Experimental article

Paracrine Effects of Adipose-Derived Stem Cell Promote Lymphangiogenesis in Irradiated Lymphatic Endothelial Cells

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Effects of ADSC on irradiated LECs

Abstract

Background: There is currently no reliable treatment for secondary lymphedema caused by lymph node dissection or radiotherapy; however, stem cell-based regenerative medicine is emerging as a promising remedy for such complications. Adipose-derived stem cells (ADSC) have recently attracted attention as they are easily accessible. The purpose of this study was to examine the effects of ADSC on lymphangiogenesis involving human dermal lymphatic endothelial cells (HDLEC) exposed to ionizing radiation.

Methods: Proliferation, migration, and tube-formation were analyzed in HDLEC that were co-cultured with ADSC or cultured in ADSC-conditioned medium. The levels of lymphangiogenic factors secreted from ADSC were analyzed by Enzyme-linked immunosorbent assays (ELISA) and Western blotting.

Results: Co-culturing with ADSC and the use of ADSC-conditioned medium both significantly promoted proliferation, migration, and tube-formation in non-irradiated HDLEC. We also found that irradiated ADSC had similar alleviative effects on irradiated HDLEC. ELISA and Western blotting analysis revealed that irradiating ADSC increased their secretion of basic fibroblast growth factor (bFGF) in a dose-dependent manner, while it caused no detectable change in their secretion of vascular endothelial growth

factor (VEGF)-A, VEGF-C, or hepatocyte growth factor.

Conclusion: These results demonstrated that factors secreted by ADSC contribute to the promotion of lymphangiogenesis in irradiated HDLEC. Our findings also suggest that radiation potentiates the paracrine effects of ADSC by stimulating bFGF protein expression.

Introduction

Lymphedema mainly affects the limbs and causes pain, deformities, and bacterial infections, which markedly reduce patients' quality of life (1-3). The most common causes of secondary lymphedema in the clinical setting are surgical lymph node dissection and radiotherapy for malignant tumors (4, 5). In secondary lymphedema caused by radiotherapy, the radiation directly damages lymphatic vessels, but fibrosis in the surrounding soft tissue also accelerates the development of the condition (6).

Several conservative treatments, such as physical therapy or compression techniques, are commonly employed for secondary lymphedema, but they are not curative, and their effects are limited (7). Microsurgical treatments, such as lymphaticovenular anastomosis or lymph node transfer, are often employed in severe cases of secondary lymphedema, but such surgery requires complicated microvascular procedures, and the efficacy of these techniques, including their effects on long-term prognosis, are unclear (8). In recent years, it has been reported that the administration of adipose-derived stem cells (ADSC) promoted lymphatic vascular network formation and improved lymphedema in rodent models of secondary lymphedema (9, 10). It is considered that ADSC do not directly differentiate into lymphatic vessel cells, but rather release vascular endothelial growth factor-C (VEGF-C), which strongly promotes lymphangiogenesis due

to its paracrine effects. On the other hand, Takeda et al. reported that culturing human dermal lymphatic endothelial cells (HDLEC) in ADSC-conditioned medium prepared with endothelial basal medium resulted in better cell proliferation, migration, and tube-formation than culturing them in basal medium supplemented with VEGF-C alone (11). Therefore, it is suggested that ADSC secrete not only VEGF-C, but also other lymphangiogenic growth factors with synergistic effects, although the interactive effects between these growth factors on lymphangiogenesis remain to be elucidated (12). The aim of this study is to examine the lymphangiogenic effect of ADSC on irradiated HDLEC.

Materials and Methods

Cell culture, reagents and irradiation

HDLEC (from an adult donor; PromoCell, Heidelberg, Germany) were cultured in endothelial cell growth medium MV2 (EGM MV2; PromoCell). ADSC (Lonza Japan, Tokyo, Japan) were cultured in a mixture of mesenchymal stem cell growth medium (MSCGM; Lonza Japan) and primate ES cell medium (ReproCELL, Kanagawa, Japan), supplemented with 50 μg recombinant human basic fibroblast growth factor (bFGF; DS Pharma Biomedical, Osaka, Japan) (1:1). HDLEC and ADSC were used for all experiments at passages 4 through 6. The number of cells was counted by the TC20 automated cell counter (Bio-Rad, Tokyo, Japan). In the proliferation, migration, and tube-formation assays, EGM MV2 without supplemental growth factors (epidermal growth factor (EGF), bFGF, insulin-like growth factor-1 (IGF-1), and VEGF-A) contained in EGM MV2 kit was used as the cell culture medium to determine the paracrine effects of ADSC. The anti-bFGF neutralizing antibody (clone bFM-1, 05-117; Merck Millipore, Tokyo, Japan) was used at a concentration of 2.5 μg/ml. Irradiation was performed at room temperature in a ^{137}Cs γ-ray irradiator at a dose rate of 1 Gy/min.

ADSC-conditioned medium

ADSC were seeded on 35-mm dishes and incubated in MSCGM at 37°C under 5% CO_2. When the ADSC became confluent, the medium was aspirated, washed once with protein-buffered saline (PBS) (-), and replaced with EGM MV2 without supplemental growth factors. Then, the ADSC were divided into two groups; i.e., the cells that were and were not irradiated with 6 Gy of γ-radiation. After the irradiation procedure, the cells were incubated at 37°C under 5% CO_2 for 5 days. Finally, the medium was collected and stored at 4°C.

Proliferation assay

ADSC were seeded on 22-mm square glass coverslips and incubated in MSCGM at 37°C under 5% CO_2. When the ADSC had reached confluence, HDLEC (1×10^4 cells in 500 µl EGM MV2 without supplemental growth factors) were seeded on other 22-mm square glass coverslips and incubated at 37°C under 5% CO_2 overnight. The HDLEC and ADSC were irradiated with 1, 2, or 6 Gy of γ-radiation as required. After the irradiation procedure, each of the glass coverslips seeded with ADSC and HDLEC was placed in the same 60-mm dish (none of them overlapped), before being incubated in EGM MV2 without supplemental growth factors at 37°C under 5% CO_2 for 5 days. The medium was changed once on day 3. The HDLEC on the glass coverslips were then fixed in 4%

paraformaldehyde for 10 min at room temperature. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI), and the number of DAPI-positive nuclei was counted under a fluorescence microscope at ×10 magnification. The number of DAPI-positive nuclei was counted in 5 randomly selected fields, and the mean values for each group were compared. The number of DAPI-positive nuclei was determined using the ImageJ software (13). The number of non-irradiated HDLEC seen after 5 days of a single-cell culture was defined as 100%, and the relative numbers of cells in the other groups were calculated.

Migration assay

HDLEC were seeded onto 35-mm dishes and incubated in EGM MV2 at 37°C under 5% CO_2. When the HDLEC reached confluence, the HDLEC culture monolayer was scratched in a straight line with a 200-μl pipette tip. Then, the HDLEC were or were not irradiated with 6 Gy of γ-radiation. The EGM MV2 and any cell debris were aspirated and washed once with PBS (-). The medium was replaced with EGM MV2 without supplemental growth factors, or ADSC-conditioned medium. Immediately after replacing the medium, the scratch was aligned in the horizontal direction, and images of it were taken with a microscope (magnification: ×10) in 5 randomly selected fields of view in

each dish. After 12 hours' incubation, images of the scratch were taken again in the same manner. On each image, the area of the scratch was measured using the ImageJ software. The rate of reduction in the scratch area from 0 to 12 hours was compared among the groups.

Tube-formation assay

Thirty-five-mm dishes with glass bottoms (diameter: 12 mm) (Iwaki Glass, Tokyo, Japan) were coated with 50 μl of Corning® Matrigel® growth factor reduced basement membrane matrix, phenol red-free (Corning, Corning, New York, USA), and the dishes were incubated at 37°C for 30 min. HDLEC (3×10^5 cells in 2 ml EGM MV2 without supplemental growth factors) were seeded onto the Matrigel®-coated dishes with glass bases and incubated at 37°C under 5% CO_2. After being incubated for 1 hour to allow the HDLEC to attach to the gel, the HDLEC were or were not irradiated with 6 Gy of γ-radiation. The medium was aspirated, washed once with PBS (-), and then was replaced with EGM MV2 without supplemental growth factors, or ADSC-conditioned medium. After 12 hours' incubation, tube-formation images were taken with a microscope (magnification: ×10) in 5 randomly selected fields of view in each Matrigel®-coated glass bottom. The tube length in each field of view was measured using the ImageJ software.

