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Quiescence does not affect p53 and stress response by irradiation in human lung fibroblasts

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ABSTRACT

Cells in many organs exist in both proliferating and quiescent states. Proliferating cells are more radiosensitive, DNA damage pathways including p53 pathway are activated to undergo either G_1/S or G_2/M arrest to avoid entering S and M phase with DNA damage. On the other hand, quiescent cells are already arrested in G_0, therefore there may be fundamental difference of irradiation response between proliferating and quiescent cells, and this difference may affect their radiosensitivity. To understand these differences, proliferating and quiescent human normal lung fibroblasts were exposed to 0.10–1 Gy of γ-radiation. The response of key proteins involved in the cell cycle, cell death, and metabolism as well as histone H2AX phosphorylation were examined. Interestingly, p53 and p53 phosphorylation (Ser-15), as well as the cyclin-dependent kinase inhibitors p21 and p27, were induced similarly in both proliferating and quiescent cells after irradiation. Furthermore, the p53 protein half-life, and expression of cyclin A, cyclin E, proliferating cell nuclear antigen (PCNA), Bax, or cytochrome c expression as well as histone H2AX phosphorylation were comparable after irradiation in both phases of cells. The effect of radioprotection by a glycogen synthase kinase 3β inhibitor on p53 pathway was also similar between proliferating and quiescent cells. Our results showed that quiescence does not affect irradiation response of key proteins involved in stress and DNA damage at least in normal fibroblasts, providing a better understanding of the radiation response in quiescent cells, which is crucial for tissue repair and regeneration.

1. Introduction

Ionizing radiation is an invaluable diagnostic and treatment tool used in various clinical applications [1,2]. However, assessing normal tissue injury during radiological treatments and accidental radiation exposure, such as nuclear power plant malfunctions, terrorist attacks with “dirty bombs,” or detonation of nuclear weapons, are of great interest [3–6]. Extensive epidemiological and toxicological research over the past decades has been focused on the health effects of radiation exposure to both the public and the workforce [7–9]. The past Chernobyl and Fukushima Daiichi disasters have demonstrated that first responders to such accidental radiation emergencies have high risks of radiation exposure [10–12].

Radiation causes injury to normal tissues by inducing cell death, or altering cell-to-cell communication, inflammatory responses, compensatory tissue hypertrophy, and repair processes [12–16]. Damage responses vary from organ to organ [17]. Because most organs are composed of both proliferating and quiescent (out of the cell cycle with a reversible state of growth and lower metabolic rate) cells, understanding how these cells differ in their radiation sensitivity will help prevent or reduce radiation injury. Proliferating cells are more sensitive than quiescent cells to radiation-induced cell death due to various reasons such as getting severe DNA damage if radiation occurs during DNA synthesis and mitosis, less time to repair DNA damage [18,19]. Lemons et al. [20] reported high antioxidant activity in quiescent primary human skin fibroblasts (as

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shown by NADPH production) and suggested that this process may help cells detoxify free radicals or synthesize fatty acids. In an earlier study [21], we reported specific activation of protein kinase C (PKC) isoforms in quiescent fibroblasts (MRC-5) after exposure to different doses of γ-irradiation.

Emerging evidence indicates that both quiescent and active cell populations coexist in several tissues, in separate yet adjoining locations [22]. Normal mammalian cells possess unique regulatory mechanisms to shift from a quiescent state to a proliferative state [23], and dysregulation of these mechanisms might result in malignant transformation [24]. Cellular quiescence and the capacity to enter the proliferation cycle are critical for maintaining tissue homeostasis [25,26]. However, radiation responses in quiescent fibroblast cells are still poorly understood. In the present study, we investigated the key proteins involved in cell cycle, DNA damage response, and cell death in both the human normal lung fibroblasts after exposure to 0.10–1 Gy of γ-irradiation. We further evaluated the radioprotection of the small molecule SB216763, a GSK-3β inhibitor [27]. We report that quiescent cells treated with γ-irradiation affect key proteins involved in stress and DNA damage similar to that of proliferating cells treated with γ-irradiation. Our results are applicable in the medical field as well as accidental or intentional radiation exposures.

