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Involvement of retinoic acid-inducible gene-I in radiation-induced senescence of human umbilical vein endothelial cells

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Abstract. In 2012, the threshold radiation dose (0.5 Gy) for cardiovascular and cerebrovascular diseases was revised, and this threshold dose may be exceeded during procedures involving radiation such as interventional radiology. Therefore, in addition to regulating radiation dose, it is necessary to develop strategies to prevent and mitigate the development of cardiovascular disease. Cellular senescence is irreversible arrest of cell proliferation. Although cellular senescence is one of the mechanisms for suppressing cancer, it also has adverse effects. For example, senescence of vascular endothelial cells is involved in development of vascular disorders. However, the mechanisms underlying induction of cellular senescence are not fully understood. Therefore, the present study explored the factors involved in the radiation-induced senescence in human umbilical vein endothelial cells (HUVECs). The present study reanalyzed the gene expression data of senescent normal human endothelial cells and fibroblast after irradiation (NCBI Gene Expression Omnibus accession no. GSE130727) and microarray data of HUVECs 24 h after irradiation (NCBI Gene Expression Omnibus accession no. GSE76484). Numerous genes related to viral infection and inflammation were upregulated in radiation-induced senescent cells. In addition, the gene group involved in the retinoic acid-inducible gene-I (RIG-I)-like receptor (RLR) signaling pathway, which plays an important role to induce anti-viral response, was altered in irradiated HUVECs. Therefore, to investigate the involvement of RIG-I and melanoma differentiation-associated gene 5 (MDA5), which are RLRs, in radiation-induced senescence of HUVECs, the protein expression of RIG-I and MDA5 and the activity of senescence-associated β-galactosidase (SA-β-gal), a representative senescence marker, were analyzed. Of note, knockdown of RIG-I in HUVECs significantly decreased radiation-increased proportion of cells with high SA-β-gal activity (i.e., senescent cells), whereas this phenomenon was not observed in MDA5-knockdown cells. Taken together, the present results suggested that RIG-I, but not MDA5, was associated with radiation-induced senescence in HUVECs.

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Introduction

In International Commission on Radiological Protection (ICRP) Publication 118, the threshold radiation dose for cataract development in the lens of the eye was revised, and the threshold radiation dose (0.5 Gy) for cardiovascular and cerebrovascular diseases was newly introduced (1). The threshold dose may be exceeded during procedures involving radiation such as interventional radiology and radiation therapy (2). Therefore, in addition to regulating radiation dose, it is necessary to develop strategies to prevent and mitigate development of cardiovascular diseases.

Cellular senescence is the irreversible arrest of cell proliferation and includes replicative and premature senescence (3). Replicative senescence is caused by telomere shortening, while various stimuli, such as oncogenic stress and DNA-damaging agents, including ionizing radiation, induce premature senescence. Senescent cells exhibit morphological changes such as flattening and hypertrophy, activation of senescence-associated β-galactosidase (SA-β-gal), induction of cell cycle-associated factors such as p16 and p21 and resistance to apoptosis (4-7). Additionally, senescent cells secrete factors such as inflammatory cytokines and proteases (8); this phenomenon is termed SA secretory phenotype (SASP) and affects surrounding cells or tissue (9).

As proliferation of cells with DNA damage may lead to the development of cancer cells, cellular senescence may be a tumor-suppressing mechanism (10). However, evidence has shown that senescent cells are also involved in age-associated diseases (11). Senescent cells cause chronic inflammation, neurodegenerative disease and rheumatoid arthritis (12-14). Furthermore, senescence of vascular endothelial cells (VECs) increases risk of not only vascular dysfunction but also development of diseases such as hypertension and atherosclerosis (15). Therefore, regulating senescence of VECs may

prevent and mitigate cardiovascular disease in individuals exposed to radiation.

The p53 pathway, which serves an important role in tumor suppression, is involved in induction of premature cellular senescence (16). Following DNA damage, p53 is activated and transcriptionally upregulates p21, which is a cyclin-dependent kinase (CDK) inhibitor that binds to the cyclin-CDK dimer to form inactive trimeric complexes, resulting in G1 arrest that leads to cellular senescence (16,17). In addition to cellular senescence, p53 can also induce apoptosis, thereby contributing to maintenance of genome integrity by eliminating cells with unrepairable DNA damage (18). These facts suggest that although p53 may be a potential target to regulate cellular senescence, p53 inhibition may disrupt genome integrity. Therefore, it is key to identify factors that regulate senescence of VECs without causing undesirable effects such as inhibiting apoptosis and downregulating EC function.

Retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) serve an important role to induce anti-viral response (19,20). RLRs are located in the cytoplasm and recognize virus-derived RNA. RLRs include RIG-I, melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). RIG-I and MDA5 contain an N-terminal domain consisting of tandem caspase activation and recruitment domains (CARDs), a central DExD/H box RNA helicase domain and a C-terminal regulatory domain (21). Although RIG-I and MDA5 are structurally and functionally similar, they recognize different types of RNA virus (21). They independently and synergistically induce anti-viral responses (22), although it is suggested that RIG-I and MDA5 do not directly interact with each other (23). By contrast, LGP2 can interact with RIG-I and MDA5 (24), but lacks CARDs, which are responsible for initiating downstream signaling pathways leading to production of type I interferon (IFN) (19). Although it is well-known that RLRs serve important roles in anti-viral responses (19,20), it remains unknown whether they are involved in radiation-indued cellular senescence.

To identify factors involved in radiation-induced senescence of VECs, the present study aimed to perform *in silico* analysis using the NCBI Gene Expression Omnibus (GEO, ncbi.nlm.nih.gov/geo/) to search for candidate factors. Since *in silico* analysis suggested that RLRs may be candidate factors for radiation-induced senescence of VECs, the present study further investigated the involvement of RLRs in radiation-induced senescence of human umbilical (HU) VECs.

Materials and methods

Reagents. PBS(-) (Ca^{2+}, Mg^{2+}-free Dulbecco's) was purchased from Wako Pure Chemical Industries. Rabbit anti-human MDA5 (cat. no. #5321), RIG-I (cat. no. #4200), β-actin (cat. no. #4967) and horseradish peroxidase-conjugated anti-rabbit IgG (cat. no. #7074) and Senescence β-Galactosidase Activity Assay kit (cat. no. #35302) were purchased from Cell Signaling Technology, Inc. Silencer® Select pre-designed RIG-I (#1, cat. no. s223615; #2, cat. no. s24144) and MDA5 small interfering (si)RNA (#1, cat. no. s34498; #2, vat. no. s34499) and Negative Control #1 (cat. no. 4390843) were purchased from Thermo Fisher Scientific, Inc. PI and FBS were purchased from Sigma-Aldrich (Merck KGaA). FITC-annexin V and annexin V binding buffer were purchased from BioLegend, Inc.

Cell culture and treatment. HUVECs (cat. no. 200-05n, population doubling level, 15) were purchased from Cell Applications, Inc. After 3-5 passages (about 1-2 weeks) at 37˚C in a humidified atmosphere of 5% CO_2, cells were used for experiments. HUVECs were seeded onto collagen I-coated dishes (35 mm; Iwaki Science Products Dept.; AGC Techno Glass Co., Ltd.) at a density of $3.0x10^4$ cells/dish and cultured with Endothelial Cell Growth Medium kit (cat. no. C-22110; Takara Bio, Inc.) at 37˚C in a humidified atmosphere of 5% CO_2/95% air. After 6 h incubation and once cells adhered to the dish, irradiation was performed. Cells were irradiated with an X-ray generator (cat. no. MBR-1520R-3; Hitachi, Ltd.) at 450 mm from the focus and at a dose rate of 0.99-1.03 Gy/min (150 kVp; 20 mA; 0.5-mm Al filter and 0.3-mm Cu filter). Cells were harvested 5 or 10 days after irradiation and used for subsequent experiments. For cells cultured for 10 days, non-irradiated cells were harvested on day 5 and reseeded onto new 35-mm dishes at a density of $3.0x10^4$ cells/dish to avoid overproliferation. The irradiated cells were washed with PBS(-) and the medium was replaced on day 5.

siRNA transfection. Knockdown of RIG-I and MDA5 was achieved using Silencer® Select Pre-designed siRNA and RNAiMAX (Invivogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The sense sequences for RIG-I #1 and #2 were 5'-GAAGCAGUAUUUAGGGAA ATT-3' (antisense: 5'-UUUCCCUAAAUACUGCUUCGT-3') and 5'-CCAGAAUUAUCCCAACCGATT-3' (antisense: 5'-UCGGUUGGGAUAAUUCUGGTT-3'), respectively. The sense sequences for MDA5 #1 and #2 were 5'-GUAACAUUG UUAUCCGUUATT-3' (antisense: 5'-UAACGGAUAACA AUGUUACAT-3') and 5'-GGUGUAAGAGAGCUACUA ATT-3' (antisense: 5'-UUAGUAGCUCUCUUACACCTG-3'), respectively. Silencer® Select Negative Control #1 (sequence not available) was used as the negative control. The final concentration of all siRNAs was 10 nM. After 48 h transfection at 37˚C in a humidified atmosphere of 5% CO_2/95% air, cells were harvested. The cells were immediately seeded onto collagen I-coated dishes for subsequent experiments.