The mean tube lengths were compared among the groups.

Enzyme-linked immunosorbent assay

ADSC were incubated in MSCGM at 37°C under 5% CO_2 until they became confluent. Then, the ADSC were irradiated with 2, 6, or 12 Gy of γ-radiation. After being irradiated, the ADSC were washed twice with PBS (-) and replenished with serum-free MSCGM. The cell culture supernatants were collected 7 days after the irradiation procedure and centrifuged at 1,500 rpm for 10 min. Among the lymphangiogenic factors secreted by the irradiated ADSC, the concentrations of bFGF, hepatocyte growth factor (HGF), IGF-1, VEGF-A, and VEGF-C were measured using Quantikine enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, Minnesota, USA).

Western blotting

ADSC that had been irradiated with 12 Gy of γ-radiation were incubated in MSCGM at 37°C under 5% CO_2 for 7 days. The ADSC were collected and lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.2], 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]) containing protease inhibitor cocktail (Roche Diagnostics Japan, Tokyo, Japan). The cell

lysate was cleared by centrifuging it at 15,000 rpm for 10 min at 4°C. Then, the supernatant was collected and used as the total cellular protein fraction. The total protein concentration was determined using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, Illinois, USA). The proteins were electrophoresed through SDS-polyacrylamide gel and were electrophoretically transferred to a polyvinyl difluoride membrane in transfer buffer (100 mM Tris and 192 mM glycine). After 30 minutes' incubation with blocking solution (10% skimmed milk), the membrane was incubated with the primary antibodies, biotinylated anti-mouse or rabbit IgG antibodies, and streptavidin-alkaline phosphatase. To visualize the resultant bands, the membrane was incubated in a detection solution containing nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as a substrate. Each blot was scanned by a high-resolution scanner, and the relative intensity of each band was quantified using the ImageJ software. Then, the expression level of each growth factor was normalized to the expression level of β-actin, and the relative post-irradiation expression levels of each growth factor compared with those seen on day 0 were calculated. The primary antibodies used in this study were anti-human bFGF (ab92337; Abcam, Tokyo, Japan), anti-human VEGF-A (ab46154; Abcam), anti-human VEGF-C (ab83905; Abcam), and anti-human IGF-1 (ab134140; Abcam).

Statistical analysis

Statistical analyses were carried out with one-way analysis of variance, followed by the post-hoc Tukey test. *P*-values of <0.05 were defined as significant. All statistical analyses were carried out with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan) (14).

Results

Cell proliferation, migration and tube-formation

In the single-cell cultures, the proliferation of non-irradiated HDLEC was markedly suppressed by radiation in a dose-dependent manner, and the administration of 2 Gy of γ-radiation resulted in the relative number of cells decreasing by approximately 50% compared with the number of non-irradiated cells (Figure 1). The proliferation of the non-irradiated HDLEC was significantly promoted by co-culturing them with non-irradiated ADSC, and this technique also ameliorated the suppressive effects of radiation on HDLEC proliferation. It should be noted that the γ-irradiated ADSC had slightly stronger protective effects on HDLEC proliferation than the non-irradiated ADSC.

Compared with those seen in the presence of EGM MV2 without supplemental growth factors, the addition of ADSC-conditioned medium resulted in significantly smaller scratches (Figure 2) and about two-fold longer tubes (Figure 3). There were no differences between the migration or tube-formation promoting effects of the non-irradiated ADSC and the irradiated ADSC-conditioned medium, indicating that the secretory ability of the ADSC was maintained after radiation exposure.

Since the EGM MV2 kit contained various exogenous growth factors, their influence on the HDLEC proliferation, migration, and tube-formation was checked by

removing each one of the exogenous growth factors from the EGM MV2 kit. We found that the proliferation of HDLEC cultured in EGM MV2 without bFGF was significantly decreased but the effects were not obvious on migration and tube-formation [see Figure, Supplemental Digital Content 1, which shows proliferation, migration, and tube-formation assay using EGM MV2 administered with supplemental growth factors in combination. (Above: Proliferation assay of HDLEC cultured for 5 days) The number of cells seen in each field when HDLEC cultured in EGM MV2 without all supplemental growth factors were subjected to single-cell cultures for 5 days was defined as 100%. Error bars represent standard deviation. $n = 3$. ***$P < 0.005$ (Center: Migration assay of HDLEC cultured for 12 hours) The area of the scratch was measured from 5 randomly selected fields of view. The percentage reduction was compared among the groups. Error bars represent standard deviation. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$ (Below: Tube-formation assay of HDLEC cultured for 12 hours) The tube length was measured from 5 randomly selected fields of view. Error bars represent standard deviation. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$]. The impact of radiation on cell number was also confirmed. The confluent ADSC in T-75 flasks were incubated for 5 days with or without 6 Gy of γ-irradiation, and the number of ADSC was measured. Because ADSC predominantly induced senescence-like cell death (data

not shown), there was no apparent difference between 6 Gy irradiated ADSC and non-irradiated ADSC [see Figure, Supplemental Digital Content 2, which shows the cell numbers of the confluent ADSC incubated for 5 days with or without 6 Gy of γ-irradiation. The confluent ADSC in T-75 flasks were incubated for 5 days with or without 6 Gy of γ-irradiation, and the number of ADSC was measured using the automated cell counter. Error bars represent standard deviation. n.s., not significant].

Detection of lymphangiogenic factors and their effects

ELISA showed that the bFGF level increased significantly as the radiation dose increased (Figure 4). While the ADSC secreted significant amounts of VEGF-A, VEGF-C, and HGF, the levels of these substances were not affected by exposing the ADSC to radiation. The ADSC did not secrete significant amounts of IGF-1 in any of the experiments. The levels of lymphangiogenic factors were also determined by Western blotting (Figure 5). The results demonstrated that bFGF was expressed in the control ADSC, and its level was significantly augmented in a time-dependent manner by the administration of 12 Gy of γ-radiation. Both VEGF-A and VEGF-C were also detectable in the non-irradiated ADSC, and their levels were not affected by radiation. In contrast to these factors, almost no IGF-1 expression was detected in the non-irradiated ADSC,

whereas IGF-1 expression became detectable at 5 to 7 days after γ-irradiation in some of the experiments (data not shown).

Thus, the results indicated that an increase in cell proliferation, migration, and tube-formation of HDLEC co-cultured with ADSC might be mediated by bFGF. Therefore, we tested the possibility by using anti-bFGF neutralizing antibody. In the non-irradiated and 2 Gy irradiated co-culture groups, we found that the daily administration of the anti-bFGF neutralizing antibody suppressed HDLEC proliferation [see Figure, Supplemental Digital Content 3, which shows proliferation, migration, and tube-formation assay using the anti-bFGF neutralizing antibody (clone bFM-1, 05-117). (Above: Proliferation assay involving the co-culturing of irradiated HDLEC and ADSC for 5 days) The number of cells seen in each field when non-irradiated HDLEC cultured in EGM MV2 without supplemental growth factors were subjected to single-cell cultures for 5 days was defined as 100%. The anti-bFGF neutralizing antibody was administered daily at a concentration of 2.5 μg/ml and the medium was changed once on day 3. Error bars represent standard deviation. $n = 3$. ***$P < 0.005$], although there was no difference in the migration and tube-formation ability when it was administered into ADSC-conditioned medium 1 hour before the assays [see Figure, Supplemental Digital Content 3, which shows proliferation, migration, and tube-formation assay using

the anti-bFGF neutralizing antibody(clone bFM-1, 05-117). (Center: Migration assay of HDLEC cultured in ADSC-conditioned medium (ADSC-CM) for 12 hours) The area of the scratch was measured from 5 randomly selected fields of view. The percentage reduction was compared among the groups. The anti-bFGF neutralizing antibody was added to ADSC-CM at a concentration of 2.5 μg/ml and incubated at 37 °C for 1 hour before the assays. Error bars represent standard deviation. *P <0.05; ***P <0.005 (Below: Tube-formation assay of HDLEC cultured in ADSC-CM for 12 hours) The tube length was measured from 5 randomly selected fields of view. The anti-bFGF neutralizing antibody was added to ADSC-CM at a concentration of 2.5 μg/ml and incubated at 37 °C for 1 hour before the assays. Error bars represent standard deviation. ***P <0.005].