2. Materials and methods

2.1. Cell culture and treatment

Human normal lung fibroblasts (IMR-90) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Fibroblasts are widely used as a cell model to study cell quiescence and cell cycle-dependent cellular changes [20,23,28]. Cells were routinely maintained in Eagle's minimal essential media (E-MEM) supplemented with 15% fetal bovine serum (FBS), vitamins, essential amino acids, non-essential amino acids, and antibiotics (GIBCO, Carlsbad, CA, USA) as described previously [29–31]. Cells were synchronized by growing them to confluence. Cells can be rendered quiescent due to contact inhibition [3,29] and were maintained for two weeks with media changes every 3 days. Cells rest in this stage (G_0) for a few weeks and can be grown normally when plated into new culture flasks [29].

Cells were irradiated with 0, 0.10, 0.20, 0.40, 0.50, 0.75, or 1 Gy of γ-rays using a ^{137}Cs source at a dose rate of 0.85 Gy/min (Gamma cell 40 Exactor, MDS Nordion, Ontario, Canada). For radioprotection experiments, cells were treated with 15 μM SB216763 [3- (2,4-dichlorophenyl) − 4 − (1-methyl-1H-indol-3-yl) − 1H − pyrrole − 2, 5-dione; dissolved in DMSO] (Sigma, St Louis, MO, USA) for 16 h before irradiation. Dose and time intervals were selected based on the preliminary studies (data not shown). For protein analyses, cells were harvested 1 h and 2.5 h after irradiation.

2.2. Measurement of cell cycle distribution

For cell cycle analyses, we used proliferating and quiescent cells cultured in 75 cm^2 tissue culture flasks (Nunc, Roskilde, Denmark). Cell cycle was assessed as previously described [30].

2.3. Analyses of micronuclei (MN) formation

We used the common biological endpoint of MN formation to assess the effects of irradiation on the DNA damage response. Proliferating and confluent cells grown on cover slips were irradiated with 0, 0.5, and 1 Gy. MN formation was assessed *in situ* as previously described [30].

2.4. Cycloheximide (CHX) treatment

Cycloheximide (CHX), a protein synthesis inhibitor, was used to study radiation-induced steady state levels of protein synthesis. CHX (Sigma, St Louis, MO, USA) was added (10 μg/ml) to all cultures; after 30 min, cells were either left un-irradiated or exposed to 1 Gy of γ-radiation. Cells were harvested at 0, 0.5, 2, 4, and 6 h (proliferating cells) and 0, 0.5, 2, and 6 h (quiescent cells). Steady state levels of p53 and p21 were assessed by immunoblot analyses.

2.5. Protein isolation, SDS-PAGE, and Western blot analyses

Total cellular proteins were isolated from control and irradiated cells using RIPA (radioimmunoprecipitation assay) buffer (1% w/v Nonidet P-40, 1% w/v sodium deoxycholate, 0.1% w/v SDS, 0.15 M NaCl, 0.01 M sodium phosphate, 2 mM EDTA, 50 mM sodium fluoride, 0.2 mM sodium vanadate, and 100 U/ml aprotinin, pH 7.2) as previously described [30]. Western blot analyses of p53, p21, p27, cyclin D, cyclin A, cyclin E, proliferating cell nuclear antigen (PCNA), Bax, cytochrome c, phosphorylated histone H2AX (γ-H2AX), actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phosphorylated p53 (Ser-15), GSK-3β, and phosphorylated GSK-3β (Cell Signaling Technology, Boston, MA, USA) were performed using specific antibodies.

Cell cycle analysis, MN formation and Western blots shown in the figures are representative of three independent experiments.

3. Results

Cell cycle analyses of proliferating cells showed 61.08%, 23.6% and 15.3% cells were in the G_0/G_1, S, and G_2/M phases, respectively. In quiescent cells that were maintained for two weeks after cells reached confluence, 91%, 0.97%, and 7.03% of cells were in the G_0/G_1, S, and G_2/M phases, respectively. A dose-dependent increase in micronuclei (0 Gy − 1.51%; 0.50 Gy − 2.0%; 1 Gy − 5.36%) was observed only in the proliferating cells. These data indicate that quiescent cells are in the resting state (G_0) and do not have any microscopically-visible DNA damage at the level of radiation doses used.

