Involvement of TRPM8 Channel in Radiation-Induced DNA Damage Repair Mechanism Contributing to Radioresistance of B16 Melanoma

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Radiation is an effective cancer treatment, but cancer cells can acquire radioresistance, which is associated with increased DNA damage response and enhanced proliferative capacity, and therefore, it is important to understand the intracellular biochemical responses to γ-irradiation. The transient receptor potential melastatin 8 (TRPM8) channel plays roles in the development and progression of tumors, but it is unclear whether it is involved in the DNA damage response induced by γ-irradiation. Here, we show that a TRPM8 channel inhibitor suppresses the DNA damage response (phosphorylated histone variant H2AX-p53-binding protein 1 (γH2AX-53BP1) focus formation) and colony formation of B16 melanoma cells. Furthermore, the TRPM8 channel-specific agonist WS-12 enhanced the DNA damage response and increased the survival fraction after γ-irradiation. We found that the TRPM8 channel inhibitor enhanced G2/M phase arrest after γ-irradiation. Phosphorylation of ataxia telangiectasia mutated and p53, which both contribute to the DNA damage response, was also suppressed after γ-irradiation. In addition, the TRPM8 channel inhibitor enhanced the γ-irradiation-induced suppression of tumor growth in vivo. We conclude that the TRPM8 channel is involved in radiation-induced DNA damage repair and contributes to the radioresistance of B16 melanoma cells. TRPM8 channel inhibitors might be clinically useful as radiosensitizers to enhance radiation therapy of melanoma.

Key words γ-ray; DNA damage response; radioresistance; transient receptor potential melastatin 8 channel

INTRODUCTION

Radiation therapy (RT) is widely used to treat both primary and malignant cancers, and more than half of cancer patients will receive RT alone or in combination with chemotherapy and/or surgery. However, RT is less effective against radiation-resistant cancer, and damage to normal cells due to high-dose irradiation is a problem. Radiosensitizers are thus a promising option to improve the effectiveness of RT against radioresistant cancer.

Melanoma is a highly malignant skin cancer, and its incidence is increasing.1,2 The 5-year survival rates are 98.2% at stage I/II, 68.2% at stage III, and 15.2% at stage IV. Thus, stage IV has a particularly poor prognosis, and new treatment methods are required.3 Cancer immunotherapy can improve the survival rate of malignant melanoma patients, and therapeutic efficiency in combination with radiotherapy is expected.4,5 However, melanoma cells are resistant to low doses of 1–2 Gy in vitro, suggesting that they have acquired high DNA repair ability.6

Ionizing radiation (IR) induces double-strand breaks (DSBs), which are the most serious type of damage to DNA. Cells damaged by ionizing radiation initiate DNA repair through DNA damage response (DDR) to prevent genomic instability.7,8 The DDR consists mainly of three processes: (i) DNA damage sensing, (ii) mediator accumulation, and (iii) cellular response via signal transduction. First, the complex of Mre11/Rad50/Nbs1 (MRN) and ataxia telangiectasia mutated (ATM) senses double-strand breaks and moves to the nucleus, accumulating at sites of DNA damage.9,10 Subsequently, ATM phosphorylates histone H2AX (γH2AX) and recruits p53-binding protein 1 (53BP1) at sites of DNA damage. Thereafter, DNA repair factors such as Artemis and Ku are induced, and DNA repair proceeds.11,12 At the same time, ATM functions as a cell cycle check point protein, and induces cell cycle arrest through activation of p53 and Chk2.13,14 Therefore, ATM is a master protein of DDR, playing a key role in cell proliferation and survival. ATM or 53BP1 deficiency results in high sensitivity to cytotoxic drugs and ionizing radiation.15 Furthermore, DNA repair factors including ATM and 53BP1 are highly expressed in cancer cells.16 Thus, DDR contributes to the radioresistance of cancer cells, and so it is important to establish the mechanisms involved in order to develop new therapeutic options.