Discussion

Among mesenchymal stem cells, ADSC are the easiest to collect, and so they have attracted much attention in plastic, reconstructive and esthetic surgery. ADSC promote wound healing by reducing the levels of factors that impair wound healing, e.g., by reducing the amount of cytokines released by local inflammatory cells or in response to angiogenic disorders (15). Although there have not been any reports about the effects of ADSC on HDLEC that have been damaged by ionizing radiation, the current study demonstrated that co-culturing HDLEC with ADSC or culturing HDLEC in ADSC-conditioned medium restored the lymphangiogenic ability of irradiated HDLEC. Previously, Haubner et al. found that the impaired proliferation exhibited by the HDMEC after they had been exposed to radiation was significantly ameliorated by co-culturing them with ADSC and the expression of bFGF increased 48 hours after irradiation in the supernatants of ADSC cultured in HDMEC medium (16). Thus, it can be concluded that ADSC co-culturing has protective effects on both lymphatic and microvascular endothelial cells.

The ameliorative effects of ADSC were observed not only when ADSC were co-cultured with HDLEC, but also when ADSC-conditioned medium was used, suggesting that these effects were induced via paracrine mechanisms. ELISA showed that the ADSC

released lymphangiogenic factors, including bFGF, HGF, VEGF-A, and VEGF-C. Among them, bFGF expression was markedly enhanced by radiation exposure. Interestingly, radiation dose-dependent increases in bFGF expression were detected in both the ELISA- and Western blotting-based experiments, indicating that the augmented expression of bFGF could represent an *in vivo* cellular response to ionizing radiation. Although IGF-1 has been reported to be one of the growth factors secreted by ADSC (17), no significant IGF-1 expression was detected in MSCGM in our experimental conditions.

Among the various lymphangiogenic factors, VEGF-C is the most important regulator of lymphangiogenesis, and VEGF-C/VEGF receptor 3 signaling is essential for proliferation, migration, and sprouting in LEC, as it induces downstream signaling involving mitogen-activated protein kinase extracellular signal-regulated kinase (ERK) 1/2 and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt (18-21). The interactions between other lymphangiogenic factors are still incompletely understood; however, it has been reported that bFGF, HGF, and IGF-1 promote lymphangiogenesis indirectly via the activation of VEGF-C and VEGF-D (22). Previous reports have suggested that bFGF cooperates with VEGF-C to promote lymphangiogenesis, not only by inducing upregulated expression of VEGF-C and VEGF-D, but also by directly acting on LEC by activating the FGF receptor 1 (FGFR-1) signaling pathway to promote the

proliferation and migration of LEC (23). We confirmed that the anti-bFGF neutralizing antibody and EGM MV2 without bFGF abolished HDLEC proliferation effects, indicating that bFGF plays a primary role in HDLEC proliferation. It is considered that the increase in bFGF expression induced by irradiating the ADSC helped to increase the viability of the HDLEC after radiation exposure and that these effects were induced via the FGFR signaling pathway and the secondary induction of VEGF-C expression.

The secretory responses of ADSC have been reported previously. For example, it was suggested that cell stress, such as hypoxia, might stimulate mesenchymal stem cells to secrete growth factors and proliferate at a faster rate (24). Kakudo et al. reported that hypoxia enhanced the proliferation of ADSC by activating hypoxia-inducible factor (HIF)-1α, and the mRNA and protein expression levels of VEGF and FGF-2 were significantly enhanced in hypoxic conditions (25). Mesenchymal stem cells are resistant to irradiation and retain stem cell characteristics, such as stem cell surface marker expression and multilineage potential, after being irradiated (26). However, few previous studies have examined the effects of radiation on the secretory responses of ADSC. Further studies are needed to elucidate the molecular mechanisms by which radiation exposure induces bFGF expression in ADSC.

There are a couple of points should be considered by interpreting the results. The

first one is the influence of exogenous growth factors included in the EGM MV2, so that we checked the proliferation, migration, and tube-formation of HDLEC by removing the factors one by one. It was found that withdrawal of bFGF significantly affected the proliferation of HDLEC, indicating that increased secretion of bFGF after radiation exposure promoted HDLEC proliferation. Its conclusion was also confirmed by the experiments using the anti-bFGF neutralizing antibody. The second one is the influence of growth factor consumption dependent on cell growth condition. It is well documented that radiation exposure induces cell death, so that the number of cells was thought to be different. However, the mode of cell death induced after γ-irradiation is primarily non-apoptotic cell death. Therefore, the confluent ADSC showed no difference in cell number with or without irradiation, and it is considered that the influence of growth factor consumption was not so significant. The third one will be the disadvantage of the usage of commercially purchased ADSC and HDLEC. The commercial cell products are easily available, but the cell origin and isolation method are unclear. Thus, it is necessary to confirm the characterization of the cells, such as differentiation assay of ADSC and immunostaining for lymphatic endothelial markers.

Our study showed that ADSC promoted proliferation, migration, and tube-formation in radiation-damaged HDLEC via paracrine effects. This is the first *in vitro*

study to suggest that a treatment strategy involving ADSC might be effective against secondary lymphedema of the limbs caused by irradiation. Moreover, since irradiation did not attenuate the paracrine effects of ADSC and increased in bFGF expression, it is possible that the prophylactic local administration of ADSC at sites that might be affected by radiation could be an effective strategy against conditions such as radiation-induced skin ulcers.

Conclusion

ADSC promoted cell proliferation, migration, and tube-formation in irradiated HDLEC via paracrine effects. Augmented expression of bFGF might have been responsible for these effects.

Figure legends

Figure 1. Proliferation assay involving the co-culturing of irradiated HDLEC and ADSC for 5 days

The number of cells seen in each field when non-irradiated HDLEC cultured in EGM MV2 without supplemental growth factors were subjected to single-cell cultures for 5 days was defined as 100%. The co-cultured ADSC were or were not irradiated (at the same radiation dose as the irradiated HDLEC). Experiments were performed in triplicates. Error bars represent standard error. $n = 3$. *$P < 0.05$; ***$P < 0.005$

Figure 2. Migration assay in ADSC-conditioned medium (ADSC-CM)

(A) The micrographs show the closure of the scratch after HDLEC had been cultured in EGM MV2 without supplemental growth factors, or ADSC-CM for 12 hours.

(B) HDLEC were irradiated with 6 Gy of γ-radiation, and the ADSC used to prepare the conditioned media were or were not irradiated with 6 Gy of γ-radiation.

(C) The area of the scratch was measured from 5 randomly selected fields of view. The percentage reduction was compared among the groups. Experiments were performed in triplicates. Error bars represent standard error. *$P < 0.05$

Figure 3. Tube-formation assay in ADSC-conditioned medium (ADSC-CM)

(A) The micrographs show the tube-formation seen after HDLEC had been cultured in EGM MV2 without supplemental growth factors, or ADSC-CM for 12 hours.

(B) HDLEC were irradiated with 6 Gy of γ-radiation, and the ADSC used to prepare the conditioned media were or were not irradiated with 6 Gy of γ-radiation.

