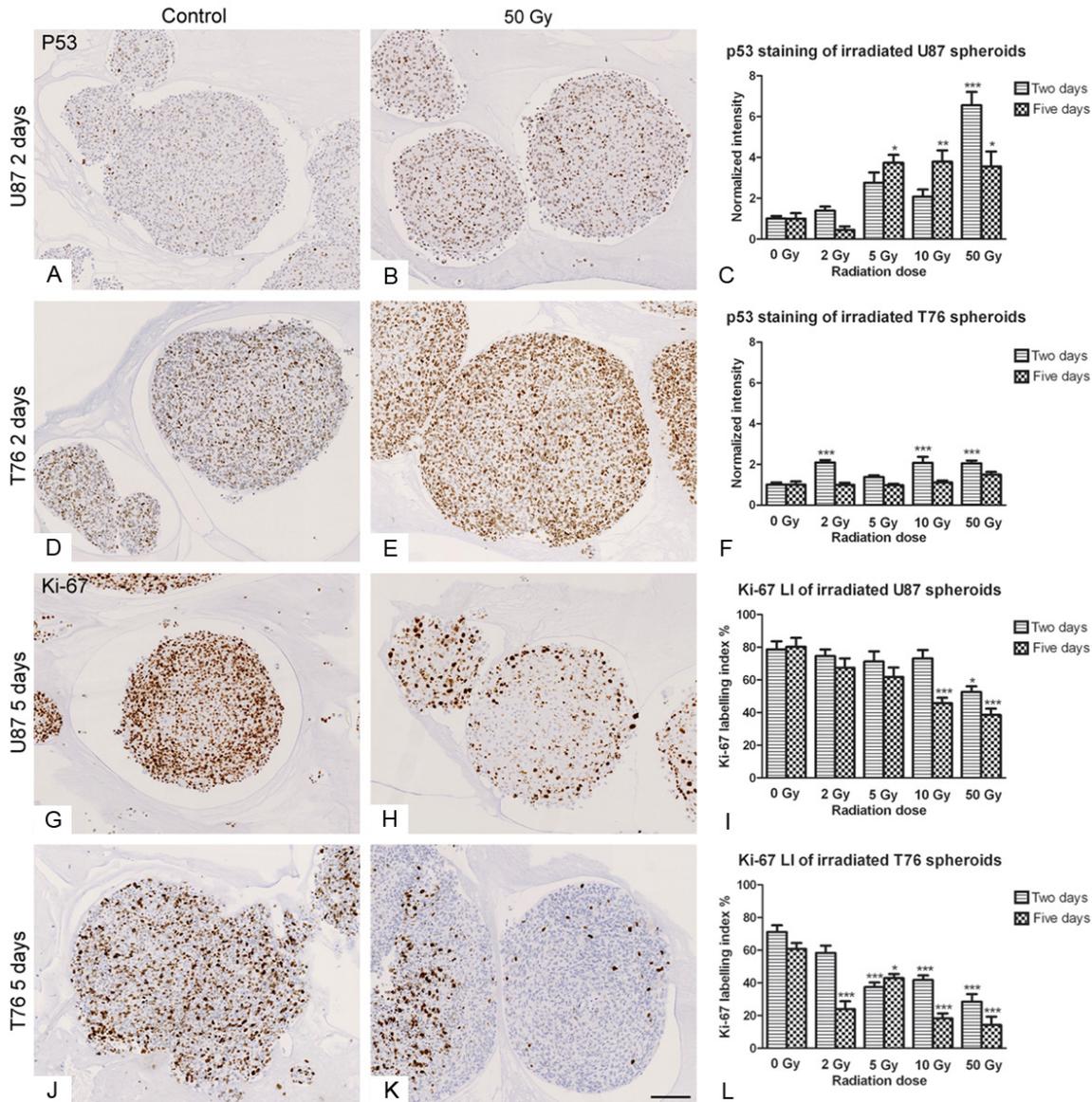
**Figure 3.** U87 and T76 spheroids were fixed, paraffin embedded, sectioned (3 μm) and immunohistochemically stained for P53 and KI-67. P53 expression increased after radiation in both U87 and T76 as shown for spheroids given 50 Gy (B, E) compared to control (A, D). Quantifying the expression a significant increase was found two days after radiation (50 Gy) and five days after radiation (5, 10 and 50 Gy) in U87 (C). P53 expression increased two days after exposure (2, 10 and 50 Gy) in T76 (F). No significant expression was found five days after exposure (F). KI-67 expression decreased after radiation in both U87 and T76 as shown for spheroids given 50 Gy (H, K) compared to control (G, J). The KI-67 labelling index (LI) in U87 spheroids decreased significantly two and five days after exposure at 10-50 Gy (I). T76 spheroids already showed decreased KI-76 LI two-five days after exposure at 2-5 Gy (L). Scalebar: 100 μm.