Western blotting. SDS-PAGE and western blot analysis were performed as previously reported (28). The following primary antibodies diluted in Can Get Signal® Immunoreaction Enhancer Solution 1 (Toyobo Life Science) were used: Anti-RIG-I (1:3,000), anti-MDA5 (1:3,000) and anti-β-actin (1:4,000). Following overnight dilution at 4˚C, the membrane was reacted with secondary antibody (1:10,000) diluted in Can Get Signal® Immunoreaction Enhancer Solution 2 (Toyobo Life Science) for 1 h at room temperature and detected using Clarity Western ECL Substrate (Bio-Rad Laboratories, Inc.). Images were captured by cool saver AE-6955 (ATTO Corporation) or iBright 1500 Image system (Thermo Fisher Scientific, Inc.).

Analysis of SA-β-gal activity. SA-β-gal activity was analyzed using the Senescence β-Galactosidase Activity Assay kit

according to the manufacturer's instructions. For analysis of SA-β-gal activity by flow cytometry, after culture of irradiated cells, the culture medium was replaced with Endothelial Cell Growth Medium containing bafilomycin A1 (100 nM). Following incubation for 1 h at 37˚C, SA-β-Gal substrate solution (33 μM) was added to the cell culture and incubated for 2 h at 37˚C. After washing three times with PBS(-), cells were harvested, washed with PBS(-) and suspended in cold PBS(-) containing 2% FBS. Fluorescence intensity of SA-β-Gal substrate was analyzed using a flow cytometer (CytoFLEX with CytExpert software version 2.4.0.28; Beckman-Coulter, Inc., https://www.beckman.jp/).

For analysis of SA-β-gal activity by a confocal microscope, HUVECs seeded onto collagen coated-glass bottom dish (cat. no. D11134H; Matsunami Glass Ind., Ltd.) were irradiated with 10 Gy and cultured for 5 days, as aforementioned. Culture medium was replaced with Endothelial Cell Growth Medium containing bafilomycin A1 (100 nM). Following incubation for 1 h at 37˚C, SA-β-Gal substrate solution (33 μM) was added to the cell culture and incubated for 2 h at 37˚C. After washing three times with PBS(-), cold PBS(-) containing 2% FBS was added to the dish. After that, cells were treated with 5 μg/ml Hoechst 33342 (Thermo Fisher, Scientific, Inc.) for 5 min at room temperature in the dark. Following washing with PBS(-), images were captured by a confocal laser microscope (LSM710; Carl Zeiss AG).

Analysis of apoptosis. Apoptosis was analyzed using FITC-annexin V and PI staining according to the manufacturer's instructions. Briefly, irradiated cells were harvested, washed twice with PBS(-), centrifuged at 200 x g for 5 min at room temperature and suspended in 100 μl annexin V binding buffer. Five μl of FITC-annexin V (90 μg/ml) and PI (1 mg/ml) were added to cell suspension and cells were incubated for 15 min at room temperature in the dark. After adding annexin V binding buffer, samples were analyzed by flow cytometer as aforementioned (CytoFLEX, Beckman-Coulter).

Statistical analysis. Data are presented as the mean ± SD of three independent experiments. Comparison of multiple groups was performed using one-way analysis of variance followed by Tukey-Kramer post hoc test. Statistical analysis was performed using Excel 2016 (Microsoft Corporation) with the Statcel 4 (OMS Publishing) add-in. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Characteristics of genes commonly upregulated in radiation-induced senescent cells. Gene expression in HUVECs, HAECs, WI-38 and IMR-90 cells, which underwent irradiation to induce senescence (25), was re-analyzed to determine the commonly upregulated genes in senescent cells, revealing 236 genes (Fig. 1A). To investigate the characteristics of these genes, GO terms and signaling pathways were analyzed (Fig. 1B and C). As shown in Fig. 1B, commonly upregulated genes in GO terms were associated with viral infections ('Response to virus') and inflammation ('Type I interferon signaling pathway' and 'Response to type I interferon'; Fig. 1B). Similarly, pathway analysis (Fig. 1C) showed that commonly upregulated genes were associated with viral infections ('Epstein-Bar virus infection' and 'Human papillomavirus infection') and inflammation ('Interferon Signaling', 'Interferon alpha/beta signaling', 'Cytokine Signaling in Immune system', 'Interferon gamma Signaling', 'Immune System').

Characteristics of genes upregulated in HUVECs following irradiation. The present study re-analyzed the microarray data of HUVECs 24 h after irradiation (26). Similar to those observed in senescent cells, genes associated with viruses and inflammation were upregulated in irradiated HUVECs (Fig. 1D). RIG-I-like receptor (RLR) signaling pathway, which plays an important role in the anti-viral response (29), was altered (Fig. 1E).

