

Spatially fractionated mini-GRID radiotherapy differentially modulates radiation-induced senescence in murine glioma and normal cells

M. Isabel Acuña^{1,*}, Miguel Ángel Prados^{2,*}, Sabela Da Silva-Alvarez^{2,3}, Víctor Luna-Vega⁴, Eva G. Kölmel⁴, Manuel Sánchez-García⁴, Manuel Collado^{2,5}, Yolanda Prezado^{1,6}

¹New Approaches in Radiotherapy Lab, Center for Research in Molecular Medicine and Chronic Diseases (CiMUS), University of Santiago de Compostela, Health Research Institute of Santiago de Compostela (IDIS), Santiago de Compostela, A Coruña, 15782 Spain

²Laboratory of Cell Senescence, Cancer and Aging, Center for Research in Molecular Medicine and Chronic Diseases (CiMUS), University of Santiago de Compostela, Health Research Institute of Santiago de Compostela (IDIS), Santiago de Compostela, A Coruña, 15782 Spain

³Department of Biochemistry and Molecular Biology, Center for Research in Biological Chemistry and Molecular Materials (CiQUS), University of Santiago de Compostela, Santiago de Compostela, A Coruña, 15782 Spain

⁴Department of Radiophysics and Radiological Protection, University Hospital of Santiago de Compostela, Santiago de Compostela, A Coruña, 15701 Spain

⁵Department of Immunology and Oncology (DIO), National Centre for Biotechnology (CNB-CSIC), Madrid, 28049 Spain

⁶Oportunus Program, Galician Agency of Innovation (GAIN), Xunta de Galicia, Santiago de Compostela, 15700 Spain

*Equal contribution

Correspondence to: Manuel Collado, Yolanda Prezado; email: manuel.collado@usc.es, yolanda.prezado@usc.es

Keywords: cellular senescence, spatially fractionated radiotherapy, mini-GRID radiotherapy, conventional radiotherapy, cancer cells

Received: March 2, 2026

Accepted: July 16, 2026

Published: September 10, 2026

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ABSTRACT

Background: Spatially fractionated radiotherapy (SFRT) is an emerging therapeutic strategy that enhances tumor control while reducing normal tissue toxicity. Although conventional radiotherapy is known to induce therapy-induced senescence (TIS), its induction by SFRT—particularly mini-GRID radiotherapy—remains poorly characterized. Addressing this question is essential to better understand SFRT-mediated biological responses and optimize its therapeutic potential.

Methods: Two rat glioma cell lines (F98, RG2), immortalized astrocytes (CTX-TNA2), and primary mouse embryonic fibroblasts (MEFs) were exposed to single radiation doses ranging from 5 to 20 Gy using conventional radiotherapy (Conv-RT) or mini-GRID RT. Seven days post-irradiation, senescence was evaluated by SA- β -galactosidase activity (X-Gal and FDG staining) and cell size analysis. Senescence markers (p53, p21, and p16) were analyzed by RT-qPCR and Western blotting, and SASP-related genes (*Il1a*, *Il6*, *Serpine1*, and *Cxcl1*) were quantified by RT-qPCR.

Results: In F98 and RG2 glioma cells, Conv-RT at 20 Gy induced a robust senescent phenotype, characterized by increased cell size, elevated SA- β -Gal activity, persistent DNA damage signaling, and upregulation of p53, p21, p16, and SASP-related genes. In contrast, mini-GRID RT achieved comparable cell number reduction while significantly limiting senescence, DNA damage signaling, and SASP activation, with expression levels

approaching non-irradiated controls. In CTX-TNA2 astrocytes and MEFs, radiation induced senescence in a dose-dependent manner, with no significant differences between irradiation modalities.

Conclusions: Mini-GRID RT maintains the cytotoxic effectiveness of conventional irradiation in glioma cells while reducing senescence and SASP induction. These findings highlight its potential to improve the therapeutic ratio and support further investigation.

INTRODUCTION

Radiotherapy (RT) remains a cornerstone of cancer treatment, providing effective local control across many tumor types. Nonetheless, bulky, radioresistant or recurrent tumors still present a therapeutic challenge because homogeneous delivery limits dose escalation by causing damage to normal tissues. This inability to ablate the tumor without excessively injuring critical tissues constrains long-term outcomes and patient quality of life [1].

Increasing evidence suggests that RT plays a multifaceted role in treatment efficacy. While it effectively targets and eliminates tumor cells, emerging data indicate that it can also induce cellular senescence—a state of permanent cell-cycle arrest traditionally viewed as a barrier to tumor progression [2, 3]. However, senescent cells can persist within tissues and secrete a variety of factors collectively known as the senescence-associated secretory phenotype (SASP) [3–5]. SASP has been associated with promoting inflammation, contributing to the major pathology of radiation-induced normal tissue and organ injury, altering the composition of the tumor microenvironment (TME), and potentially facilitating tumor growth under certain conditions [3, 4, 6, 7].

Spatially fractionated radiotherapy (SFRT) has emerged as an innovative strategy that delivers intentionally heterogeneous doses—high-dose “peaks” interspersed with low-dose “valleys” within the tumor [8]. By doing so it can treat large and bulky tumors with less toxicity to surrounding organs at risk [8]. SFRT exhibits radiobiological responses that differ fundamentally from those elicited by conventional uniform irradiation, including a differential immunomodulation [9, 10], vascular impact [11] and bystander-like effects [12] among others. However, a full understanding of the biology of SFRT is still missing. SFRT encompasses modalities such as GRID, LATTICE, microbeam or minibeam radiotherapy, which differ in their spatial pattern and peak-to-valley ratios [8]. Among these approaches, mini-GRID radiotherapy combines the advantages of narrow-beam irradiation with the widespread availability of medical linear accelerators [13, 14]. Preclinical studies have demonstrated that mini-GRID radiotherapy can generate highly modulated dose

gradients within a single fraction while better preserving normal tissue, making it a promising alternative to homogeneous irradiation for the treatment of radioresistant tumors [13, 14].

Consequently, it is critical to assess the impact of novel radiotherapy modalities on senescence induction in order to evaluate both therapeutic efficacy and long-term biological safety. While therapy-induced cellular senescence has been well-characterized following conventional radiotherapy [6, 15], its induction and phenotypic consequences in SFRT—particularly in mini-GRID—remain largely unstudied. Preclinical data suggest that the spatial distribution of radiation may modulate the frequency and characteristics of senescent cells [16], highlighting a critical gap in understanding the biological impact of SFRT.

The present study aimed to determine how radiation dose and irradiation modality—mini-GRID radiotherapy (mini-GRID RT) versus conventional radiotherapy (Conv-RT)—influence the induction of cellular senescence across distinct cellular contexts. To this end, we investigated two rat glioma cell lines, immortalized astrocytes, and primary mouse embryonic fibroblasts, and evaluated multiple senescence-associated features seven days post-irradiation.