3.1. Response of cell cycle, DNA damage and cell death proteins

To examine the cellular responses of proliferating and quiescent cells, we first examined the time dependent induction of p53 in the proliferating cells (Fig. 1A). Based on this data, we investigated several cell cycle regulatory, DNA damage and cell death response proteins 1 h and 2.5 h after irradiation. As shown in Figs. 1B and C and 2A and B the dose and time courses of p53, phosphorylated p53 (Ser-15), γ-H2AX, p21, p27, PCNA, cyclin A, cyclin D, cyclin E, Bax, and cytochrome c expression were investigated. Among the cell cycle and cell death proteins investigated, p53 and phosphorylated p53 (Ser-15) were induced in both proliferating (Figs. 1B and 2A) and quiescent (Figs. 1C and 2B) cells at both time intervals. In proliferating cells 1 h after irradiation, p53, p21, p27, and cyclin D were induced (Fig. 1B). However, in quiescent cells 1 h after irradiation, p53, p21, and p27 were induced but cyclin D was not (Fig. 1C). DNA damage, as assessed by histone H2AX phosphorylation, was increased in both proliferating and quiescent cells 2.5 h after irradiation with 1 Gy (Fig. 2A and B). In the present study, western blot analyses showed cyclin A, cyclin E, PCNA, Bax, and cytochrome c did not increase in proliferating or quiescent cells 1 h or 2.5 h after irradiation (Figs. 1B and C and 2C–F).

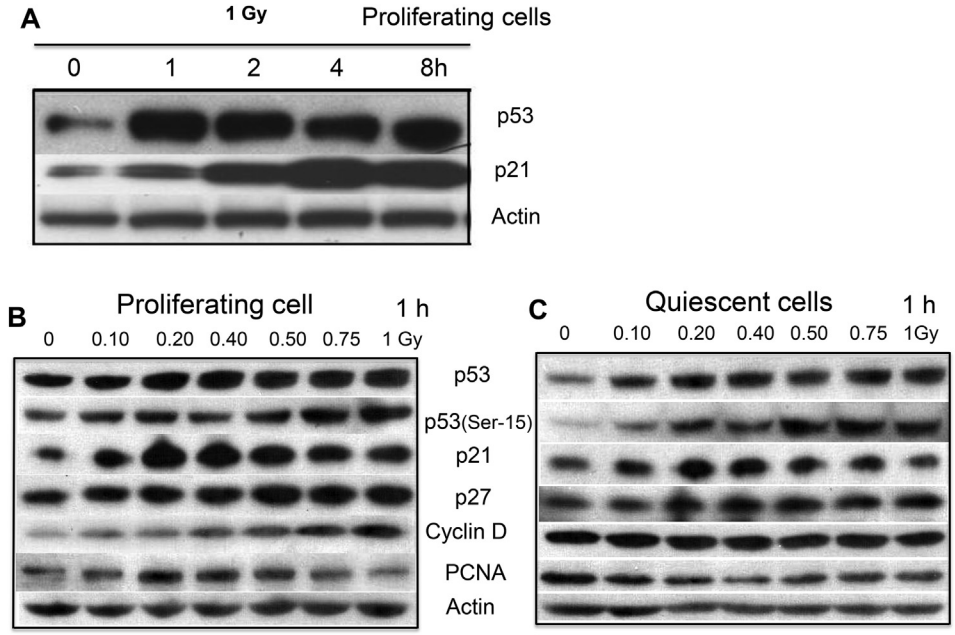

Fig. 1. Response of p53 pathway and cell cycle proteins after irradiation in proliferating and quiescent cells: (A) Proliferating normal lung fibroblasts (IMR-90) were irradiated with 1 Gy of radiation. Cells were harvested 0, 1, 2, 4, and 8 h after irradiation and whole cells were lysed. The levels of p53 and p21 were determined by western blotting. p53 showed higher expression at earlier time points, whereas p21 showed higher expression at later time points. Therefore, we selected earlier time points (1 h and 2.5 h) for further studies. Response of cell cycle, DNA damage, and cell death proteins one hour after 0–1 Gy to irradiation: p53, phosphorylation of p53 (Ser-15), p21, p27, cyclin D, PCNA levels were examined in proliferating cells (B) or quiescent cells (C). Actin was used as a loading control.