Expression of RLRs and SA-β-gal activity in irradiated HUVECs. The association between RLRs and senescence in HUVECs was analyzed *in vitro*. The present study focused on RIG-I and MDA5 because they activate downstream signaling pathways (23). Irradiation (4 Gy) increased RIG-I and MDA5 expression on post-irradiation day 5 (Fig. 2). Additionally, RIG-I and MDA5 expression remained high 10 days post-irradiation. However, RIG-I and MDA5 expression 24 h post-irradiation was not notably changed from that at baseline (Fig. 2B).

To confirm cellular senescence, SA-β-gal activity of irradiated HUVECs was analyzed (30). At 10 days after 4 Gy-irradiation, RIG-I and MDA5 expression as well as the proportion of HUVECs with high SA-β-gal activity were higher than those in non-irradiated cells (Fig. 2C). Additionally, 10 Gy irradiation effectively increased not only the expression of RIG-I and MDA5 but also the proportion of cells with high SA-β-gal activity at 5 days post-irradiation (Fig. 2D and E). These results showed that irradiation induced senescence of HUVECs accompanied by upregulation of RIG-I and MDA5 expression.

Association between radiation-induced senescence and RIG-I or MDA5 expression in HUVECs. To investigate involvement of RIG-I and MDA5 in radiation-induced senescence in HUVECs, RIG-I- or MDA5-knockdown HUVECs were constructed. Transfection of siRNA targeting RIG-I or MDA5 decreased their expression in HUVECs (Fig. 3A). Proportion of cells with high SA-β-gal activity at 10 days following 4 Gy irradiation was significantly lower in the RIG-I knockdown group than in the control group; this was not observed in the MDA5 knockdown group (Fig. 3B). Similar effects by RIG-I knockdown were observed at 5 days after 10 Gy irradiation (Fig. 3C). Furthermore, 10 Gy-irradiated HUVECs showed morphological changes such as enlargement and high fluorescence intensity for SA-β-gal substrate compared with non-irradiated cells (Fig. 3D). In addition, in line with the results of flow cytometric analysis, the number of cells with high fluorescence intensity was low in RIG-I knockdown group compared with 10 Gy-irradiated cells transfected with control siRNA (Fig. 3D).

Association between RLRs and radiation-induced apoptosis in HUVECs. The present study investigated the effects of