(**Figure 2A**). The T76 spheroid size was significantly, but only weakly reduced five days after treatment with 50 Gy (**Figure 2B**). The PI uptake in both U87 (**Figure 2G**, **2H**) and T76 (**Figure 2I**, **2J**) spheroid cultures was weak to moderate compared to control spheroids (**Figure 2C-F**). When measuring PI intensity, a significant increase in uptake was found in U87 starting at

2 Gy (**Figure 2K**) and in T76 at 50 Gy (**Figure 2L**).

*Immunohistochemical P53 and Ki-67 staining*

The irradiated spheroids were fixed, paraffin embedded, sectioned and immunohistochemically stained with P53 and Ki-67. Two days after
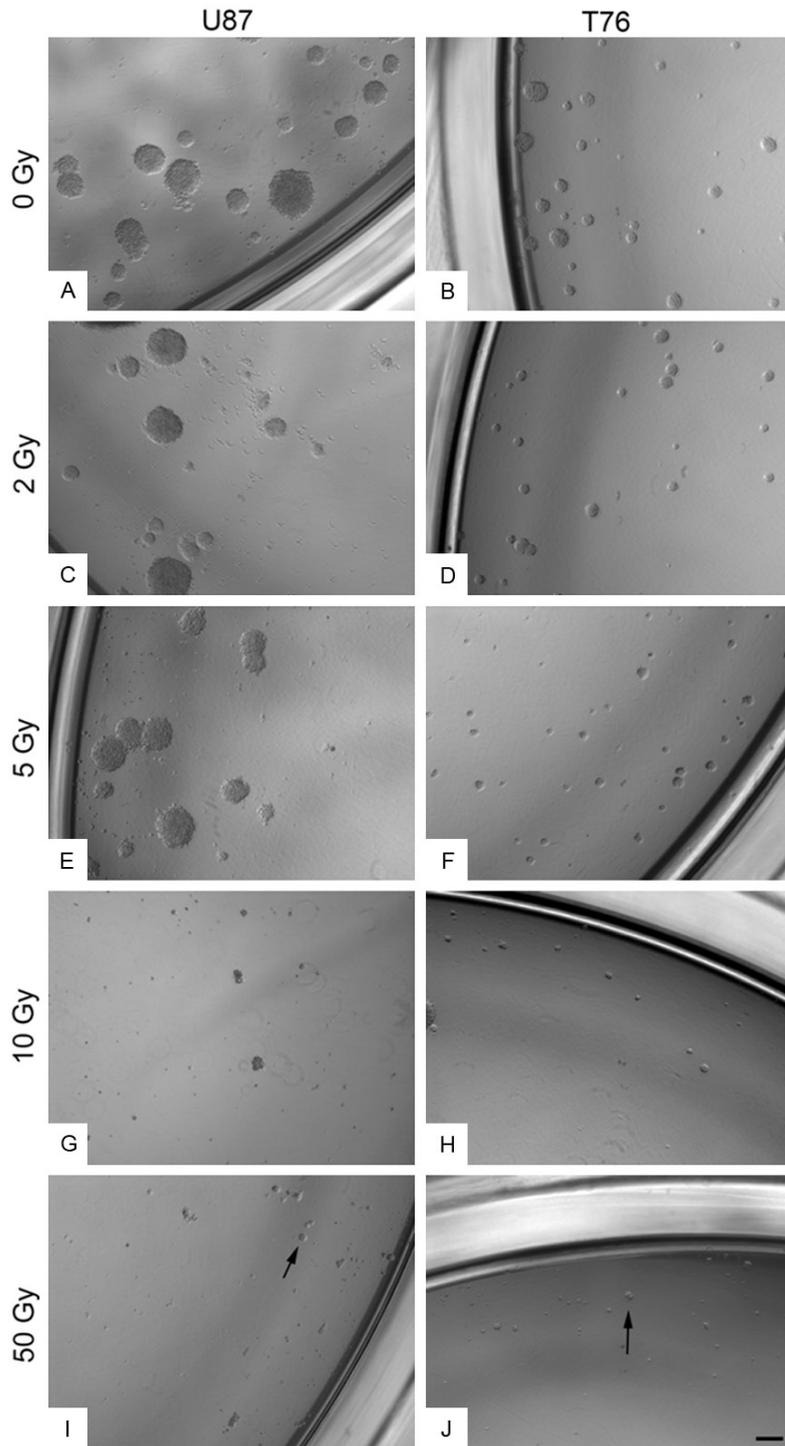
**Figure 4.** Formation of secondary spheroids at high cellular concentrations after increasing doses of irradiation. U87 and T76 primary spheroids were dissociated two (images not shown) and five days after radiation and allowed to form U87 (A, C, E, G, I) and T76 (B, D, F, H, J) secondary spheroids. Size and number of secondary spheroids seemed to be reduced in a dose dependent manner. Even at 50 Gy both U87 (I, arrow) and T76 spheroids (J, arrow) were formed. Scalebar: 100 μm.