Using complementary morphological, biochemical, and molecular readouts—including senescence-associated β -galactosidase (SA- β -Gal) activity, the most widely used marker of cell senescence [17], senescence-associated cell-cycle regulators (p53, p21, p16), DNA damage signaling (γ H2AX), and a panel of SASP factors (*Il1a*, *Il6*, *Serpine1*, and *Cxcl1*) [3, 18–20]—we show that mini-GRID RT does not exacerbate senescence induction relative to conventional irradiation. Notably, in glioma cells, mini-GRID RT preserved the antiproliferative efficacy of Conv-RT while significantly attenuating the establishment of a stable senescent phenotype and associated SASP activation, without exacerbating senescence induction in the non-tumor cell models tested.

These findings are consistent with preclinical evidence showing improved preservation of normal brain tissue following mini-GRID irradiation [13], supporting a

favorable biological safety profile. Our results reinforce mini-GRID RT as a promising therapeutic strategy for glioma and other central nervous system tumors, where effective tumor control must be balanced against long-term preservation of functional tissue and minimization of senescence-associated adverse effects.

RESULTS

F98 and RG2 glioma cells exhibit dose-dependent responses with attenuated senescence under mini-GRID irradiation

Seven days after irradiation, both F98 and RG2 glioma cells showed a clear dose-dependent reduction in cell number under Conv-RT and mini-GRID RT, with the most pronounced effect at 20 Gy (Figure 1A–1D). At this highest dose, no significant differences in cell viability were detected between Conv-RT and mini-GRID RT in either cell line, indicating that both modalities exerted a comparable antiproliferative effect.

Morphological analysis revealed irradiation-induced enlargement and flattening of cells (Figure 1A, 1B), consistent with a senescent-like phenotype, predominantly in the 20 Gy Conv-RT groups. In both F98 and RG2, a significant increase in mean cell size was observed only after 20 Gy Conv-RT compared with non-irradiated controls (Figure 1C, 1D), whereas mini-GRID RT did not induce significant cell enlargement, suggesting a milder stress response under spatially fractionated conditions.

SA- β -Gal staining further supported a modality-dependent modulation of senescence (Figure 1E, 1F). In F98 cells, Conv-RT at 20 Gy induced a marked increase in SA- β -Gal positive cells compared with both control and mini-GRID RT, while mini-GRID-irradiated F98 cells showed SA- β -Gal activity comparable to non-irradiated controls (Figure 1G). In RG2 cells, both Conv-RT and mini-GRID RT increased the proportion of SA- β -Gal positive cells relative to controls in a dose-dependent manner. However, senescence levels at 20 Gy remained significantly lower under mini-GRID RT than under Conv-RT (Figure 1H).

Overall, these effects were statistically significant in both cell lines, with 20 Gy Conv-RT consistently increasing cell size and SA- β -Gal positivity compared with controls and mini-GRID RT, while reductions in cell number did not differ between modalities at this dose. Together, these results indicate that mini-GRID RT preserves the overall effectiveness of irradiation while attenuating key features of therapy-induced senescence in glioma cells, in line with a potential therapeutic advantage for spatially fractionated irradiation.

Mini-GRID irradiation attenuates transcriptional, protein and SASP markers of senescence in F98 and RG2 glioma cells

Seven days after irradiation, RT-qPCR analysis of p21 and p16 revealed a modality-dependent regulation of senescence-associated genes in both glioma cell lines (Figure 2A, 2B). Exposure to 20 Gy Conv-RT induced a clear upregulation of p21 and p16 compared with non-irradiated controls, consistent with the activation of a senescence program. In contrast, cells irradiated with mini-GRID RT at 20 Gy showed p21 and p16 expression levels comparable to those of non-irradiated cells, indicating an absence of sustained transcriptional activation of these pathways under spatially fractionated irradiation.

These findings were corroborated at the protein level (Figure 2C). Western blot analysis of F98 and RG2 cell lysates collected seven days post-irradiation showed increased levels of p53, p21, p16 and γ H2AX following 20 Gy Conv-RT, in line with persistent DNA damage signaling and senescence induction. In mini-GRID-treated cells, the accumulation of these proteins was reduced compared with Conv-RT and reached similar levels to the ones observed in non-irradiated controls. Notably, F98 cells exhibited a stronger protein response to conventional irradiation than RG2, particularly in p16 and p21 levels, suggesting intrinsic differences in their sensitivity to radiation-induced senescence.

To assess the functional consequences of these senescence programs, we quantified several SASP-related transcripts (Il1a, Il6, Serpine1 and Cxcl1). Conv-RT induced a robust SASP response in both glioma cell lines, although the magnitude and composition of this response were cell line-dependent (Figure 2D, 2E). Il1a, Il6 and Serpine1 were markedly upregulated after 20 Gy Conv-RT in both F98 and RG2 cells, whereas Cxcl1 showed a more restricted response, with strong induction observed only in RG2 cells. Mini-GRID RT markedly attenuated the induction of these SASP genes, bringing their expression closer to control levels. Together, these results indicate that mini-GRID RT mitigates not only canonical senescence- and damage-associated pathways but also the pro-inflammatory SASP response observed under conventional irradiation, in line with the attenuated senescence phenotype detected by morphological and SA- β -Gal assays.

Non-tumor cell lines exhibit comparable senescence responses under conventional and mini-GRID irradiation

To determine whether the modality-dependent effects observed in glioma cells were tumor-specific, we analyzed two non-tumor cell models under the same