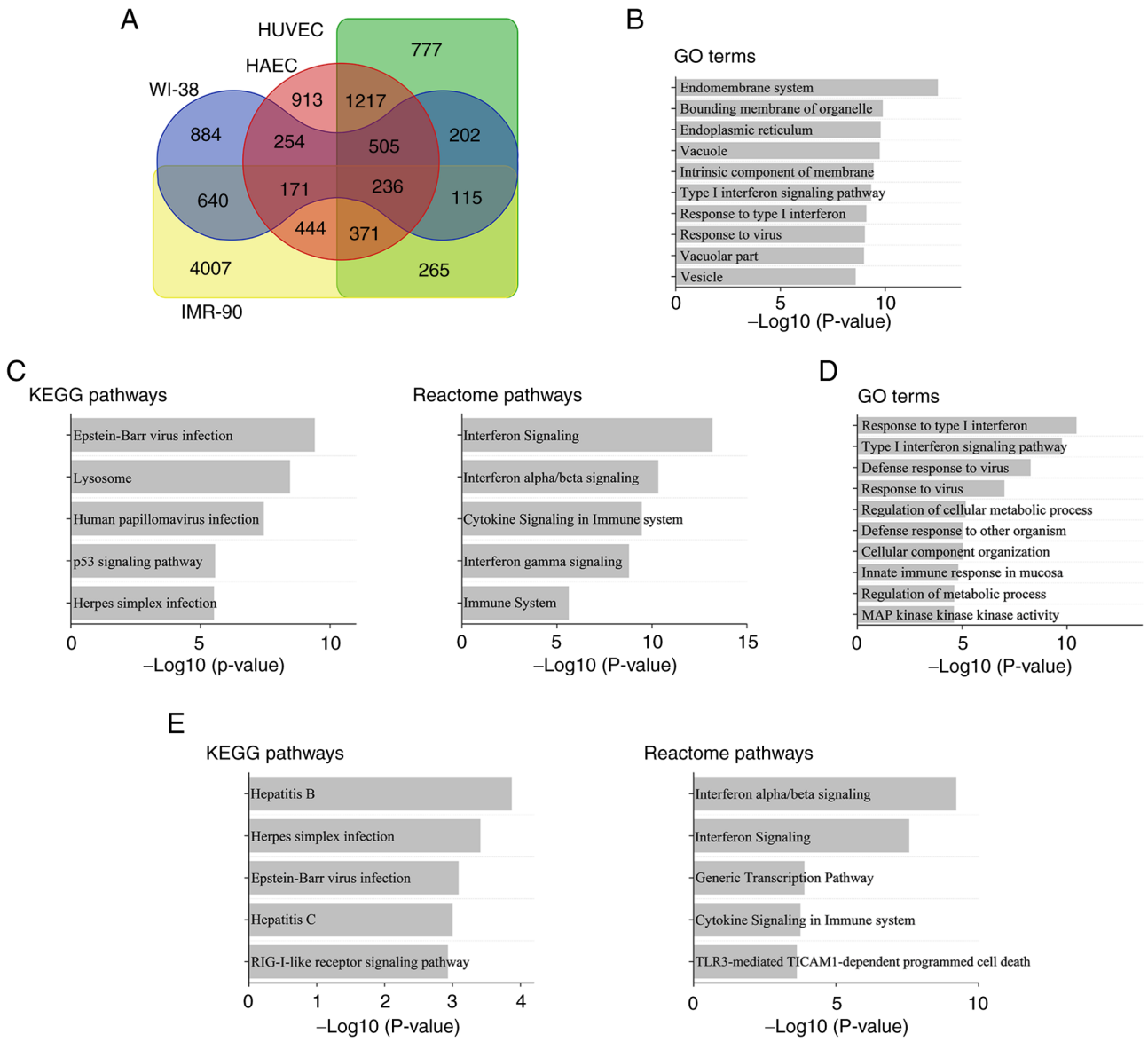

Figure 1. Characteristics of genes upregulated in irradiated cells. (A) Venn diagram of genes commonly upregulated in each cell type during senescence. Red, HAEC; blue, HUVEC; green, WI-38; yellow, IMR-90. (B) GO and (C) pathway analysis of commonly upregulated genes in senescent cells using ConsensusPathDB. (D) GO and (E) pathway analysis of upregulated genes in irradiated HUVECs using ConsensusPathDB. Pathway analyses were performed for both KEGG and Reactome pathways. HAEC, human aortic endothelial cell; HUVEC, human umbilical vein endothelial cell; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

RIG-I or MDA5 knockdown on radiation-induced apoptosis in HUVECs 10 days after 4 Gy-irradiation. Although 4 Gy irradiation statistically increased the proportion of annexin V-positive apoptotic cells (Fig. 4A and B), no significant difference in the proportion of annexin V-positive apoptotic cells in 4 Gy-irradiated cells was observed between control group and RIG-I or MDA5 knockdown group (Fig. 4B).

Discussion

Although radiotherapy is one of the primary treatments for cancer, it has adverse effects (31). Exposure of the heart to radiation during radiotherapy for breast cancer increases risk of heart disease (32). As senescence of VECs is associated with vascular diseases (15), regulating cell senescence may prevent development of vascular diseases due to radiation exposure. The present study aimed to identify factors that regulate senescence of irradiated HUVECs. *In silico* analysis suggested that RLRs may be involved in radiation-induced senescence of HUVECs. Additionally, *in vitro* analysis showed that radiation induced not only senescence of HUVECs but also upregulated RIG-I and MDA5 expression and that RIG-I knockdown, but not MDA5 knockdown, inhibited senescence of irradiated HUVECs. These results suggested that the role of RIG-I in responses of HUVECs to radiation was different from that of MDA5, and that RIG-I was involved in radiation-induced senescence of HUVECs.

Although it is well-known that RLRs serve important roles in anti-viral responses (23,24), there is a little information about their involvement in cellular senescence. RIG-I is induced in replicative senescent cells via the ataxia telangiectasia mutated-interferon regulatory factor