irradiation, U87 spheroids showed a significantly increase in P53 expression when exposed to 50 Gy (**Figure 3A-C**), whereas five days after irradiation, spheroids showed a significantly increase in P53 expression already at 2 Gy (**Figure 3C**). T76 spheroids showed a significant increase in P53 expression two days after exposure at all radiation doses, except 5 Gy (**Figure 3D-F**). Five days after radiotherapy P53 expression was at the control level for all doses (**Figure 3F**).

Ki-67 LI in U87 and T76 spheroids decreased after irradiation. Two days after U87 spheroids were exposed to 50 Gy, the Ki-67 LI was significantly reduced (**Figure 3I**). Similar results were seen five days after exposure at 10-50 Gy (**Figure 3G-I**). In T76 spheroids, a significant decrease in Ki-67 LI was observed at 5-50 Gy two days after radiation (**Figure 3L**), and at all doses five days after exposure (**Figure 3J-L**).

*Spheroid proliferation assay*

Two and five days after irradiation, spheroids were dissociated and allowed to form secondary spheroids. For both U87 and T76, the size and number of spheroids were significantly reduced in a dose dependent manner (**Figures 4, 5**). The number of U87 spheroids was significantly reduced two days after treatment with 2 Gy and five
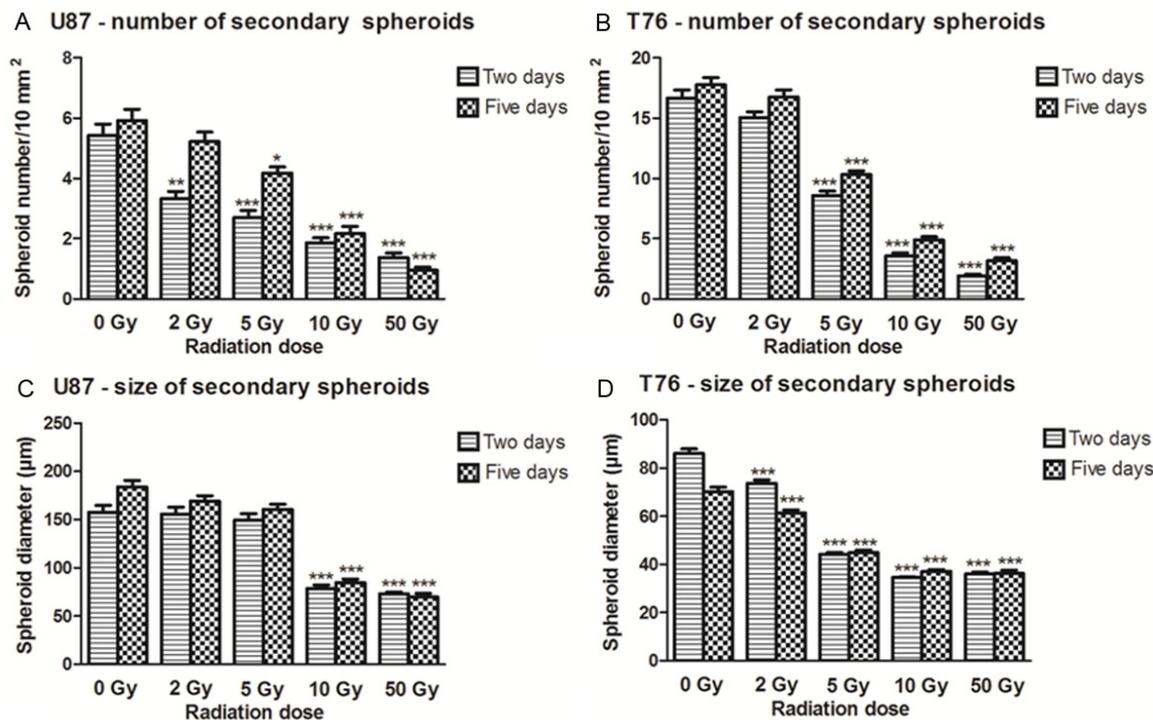
**Figure 5.** Formation of secondary spheroids at high cellular concentrations after increasing doses of irradiation. U87 and T76 primary spheroids were dissociated two and five days after radiation and allowed to form U87 (A, C) and T76 secondary spheroids (B, D). The number of U87 secondary spheroids decreased significantly two days after exposure to 2 Gy (A), whereas the number of T76 spheroids decreased significantly both two and five days after exposure to 5 Gy (B). Exposure to 10 Gy significantly decreased U87 spheroid size two and five days after irradiation (C). For T76 spheroids, exposure to 2 Gy significantly decreased spheroid size two and five days after irradiation (D).