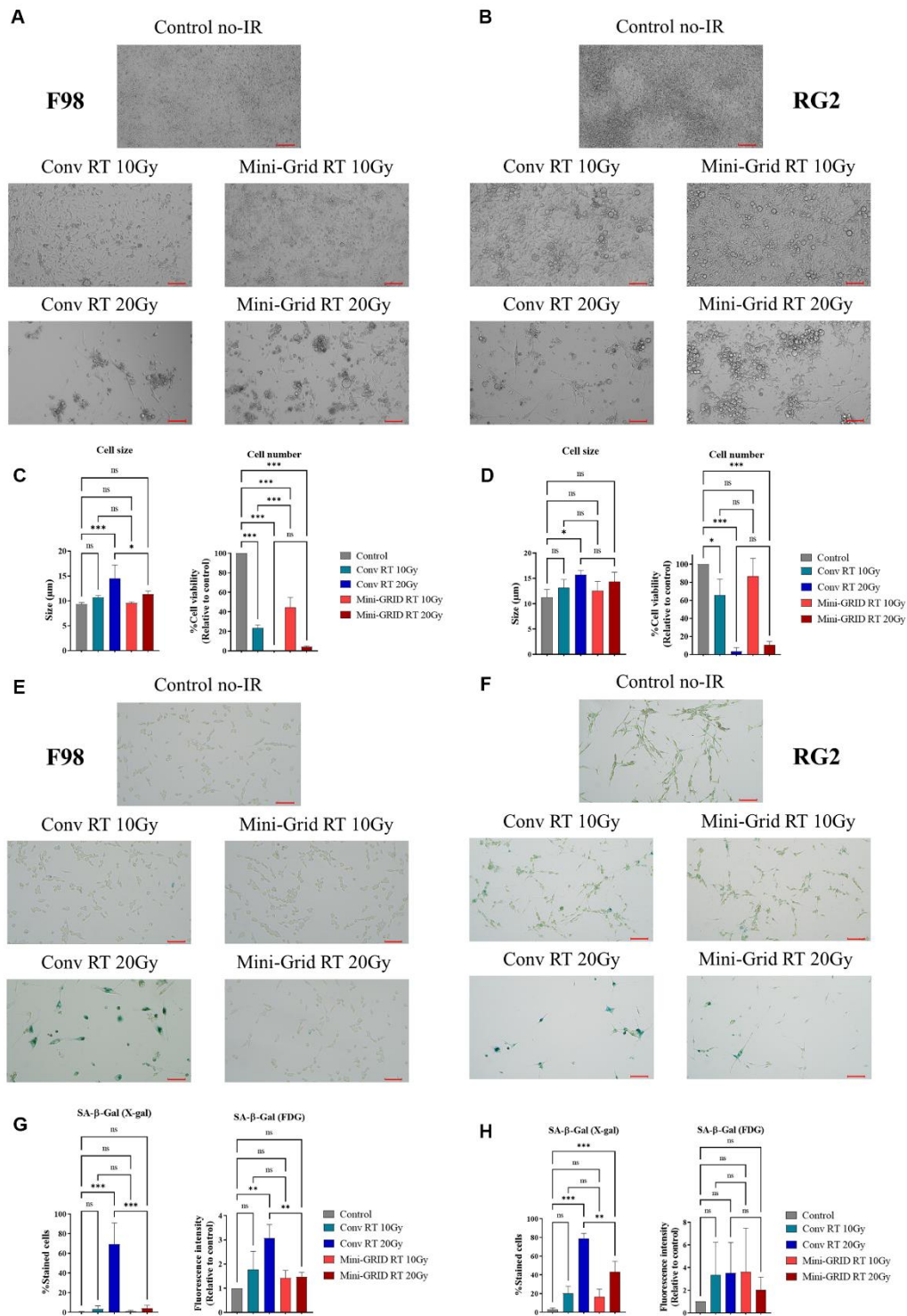


Figure 1. Mini-GRID radiotherapy preserves growth inhibition while softening senescence features in glioma cells. (A, B) Representative phase-contrast images of F98 (A) and RG2 (B) glioma cells 7 days after irradiation with the indicated doses (0–20 Gy) delivered as conventional radiotherapy (Conv-RT) or mini-GRID RT. These images show adherent cultures and are intended to illustrate overall morphology and culture density after treatment. Irradiated cultures, particularly those exposed to 20 Gy Conv-RT, exhibit enlarged and flattened cells consistent with a senescent-like morphology. (C, D) Quantification of radiation responses in F98 (C) and RG2 (D) cells, showing dose-dependent reductions in cell number and increased mean cell size 7 days after irradiation. Cell-size quantification was performed independently on individualized detached cells using a LUNA-II automated cell counter. A significant

enlargement of cell size is observed predominantly after 20 Gy Conv-RT, whereas mini-GRID RT at the same nominal dose induces a milder increase in cell size despite a comparable loss of cell number. (E, F) Representative images of senescence-associated β -galactosidase (SA- β -Gal) staining in F98 (E) and RG2 (F) cells 7 days after treatment. (G, H) Quantification of SA- β -Gal-positive cells in F98 (G) and RG2 (H) cultures. In both cell lines, Conv-RT at 20 Gy strongly increases SA- β -Gal positivity compared with non-irradiated controls, whereas mini-GRID RT results in lower senescence levels at the same dose, with F98 mini-GRID samples approaching control values. Data are presented as mean $\pm$ SD of at least three independent experiments. Statistical significance was assessed by one-way ANOVA (* p < 0.05; ** p < 0.01; *** p < 0.001. ns, not significant).

experimental conditions: CTX-TNA2 astrocytes and primary murine embryonic fibroblasts (MEFs). CTX-TNA2 cells were exposed to 5, 10, and 15 Gy, whereas MEFs received 10 and 20 Gy, delivered either as Conv-RT or mini-GRID RT.

In MEFs, representative images illustrate irradiation-induced morphological changes 7 days after treatment (Figure 3A), paralleled by increased SA- β -Gal staining at higher doses (Figure 3B). Quantitatively, MEFs displayed a clear dose-dependent increase in mean cell