3.2. Half life of p53 was similarly increased after irradiation in proliferating and quiescent cells

To determine whether p53 accumulation was attributed to increased protein stability, we performed cycloheximide (CHX) chase experiments in the presence and absence of 1 Gy of irradiation in proliferating (Fig. 3A) and quiescent (Fig. 3B) cells. Cells were harvested at various times after irradiation, and decay in the levels of p53 were analyzed by immunoblotting. In the presence of CHX,

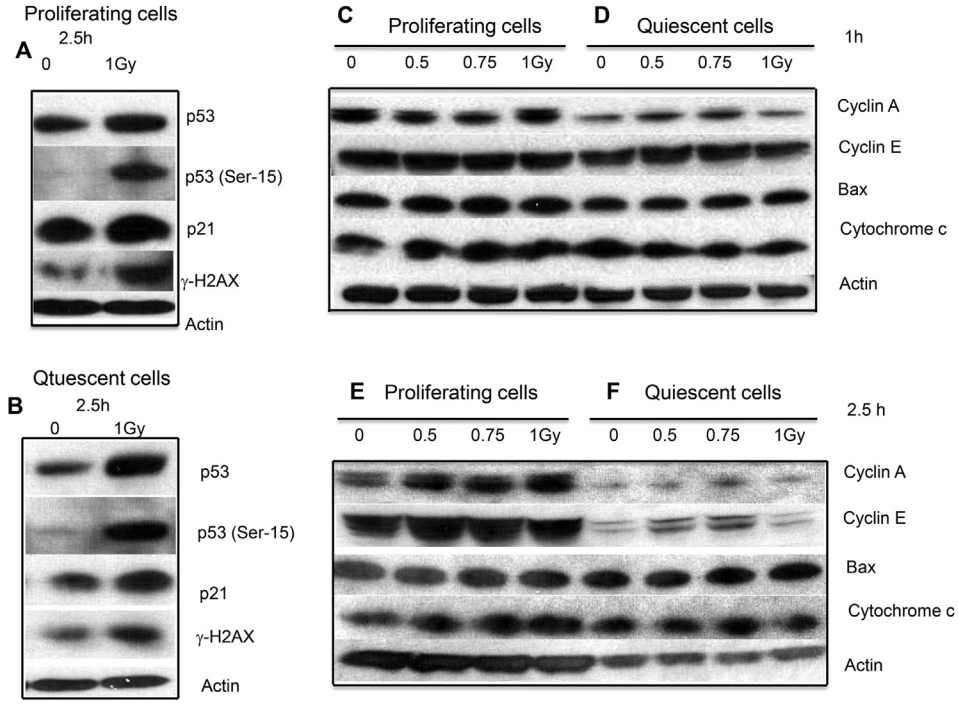

Fig. 2. Response of γ-H2AX, cell death and cell cycle proteins after irradiation in proliferating and quiescent cells: A and B – The levels of p53, phosphorylation of p53 (Ser-15), p21, and γ-H2AX in proliferating and quiescent cells 2.5 h after 0–1 Gy γ-irradiation. C–F: The levels of cyclin A, cyclin E, Bax, and cytochrome c in proliferating and quiescent cells either 1 h or 2.5 h after 0–1 Gy of irradiation.

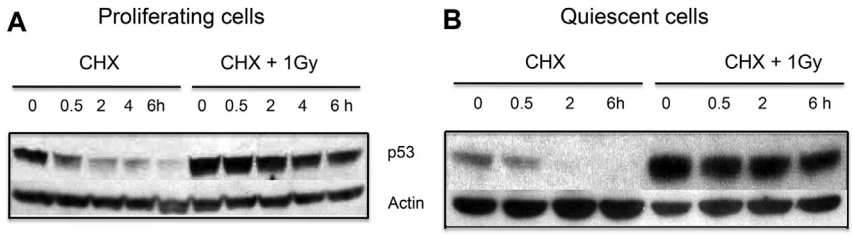

Fig. 3. A half life assay of p53 in proliferating and quiescent cells after γ-irradiation. Representative western blots of cells treated with cycloheximide (CHX) alone or after 1 Gy of γ-irradiation. A. Proliferating cells. B. Quiescent cells. After 30 min of incubation with CHX (10 μg/ml), cells were left un-irradiated or exposed to 1 Gy of radiation. Cells were harvested after 0, 0.5, 2, 4, and 6 h (proliferating cells) or 0, 0.5, 2, and 6 h (quiescent cells).

radiation reduced the rate of p53 degradation in both proliferating and quiescent cells.