days after treatment with 5 Gy (**Figure 5A**). A significant reduction in the number of T76 spheroids was seen after exposure starting at 5 Gy both after two and five days (**Figure 5B**). In line with these results, U87 secondary spheroid size was significantly reduced when exposed to 10 and 50 Gy after both two and five days (**Figure 5C**). In T76 this decrease was observed already at 2 Gy after both two and five days (**Figure 5D**).

*Limiting dilution assay*

Irradiated primary spheroids were dissociated two and five days after treatment and the clonogenic potential was evaluated using a limiting dilution assay. In general, the spheroid formation capacity upon irradiation was higher for T76 compared to U87.

Primary U87 spheroids irradiated with 10-50 Gy exhibited a significant lower capacity to form secondary spheroids, both two and five days after treatment compared to control (**Figure 6A**, **6C**). Primary T76 spheroids showed a sig-

nificant decreased clonogenic potential at all radio-doses two days after exposure (**Figure 6B**), whereas five days after exposure only the 50 Gy dose was found to be significant (**Figure 6D**).

*Gene expression upon radiation*

Hierarchical cluster analysis of qPCR results showed two distinct clusters corresponding to U87 and T76 spheroids (**Figure 7**). U87 had an upregulation of Hypoxia-inducible factor 1/2-α, CD15 and c-Myc, whereas T76 had an upregulation of CD133, Oct-4, podoplanin, Nestin, Mushashi, Nanog and Sox-2. There were no specific clusters for non-irradiated and irradiated cells as well as primary and secondary spheroids, neither two nor five days after irradiation.