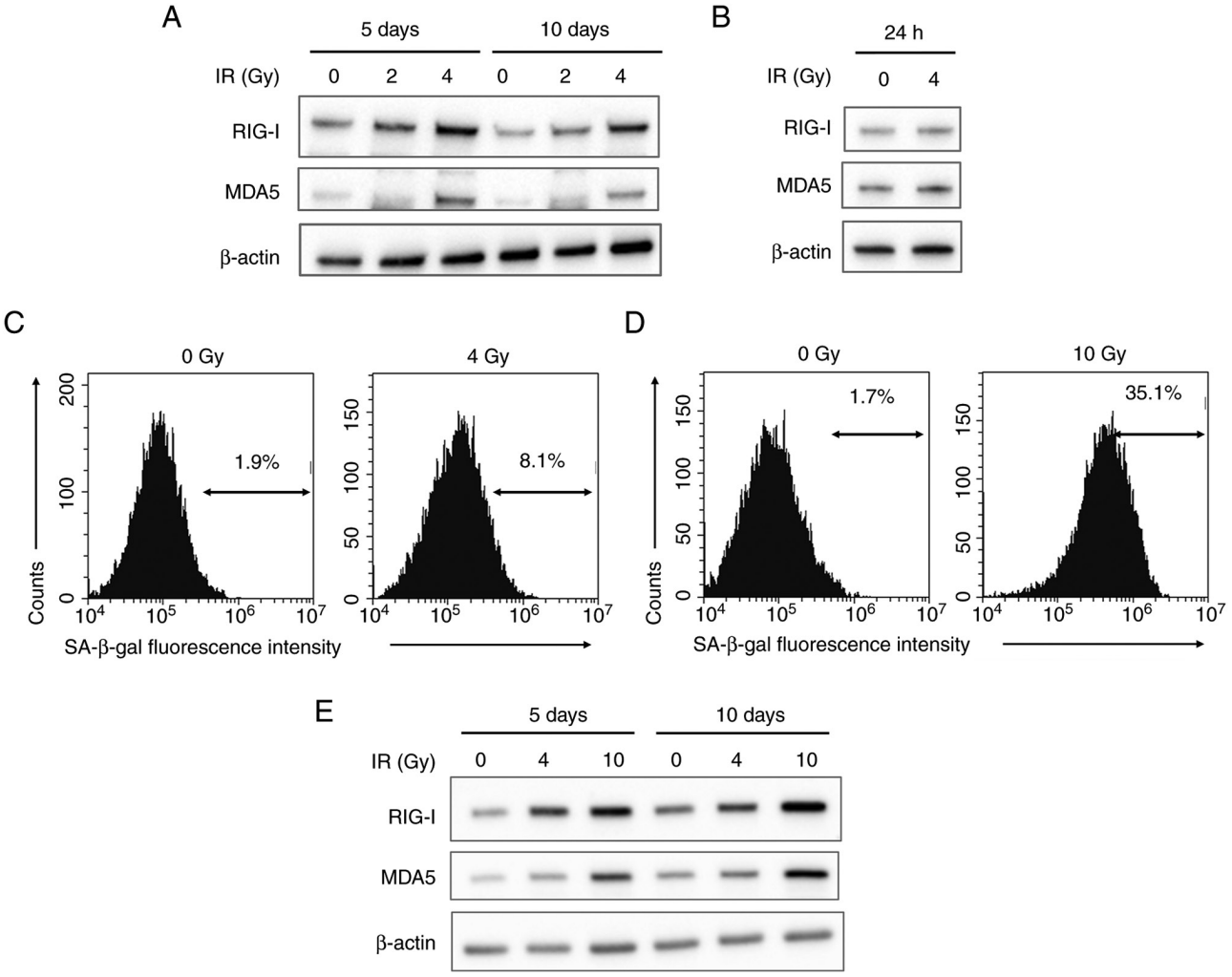

Figure 2. RIG-I and MDA5 expression and SA-β-gal activity in IR HUVECs. Expression of RIG-I and MDA5 in HUVECs at (A) 5 and 10 days and (B) 24 h post-IR were analyzed by western blot. HUVECs treated with (C) 4 and (D) 10 Gy were cultured for 10 and 5 days, respectively. After culture, cells were harvested to analyze SA-β-gal activity. Representative histograms of SA-β-gal activity are shown. Inset number indicates the proportion of cells with high SA-β-gal activity. (E) Expression of RIG-I and MDA5 in 4 or 10 Gy-IR HUVECs at 5 and 10 days post-IR were analyzed by western blot. β-actin was used as a loading control. RIG-I, retinoic acid-inducible gene I; MDA5, melanoma differentiation-associated gene 5; SA-β-gal, senescence-associated β-galactosidase; IR, ionizing radiation; HUVEC, human umbilical vein endothelial cell.

1 axis (33). In addition, Zeng *et al* (34) reported that in the senescence-accelerated mouse prone-8 mouse model of premature aging, the RIG-I/NF-κB signaling pathway is activated. In line with the aforementioned reports, the present study also showed upregulation of RIG-I expression in irradiated HUVECs, accompanied by increased SA-β-gal activity. These data suggested that the upregulation of RIG-I expression is a feature, and may be a useful marker, of senescent cells.

Here, RIG-I knockdown attenuated radiation-induced increase in SA-β-gal activity in cells, suggesting that RIG-I promoted radiation-induced senescence in HUVECs. To the best of our knowledge, the present study is the first to show the involvement of RIG-I in radiation-induced cellular senescence. By contrast, Zhao *et al* (35) reported the anti-aging effects of RIG-I. In the aforementioned study, RIG-I$^{-/-}$ mice showed age-related features such as alopecia and shortened survival. Additionally, continuous passage of RIG-I$^{-/-}$ mouse embryonic fibroblast results in premature replicative senescence (35). Although the reasons for the difference in the roles of RIG-I between the present results and those of the aforementioned study remain unclear, the difference in types of cells or senescence (replicative or premature senescence) may be involved due to the effects of senolytic drugs that eliminates senescent cells depending on cell or type of senescence-induced stimuli (36,37).