(C) The tube length was measured from 5 randomly selected fields of view. Experiments were performed in triplicates. Error bars represent standard error. $^{**}P < 0.01$

Figure 4. ELISA of lymphangiogenic factor expression in ADSC to examine paracrine effects

The levels of lymphangiogenic factors in the cell culture supernatants of ADSC at 7 days after the ADSC had been irradiated with 2-12 Gy of γ-radiation were analyzed. The levels of (A) bFGF, (B) VEGF-A, (C) VEGF-C, (D) HGF, and (E) IGF-1 are shown. Experiments were performed in triplicates. Error bars represent standard error. $n = 3$. $^{*}P < 0.05$; $^{***}P < 0.005$

Figure 5. Western blotting analysis of bFGF, VEGF-A, and VEGF-C expression

(A) ADSC cell lysates were obtained at indicated days after the ADSC had been irradiated with 12 Gy of γ-radiation. The associated blots were probed with the indicated antibodies.

(B) The graphs show the results of quantitative analyses of the relative expression levels of each growth factor. Band density was quantified using the ImageJ software, and the expression levels of each growth factor were normalized to the expression level of β-actin. Then, the relative expression level of each growth factor compared with that seen on day 0 was calculated at each post-irradiation time point. Error bars represent standard deviation. $n = 3$. *$P < 0.05$; **$P < 0.01$

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26. Nicolay, N. H., Sommer, E., Lopez, R., et al. Mesenchymal stem cells retain their defining stem cell characteristics after exposure to ionizing radiation. *Int J Radiat Oncol Biol Phys* 2013;87:1171-1178.

Figure 1.

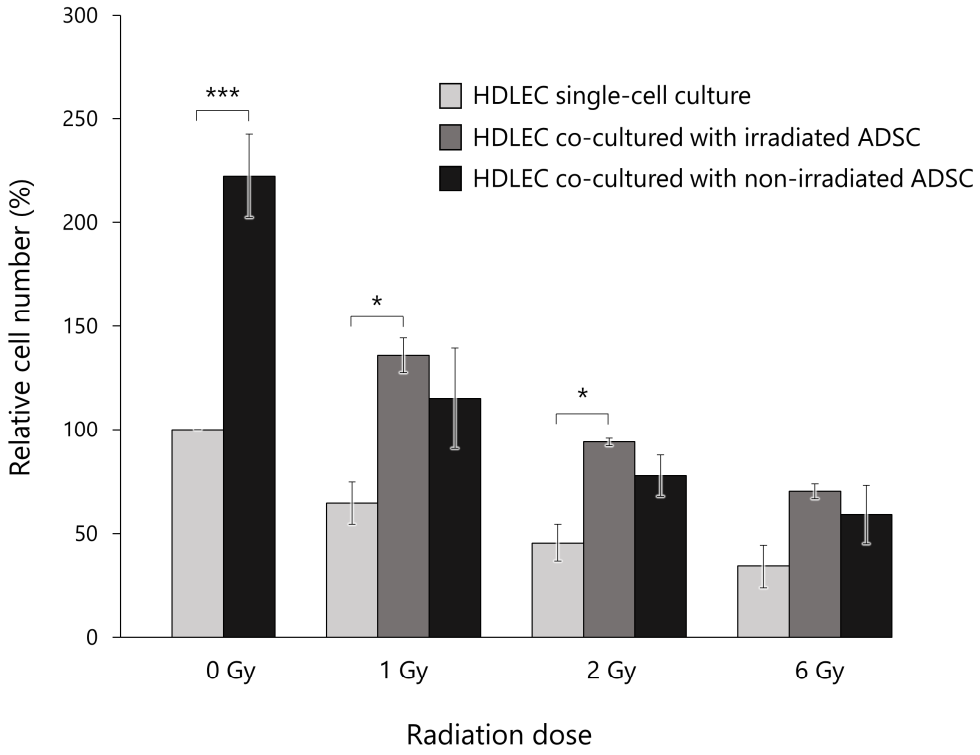
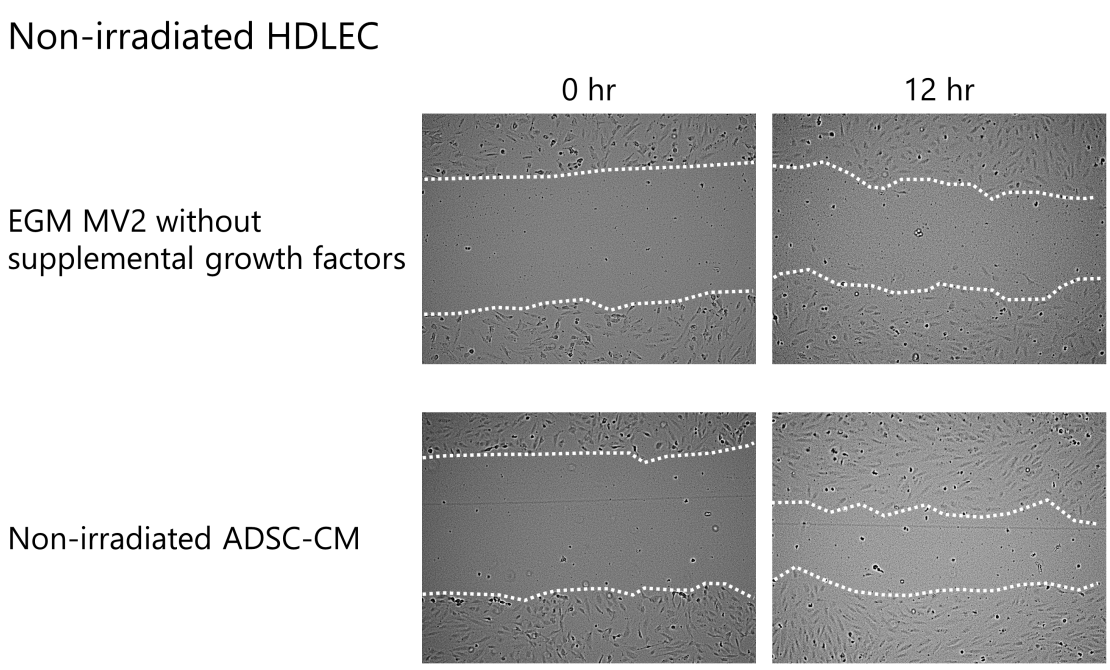
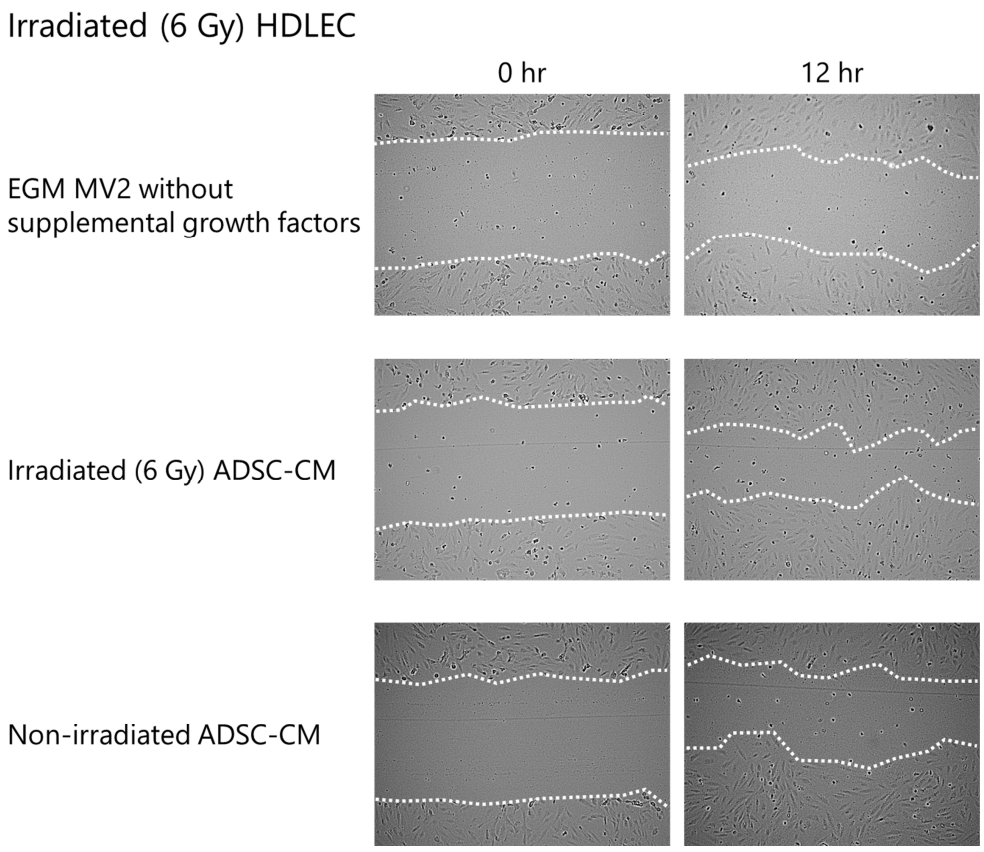

300
250
200
150
100
50
0
Relative cell number (%)
***
*
*
HDLEC single-cell culture
HDLEC co-cultured with irradiated ADSC
HDLEC co-cultured with non-irradiated ADSC
0 Gy
1 Gy
2 Gy
6 Gy
Radiation dose

Figure 2.