The transient receptor potential melastatin 8 (TRPM8) channel is a calcium-permeable non-selective cation channel belonging to the TRPM channel family. It is activated by cold stimulation (below 26°C), menthol and icilin, serving as a sensor of extracellular stimuli.17 In some cancer cell lines, TRPM8 is involved in cancer progression, survival, proliferation and migration ability. In prostate cancer, TRPM8 channel expression level and cancer malignancy are negatively correlated, while a specific inhibitor of TRPM8, N-(3-aminopropyl)-2-[(3-methylphenyl)methoxy]-N-(2-thienylmethyl)benzamide (AMTB) and JNJ41876666, which are specific inhibitors of TRPM8, decrease cancer cell survival and migration without affecting normal cell survival.18,19 Moreover, TRPM8 contributes to ionizing-radiation resistance via CaMKII-cdc25-cdc2 in glioblastoma, and is involved in the migration and chemotaxis of glioblastoma cells, suggesting that it is a promising target for glioblastoma treatment.20 As regards other cancers, TRPM8 is involved in replicative senescence of pancreatic carcinoma, cell
proliferation and invasion of lung carcinoma, cell cycle progression and survival of osteosarcoma, and cell migration of breast adenocarcinoma. On the other hand, several studies indicate that TRPM8 is involved in cell survival of melanoma cell lines. Activation of TRPM8 by l-menthol, which is a TRPM8 channel agonist, decreased the survival fraction of human melanoma G-361 cells. l-Menthol is also cytotoxic to malignant melanoma A375 cells. However, it remains unclear whether TRPM8 influences DDR after γ-irradiation.

We have already established that TRPV1 and TRPM2 channels are involved in DDR. Therefore, in this study, we investigated a role of TRPM8 in the radioresistance of B16 mouse melanoma in vitro and in vivo by using AMTB. We examined the DDR after γ-irradiation by means of immunofluorescence and the cell viability by evaluating colony formation, and found that the inhibitor suppressed the DDR and enhanced the γ-irradiation-induced decrease in survival. Furthermore, the inhibitor enhanced the inhibitory effect of γ-irradiation on tumor growth of B16 melanoma in mice, supporting the idea that the TRPM8 channel is involved in radiation resistance of melanoma in vivo.

MATERIALS AND METHODS

Mice, Reagents and Antibodies Pathogen-free male C57BL/6 mice were purchased from Sankyo Labo Service (Tokyo, Japan) and used at 5 weeks of age. The mice were treated and handled according to the Tokyo University of Science’s institutional ethical guidelines for animal experiments and with the approval of Tokyo University of Science’s Institutional Animal Care and Use Committee (Permission Nos. S19007, S18009).

Dulbecco’s modified Eagle’s medium (DMEM) was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) and Gibco fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (U.S.A). AMTB hydrochloride, a specific inhibitor of TRPM8, was purchased from FUJIFILM Wako Pure Chemical Corporation, and WS-12, a chloride, a specific inhibitor of TRPM8, was purchased from Thermo Fisher Scientific (U.S.A). AMTB hydrochloride (10H.E12) antibody (Abcam, U.K., #ab36810), rabbit anti-TRPM8 antibody (Sigma, U.S.A. #HPA024117), p53 (D2H90) rabbit monoclonal antibody (mAb) (Cell Signaling Technology, U.S.A., #3253S), phospho-p53 (Ser15) antibody (Cell Signaling Technology #9284), 53BP1 antibody rabbit polyclonal antibody (Novus Biological, U.S.A., #NB100-305), purified mouse anti H2AX phosphorylated (Ser139) (2F3) antibody (Bio Legend, U.S.A., #613402) and phospho-histone H2AX (Ser139) rabbit monoclonal antibody (Cell Signaling Technology #9718S). The secondary antibodies used were goat anti-rabbit immunoglobulin G (IgG)-fluorescein isothiocyanate (FITC) (Sigma-Aldrich, #9887-1ML), Alexa Fluor 594 goat anti-mouse IgG (H + L) (Invitrogen, U.S.A., #A-11005), anti-rabbit IgG horseradish peroxidase (HRP)-linked antibody (Cell Signaling Technology #7074S) and anti-mouse IgG HRP-linked antibody (Cell Signaling Technology #7076).

Cell Culture and Irradiation Cells were cultured and irradiated as described previously. Cells were grown in DMEM supplemented with 10% FBS, penicillin (100 units/mL) and streptomycin (100 µg/mL) in a humidified atmosphere of 5% CO2 in air at 37°C. The cells were then irradiated with γ-rays from a Gammacell 40 (137Cs source) (Nordion International, Inc., Canada; 0.827 Gy/min) at room temperature for a suitable time.

