Changing the gravity vector direction by inverted culture enhances radiation-induced cell damage

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ABSTRACT

In recent years, it has become clear that the cytotoxicity of γ-irradiation of cells is increased under microgravity conditions. However, there has been no study of the effect of the gravity vector direction, rather than the magnitude, on γ-ray-induced cytotoxicity. Therefore, in this study, we inverted cultures of human bronchial epithelium BEAS-2B cells and human lung cancer A549 cells in order to change the gravity vector direction by 180° with respect to the cells and observed the cellular response to radiation in this state. We found that cells in inverted culture showed increased irradiation-induced production of reactive oxygen species and decreased expression of the antioxidant protein thioredoxin-1 compared to cells in normal culture. Furthermore, the DNA damage response was delayed in γ-irradiated cells in inverted culture, and the number of unrepaired DNA sites was increased, compared to irradiated cells in normal culture. γ-Ray-induced cell death and the number of G_2-M arrested cells were increased in inverted culture, in accordance with the decreased capacity for DNA repair. Our findings suggest that the gravity vector direction, as well as its magnitude, alters the cellular response to radiation.

1. Introduction

In recent years, biological experiments under microgravity conditions have been conducted in order to examine the feasibility of creating long-term extraterrestrial habitats. Particularly in the space environment, microgravity and cosmic radiation are major challenges that can cause major physical disabilities [1,2] Osteoporosis is a typical example, and countermeasures include weighted exercise and eating a healthy diet [3,4]. Furthermore, humans in space are exposed to high-energy ionizing radiation that is different from that on Earth [5], resulting in decreased bone marrow cell function and skin inflammation [6]. Recent studies have shown that radiation exposure in microgravity causes greater cell damage than the same dose in normal gravity [7,8]. Therefore, it is necessary to understand the radiation cell response in