**Discussion**

In this study we investigated the short-term effects of radiation on glioblastoma cells. We found that irradiation induced a dose-depen-
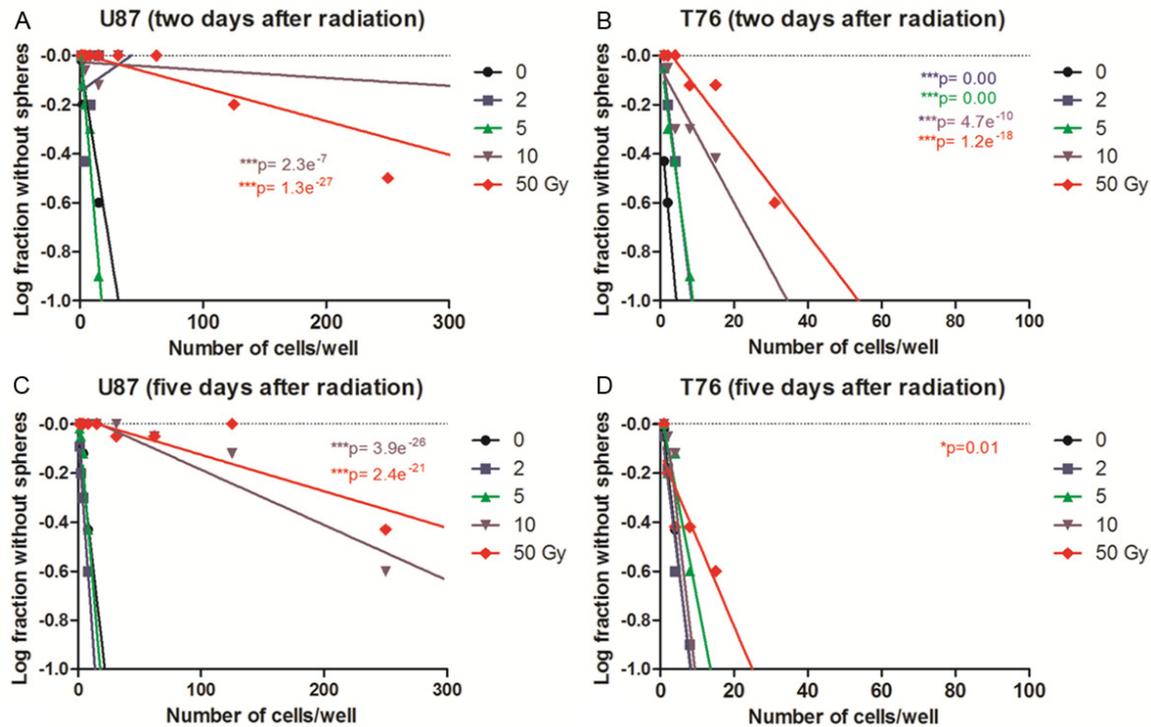
**Figure 6.** Irradiated primary spheroids were dissociated after treatment and re-plated in 96-well plates in decreasing concentrations going down to 1 cell/well (limiting dilution assay). U87 spheroids exposed to 10-50 Gy (A, C), and T76 spheroids exposed to 2-50 Gy (B, D) exhibited a lower capacity to form secondary spheroids compared to control spheroids. The spheroid formation capacity was highest for T76 both two and five days after irradiation.

dent decrease in proliferation and spheroid formation capacity in both T76 and U87 cells. Gene expression analysis including nine stem cell-related and two key hypoxia genes did not reveal any changes upon radiation. In general we observed no profound differences in spheroids dissociated either two or five days after radiation.

We found a minor decrease in primary T76 spheroid size after radiation (50 Gy) accompanied by a weakly to modestly increased PI uptake, suggesting a limited acute effect on cell viability. Despite radiation, U87 spheroid size did not decrease, but we observed some PI uptake. In line with our results, another study also observed a decrease in size of primary radiated U87 and patient-derived spheroids 15 days after exposure [7]. A previous study investigated the acute effect of irradiation in U87 cells 1-72 hours after exposure [29]. The results suggested that apoptosis was induced in a time-dependent manner after irradiation, thereby supporting our observation of cell death already within the first days after irradiation.