(A)


Non-irradiated HDLEC
0 hr
12 hr
EGM MV2 without supplemental growth factors
Non-irradiated ADSC-CM

(B)


Irradiated (6 Gy) HDLEC
0 hr
12 hr
EGM MV2 without supplemental growth factors
Irradiated (6 Gy) ADSC-CM
Non-irradiated ADSC-CM

(C)

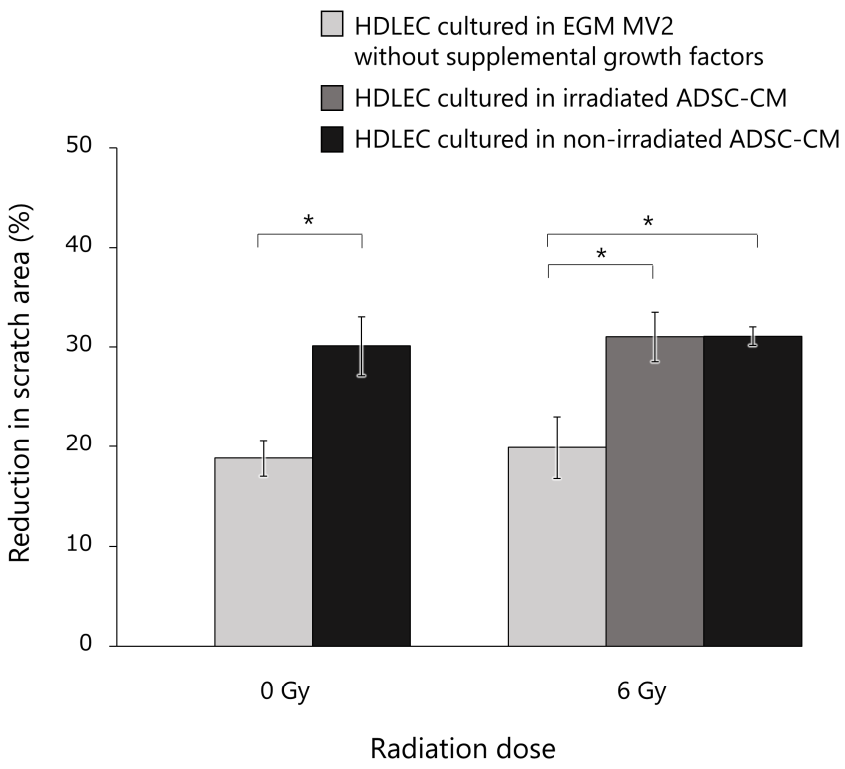
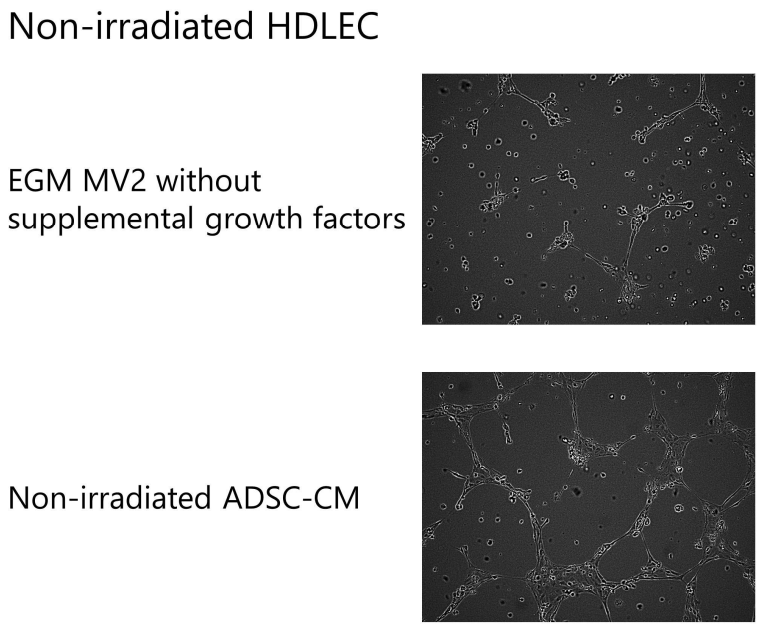
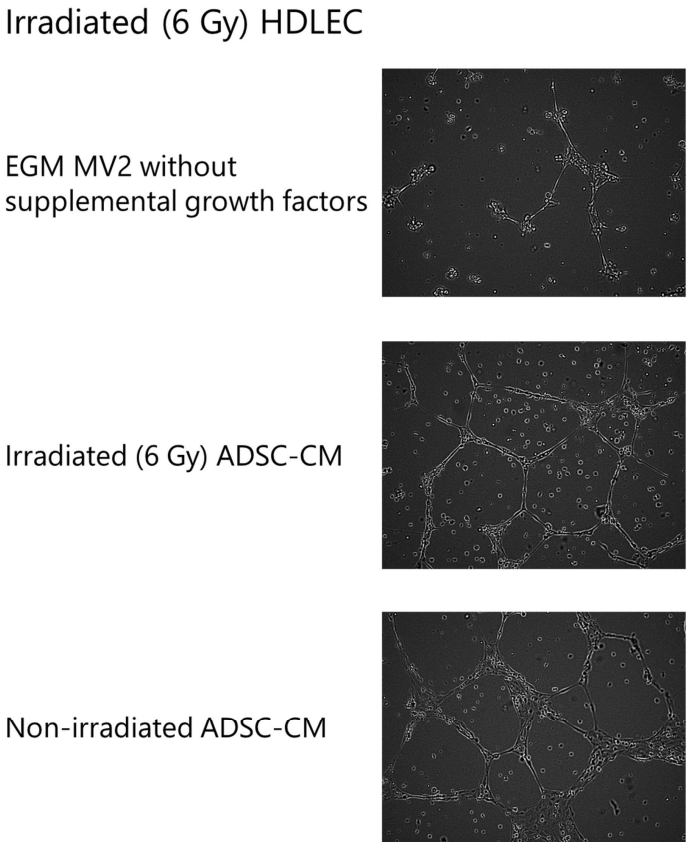

HDLEC cultured in EGM MV2
without supplemental growth factors
HDLEC cultured in irradiated ADSC-CM
HDLEC cultured in non-irradiated ADSC-CM
Reduction in scratch area (%)
50
40
30
20
10
0
*
*
*
0 Gy
6 Gy
Radiation dose

Figure 3.