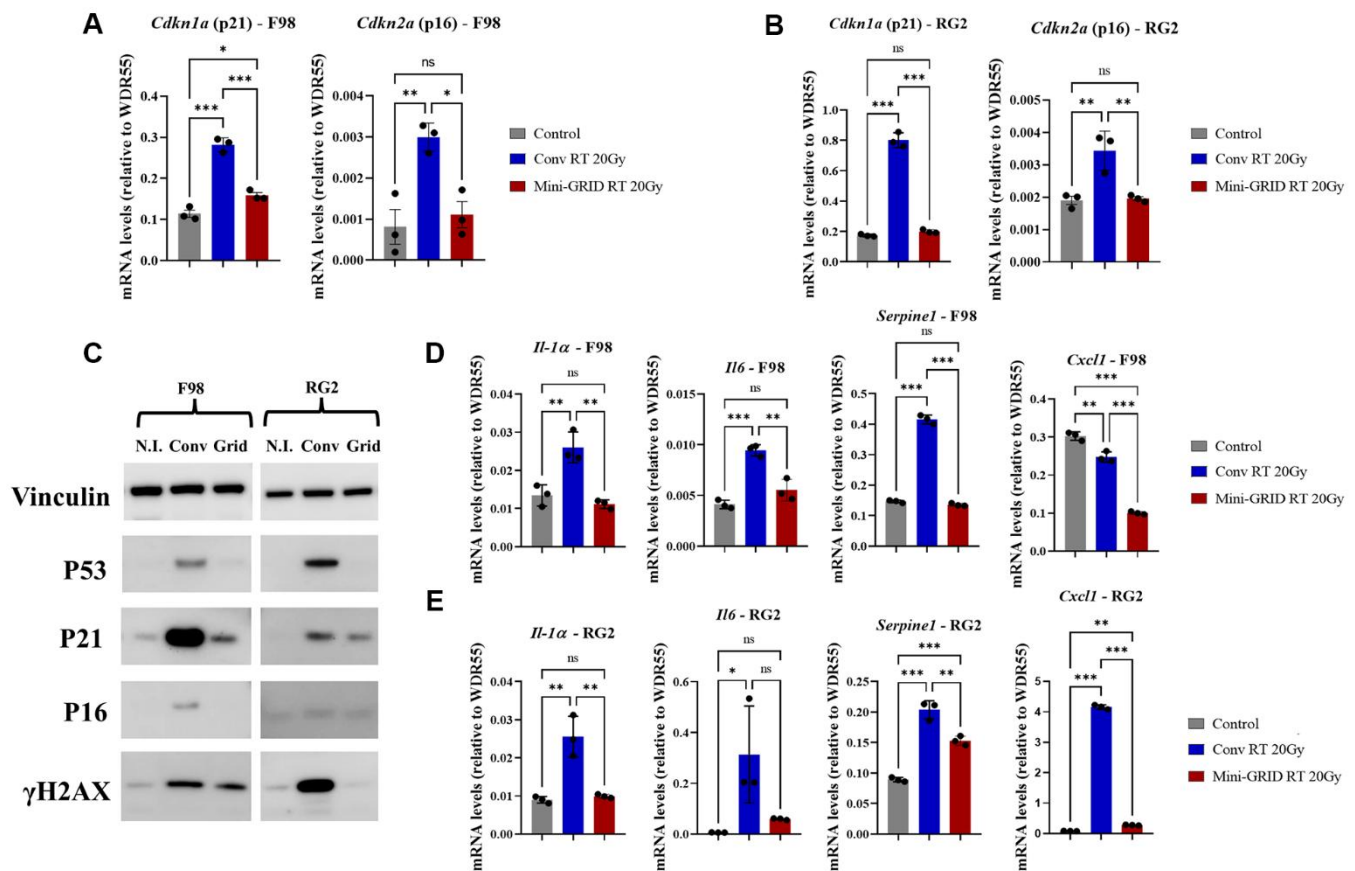


Figure 2. Molecular and secretory profiling reveals a reduced senescence burden after mini-GRID irradiation in F98 and RG2 cells. (A, B) Relative mRNA expression of *Cdkn1a* (p21) and *Cdkn2a* (p16) in F98 (A) and RG2 (B) cells 7 days after irradiation with 20 Gy delivered as Conv-RT or mini-GRID RT, normalized to the reference gene *Wdr55* and expressed as fold-change versus non-irradiated controls ($2^{-\Delta\Delta Ct}$). Conv-RT induces a robust upregulation of p21 and p16, whereas expression levels in mini-GRID-treated cells remain close to those of controls. (C) Representative Western blot analysis of p53, p21, p16 and γ H2AX protein levels in F98 and RG2 cells 7 days after treatment. N.I., non-irradiated. Conv-RT at 20 Gy leads to accumulation of DNA damage and senescence markers, whereas mini-GRID RT reduces the induction of these proteins and brings their levels closer to those observed in non-irradiated cells. Vinculin is shown as a loading control. (D, E) Expression of selected senescence-associated secretory phenotype (SASP) factors *Il1a*, *Il6*, *Serpine1* and *Cxcl1* in F98 (D) and RG2 (E) cells 7 days after irradiation, quantified by RT-qPCR and expressed as fold-change versus non-irradiated controls. Conv-RT elicits strong induction of all four SASP genes, whereas mini-GRID RT markedly attenuates this response, with *Il1a*, *Il6* and *Serpine1* approaching baseline levels and *Cxcl1* induction being strongly blunted or almost abolished. Data are mean $\pm$ SD of at least three independent experiments. One-way ANOVA was used to assess statistical significance (* p < 0.05; ** p < 0.01; *** p < 0.001. ns, not significant).

size and a reduction in cell number (Figure 3C). SA- β -Gal activity assessed by both X-Gal staining and FDG fluorescence increased significantly at the highest dose compared with controls (Figure 3D). Importantly, across all readouts, no significant differences were detected between Conv-RT and mini-GRID RT at matched doses.

Similarly, CTX-TNA2 astrocytes showed a dose-dependent response in morphology (Figure 3E) and SA- β -Gal readouts (Figure 3F). Quantification confirmed increased mean cell size and reduced cell number with escalating doses (Figure 3G), while SA- β -Gal activity measured by X-Gal and/or FDG followed the same dose-dependent pattern (Figure 3H). Again, no modality-dependent differences were observed.

Together, these findings indicate that, unlike glioma cells, non-tumor cell lines do not display a modality-dependent modulation of senescence, supporting the notion that mini-GRID RT elicits reduced senescence responses in tumor cells while exerting similar effects to conventional irradiation in non-tumor backgrounds.

DISCUSSION

Radiation-induced senescence is increasingly recognized as an important component of the cellular response to radiotherapy, with potential consequences for both tumor control and normal tissue toxicity. In this study we explored for the first time the impact of spatially fractionated mini-GRID radiotherapy on the induction of senescence and the senescence-associated secretory phenotype (SASP) in tumor and non-tumor cell lines. Our results show that, in F98 and RG2 glioma cells, conventional radiotherapy at 20 Gy induces a robust senescent response characterized by increased cell size, high SA- β -Gal activity, accumulation of damage and cell-cycle markers (p53, p21, p16, γ H2AX) and a strong activation of several SASP genes, particularly *Il1a*, *Il6* and *Serpine1*, with *Cxcl1* showing a more cell line-specific pattern. In contrast, mini-GRID irradiation preserves the cytotoxic effect, as reflected by a reduced number of tumor cells, but clearly attenuates this response. Cell morphology and SA- β -Gal activity in mini-GRID irradiated cells are similar to those of controls, p21/p16 and SASP gene expression remain close to basal levels, and DNA damage signaling is not increased. This differential effect was not observed in non-tumor cell lines (astroglial CTX-TNA2 and MEFs), where both modalities induced senescence in a dose-dependent manner without significant differences.

Our observation that mini-GRID limits the establishment of stable senescence has important radiobiological implications. Therapy-induced senescence

(TIS) is increasingly recognized as a double-edged sword [6, 7]. On the one hand it can support anti-tumor immune responses—radiation-induced senescence activates the SASP and promotes NKT cell activation and M1 macrophage polarization [21]. On the other hand, the SASP is heterogeneous, and in the long term it can create a pro-inflammatory and immunosuppressive microenvironment that favors tumor progression and resistance to therapy [21]. Persistent senescence may increase PD-L1 expression and reduce cell susceptibility to T-cell responses [22–25]. In addition, recent studies show that radiation-induced astrocyte senescence enhances glioblastoma growth and invasiveness through IL-6 release and JAK2–STAT3 activation, and that pharmacological elimination of senescent cells reduces tumor volume and prolongs survival in mouse models [26]. Our finding that mini-GRID attenuates p21/p16 expression and markedly reduces *Il1a*, *Il6* and *Serpine1* levels indicates that this modality mitigates the establishment of a full SASP in glioma cells. This could decrease the release of pro-inflammatory factors such as IL-6 and limit the pro-tumor effects of the senescent microenvironment.

Interestingly, *Cxcl1* displayed a more divergent pattern than the other SASP-associated genes analyzed. Whereas *Il1a*, *Il6* and *Serpine1* were broadly induced by Conv-RT and attenuated by mini-GRID RT, *Cxcl1* showed a clear cell line-specific response: it was not induced in F98 cells at day 7, but was strongly upregulated in RG2 cells after Conv-RT. This is consistent with the heterogeneous nature of the SASP, whose composition depends on cell type, senescence trigger, timing and genetic background [27]. *Cxcl1*, a *Cxcr2* ligand involved in inflammatory chemokine signaling and immune-cell recruitment, has been linked to senescence reinforcement, radiation-induced inflammatory responses and radioresistance in tumor models [28–30]. Thus, the strong induction of *Cxcl1* in RG2 cells after Conv-RT may reflect a more prominent chemokine-driven inflammatory SASP in this cell line, whereas F98 may rely on other SASP mediators. Importantly, mini-GRID RT did not trigger this *Cxcl1* response in RG2, supporting the conclusion that spatially fractionated irradiation attenuates selected pro-inflammatory SASP components in a cell line-dependent manner.