microgravity in detail, and to develop radioprotective agents [9]. There are three main types of cosmic radiation in low Earth orbit, where the International Space Station (ISS) travels. Galactic cosmic ray (GCR): radiation coming from outside the solar system. Solar particle Event (SPE): radiation produced by solar flares. Radiation belt particle (RBP): radiation captured by the Earth's magnetic field [10]. Since these cosmic radiations are mainly composed of protons and heavy particles, they can be shielded by the metal building materials of the ISS. However, cosmic radiation also includes γ-rays and X-rays, which are highly penetrating radiation types [11]. These two elements cannot be completely shielded by metal and could pose a health hazard to people staying on the ISS for long periods of time. Therefore, in this study, we investigated how changes in the gravitational environment affect the radiation cell response during γ-ray irradiation.
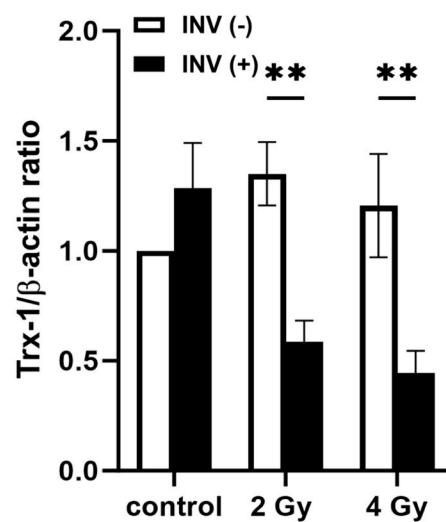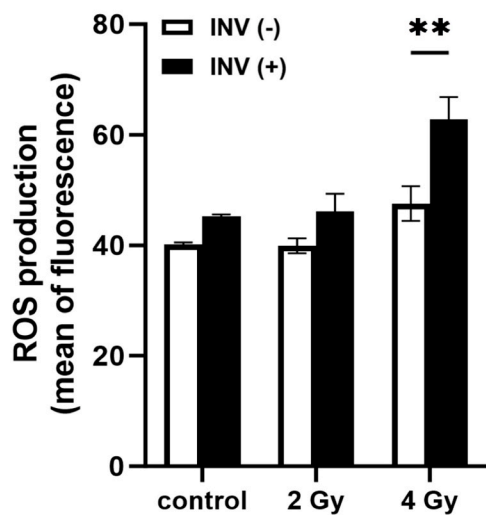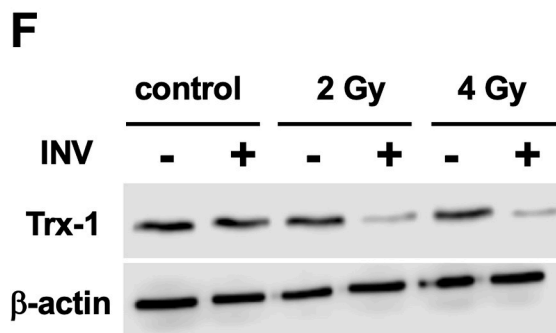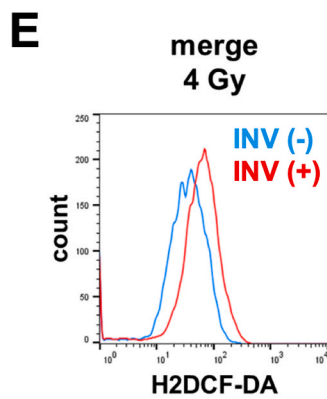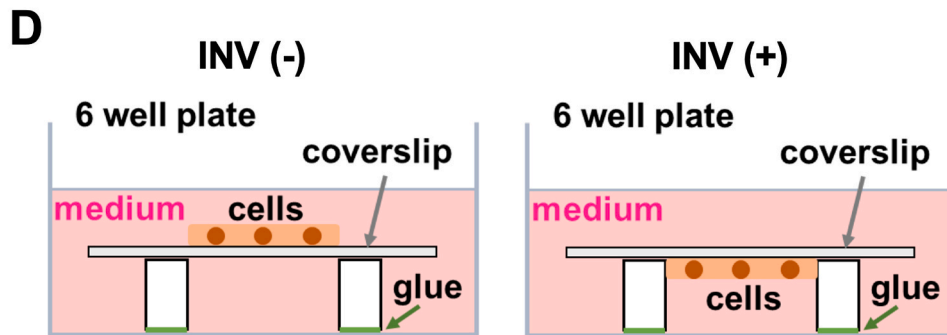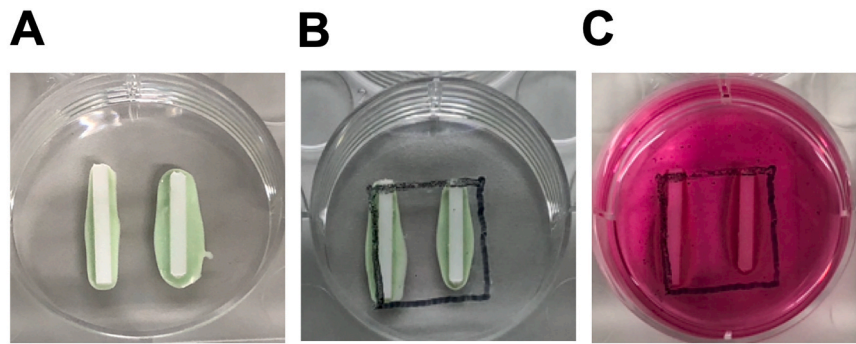
(caption on next page)

Fig. 1. Changes in γ-ray-induced ROS production and thioredoxin-1 expression in inverted culture.
Cells were seeded at a density of 2.5×10^4 cells in 6-well plates containing sterile coverslips (outlined in black in the photo) and incubated for 48 h. Then the coverslips were placed on bridge girders made of steel bars in either normal or inverted orientation and further incubated for 24 h before irradiation. (A) 6-well plate with glued bridge. DUPLICONE was confirmed to have no cytotoxicity. (B) A coverslip (outlined in black) is placed on the bridge. For normal culture, the coverslip was placed directly on the bridge girder. For the inverted culture, the cover glass was inverted before being placed on the bridge. (Note: in the actual experiments, the coverslips were not marked in black.) (C) The 6-well plate was filled with culture medium to cover the cells on the coverslip. (D) Schematic illustration of the inverted culture. (E) BEAS-2B cells were pre-incubated for 24 h in inverted culture before irradiation (INV (−): Normal culture, INV (+): Inverted culture). Cells were irradiated with various doses of γ-rays (2, 4 Gy) and further incubated under the same condition for 24 h. The data represent means ± S.E. (n = 3, three independent experiments) Significant difference from 4 Gy irradiated INV (−) cells. * *(p < 0.01). (F) At 24 h after irradiation (2, 4 Gy), expression of thioredoxin-1 (Trx-1) (12 kDa) was evaluated by western blotting. The data represent means ± S.E. (n = 4, four independent experiments) Significant difference from 2, 4 Gy irradiated INV (−) cells. **(p < 0.01).

