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# Irradiation-induced localization of IL-12-expressing mesenchymal stem cells to enhance the curative effect in murine metastatic hepatoma

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**Irradiation in conjunction with gene therapy is considered for efficient cancer treatment. Mesenchymal stem cells (MSCs), due to their irradiation-promotable tumor tropism, are ideal delivery vehicles for gene therapy. In this study, we investigated whether treatment with radiation and interleukin (IL)-12-expressing MSCs (MSCs/IL-12) exerts improved antitumor effects on murine metastatic hepatoma. HCa-I and Hepa 1-6 cells were utilized to generate heterotopic murine hepatoma models. Tumor-bearing mice were treated with irradiation or MSCs/IL-12 alone, or a combination. Monocyte chemoattractant protein-1 (MCP-1/CCL2) expression was assessed in irradiated hepatoma tissues to confirm a chemotactic effect. Combination treatment strategies were established and their therapeutic efficacies were evaluated by monitoring tumor growth, metastasis and survival rate. IL-12 expression was assessed and the apoptotic activity and immunological alterations in the tumor microenvironment were examined. MCP-1/CCL2 expression and localization of MSCs/IL-12 increased in the irradiated murine hepatoma cells. The antitumor effects, including suppression of pulmonary metastasis and survival rate improvements, were increased by the combination treatment with irradiation and MSCs/IL-12. IL-12 expression was increased in tumor cells, causing proliferation of cluster of differentiation $8^+$ T-lymphocytes and natural killer cells. The apoptotic activity increased, indicating that the cytotoxicity of immune cells was involved in the antitumor effect of the combined treatment. Treatment with irradiation and MSCs/IL-12 showed effectiveness in treating murine metastatic hepatoma. IL-12-induced proliferation of immune cells played an important role in apoptosis of tumor cells. Our results suggest that treatment with irradiation and MSCs/IL-12 may be a useful strategy for enhancing antitumor activity in metastatic hepatoma.**

**Key words:** HCC, metastasis, mesenchymal stem cell, IL-12, irradiation, gene therapy

**Abbreviations:** CCR2: C-C chemokine receptor type 2; CD: cluster of differentiation; FIR: fractionated irradiation; HCC: hepatocellular carcinoma; IFN-γ: interferon-γ; IL-12: interleukin 12; MCP-1/CCL2: monocyte chemoattractant protein-1; MSCs: mesenchymal stem cells; MSCs/IL-12: IL-12-expressing MSCs; NK cell: natural killer cell; rAD/IL-12: IL-12 encoded recombinant adenovirus; SIR: single irradiation; TAG-72: tumor-associated glycoprotein-72

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Hepatocellular carcinoma (HCC) is a major health problem worldwide, with increasing incidence rates; HCC causes almost one million deaths annually and generally develops on a background of chronic inflammatory liver disease.[1] Although HCC is the fifth most common cancer in men and seventh most common in women worldwide, its management is difficult because HCC typically arises and progresses early on without associated symptoms or signs.[2] Therefore, irradiation to treat patients with late-stage hepatoma has been increasingly selected as the therapeutic option in HCC management.[3]

Radiation therapy uses high-energy radiation to destroy cancer cells. It is employed as a component of adjuvant therapy after surgery or in treatment of a primary malignant tumor.[4] However, irradiation is used carefully in HCC treatment because liver tissues are highly susceptible to radiation damage[5] and some metastatic HCCs that originate elsewhere can recur due to radioresistance.[6] Combination treatments utilizing gene therapy with irradiation have been applied to overcome these shortcomings.[7] Recently, irradiation has also been used as a tool for enhancing the therapeutic effect by modulation of the tumor microenvironment.[8]

**What's new?**

**Mesenchymal stem cells (MSCs) are promising gene-delivery vehicles, with the potential to improve antitumor effects when used in combination with existing therapies. In the present study, the combined use of interleukin (IL)-12-expressing MSCs (MSCs/IL-12) and radiation therapy increased antitumor activity in murine metastatic hepatoma, a model that is representative of human metastatic hepatocellular carcinoma (HCC), which affects nearly half of HCC patients. Treatment with MSCs/IL-12 resulted in increased IL-12 expression in tumor cells and immune cell proliferation. Immune cell cytotoxicity, evidenced by increased apoptotic activity, appeared to play a role in MSCs/IL-12 augmentation of antitumor effects.**