These differences observed between F98 and RG2 further support the notion that radiation-induced senescence is highly context-dependent [3, 19]. Although both glioma cell lines showed attenuation of senescence-associated features after mini-GRID RT compared with Conv-RT, the magnitude and molecular pattern of this response differed between them. F98

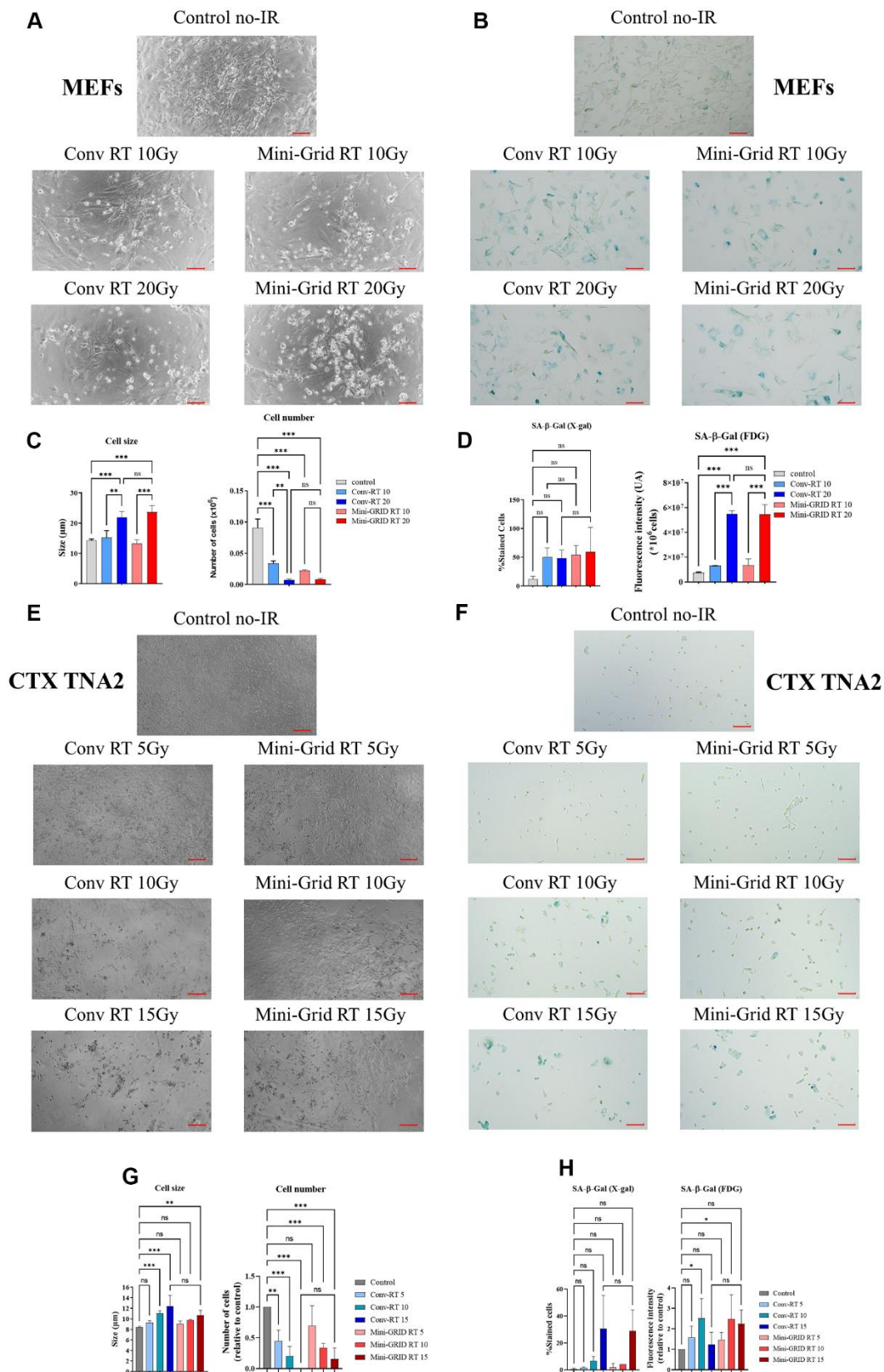


Figure 3. Non-tumor astrocytes and fibroblasts show similar senescence induction after conventional and mini-GRID irradiation. (A) Representative microscopy images of primary mouse embryonic fibroblasts (MEFs) 7 days after irradiation (0, 10, and 20 Gy) delivered as conventional radiotherapy (Conv-RT) or mini-GRID RT. (B) Representative senescence-associated β -galactosidase (SA- β -Gal; X-

Gal) staining images of MEFs under the same conditions. (C) Quantification of MEF radiation responses (mean cell size and cell number) showing a dose-dependent increase in cell size and reduction in cell number, with no significant differences between Conv-RT and mini-GRID RT at matched doses. (D) Quantification of SA- β -Gal activity in MEFs by X-Gal positivity and FDG-derived fluorescence, revealing increased senescence at the highest dose compared with controls, with similar levels across irradiation modalities. (E) Representative phase-contrast images of CTX-TNA2 astrocytes 7 days after irradiation (0, 5, 10, and 15 Gy) delivered as Conv-RT or mini-GRID RT. (F) Representative SA- β -Gal readouts in CTX-TNA2 cells. (G) Quantification of CTX-TNA2 radiation responses (mean cell size and cell number) showing a dose-dependent phenotype without modality-dependent differences. (H) Quantification of SA- β -Gal activity in CTX-TNA2 cells (X-Gal and/or FDG), confirming comparable senescence induction under Conv-RT and mini-GRID RT. Data are presented as mean $\pm$ SD from at least three independent experiments. Statistical significance was assessed by one-way ANOVA (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. ns, not significant).

cells displayed a stronger induction of several canonical senescence and DNA damage markers, particularly p16 and p21 at the protein level, whereas RG2 cells showed a distinct SASP profile response after Conv-RT. These differences may reflect intrinsic cell line-specific properties, including basal stress levels, proliferative state, DNA damage response capacity, and differential engagement of the p53/p21 and p16/Rb pathways. Therefore, rather than indicating a uniform senescence program shared by both glioma models, our data suggest that mini-GRID RT attenuates radiation-induced senescence across distinct glioma backgrounds, although the molecular composition of this response remains cell line-dependent.

The modality-dependent differences observed between conventional and mini-GRID irradiation probably reflect mechanisms intrinsic to spatial dosimetry. In mini-GRID, narrow high-dose peaks cause lethal damage in the cells beneath, leading to cell death rather than senescence, whereas valley regions receive sublethal doses that allow repair without fully activating the senescence program. This aligns with the concept of “functional dose painting,” where tumor tissue is subdivided into subpopulations exposed to lethal and sublethal damage. Infrared microscopy studies suggest that mini-GRID induces less protein denaturation and oxidative damage in tissue than conventional radiotherapy [13], which may contribute to the reduction of persistent damage signals and the lower p53/p21 activation we observed. In contrast, homogeneous radiation exposes all cells to high doses that may exceed the repair threshold and activate senescence and SASP pathways. Moreover, evidence that ionizing radiation induces senescence and SASP not only in irradiated cells but also in neighboring cells through non-targeted effects and extracellular vesicles [31] highlights the importance of reducing the burden of senescent cells in the treated volume. Mini-GRID, by limiting the number of cells exposed to intermediate doses, could reduce paracrine propagation of senescence and inflammatory signaling.