Using immunohistochemical stainings, we observed a significantly increased P53 expression in both cultures, whereas Ki-67 LI was significantly decreased, especially in T76 spheroids. These observations are most likely explained by the radiation induced DNA breaks leading to increased P53 expression, resulting in G1 arrest (reduced proliferation) and apoptotic cell death or DNA repair [29, 30]. The P53 immunohistochemical staining used in this study is adjusted so only overexpression results in staining. The T76 control spheroids seemed already before radiation to overexpress P53 compared to U87. This may be explained by already acquired P53 mutations leading to either no P53 protein or mutant protein production. Mutant P53 is more stable than wildtype P53, thereby resulting in increased expression of the protein, but loss of function [30, 31]. Since we observed a decrease in proliferation in T76 spheroids alternative mechanisms may lead to reduced proliferation in these spheroids. A previous study investigating the effect of radiation in organotypic multicellular spheroids supported our P53 and Ki-67 findings [32]. In line with our results, they also found
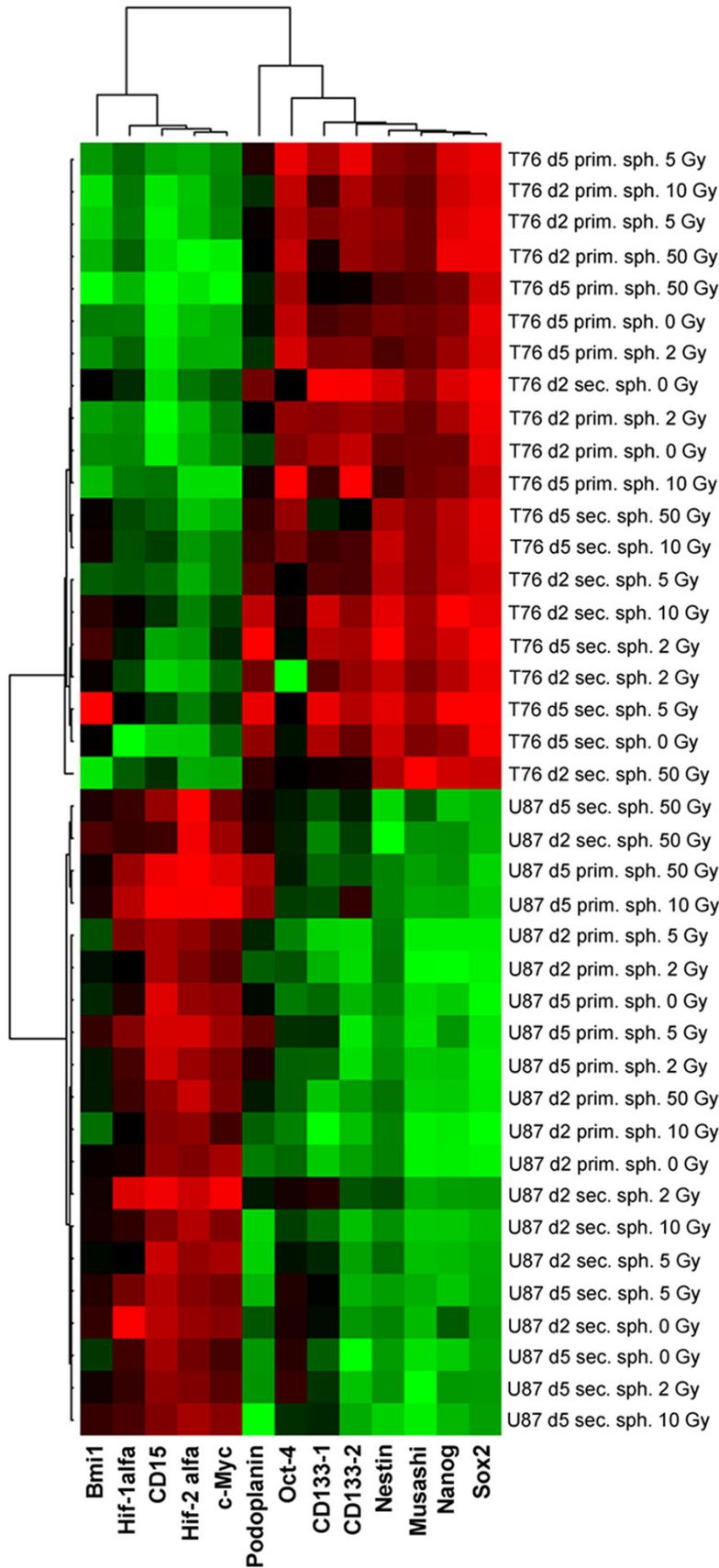
Figure 7. Hierarchical cluster analysis of quantitative PCR (qPCR) results comprising 9 stem cell-related genes and two hypoxia related genes. Two distinct clusters corresponding to U87 and T76 spheroids were identified confirming high expression of stem-cell related genes in patient-derived T76 spheroids grown in stem cell containing medium. In contrast the standard cell line U87 has for decades been grown in serum-containing medium. There were no specific clusters for non-irradiated and irradiated cells, neither two nor five days after irradiation suggesting no upregulation of stem-cell related genes after irradiation. Red corresponds to high expression and green corresponds to low expression. Abbreviations: d: days, prim: primary, sec: secondary, sph: spheroid.