Many studies have focused on the effect of the magnitude of gravity on the cytotoxicity of radiation, but there has been no study of the influence of the vector direction of gravity on cytotoxicity. However, recent reports have shown that, compared to conventional culture methods, cells cultured in a 180-degree-inverted state exhibit changes in gene expression similar to those of cells cultured in microgravity and during spaceflight [12]. Thus, we hypothesized that irradiation of cells in inverted culture may causes changes in radiation-induced cellular responses similar in those that occur under microgravity.

Here, we examined this idea by growing cells in an inverted culture system, so that the gravity vector direction is switched by 180° with respect to the cells, and evaluating the changes in levels of reactive oxygen species (ROS), expression of antioxidant protein thioredoxin-1 which contains the –SH group in its structure [13], timing of DNA damage response, number of unrepaired DNA sites, cell death, and number of G_2-M arrested cells compared to cells in normal culture. Our results support the idea that not only the magnitude of gravity but also its vector direction influences the extent of cellular radiation damage.

2. Materials and methods

2.1. Cell culture and irradiation

BEAS-2B cells was grown in RPMI-1640 (FUJIFIIM Wako Pure Chemical Corporation, Osaka, Japan) and A549 cells was grown in DMEM (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10 % Gibco® fetal bovine serum (FBS) (Gibco, MA, USA), penicillin (100 U/mL) and streptomycin (100 μg/mL) in an atmosphere of 5 % CO_2 in air at 37 °C, as described previously [14,15]. Irradiation of cells was performed as described previously [14,15].

2.2. Inverted culture

The inverted culture procedure was based on previous reports [12, 16]. Cells were seeded at a density of 2.5×10^4 cells in 6-well plates containing sterile coverslips and incubated for 48 h. Then the coverslips were placed on bridge girders made of steel bars in either normal or inverted orientation (Fig. 1A–D) and further incubated for 24 h prior to irradiation. The girders were fixed with DUPLICONE (Shohu Inc., Kyoto, Japan). The irradiated cells were incubated under the same conditions for the indicated time.

2.3. Measurement of reactive oxygen species (ROS) production by flow cytometry

BEAS-2B cells were harvested by trypsinization, washed with PBS, and incubated with H2DCFDA (10 μM) for 40 min at 37 °C. Then the cells were washed and the fluorescence of H2DCFDA was measured with a FACSCalibur Flow Cytometer (Bd Biosciences, Systems And Reagents, Inc, CA, U.S.A). The data was analyzed with FlowJo software (FlowJo, LCC).

2.4. Western blotting

BEAS-2B cells were lysed in buffer on ice for 30 min after irradiation. The buffer contained 1 % TritonX-100, protease inhibitor (Sigama-Aldrich, MO, U.S.A). Cell debris was removed by centrifuging the cell lysate at 15 min. Protein were fractionated by SDS-PAGE and transfected to PVDF membrane. Membranes were blocked overnight at 4 °C with 10 % bovine serum albumin. The membranes were incubated overnight at 4 °C with the primary antibody, rabbit anti-thioredoxin-1 (1:1000) (#2429S) (Cell signaling Technology, Inc., MA, USA) or anti-β-actin mouse mAb (1:50000) (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Blots were also incubated with anti-rabbit IgG, HRP-linked antibody (1:20000) or anti-mouse IgG, HRP-linked antibody (1:50000) (Cell signaling Technology, Inc., MA, U.S.A) for 1 h at room temperature (RT). These blots were imaged by using ImunoStar® LD (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

2.5. Immunofluorescence staining

The site of γH2AX was detected as described previously [14]. At 0.5–24 h post irradiation, cells were fixed in 4 % paraformaldehyde in PBS for 10 min at RT. Cells were permeabilized by using 0.1 % Triton-X 100 and then blocked by 10 % FBS. After blocking, cells were incubated with primary antibody (mouse anti-H2AX phosphorylated (Ser139) antibody (#613042) (Bio Legend, CA, U.S.A.) 1: 400 for 24 h at 4 °C. After incubation, cells were incubated with secondary antibody (Alexa Fluor 594 goat anti-mouse immunoglobulin G) (1:200) (Invitrogen, CA, U.S.A.) and Counterstaining with Hoechst 33342 (1 μg/mL) (1:100) (FUJIFILM Wako Pure Chemical Corporation, Osaka, U.S.A) for 1h at RT. The images were obtained with a laser-scanning confocal microscope (FV1000 IX81; Olympus, Tokyo, Japan).