Bone marrow-derived mesenchymal stem cells (MSCs) are adult pluripotent progenitor cells with the potential to differentiate into osteoblasts, chondrocytes or adipocytes.[9] MSCs are emerging as cellular vehicles for anticancer gene delivery. Representative antitumor molecules such as tumor necrosis factor (TNF)-related apoptosis-inducing ligand or interleukin (IL)-12 can easily transduce into the MSCs.[10–12] Expression of transgenes from engineered MSCs can be maintained for up to 6 months because of their hypoimmunogenic properties. Moreover, MSCs themselves have an ability to migrate which can be enhanced by irradiation into the neoplasm. The therapeutic efficacy of engineered MSCs is currently being assessed in various cancer models.[11,12]

Gene-modified MSCs have been applied to HCC treatment as a therapy directed toward the tumor stroma and angiogenesis.[13] However, the investigation of gene-modified MSCs in HCC treatment is a new area of research, compared to its study and application in other cancers.[11,12] There are also no reports addressing the therapeutic effects of genetically modified MSCs in extrahepatic metastasis, despite the occurrence of metastasis in almost half of HCC patients.[14] Therefore, further studies are needed for establishing optimal strategies that utilize gene-modified MSCs for treating metastatic HCC.

The IL-12 signaling pathways strongly highlight the potential of this cytokine and its related molecules as rational candidates for antitumor therapies. IL-12 expression at a tumor site could attract activated lymphocytes. In addition, IL-12 is critical for the development of the peritumoral stroma required for accepting tumor-migrating T cells. Thus, the level and duration of IL-12 at the tumor site may have a major role in triggering tumor-specific immune responses.[15]

In this study, we generated gene-modified MSCs which expressed HCC-targeted IL-12 selectively. By applying the IL-12-expressing gene-modified MSCs (MSCs/IL-12) and irradiation to murine metastatic hepatoma, we investigated whether irradiation improved the therapeutic efficacy of MSCs/IL-12. Furthermore, we investigated whether this combination therapy was efficacious as an antitumor therapy by monitoring the metastases, tumor formation and survival rate in an extrahepatic metastatic model.

## Material and Methods

### Mice and tumor lines

Animal facilities were approved by the Association of Assessment and Accreditation of Laboratory Animal Care (AAALAC), and all experiments were executed under the institutional guidelines established by the Institutional Animal Care and Use Committee at Yonsei University (IACUC-2012-0177). All mice were raised with liberal access to food and water under specific pathogen-free conditions in a room maintained on a 12-hr light/dark cycle. All *in vivo* extractions were performed under deep anesthetization or after euthanasia. Six- to seven-week-old male C3H/HeN and C57BL6 mice were purchased from Orientbio (Seong-Nam, South Korea). HCa-I[16] and Hepa 1–6,[17] murine hepatoma cell lines, were used for tumor growth. And, mice were divided into six groups for the experiment considering statistical significance: Tumor control, Single Irradiation (SIR), Fractionated Irradiation (FIR), MSCs/IL-12, SIR + MSCs/IL-12, FIR + MSCs/IL-12.

### Irradiation

The tumor bearing mouse was placed in an acrylic chamber in order to immobilize the right leg without anesthesia. Lead shields were used to avoid unwanted irradiation to other body parts. Tumor irradiation was performed by an X-ray irradiator (X-RAD 320, Precision X-Ray) using 2.0 mm Al filtration (300 kVp) at a dose of 10 Gy single or 2 Gy $\times$ 5 (total 10 Gy) fractionation.

### Preparation of gene-modified MSCs

MSCs/IL-12 were made with reference to the method that has been established.[12]

### Noninvasive in vivo bio-imaging system

MSCs/IL-12 were labeled with Dye eFluor® 670 Cell Labeling (5 $\mu$M/1 $\times$ $10^6$ cells, e-Bioscience, San Diego, CA), and *in vivo* image was acquired using Xenogen *In Vivo* Imaging System (IVIS® 100 series, Caliper Life Science, Hopkinton, MA).