(A)


Non-irradiated HDLEC
EGM MV2 without
supplemental growth factors
Non-irradiated ADSC-CM

(B)


Irradiated (6 Gy) HDLEC
EGM MV2 without supplemental growth factors
Irradiated (6 Gy) ADSC-CM
Non-irradiated ADSC-CM

(C)

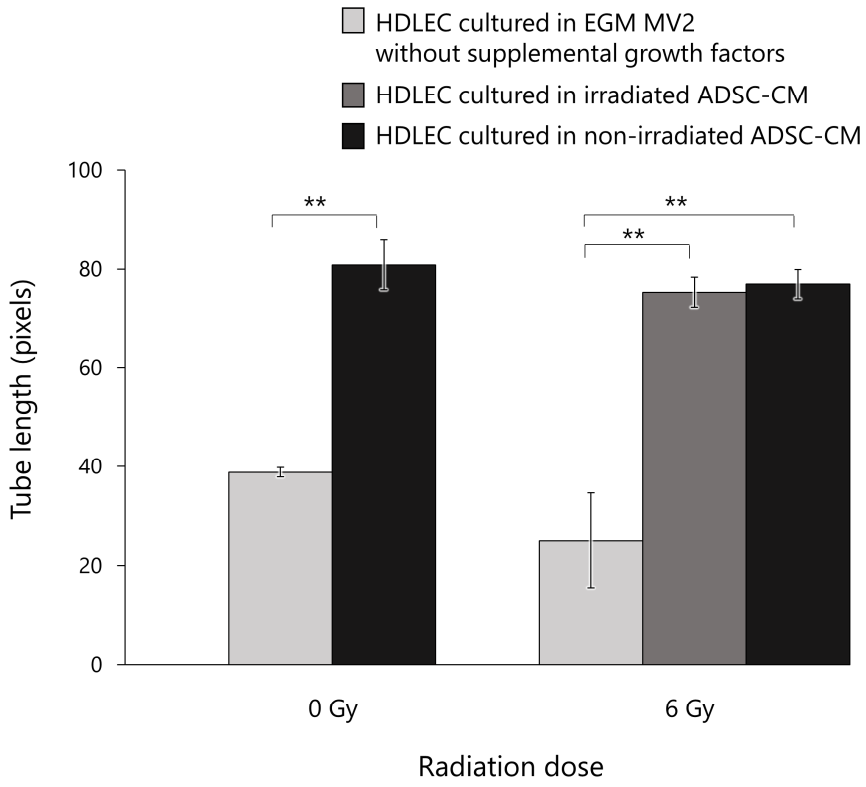
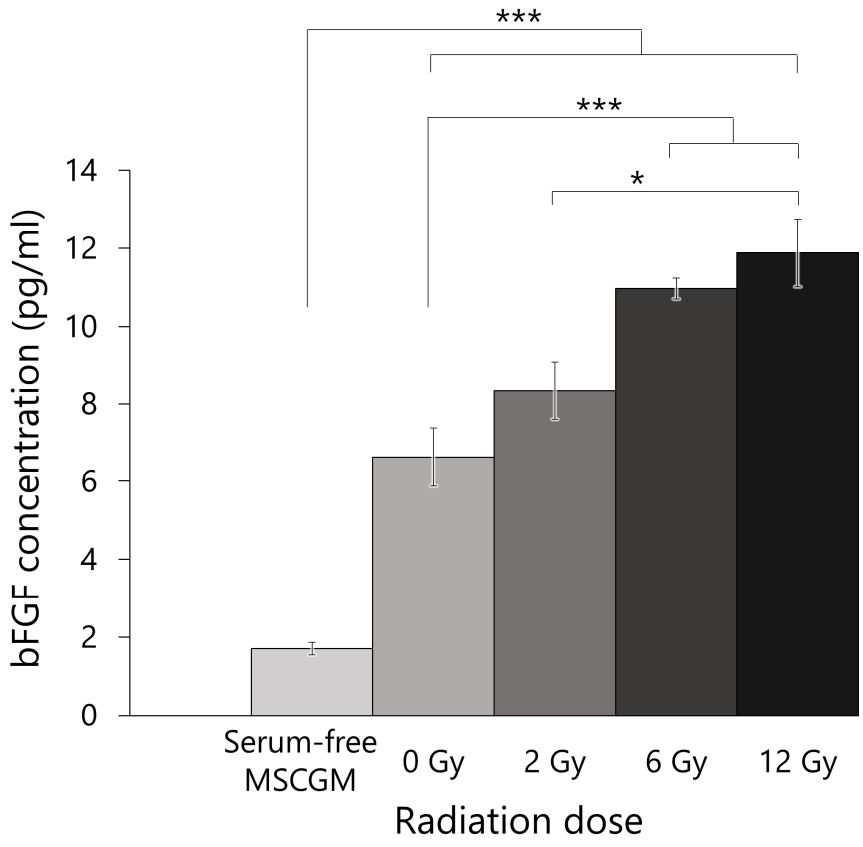
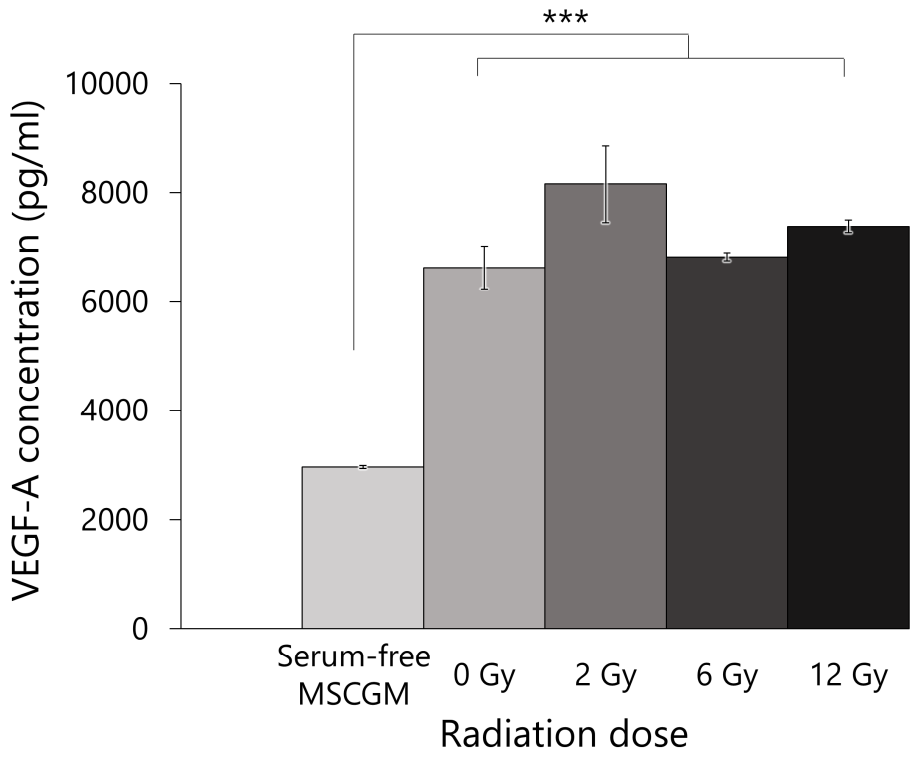

HDLEC cultured in EGM MV2
without supplemental growth factors
HDLEC cultured in irradiated ADSC-CM
HDLEC cultured in non-irradiated ADSC-CM
Tube length (pixels)
100
80
60
40
20
0
**
**
**
0 Gy
6 Gy
Radiation dose

Figure 4

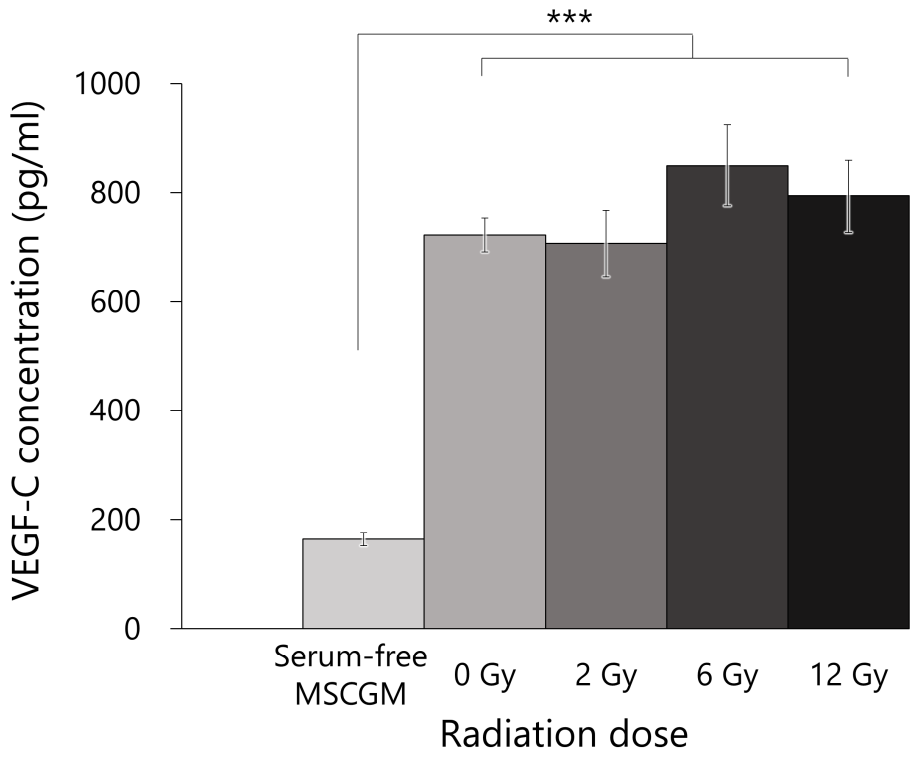
(A)