Immunofluorescence We detected formation of γH2AX and 53BP1 foci at DSB sites as described previously. B16 melanoma cells (8.0 × 104 cells/mL) were seeded in a 40 mm dish and incubated for 24h in a 5% CO2 atmosphere at 37°C, followed by exposure to the indicated dose of γ-rays for the indicated time. B16 melanoma cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10min at room temperature and permeabilized in 0.1% Triton X-100 for 5min on ice. After incubation in blocking buffer (10% FBS in PBS) for 1h, the fixed cells were incubated with primary antibody (γH2AX 1:200, 53BP1 1:200, ATM 1:1000) for 24h at 4°C and with secondary antibody (1:200) for 1h. Counterstaining with Hoechst 33258 (1µg/mL) was used to verify the location and integrity of nuclei. Fluorescence images (magnification: ×600) were obtained with a laser scanning confocal microscope (FV1000 IX81; Olympus, Japan).

Colony Formation Assay Colony formation assay was performed as described previously. 4.0 × 102 cells were seeded in a 60 mm dish. After incubation for 1 week, the cells were stained with 0.5% crystal violet (Wako). Colonies containing more than 50 cells were counted. The average was obtained for each sample. The results were normalized with respect to corresponding non-irradiated cells, and the survival fraction was calculated. Inhibitors and agonist were added to the medium at 30min before γ-irradiation.

Transplantation Model Irradiation of B16 melanoma-transplanted mice was performed based on our previous reports. Irradiation of 104 cells/mL was administered to B16 melanoma-bearing mice (23.5 ± 1.83 g) on the 14th, 18th, 21st and 23rd days after transplantation. Mice were irradiated with 1 Gy of γ-rays at 2h after treatment with AMTB hydrochloride on the 14th, 18th, 21st and 23rd days after transplantation. The size of the solid tumor was measured with a caliper twice a week for 24d, and the volume was calculated according to the following equation;

\[ V(\text{mm}^3) = \text{length}(\text{mm}) \times \text{width}(\text{mm}) \times \text{width}(\text{mm}) \times 0.5 \]

Cell Cycle Analysis by Flowcytometry Cells were harvested with ethylenediaminetetraacetic acid (EDTA), washed with PBS, and fixed in cold 70% ethanol on ice for 1h. Cell suspensions were washed twice with PBS, treated with ribonuclease A (0.34 mg/mL) (Roche, Switzerland) and stained with propidium iodide (PI) (50 µg/mL) (Sigma-Aldrich) for 30min. Flow cytometry was performed to determine DNA content and cell cycle distribution.

Small Interfering RNA (siRNA) Transfection for Knockdown of TRPM8 siRNA targeting mouse TRPM8 and negative control siRNA (TriFECTa Kit® DsIRNA Duplex) were purchased from Integrated DNA Technology (U.S.A.). Cells (5.0 × 104 cells in a 24-well plate) were incubated in culture medium for 16h. The siRNA duplex oligonucleotides (25 nM) for knockdown of mouse TRPM8 channel (mm.R1:Trpm8.13.3) were transfected into B16 melanoma cells by using Lipo-
fectamine® RNAiMAX (Invitrogen) according to the manufacturer’s instructions. At 48h after transfection, the expression of TRPM8 was confirmed by Western blotting. The expression level of TRPM8 in the knockdown cells was decreased to 32% of that in the cells transfected with scrambled siRNA.

**Western Blotting** We performed Western blotting as described previously. Cells were washed twice with ice-cold PBS and lysed in PBS containing 10mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES)-NaOH (pH 7.4), 1% Triton X-100, 5mM EDTA, 30mM sodium pyrophosphate, 50mM sodium fluoride, 1mM sodium orthovanadate, 1.04mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.8µM aprotinin, 21µM leupeptin, 36µM bestatin, 15µM pepstatin A and 14µM E-64 for 30min on ice. The samples were mixed

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![Western Blotting](image)

**Fig. 1.** Change of TRPM8 Expression after γ-Irradiation and Effects of TRPM8 Channel Inhibitor and Agonist on the Number of γH2AX-53BP1 Foci at 30min after γ-Irradiation