Upon activation, RLRs induce production of anti-viral cytokines such as type I IFN-β (38), which promotes cellular senescence (39). Ranoa *et al* (40) reported that ionizing radiation induces IFN-β production in both mouse fetal fibroblasts and the human glioma cell line D54 via the endogenous RNA/RIG-I pathway. Therefore, it is possible that RIG-I is involved in radiation-induced senescence via regulation of IFN-β. Additionally, as IFN-β can induce RLR expression (41), it may also upregulate RLR expression in irradiated HUVECs. To confirm this, further studies regarding the role of IFN-β in irradiated HUVECs are needed.

MDA5 knockdown failed to attenuate radiation-induced senescence in HUVECs, although its activation induces production of type I IFN (42). The type of RNA detected

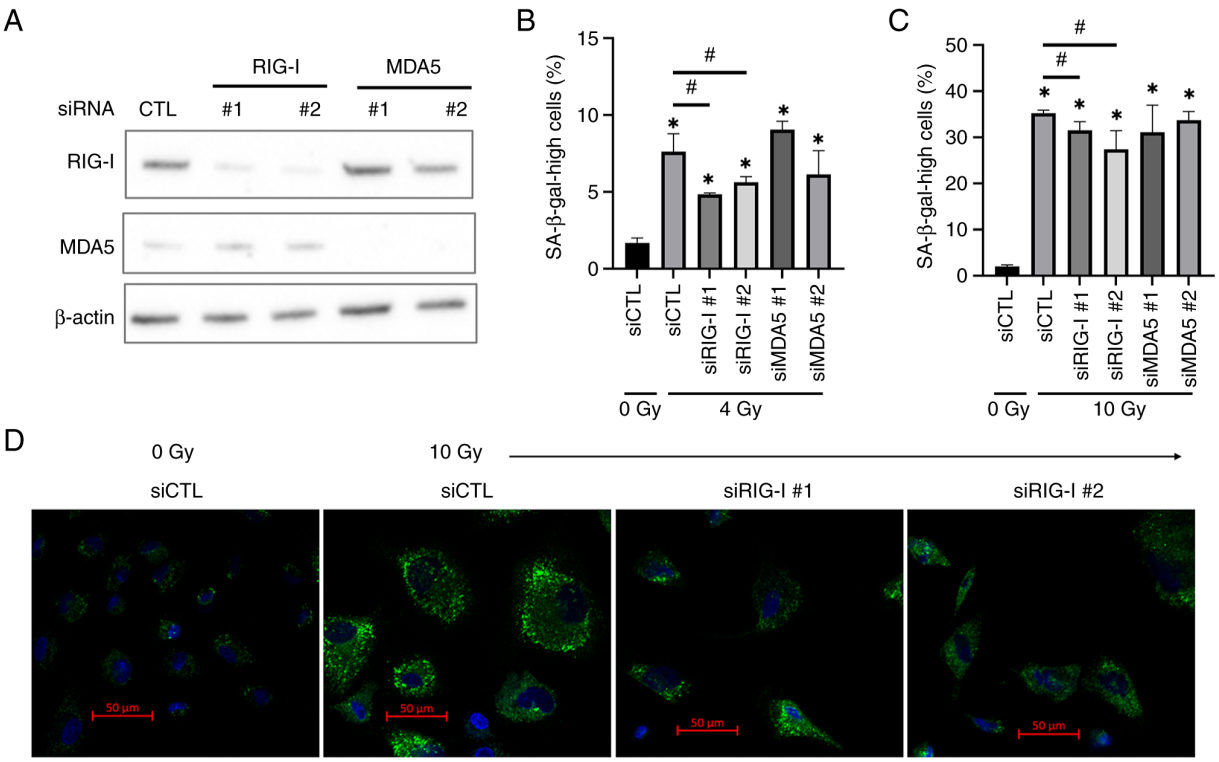

Figure 3. Effect of RIG-I or MDA5 knockdown on radiation-induced cellular senescence in HUVECs. (A) Expression of RIG-I and MDA5 in HUVECs transfected with siRNA targeting RIG-I or MDA5. β-actin was used as a loading control. RIG-I- or MDA5-knockdown HUVECs were exposed to (B) 4 or (C) 10 Gy irradiation. After culture, cells were harvested to analyze SA-β-gal activity. (D) RIG-I-knockdown HUVECs were exposed to 10 Gy irradiation and cultured for 5 days. SA-β-gal activity was analyzed. Blue and green fluorescence indicate Hoechst 33342 and SA-β-gal, respectively. *P<0.05 vs. 0 Gy; #P<0.05. RIG-I, retinoic acid-inducible gene I; MDA5, melanoma differentiation-associated gene 5; HUVEC, human umbilical vein endothelial cell; SA-β-gal, senescence-associated β-galactosidase; si, small interfering RNA; CTL, control.