***
***
*
14
12
10
8
6
4
2
0
bFGF concentration (pg/ml)
Serum-free MSCGM
0 Gy
2 Gy
6 Gy
12 Gy
Radiation dose

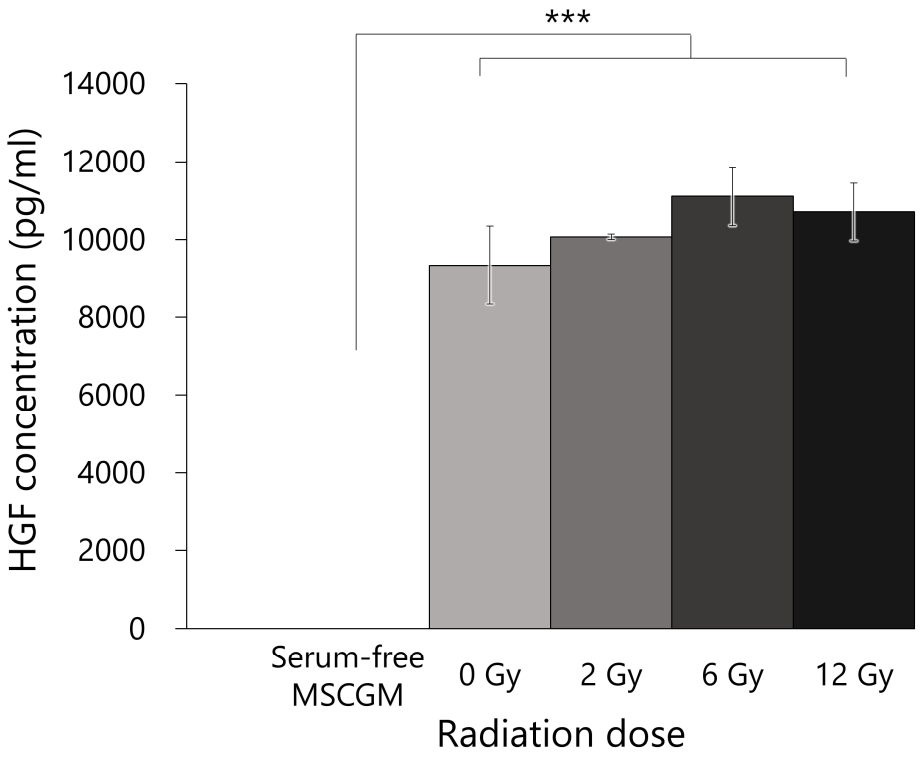
(B)


***
10000
8000
6000
4000
2000
0
VEGF-A concentration (pg/ml)
Serum-free MSCGM
0 Gy
2 Gy
6 Gy
12 Gy
Radiation dose

(C)


***
1000
800
600
400
200
0
VEGF-C concentration (pg/ml)
Serum-free MSCGM
0 Gy
2 Gy
6 Gy
12 Gy
Radiation dose

(D)


***
14000
12000
10000
8000
6000
4000
2000
0
HGF concentration (pg/ml)
Serum-free MSCGM
0 Gy
2 Gy
6 Gy
12 Gy
Radiation dose

(E)

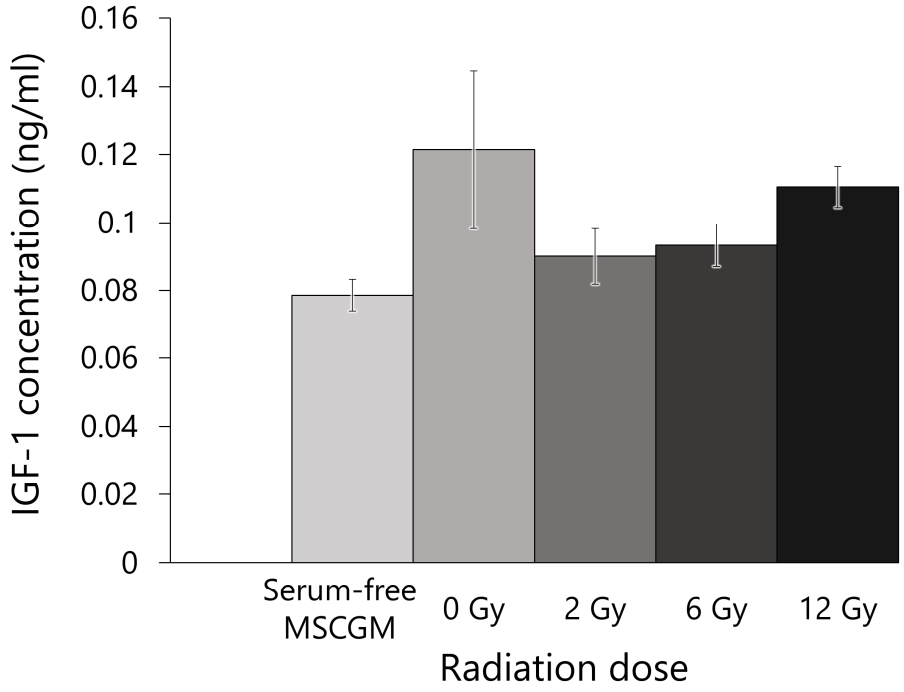
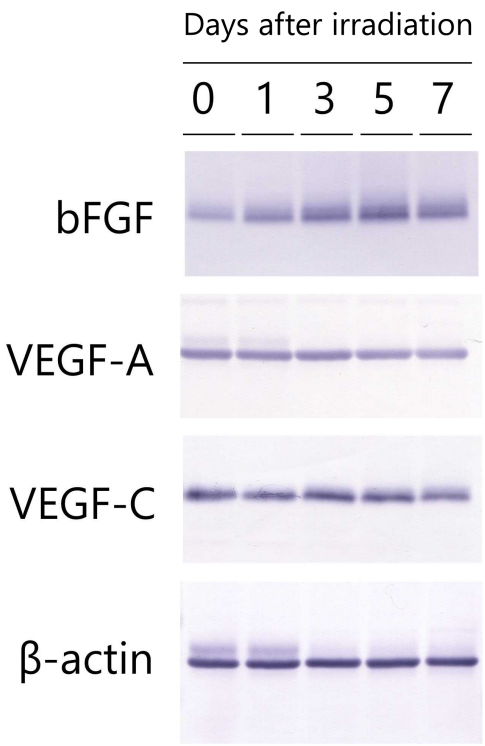
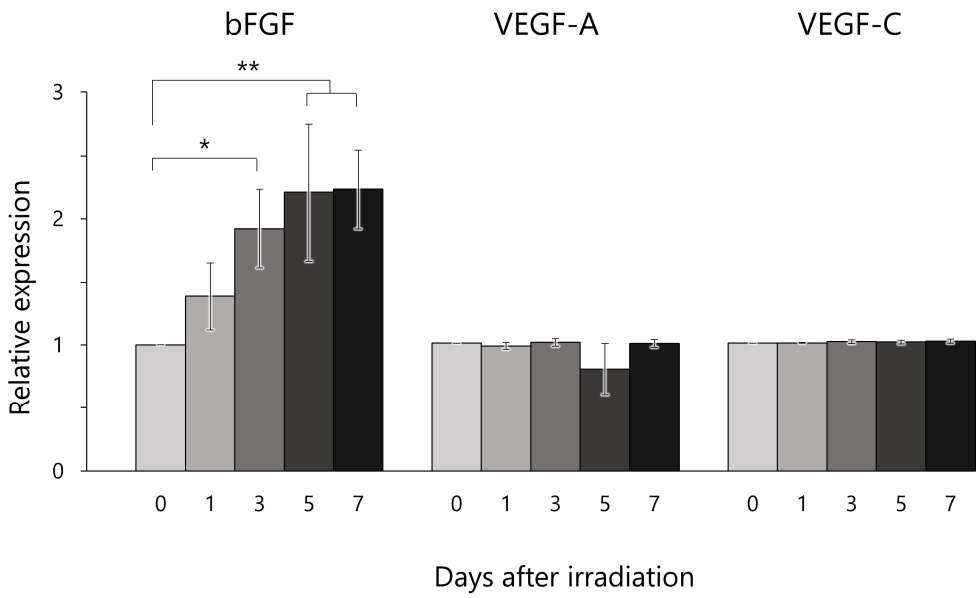