Although our data highlight the potential of mini-GRID to improve the therapeutic ratio, they also show that this benefit appears specifically in tumor cells. In the

non-tumor models (astroglia CTX-TNA2 and MEFs), both conventional radiotherapy and mini-GRID induced senescence in a dose-dependent fashion without notable differences. This finding suggests that some normal cells are less sensitive to spatial dose modulation, possibly because they have a greater capacity for repair or a lower dependence on the p53/p16 pathway. In addition, our study was limited to a panel of murine cell lines in 2D culture, to single doses and to a single time point. Importantly, 2D monolayer cultures cannot fully recapitulate the paracrine propagation of senescence within a structured tissue microenvironment. In this setting, SASP factors, extracellular vesicles and bystander signals diffuse freely in shared culture medium, whereas in 3D or *in vivo* models their distribution may be shaped by extracellular matrix, tissue architecture, vascularization, immune cells and oxygen/nutrient gradients. We also did not evaluate the temporal dynamics of senescence, the long-term persistence of the SASP, or interactions with other components of the tumor microenvironment. Similarly, we explored only one mini-GRID configuration. Other geometries, fractionation schemes or peak-to-valley ratios could produce different effects. These limitations underscore the need for studies in three-dimensional and *in vivo* models that better reproduce the complexity of the tumor microenvironment, including vascularization and immune responses [8, 32]. The 7-day endpoint was selected to evaluate an established senescence-associated phenotype rather than an acute DNA damage response. Therapy-induced senescence typically requires several days after treatment to develop characteristic morphological changes, SA- β -Gal activity and sustained activation of cell-cycle arrest/SASP markers. Senescence-associated markers may also follow different temporal kinetics after irradiation: early responses can include DNA damage signaling and p53/p21 activation, whereas later stages are generally associated with sustained cell-cycle arrest, increased SA- β -Gal activity, morphological enlargement and progressive SASP remodeling. Previous studies in glioma/astrocyte models have linked early p21 responses at 24 h with SA- β -Gal positivity at later time points such as days 5–7, supporting the use of this window to assess established radiation-induced senescence [33, 34]. Thus, day 7 was chosen as a

biologically relevant endpoint to assess persistent radiation-induced senescence in surviving cells after Conv-RT or mini-GRID RT, while recognizing that individual markers may peak, decline or evolve at different times after irradiation.

Looking ahead, our results support mini-GRID as a promising strategy for high-grade tumors of the central nervous system. By attenuating senescence and the SASP, mini-GRID could reduce the need for complementary senolytic or senomorphic drugs currently proposed to mitigate the adverse effects of therapy-induced senescence [21]. The immune context also deserves attention. Spatially fractionated mini-GRID radiotherapy can stimulate systemic anti-tumor immunity: localized high-dose peaks induce immunogenic cell death while low-dose valleys spare resident immune cells, allowing tumor antigens and chemokines to recruit and activate T cells [9, 10]. Emerging evidence suggests that this combination of high-dose and low-dose subregions may synergize with immunotherapies [35, 36] and generate abscopal or distant tumor responses that are rarely seen after uniform radiotherapy. Minibeam radiotherapy was able to turn the irradiated tumor into an “*in situ* vaccine” [9] by releasing tumor-specific antigens and stimulating the innate and adaptive immune responses. Further preclinical and clinical research is needed to identify optimal doses, sequencing and combinations for maximizing synergy with checkpoint inhibitors. Future work should also evaluate how the reduction of the senescent burden by mini-GRID influences immune activation or suppression, the incidence of abscopal effects, and the long-term need for senolytics. In summary, mini-GRID radiotherapy emerges as a tool capable of decoupling the cytotoxic efficacy of radiation from the induction of senescence and the SASP in tumor cells. This, combined with its potential to enhance immunotherapy, could translate into better clinical outcomes and a reduction in long-term detrimental effects.

Future work in 3D models and *in vivo*, incorporating longitudinal analyses and functional readouts of SASP and immune interactions, will be essential to validate and extend these observations and to define how best to exploit mini-GRID RT within therapeutic strategies aimed at minimizing senescence-associated adverse effects.

In conclusion, our findings show that radiation-induced senescence scales strongly with dose in both glioma and non-tumor cells. In glioma cell lines F98 and RG2, the mini-GRID modality achieves a comparable reduction in cell number to conventional radiotherapy, yet markedly results in reduced

canonical senescence markers, persistent DNA damage and SASP activation. In contrast, for non-tumor CTX-TNA2 astrocytes and mouse embryonic fibroblasts, senescence increases with dose regardless of the irradiation technique, and no significant differences emerge between conventional and mini-GRID delivery under the conditions tested.

These observations indicate that spatially fractionated mini-GRID RT can alter the qualitative nature of radiation-induced stress responses in tumor cells without exacerbating senescence in healthy tissues. By preserving cytotoxic efficacy while limiting senescent signaling, mini-GRID holds promise for improving the therapeutic ratio and potentially reducing the need for senolytic interventions. Further preclinical studies in more complex models are warranted to validate these benefits and to optimize clinical translation.

MATERIALS AND METHODS

Cell lines and culture conditions

The cell lines included in this study were two rat glioma tumor cell lines (F98 and RG2), one immortalized rat astrocyte cell line, CTX TNA2 and cells from primary cultures of mouse embryonic fibroblasts, MEFs. Cells were maintained in Dulbecco's Modified Eagle Medium, High Glucose (11965092, Gibco), supplemented with 10% foetal bovine serum ((21523002, Corning), 1% penicillin–streptomycin (15140-122, Gibco), and sodium pyruvate (11360-039, Gibco). For the RG2 cell line, the medium was additionally supplemented with HEPES buffer (15630-049, Gibco). All cultures were incubated at 37° C in a humidified atmosphere containing 5% CO₂ and grown to approximately 80% confluence before use. Routine mycoplasma testing was performed for all cell lines to ensure contamination-free cultures.

The day before irradiation, cells were seeded in 48-well plates at a density of 20,000 cells/well (F98, RG2, MEFs) or 12,000 cells/well (CTX-TNA2). Due to the limited size of the irradiation field in the mini-GRID modality, only four wells on each side of a 48-well plate (MW48) were designated for sample irradiation. To maintain consistency, the same well layout was used for conventional irradiation. Surrounding wells and interstitial spaces were filled with 200 µL of sterile water, matching the sample medium volume, to minimize edge effects and ensure uniform dose distribution (Supplementary Figure 1). The day of the irradiation, cells were at 60% of confluency. Immediately before irradiation, the culture medium was replaced and the volume reduced from 500 µL to 200 µL per well. Twenty-four hours after irradiation, the

medium was changed again and cells were maintained in the incubator for 7 days.