2.6. Colony formation assay

Colony assay was performed as described previously [14]. 24 h after irradiation, 200 cells were seeded into per well of a 6-well plate. After culture for 1 week, colonies were stained with 0.5 % crystal violet. After the staining solution has dried, number of colonies were counted and the survival rate was normalized using the corresponding non-irradiated group.

2.7. Cell cycle analysis

Cell cycle analysis was performed as described previously [14]. Cell cycle was determined from DNA content using flow cytometry analysis (FACSCalibur Flow Cytometer Bd Biosciences, Systems And Reagents, Inc., CA, U.S.A). The data were analyzed with FlowJo software (FlowJo, LCC).

2.8. Statistics

Results are expressed as mean ± standard error (S.E.). The data were obtained from three or more independent experiments. The statistical significance of differences between control (INV-) and other groups was
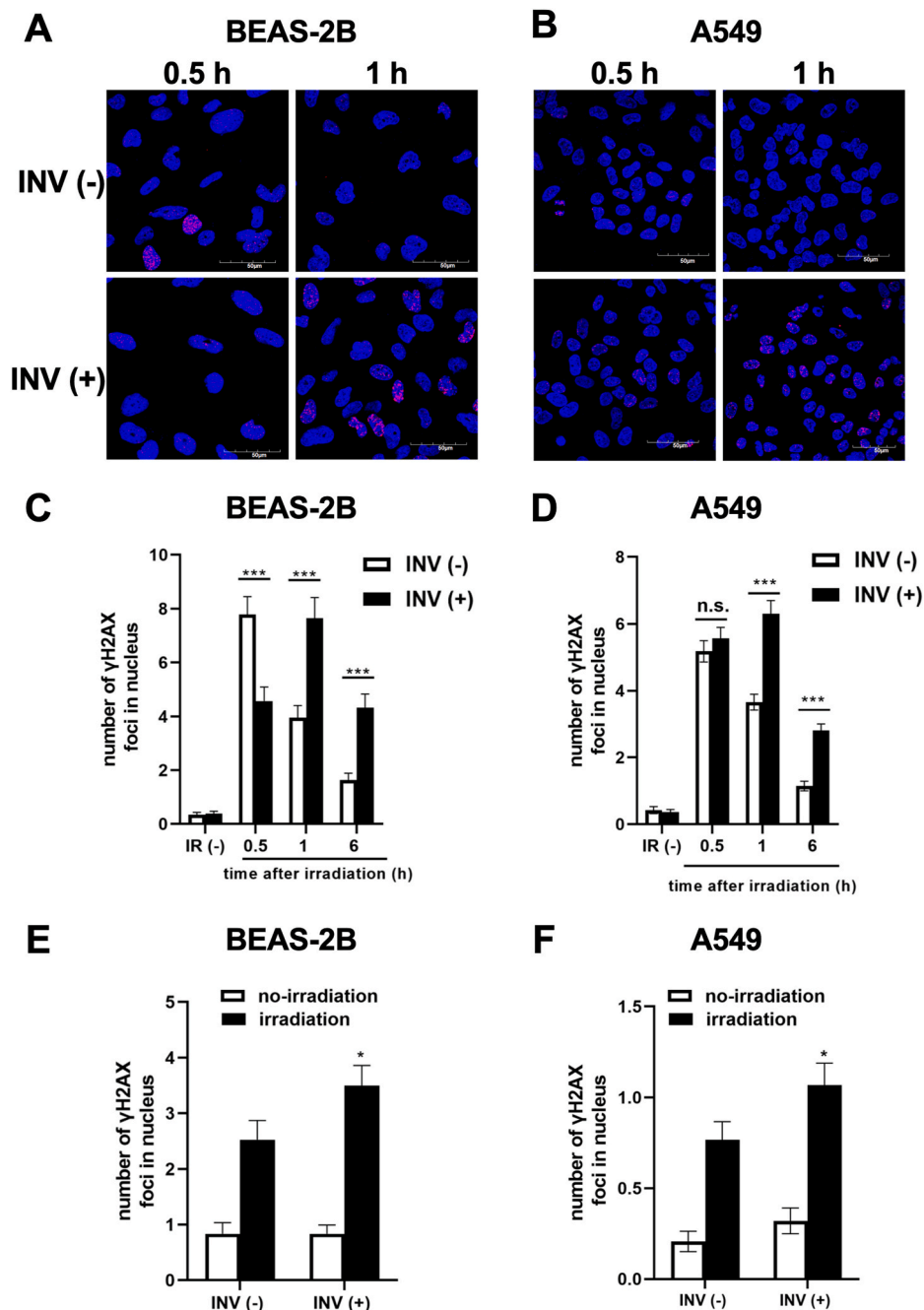
Fig. 2. Inverted culture suppresses the DNA damage response.