3.3. GSK-3β inhibitor similarly abrogates radiation-induced p53 and p21 accumulation in proliferating and quiescent cells

GSK-3β inhibitor has been known to prevent radiation-induced cell death in normal cells but less do so in cancer and it is a useful tool for patients treated with radiation therapy. One of the mechanisms of radiation protection of GSK-3β inhibitor is attenuation of p53-dependent cell death by inhibiting p53 [32]. Since GSK-3β plays a critical role in cellular metabolism, metabolic difference between proliferating and quiescent cells may cause different levels of attenuation of p53 mediated by GSK-3β inhibitor after irradiation. To test this, we evaluated p53 and p21 expression in response to radiation in the presence of a GSK inhibitor in proliferating and quiescent cells. Cells were treated with SB216763, a GSK-3β inhibitor and further irradiated with 0.50–1 Gy. We then examined the levels of p53, p21, GSK-3β, and phosphorylated form of GSK-3β. Proliferating and quiescent cells that were treated with SB216763 showed similar attenuation of p53 and p21 expression after irradiation compared to cells exposed to radiation alone (Fig. 4A and B). These results suggested that although quiescent cells have been known to exhibit low metabolism, the radiation protection mediated by p53 attenuation by GSK-3β inhibitor is similar between proliferating and quiescent cells.

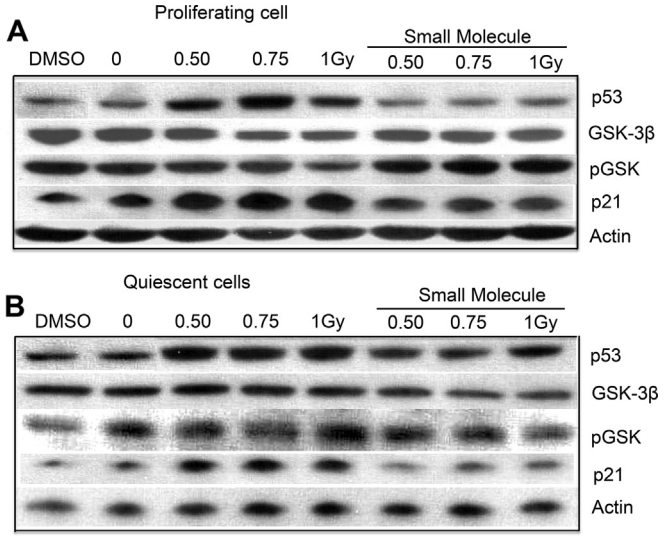

Fig. 4. GSK-3β inhibition abrogates radiation-induced p53 and p21 accumulation: A and B-Proliferating and quiescent cells were treated with 15 μM of SB216763 for 16 h before γ-irradiation. For protein analyses, cells were harvested 2.5 h after 0.50, 0.75, or 1 Gy of irradiation. The levels of p53, GSK-3β, phosphorylation of GSK (pGSK) and p21 in proliferating and quiescent cells were assessed.

4. Discussion

The concept of cellular quiescence has changed over time. Previously, it was believed that cells become quiescent by default due to nutrient deprivation or contact inhibition. Recently, it has been hypothesized that in the absence of signals that favor proliferation, mitotic cells either arrest growth in a reversible state termed quiescence or stay in the G_0 phase with reduced cell size and metabolic activity [23,33]. Because quiescence is a state of growth cessation and cells retain the ability to re-enter the cell cycle in response to normal physiological stimuli, we investigated quiescent normal lung fibroblast cells response to radiation doses $\leq$1 Gy. These doses are likely to occur in populations during medical field interventions as well as during accidental or intentional radiation exposure. Interestingly, our proteomic results show that key proteins involved in stress responses in quiescent lung fibroblasts respond similarly to those in proliferating cells.

Biological and health effects from low-dose radiation exposure have recently been proven to be more mutagenic and carcinogenic than originally thought. Radiation sensitivity varies with different phases of the cell cycle, and it is known that proliferating cells are radiosensitive; however, the effects on quiescent cells are not clear. Most eukaryotic cells spend the majority of their life in quiescence [34], a state defined as a temporary absence of proliferation [33,35]. However, quiescence encompasses various cellular situations, and multiple mechanisms are activated to protect normal cells from differentiation, senescence, and death [28,36,37]. It is still unclear what causes cells to remain in the quiescent state. Low levels of cyclins, particularly cyclin A and cyclin E are proposed to be a hallmark of cellular quiescence [24]. In an earlier study, we reported specific activation of PKC isoforms in both proliferating and quiescent human lung fibroblasts (MRC-5) in response to γ-radiation (0–1 Gy) [31]. Activation of PKC in quiescent cells suggests that the induction of signaling cascades can occur even in the absence of active cellular proliferation [31]. It is known that radiation alters the genome of proliferating cells to cause them to become cancerous; however, the mechanisms involved after irradiation in non-proliferating cells are poorly understood. Atsumi et al. [38] reported low levels of total H2AX in human and murine quiescent fibroblasts compared to actively proliferating cells; we did not see marked difference in H2AX phosphorylation, a DNA damage marker, after 1 Gy of irradiation, indicating similar response of DNA damage of quiescent cells.