Mouse Embryonic Fibroblast (MEF) isolation

MEFs were obtained from C57BL/6 WT embryos at embryonic day 13.5 (E13.5). Pregnant females were euthanized by cervical dislocation, and uteri were collected under sterile conditions. Embryos were isolated, euthanized by thermal shock, and maintained in PBS containing 1% penicillin/streptomycin (Sigma-Aldrich) at 4° C. Heads and internal organs were removed, and the remaining tissue was washed in PBS, minced with sterile scalpels, and incubated in 1.5 mL of trypsin-EDTA (Sigma-Aldrich) at 37° C for 10 min. The suspension was gently pipetted to promote dissociation and further incubated for an additional 10 min. Cells were then resuspended in standard culture medium and plated in 100 mm dishes (Corning). Cultures were expanded to 150 mm dishes upon reaching confluence. Cells were cryopreserved in aliquots of 4×10^6 cells in freezing medium at -80° C for 24 h and subsequently transferred to liquid nitrogen for long-term storage.

Irradiation setup and dosimetry

Irradiations were carried out using a 6 MV Varian TrueBeam linear accelerator operating in flattening filter-free (FFF) mode at the Hospital Clínico de Santiago de Compostela. To implement the mini-GRID technique for *in vitro* irradiation, a custom-designed collimator previously described [13] (Supplementary Figure 1A) was mounted on the accelerator head. For conventional radiotherapy (Conv-RT), this collimator was removed (Supplementary Figure 1C).

F98, RG2, and mouse embryonic fibroblast (MEF) cell lines were irradiated with single doses of 10 Gy and 20 Gy. The immortalized astrocyte line CTX TNA2, expected to be more radiosensitive, was exposed to 5 Gy, 10 Gy, and 15 Gy.

The irradiation setup consisted of a phantom composed of 2 cm of solid water slabs (RW3), the MW48 culture plate, and an additional 5 cm of RW3 slabs placed on top. For mini-GRID RT, the phantom surface was positioned at the isocentre and irradiated using a single field at a gantry angle of 0°. For Conv-RT, the MW48 plate was positioned close to the isocentre to ensure dose homogeneity and irradiated using two opposing fields at gantry angles of 0° and 180°.

The dose prescription was 5, 10, 15 and 20 Gy mean dose in both Conv-RT and mini-GRID. The mean dose was calculated as the mean dose of the profile between the two most extreme peaks in the central profile. Table 1

shows the dosimetry parameters at the cell position measured with Gafchromic EBT-XD films on the plate. On the day of the irradiation, radiochromic films (Gafchromic EBT-XD) were placed on the MW48 plate to verify dose delivery for both irradiation modalities (Supplementary Figure 1B, 1D). The measured average doses agreed with the prescribed values within $\pm 3\%$ for both cell lines, demonstrating excellent reproducibility and dose delivery accuracy.

Senescence studies

Senescence-Associated β -Galactosidase (SA- β -Gal) staining and quantification

Cells were fixed at room temperature with a solution containing 2% formaldehyde and 0.2% glutaraldehyde. After washing, they were incubated overnight at 37° C with a freshly prepared SA- β -Gal staining solution [17, 37] containing either 1 mg/mL 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-Gal; Fisher Scientific) or 20 mM fluorescein di- β -D-galactopyranoside (FDG), in 40 mM citric acid/sodium phosphate buffer (pH 5.6) supplemented with 5 mM $K_3Fe[CN]_6$, 5 mM $K_4Fe[CN]_6$, 150 mM NaCl, and 2 mM $MgCl_2$.

For X-Gal staining, blue-stained cells were visualized and quantified under an optical microscope, and results were expressed as the percentage of SA- β -Gal-positive cells. For FDG-based assays, the culture medium containing the released fluorescent product was collected, and fluorescence intensity was measured at 485 nm excitation and 535 nm emission using a Mithras LB 940 microplate reader (Berthold Technologies).

Cell size was determined using a LUNA-II™ automated cell counter (Logos Biosystems, Anyang, South Korea) after cell detachment, allowing the analysis of individualized cells in suspension.

Gene expression analysis (RT-qPCR)

Total RNA was extracted from cultured cells using the NucleoSpin RNA Plus kit (Macherey-Nagel) according to the manufacturer's instructions. Relative mRNA expression was quantified by one-step reverse transcription quantitative PCR (RT-qPCR) using the NZYSpeedy One-step RT-qPCR Green kit, ROX (NZYtech), on a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific).

Gene-specific primers were used for Wdr55 (reference gene), *Cdkn1a* (p21), *Cdkn2a* (p16) and for the SASP-related genes *Il1a*, *Il6*, *Serpine1* and *Cxcl1* (primer sequences in Supplementary Table 1). Each reaction was performed in triplicate for every sample and target

Table 1. Data are showing the average dose for each prescribed dose and modality of irradiation and the measured peak dose, valley dose, peak-to-valley dose ratio (PVDR) at 2 cm from the central axis in the mini-GRID irradiation.

Dose	Conv-RT		Mini-GRID RT		
	Average dose (Gy)	Average dose (Gy)	Peak dose (Gy)	Valley dose (Gy)	PVDR
5 Gy	4.96 ± 0.15	4.91 ± 0.11	10.2 ± 0.6	3.28 ± 0.15	3.10 ± 0.3
10 Gy	9.9 ± 0.4	9.8 ± 0.3	20.5 ± 1.5	7.1 ± 0.4	2.9 ± 0.4
15 Gy	14.6 ± 0.3	14.5 ± 0.3	29.7 ± 1.5	10.6 ± 0.4	2.8 ± 0.2
20 Gy	20.8 ± 1.3	19.9 ± 0.5	41.2 ± 2.6	14.4 ± 0.7	2.9 ± 0.3

gene. Relative expression levels were calculated using the 2^{−ΔΔCt} method [38], normalizing to Wdr55.

Protein expression (Western blot)

Cells were lysed in RIPA buffer supplemented with protease inhibitors, and total protein concentration was determined using the Bradford assay. Equal protein amounts (15 µg per sample) were separated by SDS-PAGE on 10% polyacrylamide gels and transferred onto PVDF membranes (0.45 µm, Cytiva). Membranes were blocked with 5% BSA in TBS-T (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 1 h at room temperature, followed by overnight incubation at 4° C with the following primary antibodies: anti-p16 (M-156, sc-1207, Santa Cruz Biotechnology), anti-p21 (F-5, sc-6246, Santa Cruz Biotechnology), anti-p53 (#2524; Cell Signaling Technology), anti-Vinculin (7F9, sc-73614, Santa Cruz Biotechnology), and anti-γH2AX (#9718, Cell Signaling Technology).

After washing, membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Immunoreactive bands were visualized using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) and imaged with an Amersham ImageQuant 800 system (Cytiva).

Statistical analysis

Data from at least three independent experiments were analyzed using one-way ANOVA. Results were expressed as mean ± standard deviation (SD), and differences were considered significant at p < 0.05. Significance levels are indicated as follows: *p < 0.05; **p < 0.01; ***p < 0.001. ns, not significant.