Cells were seeded at a density of 2.5×10^4 cells in 6-well plates containing sterile coverslips and incubated for 48 h. Then the coverslips were placed on bridge girders made of steel bars in either normal or inverted orientation and further incubated for 24 h prior to irradiation (INV (−): Normal culture, INV (+): Inverted culture). (A–D) BEAS-2B and A549 cells after γ-irradiation (2.0 Gy) were further incubated for the indicated time under the same condition. After incubation, γH2AX foci were detected by immunostaining. γH2AX foci (Red) in nuclei (Blue) were counted (30 cells/sample). The data represent means ± S.E. (n = 90, three independent experiments). A significant different between the INV (−) and INV (+) group is indicated by *** (p < 0.001).

(C, D) BEAS-2B and A549 cells after γ-irradiation (2.0 Gy) were further incubated for 24 h under the same condition. After incubation, γH2AX foci in nuclei were immunostained and counted (40 cells/sample in BEAS-2B or 50 cells/sample in A549). The data represent means ± S.E. (n = 120 or 150, three independent experiments). A significant different between the INV (−) and INV (+) group is indicated by *(p < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

calculated by using 2-way ANOVA with Bonferroni's test. Calculations were done with Prism (Graph Pad Software, MA, U.S.A). The criterion of significance was set at p < 0.05.