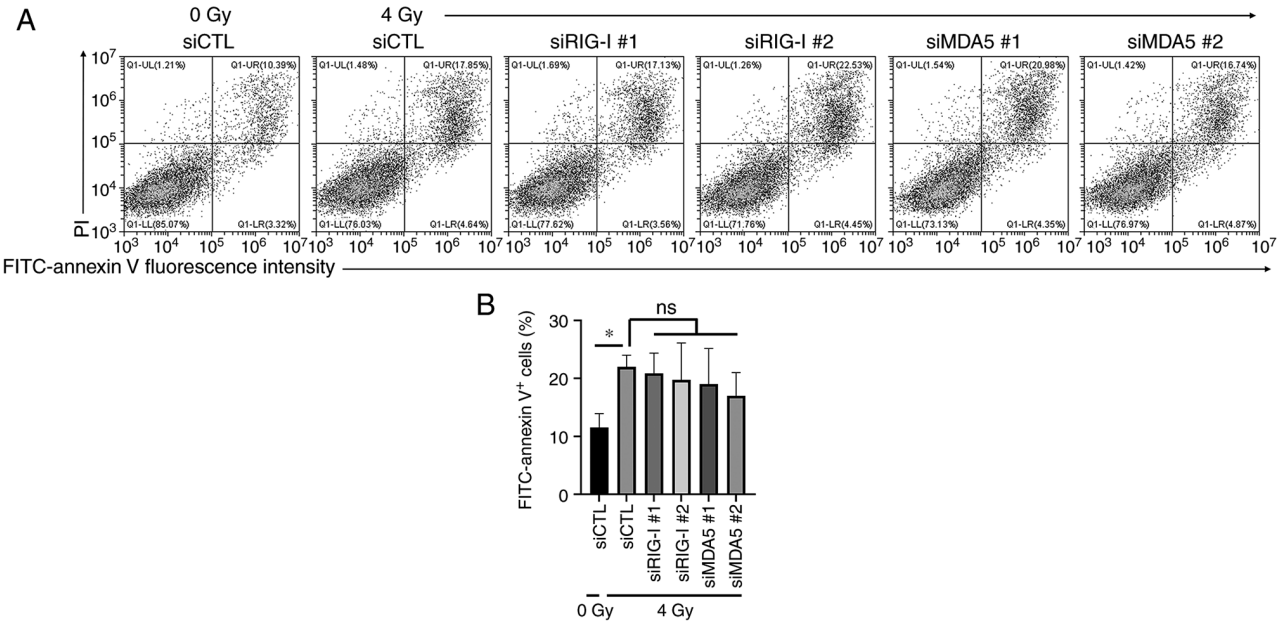

Figure 4. Effect of RIG-I or MDA5 knockdown on radiation-induced apoptosis in HUVECs. RIG-I or MDA5 knockdown HUVECs exposed to 4 Gy were harvested for FITC-annexin V/PI staining. (A) Representative cytograms of FITC-annexin V/PI staining. (B) Proportion of annexin V-positive cells in HUVECs. *P<0.05. RIG-I, retinoic acid-inducible gene I; MDA5, melanoma differentiation-associated gene 5; HUVEC, human umbilical vein endothelial cell; ns, not significant; si, small interfering RNA; CTL, control.

by MDA5 is different from that by RIG-I. Briefly, RIG-I primarily recognizes short double-stranded 5'-triphosphate RNA, while MDA5 recognizes long double-stranded RNA (22). Ranoa *et al* (40) reported that MDA5 is not

involved in radiation-induced IFN-β and that radiation enriches endogenous small RNA molecules in RIG-I complexes. Thus, it is likely that through endogenous small RNA molecules, radiation activates the RIG-I pathway, leading to cell senescence.

Although the tumor suppressor gene TP53 may be an effective target to control cellular senescence induced by DNA damage, it is also involved in apoptosis regulation (43). As proliferation of cells with DNA damage may increase the risk of genomic instability (44), suppression of p53 may be undesirable. Here, RIG-I knockdown did not affect radiation-induced apoptosis. Therefore, RIG-I may be a potential target for the regulation of senescence while maintaining genomic stability following radiation exposure.

In conclusion, the present study revealed a novel role of RIG-I in regulation of radiation-induced senescence in HUVECs. As RIG-I mediates SASP (33), regulating RIG-I may prevent and relieve SASP-mediated adverse effects as well as senescence induction. Although it is necessary to elucidate the mechanisms of RIG-I-mediated radiation-induced cellular senescence and the role of RIG-I in VEC functioning, RIG-I could be a potential target for prevention and mitigation of vascular damage after radiation exposure.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

HY conceived the study, designed the methodology, analyzed data and wrote the manuscript. FS, AK, ET and KS analyzed data. FS wrote the manuscript. ET interpreted data and edited the manuscript. All authors have read and approved the final manuscript. FS and HY confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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