ACKNOWLEDGMENTS

We acknowledge the support of the María de Maeztu program grant (CEX2024-001463-M) funded by MICIU/AEI/10.13039/501100011033, and from the

“Regional Ministry of Education, Science, Universities and Vocational Training” of the Xunta de Galicia through the CIGUS Network of Research Centres (ED431G/2023/02), and the European Union through the European Regional Development Fund (ERDF).

AUTHOR CONTRIBUTIONS

M.I.A. and M.A.P. performed experiments, analyzed data and wrote the manuscript. S.D.S.-A. contributed to experimental work and data analysis. V.L-V., E.G.K. and M.S-G. performed irradiation and dosimetry. Y.P. and M.C. conceived and supervised the study, secured funding and wrote the manuscript. All authors reviewed and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

FUNDING

Y.P. is an Oportunius Research Professor of the Galician Agency of Innovation (GAIN), Xunta de Galicia. Work in her laboratory is funded by Xunta de Galicia (2024-PN189-I). Work in the laboratory of M.C. is funded by grants from MICIU/AEI/10.13039/501100011033 and by “ERDF/EU” (PID2024-159335OB-I00) and GAIN, Xunta de Galicia (IN607B2024/13). M.A.P. was supported by pre-doctoral fellowships from GAIN, Xunta de Galicia (ED481A 2022/432) and FPU program from MCIU (FPU23/02493). S.D.S.-A. is supported by a Juan de la Cierva fellowship (JDC2022-049032-I) funded by MCIU/AEI/10.13039/501100011033 and European Union “NextGenerationEU”/PRTR”.

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 PMID:39827884

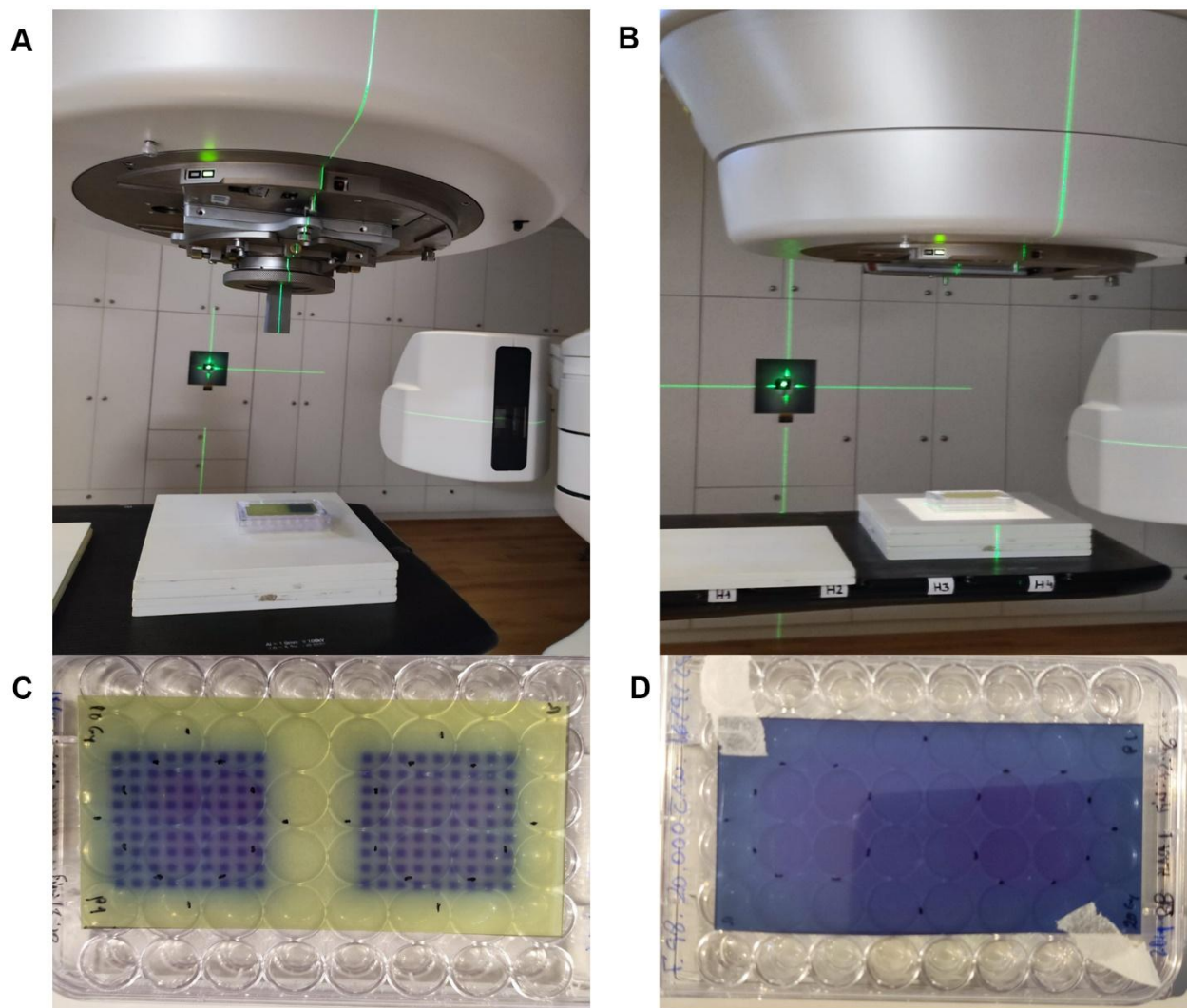
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SUPPLEMENTARY MATERIALS

Supplementary Figure



Supplementary Figure 1. Irradiation setup for 48-well plates. The custom collimator was mounted for mini-GRID irradiation (A) and removed for conventional irradiation (B). Representative radiochromic films show the spatially modulated mini-GRID dose distribution (C) and the homogeneous Conv-RT dose distribution (D).

Supplementary Table

Supplementary Table 1. Primer sequences.

Gene	Primers	Sequence (5'-3')
Wdr55	Forward (Fw)	GGGACGTGGACGGAGATGTG
	Reverse (Rv)	GAGAAGACAACAGCCCGGCA
Cdkn1a (p21)	Forward (Fw)	GTGGGTCTGACTCCAGCCC
	Reverse (Rv)	CCTTCTCGTGAGACGCTTAC
Cdkn2a (p16)	Forward (Fw)	CGTACCCCGATTCAAGGTGAT
	Reverse (Rv)	TTGAGCAGAAGAGCTGCTACGT
Il6	Forward (Fw)	GCTACCAAACCTGGATATAATCAGGA
	Reverse (Rv)	CCAGGTAGCTATGGTACTCCAGAA
Il1a	Forward (Fw)	TCCATAACCCATGATCTGGAA
	Reverse (Rv)	TTGGTTGAGGGAATCATTAT
Cxcl1	Forward (Fw)	CTGGGATTCACCTCAAGAACATC
	Reverse (Rv)	CAGGGTCAAGGCAAGCCTC
Serpine1	Forward (Fw)	GCCAGGGTTGCACTAAACAT
	Reverse (Rv)	CCAACATCTTGGATGCTGAA