3. Results & discussion

First, to investigate whether the vector direction of gravity affects the cellular radiation response, we constructed an experimental model that allows irradiation of cells in an inverted culture system (Fig. 1A–D). Previous studies have reported that the position and shape of the nucleus change depending on the vector direction of gravity with respect to the cells [12]. Cellular organelles, including the nucleus, greatly influence cellular homeostasis and metabolism, and loss of homeostasis alters cellular responses to external stresses [17,18].
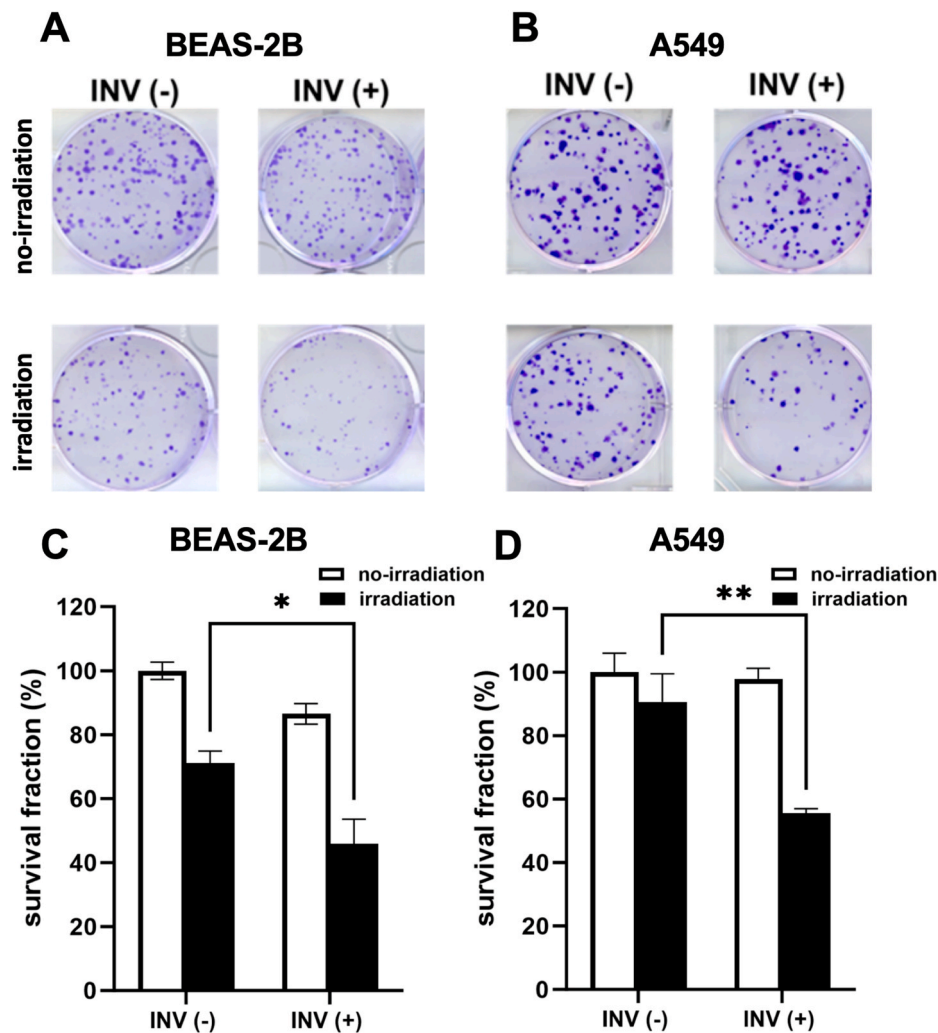
Fig. 3. Inverted culture enhances γ-ray-induced cell death.
Cells were seeded at a density of 2.5×10^4 cells in 6-well plates containing sterile coverslips and incubated for 48 h. Then the coverslips were placed on bridge girders made of steel bars in either normal or inverted orientation and further incubated for 24 h prior to irradiation (INV (−): Normal culture, INV (+): Inverted culture). (A, B) Cell survival rate was measured by colony formation assay. Cells were irradiated with 2.0 Gy of γ-rays, incubated for 24 h under the same condition, then seeded into 6-well plates (200 cells/well, triplicate), and further incubated for 7–8 days. Colonies were stained with crystal violet and colonies of >50 cells were counted. The data represent means ± S.E. (n = 3, three independent experiments). A significant difference between the INV (−) and INV (+) groups is indicated by ** ($p <$ 0.01), *($p <$ 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Reactive oxygen species (ROS) are produced by ionizing radiation and are one of the causes of DNA double-strand breaks (DSBs). When cells in inverted culture were irradiated with γ-rays, ROS production was significantly increased compared to that of cells in normal culture (Fig. 1E). Thioredoxin-1 (Trx-1) is an antioxidant protein that has a marked effect on the intracellular redox balance [13] and has been suggested that function of Trx-1 is suppressed under microgravity conditions [19]. Therefore, we investigated whether Trx-1 expression was changed under our experimental conditions, as any change would be expected to alter the susceptibility of cells to oxidative stress damage. We found that the expression level of Trx-1 was significantly reduced in inverted-culture cells exposed to γ-ray irradiation (Fig. 1F).

Next, we investigated whether the DNA damage response (DDR) after irradiation is altered by inverted culture of BEAS-2B cells (Fig. 2A–C, E) and A549 cells (Fig. 2B–D, F). Normally, DNA repair proteins are recruited to DNA damage sites, and they disappear from double-strand breaks (DSBs) site as soon as the repair is completed. In the normal-culture group, the number of γH2AX foci reached a maximum at 0.5 h after γ-ray irradiation and subsequently decreased (Fig. 2C and D). On the other hand, in the inverted culture, the number