(A) Cells were irradiated with 2Gy of γ-rays and incubated for 24h. Densitometric analysis of target bands was normalized to total protein (IR (−) TRPM8). Each value represents the mean ± standard error (S.E.) (n = 3). Significant differences between the control and irradiated cells are indicated by * (p < 0.05). (B–D) Cells were preincubated for 30min with (B) AMTB (10µM), (C) WS-12 (2µM), (D) BAPTA-AM (10µM) or Ca2+-free RPMI. Cells were irradiated with γ-rays and incubated for 30min. Co-localization of γH2AX (red) and 53BP1 (green) in nuclei (blue) was evaluated. Each value represents the mean ± S.E. (n = 43–55). Significant differences between the irradiation-only group and treated group are indicated by *** (p < 0.001). (Color figure can be accessed in the online version.)
with 2 × sample buffer and incubated at 95 °C for 10 min. Protein concentration of samples was determined with Bio-Rad Protein assay reagent (BioRad, U.S.A.). Aliquots of samples containing proteins were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and bands were transferred onto polyvinylidene difluoride (PVDF) membranes. The blots were incubated overnight with 1% bovine serum albumin (BSA) in TBST (0.1% Tween-20, 10 mM Tris–HCl, 0.1 M NaCl) or 5% non-fat dry milk in TBST, then incubated overnight with primary antibody (TRPM8 1:1000, p-ATM 1:1000, p-p53 1:1000, p53 1:1000) at 4 °C. They were then washed with TBST and incubated with the appropriate secondary antibody (1:20000, or 1:2000) for 1.5 h at room temperature. The levels of specific proteins were visualized by using enhanced chemiluminescence (ECL) Western detection reagents (GE Healthcare, U.S.A.).

Statistics Results are expressed as the mean ± standard error (S.E.). The statistical significance of differences between control and other groups was calculated using Student’s t-test or Dunnett’s test. We statistically analyzed the experiments having two factors (e.g. irradiation and inhibitor) by two-way ANOVA followed by pairwise comparisons with Bonferroni’s post hoc analysis. Calculations were done with the Instat version 3.0 statistical software package and the Prism version 5.0a (Graph Pad Software). The criterion of significance was p < 0.05.

RESULTS

Expression of TRPM8 in B16 Melanoma Cells Over-expression of the TRPM8 channel has been reported in many cancers.24,26,31,32) We confirmed that TRPM8 was expressed in B16 melanoma cells, and its expression was increased at 24 h after γ-irradiation (Fig. 1A).

Involvement of TRPM8 Channel in γ-Irradiation Induced γH2AX-53BP1 Focus Formation When DSBs are induced by γ-irradiation, γH2AX is rapidly formed and DNA repair is initiated. We previously reported that the formation of γH2AX-53BP1 foci peaked at 30 min after γ-irradiation in B16 melanoma cells.30) Here, to investigate whether TRPM8 is involved in DDR, we evaluated the formation of γH2AX-53BP1 foci after γ-irradiation by means of immunofluorescence, and examined the effects of a TRPM8 inhibitor and an agonist. We selected AMTB as the inhibitor, because it is highly selective for TRPM8 and can be used in vitro and in vivo.33–35) We examined the cytotoxicity of AMTB by means of lactate dehydrogenase (LDH) assay and found that B16 cells did not release LDH in the presence of 1–10 µM AMTB (data not shown), so we used AMTB at 10 µM for the present experiments.

The number of co-stained γH2AX-53BP1 foci was reduced by pretreatment with 10 µM AMTB and increased by 2 µM WS-12 (a TRPM8 agonist) (Figs. 1B, C). BAPTA-AM (an intracellular Ca2+ chelator) or removal of extracellular Ca2+, which suppresses TRP channel-mediated Ca2+ elevation, also decreased the formation of γH2AX-53BP1 foci after γ-irradiation (Fig. 1D).

These results indicate the involvement of the TRPM8 channel in the formation of γH2AX-53BP1 foci after γ-irradiation. Since γH2AX foci disappear when DNA damage is repaired, we measured persistent γH2AX-53BP1 foci at 24 h after γ-irradiation as a measure of unrepaired DNA damage. The number of persistent γH2AX-53BP1 foci was increased by pretreatment with 10 µM AMTB compared with 2 Gy irradiation alone (Fig. 2A). In contrast to the case of AMTB,
WS-12 decreased persistent γH2AX-53BP1 foci compared with 5 Gy irradiation alone (Fig. 2B). These results indicate that the TRPM8 inhibitor delayed DNA repair, supporting the idea that TRPM8 is involved in the DNA damage response.