increased P53 expression as well as a decrease in proliferation in two of four cultures [32].

We found-as expected from the Ki-67 LI results-a pronounced dose-dependent inhibition of U87 and T76 secondary spheroid formation at high seeding concentrations. The reduced spheroid formation capacity but limited cell death observed in the primary spheroids is most likely a consequence of late cell death following radiation. Pre-mitotic cell death rarely occurs, whereas cell death is post-mitotic in most solid tumors, and usually occurs after several mitosis attempts [33].

The spheroid formation capacity upon irradiation was higher for T76 compared to U87. This was most likely explained by an

enhanced content of tumor stem-like cells being found in T76 spheroids. Tumor stem-like cells have earlier been suggested to be highly radioresistant [12, 34, 35], thereby suggesting this subpopulation of cells to be responsible for tumor regrowth. T76 cells have since the culture was established been cultured in serum-free so-called stem cell medium, whereas U87 has been cultured in serum-containing medium for decades.

The qPCR results revealed that U87 and T76 formed two separate clusters. The increased expression of Hypoxia-inducible factor-1/2-α in U87 is most likely explained by an increased U87 spheroid size leading to central hypoxia, a phenomenon observed in many types of spheroids [36]. In contrast, T76 clearly expressed a higher level of stem cell-related genes compared to U87, which was in line with the higher spheroid formation capacity of T76 both before and after irradiation. This is in line with patient-derived cell lines better preserving the stem cell phenotype found in the parental tumor [18] and in line with the standard high-passage cell lines like U87 often losing stem cell characteristics [26]. Supporting better preserved stem cell features of T76, our results also showed that T76 formed spheroids at low clonal densities as well as patient-like infiltrating tumors in mice. However, we found no differences in stem cell-related mRNA expression between non-radiated and radiated cells for either U87 or T76 both two and five days after radiation. In contrast to our results, one study with patient-derived spheroids found increased expression of stem cell related genes: Nanog, Olig2, Nestin and Sox2, after radiation (3 Gy) [16]. However, these spheroids were allowed to dedifferentiate in 30 days before gene expression profiling. This may suggest that targeting of stem-cell related genes should be used four weeks after radiation therapy but not as part of a concomitant strategy. At present, clinical trials such as the notch-inhibitor RO4929097 trial targeting the tumor stem cell phenotype, are based on concomitant treatment with radiotherapy (ClinicalTrials.gov Identifier: NCT01119599). However based on the findings in the current study this protocol may be suboptimal. Consequently, the timing of concomitant treatment with radiation needs to be further investigated, to ensure optimal combination with stem cell targeting therapies.

In conclusion our results suggest that the short-term effects of radiation comprise limited cell death but a pronounced reduction of cell proliferation. We found no short-term upregulation of stem cell related genes suggesting limited effect of concomitant anti-tumor stem cell therapy and radiation. The optimal strategy for combining these therapies should be further investigated.

### Disclosure of conflict of interest

None.

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