of γH2AX foci peaked at 1 h, and γH2AX foci had not disappeared even at 6 h after γ-irradiation in both BEAS-2B cells and A549 cells (Fig. 2C and D), suggesting that the DDR was delayed in both cell lines in the inverted culture. It is unclear why DDR was delayed. However, this phenomenon may be due to changes in p16 protein expression occurring in the inverted culture samples. P16 protein is a factor involved in cell cycle control and cellular senescence [20]. Recent reports have shown that DDR after radiation is delayed in cells with increased p16 expression by suppressing nonhomologous end joining (NHEJ) [21,22]. There is also a report that p16 expression increases in rat lumbar disc tissue under conditions like microgravity [23]. If p16 expression was similarly elevated in the inverted culture samples, increased p16 may suppress NHEJ and delay DDR compared to normal culture. Further, the number of accumulated DSBs was increased compared to normal culture. We also counted DSB sites remaining at 24 h after γ-ray irradiation as unrepaired DNA sites (Fig. 2E and F). Cells in inverted culture showed a significantly greater number of unrepaired DNA sites than cells in normal culture, supporting the idea that DDR was suppressed (Fig. 2E and F). Alternatively, the increase in unrepaired DNA sites may simply be due to increased damage to DNA compared to cells in normal culture.
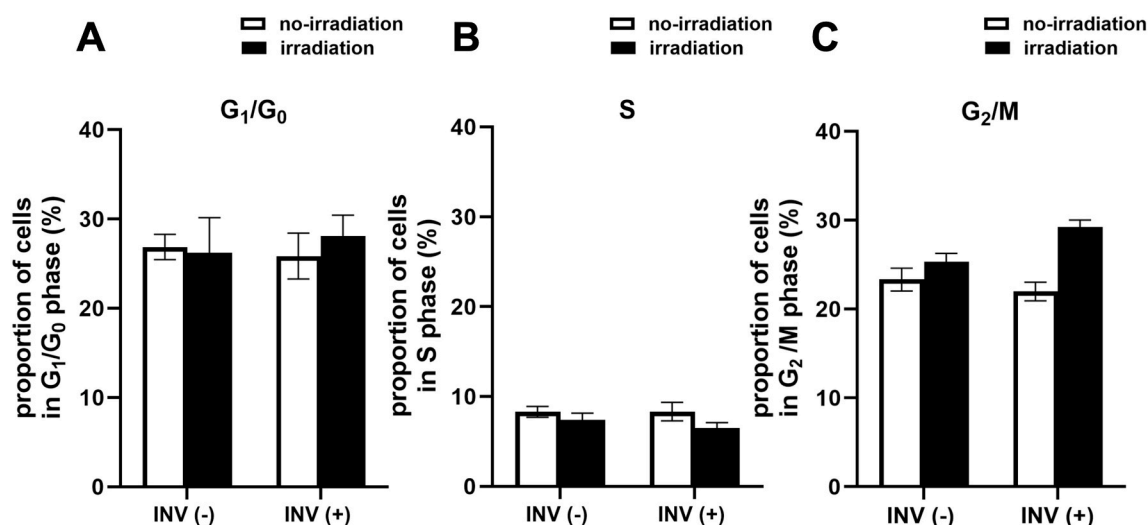
Fig. 4. Cell cycle arrest after γ-ray irradiation in inverted culture

Cells were seeded at a density of 2.5×10^4 cells in 6-well plates containing sterile coverslips and incubated for 48 h. Then the coverslips were placed on bridge girders made of steel bars in either normal or inverted orientation and further incubated for 24 h prior to irradiation (INV (−): Normal culture, INV (+): Inverted culture). (A–C) BEAS-2B cells were irradiated with 2.0 Gy of γ-rays, then further incubated for 24 h under the same condition. The percentage of cells in each stage of the cell cycle was measured by flow cytometry. The data represent means ± S.E. (n = 5, five independent experiments). A significant difference between the INV (−) and INV (+) group is indicated by *($p < 0.05$).

As shown in Fig. 1E, ROS production was increased after irradiation in the inverted-culture group and the resulting increase in DNA damage may account for the increased number of DSB sites that are not completely repaired after 24 h.

We next investigated whether the gravitational environment during irradiation affected irradiation-induced cell death. Fig. 3 shows that γ-irradiation reduced the number of colonies, and γ-ray-induced cell death was significantly enhanced in the inverted-culture group compared to normal culture in both BEAS-2B cells (Fig. 3A, C) and A549 cells (Fig. 3B, D). After exposure to radiation, cells stop dividing until DNA repair is complete. As shown in Fig. 2E and F, there are more unrepaired DNA damage sites in the inverted-culture group than in the normal-culture group, so cell division might be suppressed in the former group, leading to increased γ-ray-induced cell death.