Effect of TRPM8 Channel Inhibitor and Agonist on Cell Survival Fraction after γ-Irradiation  
Next, we investigated the effect of AMTB and WS-12 on the cell survival fraction at 1 week after γ-irradiation by means of colony formation assay. The survival fraction of B16 melanoma cells decreased in a dose-dependent manner following irradiation. Pretreatment with 10 µM AMTB significantly enhanced the decrease of survival fraction (Fig. 3A). On the other hand, pretreatment with 2 µM WS-12 attenuated the decrease of survival fraction (Fig. 3B). Pretreatment with 10 µM BAPTA-AM
enhanced the decrease of survival fraction after \( \gamma \)-irradiation (Fig. 3C). Taken together, these results indicate that the TRPM8 channel plays an important role in cell recovery from irradiation-induced DNA damage.

**Involvement of TRPM8 in \( \gamma \)-H2AX-53BP1 Focus Formation and Cell Survival after \( \gamma \)-Irradiation**

To confirm the involvement of the TRPM8 channel in the formation of \( \gamma \)H2AX-53BP1 foci and in cell survival after \( \gamma \)-irradiation, we knocked down TRPM8 with siRNA. We confirmed the decrease of TRPM8 expression in knockdown B16 melanoma cells by Western blotting (Fig. 4A). Focus formation of \( \gamma \)H2AX-53BP1 at 30 min after \( \gamma \)-irradiation was significantly suppressed in TRPM8-knockdown cells (Fig. 4B). Further, the decrease of survival fraction after \( \gamma \)-irradiation was significantly enhanced (Fig. 4C). These results strongly support the view that the TRPM8 channel is involved in DDR and cell survival after \( \gamma \)-irradiation.

**Effect of TRPM8 Channel Inhibitor on \( \gamma \)-Irradiation-Induced Cell Cycle Arrest**

Next, we examined the effect of AMTB on the \( \gamma \)-irradiation-induced cell cycle arrest. As shown in Fig. 5, 2 Gy \( \gamma \)-irradiation induced G2/M arrest in B16 melanoma cells, and the G2/M arrest was enhanced by AMTB (Fig. 5), indicating that the population of DNA-damaged cells...
was increased by blockade of the TRPM8 channel.

**Involvement of TRPM8 in Phosphorylation of ATM and p53 after γ-Irradiation** To elucidate the involvement of TRPM8 in DDR, we examined the effect of the TRPM8 channel inhibitor on phosphorylation of ATM and p53 after γ-irradiation.

First, we examined the effect of the ATM inhibitor KU55933 on the formation of γH2AX-53BP1 foci after γ-irradiation by means of immunofluorescence. KU55933 strongly suppressed the focus formation at 30 min after γ-irradiation (Fig. 6A).

Next, we examined the effect of the TRPM8 channel inhibitor on the formation of ATM-γH2AX focus after γ-irradiation. AMTB suppressed ATM-γH2AX focus formation (Fig. 6B) as well as γH2AX-53BP1 focus formation (Fig. 1B). These results indicate that TRPM8 is involved in the activation of ATM, phosphorylation of H2AX, and accumulation of 53BP1.

We further examined ATM and p53 phosphorylation after γ-irradiation by means of Western blotting. ATM was activated within 1 h after γ-irradiation (Fig. 6C). Pretreatment with AMTB suppressed the phosphorylation of ATM (Fig. 6D), and also suppressed both p53 expression (Fig. 6E) and p53 phosphorylation (Fig. 6F) after γ-irradiation. Since ATM mediates the activation of p53 after DNA damage, these results indicate that the role of the TRPM8 channel in DDR after γ-irradiation involves both ATM and p53.

**Radiosensitizing Effect of TRPM8 Channel Inhibitor in Vivo** Finally, to investigate whether the above findings have relevance in vivo, we examined the radiosensitizing effect of AMTB on the growth of B16 melanoma cells transplanted into footpads of C57BL/6 mice. The mice were pretreated with 50 µM AMTB (100 µL/head i.p.), and then given whole-body γ-irradiation (1 Gy) at the 14th, 18th, 21st and 23rd days after transplantation (total 4 Gy irradiation). We measured the tumor volume and body weight at the 14th, 18th, 21st, 23rd and 25th days after transplantation. As shown in Fig. 7, the tumor volume was slightly suppressed by AMTB alone, and moderately suppressed by 1 Gy γ-irradiation. The combination of AMTB and 1 Gy γ-irradiation significantly suppressed melanoma growth. Therefore, it is indicating that AMTB exerts a radiosensitizing effect not only in vitro, but also in vivo.
DISCUSSION