In the present study, key cell cycle regulatory and cell death proteins were investigated in both proliferating and quiescent normal lung fibroblasts. Among the cell cycle checkpoint proteins investigated, p53 and phosphorylated p53 (Ser-15) were rapidly induced in both phases of cells. Along with p53, its downstream

protein p21 was also induced in both cell phases. Our data show that doses of radiation ≤1 Gy induced p53 and its downstream target p21 in actively dividing cells as well as in the non-dividing quiescent cells. We demonstrate p53 induction, p53 phosphorylation, and histone H2AX phosphorylation following radiation doses of ≤1 Gy in quiescent cells, indicating non-dividing quiescent cells have a similar genomic response to radiation exposure compared to proliferating cells. Interestingly, p21, a cell cycle arrest protein, which was present at higher levels in quiescent cells, was further up-regulated after irradiation (Fig. 1C). This result suggests that p21 may have other protective functions in addition to cell cycle arrest in quiescent cells. p21 has been known to protect cells from apoptosis and induces cell cycle arrest such as inactivating cyclin A/Cdk2 complexes, requisite effector of apoptotic death [39]. Whether this upregulation of p21 by irradiation contributes to resistant to apoptosis even in quiescent cells remains to be investigated. The ability of proliferating cells to properly exit the cell cycle and retain viability during quiescence, and to return to the cell cycle when necessary, is an important process in complex multi-cellular processes such as growth and healing [40]. The transition from one phase of the cell cycle to the next is driven by various cyclins/cyclin-dependent kinase (CDK) proteins [41]. Therefore, cell cycle progression consists of precisely controlled events. To understand the relationship between cell proliferation, quiescence, and irradiation, proteins involved in the cell cycle, including cyclin A, cyclin D, cyclin E, and p27, were investigated. As expected, proliferation marker, cyclin A and E were reduced in quiescent cells and irradiation did not affect the levels of these proteins (Fig. 2F). However, another proliferation marker, cyclin D, was induced by irradiation in proliferating cells. This is consistent with the recent finding of the role of cyclin D in DNA repair [42]. Interestingly, cyclin D was not induced in quiescent cells, suggesting that the role of cyclin D in DNA repair may be different between proliferating and quiescent cells. p27 was induced further in the quiescent cells after irradiation (Fig. 1C). Since the levels of p27 are higher in the quiescent cells than proliferating cells to contribute to maintain quiescent state [43], the biological reason for further increase of p27 after irradiation remains to be investigated.

Radiation is delivered primarily by high energy photons (γ and X-rays) and charged particles (electrons), and the deposited energy alters tissue metabolism. GSK-3β plays a critical role in cellular metabolism [32], and we asked whether the difference of the metabolic activity in proliferating and quiescent cells may affect the radioprotection of GSK-3β inhibitor. GSK-3β is a highly conserved serine/threonine protein kinase that is ubiquitously expressed and found in all eukaryotes. Although initially identified as a regulator of glycogen metabolism [44], GSK-3β can act as a downstream regulatory switch for numerous signaling pathways [45]. Because p53 plays a pivotal role in GSK-3β-dependent apoptosis, we evaluated p53 induction and p21 expression after treatment with GSK-3β inhibitor, SB216763. p53 and p21 expression was similarly attenuated after treatment (Fig. 4), indicating a radioprotective effect is similar between proliferating and quiescent cells.

In summary, we observed p53 activation and phosphorylation, p21 activation, and histone-H2AX phosphorylation following irradiation with doses of ≤1 Gy in quiescent cells. These data may give us a better understanding regarding the radiation response in quiescent fibroblasts, which are present in many somatic tissues. Furthermore, proliferating and quiescent cells that were treated with the GSK-3β inhibitor SB216763 attenuate p53 and its downstream target p21, indicating radioprotection occurs even in quiescent cells. The exquisite sensitivity of quiescent cells to radiation doses ≤1 Gy may serve as a valuable biomarker for estimating the health effects of radiation, which is likely applicable for clinical oncology as well as for accidental or intentional radiation exposure.

Authors' contributions

RB designed, JD and RB performed the experiments, analyzed the data and wrote the paper. KI helped in the experimental design and manuscript preparation. All the authors read and approved the final manuscript.

Conflict of interest

The author declares no conflict of interest.

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