Fig. 4 illustrates the cell cycle of BEAS-2B cells after γ-ray irradiation. Radiation-induced cell cycle arrest occurs primarily in the G_2-M phase. Indeed, there was an increase in G_2-M phase-arrested cells even in normal culture (Fig. 4). In the inverted culture, the number of cells in G_2-M phase arrest was increased, which is consistent with the results in Fig. 3. These results indicate that the number of unrepaired DNA damage sites in the inverted-culture group was increased after γ-ray irradiation, leading to cell cycle arrest, loss of cell division, and enhanced radiation cytotoxicity.

In summary, the increased radiation damage of BEAS-2B cells in the experimental model we used may be related to excessive ROS generation. Normally, reactive oxygen species generated by γ-ray irradiation are suppressed by antioxidant proteins such as Trx-1. However, in the inverted-culture model, γ-ray irradiation decreased the expression of Trx-1 and increased the amount of intracellular ROS compared to normal culture. The excess ROS would cause DNA damage, leading to cell cycle arrest and increased cell death. Evaluation of DNA damage response and cell death was performed at 2 Gy, but if higher doses of γ-rays were irradiated, the number of unrepaired DNA sites may have increased and cell death may have been further enhanced. Indeed, we reported that in both BEAS-2B and A549 cells, the radiation damage observed with 2 Gy irradiation was further enhanced when doses higher than 2 Gy were used [14,24]. Radiation damage was also enhanced in A549 cells by inverted culture. The consistent results for both types of cells suggests that the enhancement of radiation cell damage by inverted

culture is not cell-type-specific, but also occurs in normal cells. It has been reported that cancer cells are more susceptible to radiation sensitization under microgravity [25]. By using our experimental conditions and comparing the radiation responses of normal cells and cancer cells, it may be possible to develop agents that specifically protect normal cells from radiation in space.

The gravitational environment has a major impact on the homeostasis of living organisms. For example, plant roots always develop in the direction of the gravity vector, and gravitational stimulation has a marked influence on the development of mammalian skeletons and muscles [26,27]. Therefore, it is not surprising that changes in the gravitational environment can disrupt the homeostasis of organisms and alter cellular responses to external stresses such as radiation [28–30]. In this context, it is noteworthy that our experiments showed similar cellular radiation responses to those found in a simulated space experiment using a microgravity generator [31]. Though epithelial cells were used in this study, it has been reported that radiation damage to cardiomyocytes and skeletal muscles is also enhanced in a microgravity environment [26,27]. Therefore, it is very likely that a similar phenomenon would occur if the same study were performed on these cells under our experimental conditions. Under our experimental conditions, involving normal gravity but an inverted gravity vector with respect to the cells, redox homeostasis may have been disrupted, leading to enhanced radiation-induced cell damage as in the microgravity experiment. Our results further support the idea that enhancing the function of antioxidant proteins may be important for protecting humans from the damaging effects of radiation in space.

There are many reports dealing with the effects of varying the magnitude of gravity. For example, organelles such as the nucleus adhere to cytoskeletal proteins with the support of gravity, which helps maintain their functional form [28,29]. However, it is expected that the adhesion between intracellular organelles and skeletal proteins will weaken in microgravity, and we think intercellular adhesion may also be affected in the inverted culture we employed [12,16,31].

Importantly, our experimental model in inverted culture might mimic radiation exposure in microgravity environments. To develop agents which protect from space radiation, it is possible that this experimental model use as an alternative experimental model of microgravity equipment. Since a reason for the increased radiation

damage in the inverted-culture model is a decrease in antioxidants in cells, treatment with antioxidants such as N-Acetyl-L-cysteine may reduce the damage, suggesting that agents that restore antioxidants could serve as space radiation protectors.

In conclusion, our inverted culture system does not require any special equipment and samples can easily be irradiated. We believe this experimental model is a promising candidate for initial screening studies in the search for radioprotective agents to assist humans to live in space.

CRediT authorship contribution statement

Yuma Mizoguchi: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Formal analysis. **Masao Kamimura:** Writing – review & editing, Resources, Methodology, Funding acquisition, Conceptualization. **Kazuki Kitabatake:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Formal analysis. **Fumiaki Uchiumi:** Writing – review & editing, Supervision. **Shin Aoki:** Writing – review & editing, Project administration, Funding acquisition. **Mitsutoshi Tsukimoto:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

Acknowledgement

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101792.

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