Our present results show that expression of TRPM8 is increased together with activation of p53 in B16 melanoma cells in vitro as a part of the cellular DDR. The TRPM8 channel is known to be a transcriptional target of p53. Thus, activation of p53 by γ-irradiation might increase the expression of TRPM8 and thereby facilitate the DDR after γ-irradiation. This idea is supported by our finding that the TRPM8 channel inhibitor AMTB has a radiosensitizing effect on B16
of DNA damage. 37) On the other hand, 53BP1 accumulates generated at DNA double-strand breaks, acting as a marker controlled, and Ca \(^{2+}\) influx into cells. However, the suppression of focus formation involved in the early stage of DDR after \(\gamma\)-irradiation. 38) Hence, by examining the persistence of foci is possible to evaluate whether DNA repair has been performed correctly. We found that the persistence of \(\gamma\)H2AX-53BP1 foci was increased by AMTB (Fig. 2A), indicating that TRPM8 is involved in the early stage of DDR after \(\gamma\)-irradiation. Further, TRPM8 may also be involved in late stages of DDR. It is known that \(\gamma\)H2AX and 53BP1 disappear when DNA repair is completed. 39) Hence, by examining the persistence of foci is possible to evaluate whether DNA repair has been performed correctly. We found that the persistence of \(\gamma\)H2AX-53BP1 foci was increased by AMTB (Fig. 2A) and decreased by WS-12 (Fig. 2B). Thus, TRPM8 channels appear to be involved in both the early and late stages of DDR. TRP channels play an important role in intracellular calcium homeostasis by regulating Ca \(^{2+}\) influx. 39–41) We observed here that the formation of \(\gamma\)H2AX-53BP1 foci after \(\gamma\)-irradiation was suppressed by an intracellular calcium ion chelator (Fig. 1D). Influx of Ca \(^{2+}\) into cancer cells contributes to migration, apoptosis, and radiation resistance in breast cancer. 42,43) Therefore, TRPM8 might contribute to DDR by mediating Ca \(^{2+}\) influx into cells. However, the suppression of focus formation by the chelator was weaker than that caused by TRPM8 channel inhibition. The intracellular Ca \(^{2+}\) concentration is strictly controlled, and Ca \(^{2+}\) influx through the TRPM8 channel may be particularly related to the DNA damage response. TRPM8 channel inhibition or TRPM8 channel knockdown appears to enhance reproductive death after \(\gamma\)-irradiation by suppressing DDR. On the other hand, WS-12 decreased the survival rate after \(\gamma\)-irradiation (Fig. 3B), indicating that TRPM8 channel activation improves survival by promoting DNA damage response and DNA repair. This raises the interesting possibility that activation of TRPM8 channels may attenuate skin damage associated with radiation treatment. On the other hand, TRPM8 channel inhibitors may be candidates for radiosensitizers.

Our findings also indicate that TRPM8 plays a role in cell cycle progression. When DNA is damaged, the cell cycle is stopped to repair the damage. We found that treatment of irradiated cells with AMTB decreased the G\(_1\)/G\(_0\) and S phase, and increased G\(_2\)/M (Fig. 5). This is consistent with reports that TRPM8 is involved in S phase progression in glioblastoma. 20) Therefore, TRPM8 may also be involved in S phase progression in melanoma cells. The S phase progression is essential for DNA replication, and S-phase abnormalities induce genomic instability. 44) Thus, treatment with AMTB would enhance genomic instability through suppression of DDR and promotion of abnormal DNA synthesis following \(\gamma\)-irradiation. As a result, reproductive death is induced due to loss of proliferative ability.