IGF-1 concentration (ng/ml)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Serum-free MSCGM
0 Gy
2 Gy
6 Gy
12 Gy
Radiation dose

Figure 5

(A)

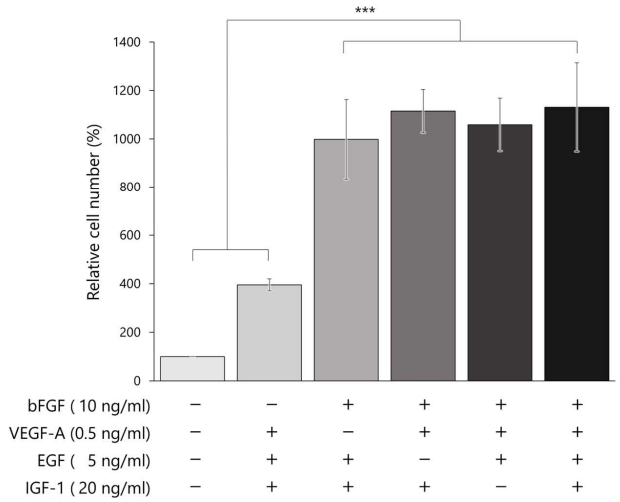


(B)

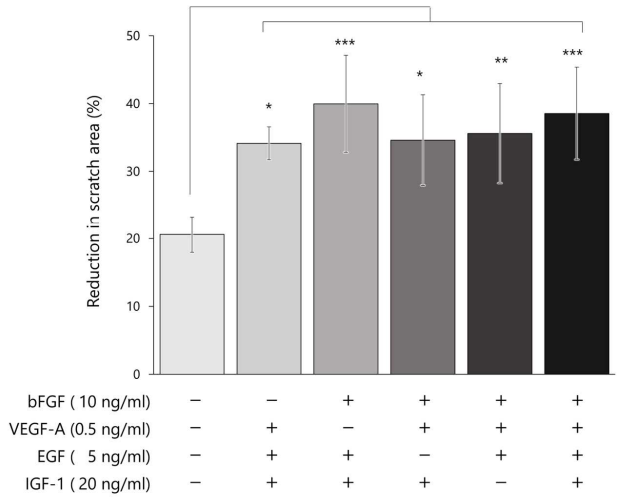

bFGF
VEGF-A
VEGF-C
**
*
Relative expression
3
2
1
0
0 1 3 5 7
0 1 3 5 7
0 1 3 5 7
Days after irradiation

Figure, Supplemental Digital Content 1

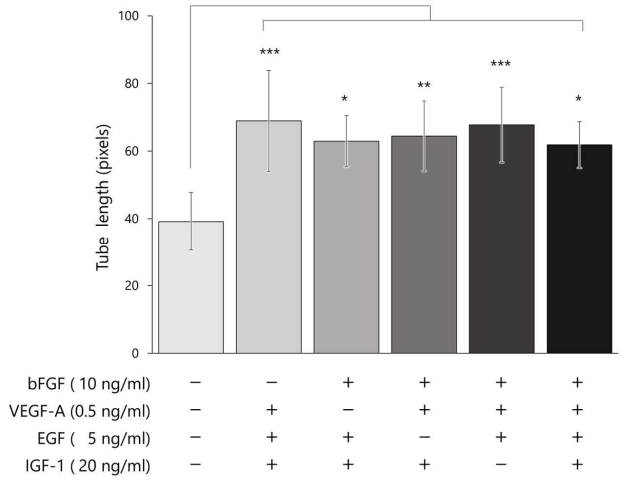
A: Proliferation assay



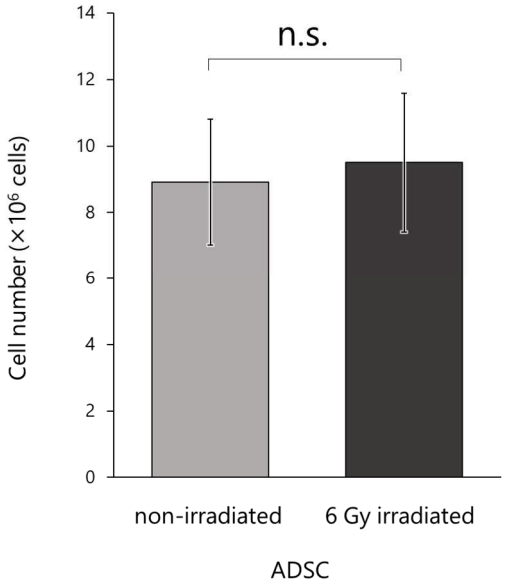
B: Migration assay



C: Tube-formation assay

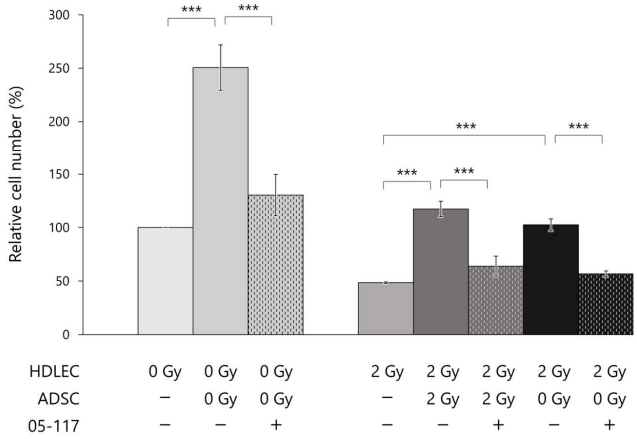


Figure, Supplemental Digital Content 2

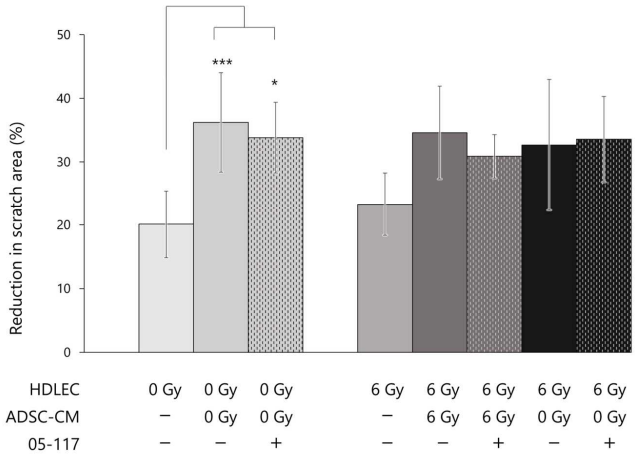


Figure, Supplemental Digital Content 3

A: Proliferation assay



B: Migration assay



C: Tube-formation assay