Our results further demonstrate the involvement of TRPM8 in phosphorylation of ATM and p53 after \(\gamma\)-irradiation. Klupp et al. reported the involvement of calmodulin-dependent protein kinase II (CaMKII) and cell division cycle 25 homolog C (cdc25C) via activation of the TRPM8 channel after exposure to ionizing radiation. 20) However, there has been no report of involvement of TRPM8 in ATM activation after \(\gamma\)-irradiation. AMTB decreased the formation of ATM-\(\gamma\)H2AX foci and the phosphorylation of ATM after \(\gamma\)-irradiation (Figs. 6B, D). We also observed suppression of phosphorylation and expression of p53 (Figs. 6E, F). ATM protein kinase is a key regulator of the DDR, being involved in the phosphorylation of H2AX, accumulation of 53BP1 and phosphorylation of p53 via suppression of mdm-2. 11–14) In addition, ATM is involved in the cell cycle progression at the spindle checkpoint and S-phase check point via Fanconi Anemia group D2 protein (FANCD2) and Nijmegen breakage syndrome 1 (Nbs1). 45) These results indicate that TRPM8 mediates DDR though the

**Fig. 7. Radiosensitizing Effect of TRPM8 Channel Inhibitor in Vivo**

B16 melanoma cells were injected into the hind footpad of 5-week-old male C57BL6 mice. AMTB was intraperitoneally administered to the B16 melanoma-bearing mice on the 14th, 18th, 21st, and 23rd day after transplantation. Mice were irradiated with \(\gamma\)-rays (1 Gy) at 2 h after treatment with AMTB on the 14th, 18th, 21st, and 23rd day after transplantation. The size of the solid tumor was measured twice a week for 24 d and the volume was calculated as described in the text. Values are mean ± S.E. Significant differences between indicated groups are indicated by * (\(p<0.05\)).
ATM-p53 pathway after γ-irradiation.

It has been reported that AMTB suppresses proliferation of prostate cancer cells in vitro and cures overactive bladder in rats.\textsuperscript{15,40} However, there has been no report on the combined use of AMTB with γ-irradiation in vivo. Melanoma growth was barely suppressed by AMTB treatment alone or 1 Gy γ-irradiation alone. However, the combination of 1 Gy γ-irradiation with AMTB strongly suppressed tumor growth, suggesting that AMTB functions as a radiosensitizer of melanoma in vivo.

Several reports have suggested that TRPM8 channels are activated by ionizing radiation. It is also well known that γ-irradiation generates reactive oxygen species (ROS), and that poly(ADP-ribose) (ADPR) is generated from nicotinamide adenine dinucleotide (NAD\textsuperscript{+}) by poly(ADP-ribose) polymerase (PARP) after DNA damage has occurred.\textsuperscript{47,48} Recently, it was reported that ROS and ADPR activate the TRPM8 channel in human kidney and prostate cancer cells.\textsuperscript{49} Thus, TRPM8 may be activated by ROS and ADPR after γ-irradiation of melanoma cells. Additionally, TRPM8 channel is known to have several isoforms. TRPM8 isoforms have been shown to regulate mitochondrial associated endothelial reticulum membrane (MAREM)\textsuperscript{50} in breast cancer cells. TRPM8 isoforms induce apoptosis in prostate cancer cells.\textsuperscript{50,51}

It was reported that TRPM8 contributes to hypoxia in the tumor environment by stabilizing hypoxia-inducible factor-1 (HIF-1), mediated by receptor of activated protein C kinase 1 (RACK1).\textsuperscript{52} Hypoxia causes resistance to radiation therapy and drug therapy, and is associated with a poor prognosis in cancer treatment. Furthermore, peptides of TRPM8 derived from prostate cancer may activate the cytotoxicity of CD8\textsuperscript{+} T-lymphocytes, so TRPM8 may be promising targets for cancer immunotherapy.\textsuperscript{33}

In conclusion, our results show that the TRPM8 channel inhibitor AMTB potentiates reproductive death of melanoma after γ-irradiation by suppressing DDR. Furthermore, it enhances the γ-radiation-induced suppression of tumor growth in vivo. Many radiosensitizers, including cell cycle checkpoint kinase inhibitors, tyrosine kinase inhibitors and gold nanoparticles have been investigated.\textsuperscript{54–56} But side effects are a problem, because these radiosensitizers affect not only cancer tissues, but also normal tissues.\textsuperscript{53} In normal tissues, TRPM8 channel is expressed in testis and prostate, while it is weakly expressed in other tissues including skin, lungs and brain.\textsuperscript{53} However, TRPM8 is highly expressed in many cancers, including glioblastoma, breast cancers, prostate cancers, and melanoma.\textsuperscript{24,31,32} TRPM8 channel inhibitors may be promising candidates as radiosensitizers for malignant cancers.

**Conflict of Interest**  The authors declare no conflict of interest.

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