### Pulmonary metastasis

*Bouin*'s solution was prepared using 75 ml saturated aqueous solution of picric acid and 25 ml of 40% aqueous formalin (Sigma, St. Louis, MO). Lungs were fixed by submersion in *Bouin*'s solution for 6 hr and fixed lungs were transferred to 70% alcohol.

### Sample preparation

Cultured medium of hepatoma cells were concentrated using VIVASPIN® (Sartorius Stedim Biotech, Goettingen, Germany). Tumor tissues were chopped to 4 $mm^2$ and

biopsies were homogenized in tissue protein lysis buffer (Pierce, Rockford, IL). Homogenates were centrifuged (13,000 RPM, 5 min), and protein supernatant was collected. Splenocytes were isolated and cultured on CD3/CD28 coated plate (BD bioscience, Franklin Lakes, NJ) for 2 days. Then, the medium were concentrated. Allowed the blood to clot by leaving it undisturbed at room temperature for 15 min. Removed the clot by centrifuging at forces in excess of 7,500*g* for 10 min, and then the serum was collected. Each protein extract was used for analyses in enzyme-linked immunosorbent assay (ELISA) and Western blot. Total RNA was extracted from hepatoma cells using a RNeasy® mini kit (Qiagen®, Hilden, Germany). cDNA was synthesized by reverse transcription system (Promega, Fitchburg, WI) for reverse transcription-PCR (RT-PCR). Tumor tissues were embedded in paraffin block or frozen Tissue-Tek® OCT™ compounds (Sakura Finetek, Alphen aan den Rijn, The Netherlands), and 5 $\mu$m sections were attached to slides for immunohistochemistry, immunofluorescence and TUNEL assay. Single cells for FACS analysis were isolated from tumors as previously described.[18]

### ELISA

Total protein concentration in each sample was determined using a BCA assay (Pierce, Rockford, IL), and quantitative analysis of monocyte chemotactic protein-1 (MCP-1/CCL2), interleukin (IL) 12, interferon (IFN)-$\gamma$ and alpha fetoprotein (AFP) expression was carried out using MCP-1/CCL2, IL-12p70, IFN-$\gamma$ and AFP ELISA Set (BD Pharmingen, Franklin Lakes, NJ) according to the manufacturer's protocols.

### Western blot

Protein lysates of tumor tissue were separated by electrophoresis in 12–15% polyacrylamide gels and electrotransferred to nitrocellulose membranes (GE Life sciences, Pittsburgh, PA). Membranes were incubated overnight at 4°C with the monocyte chemoattractant protein-1 (MCP-1/CCL2), C-C chemokine receptor type 2 (CCR2) and IL-12 antibody (1:500, 95 kDa, Bioss, Woburn, MA). Then, membranes were incubated for 1 hr at room temperature in 1:1,000 diluted goat anti-rabbit IgG, HRP conjugated (Bioss). Blots were developed using chemiluminescent peroxidase substrate (Sigma-Aldrich, St. Louis, MO) and exposed using X-ray film and developer (Agfa, Mortsel, Belgium). Densitometry measurements were made using Image-J software (freeware; rsbweb. NIH. Gov/ij).

### RT-PCR

The 1 $\mu$g/$\mu$l cDNA was used for PCR. Primer pairs for amplification of endogenous MCP-1/CCL2 and GAPDH were as follows: MCP-1/CCL2: 5′ AGGTCCCTGTCATG CTTCTG 3′ and 5′ TCTGGACCCATTCCTTCTTG 3′; GAPDH: 5′ CGACTTCAACAGCAACTCCCACTCTTCC 3′ and 5′ TGGGTGGTCCAGGGTTTCTTACTCCTT 3′.

### Immunofluorescence

About 5 $\mu$m cryo-sectioned tumor tissues were incubated overnight with the primary antibodies: polyclonal rabbit anti-MCP-1/CCL2, Cluster Differentiation (CD) 90, CD73 and IL-12R (1:500, Bioss). Sections were incubated with anti-rabbit secondary biotinylated antibody (1:1,000, Vector, Burlingame, CA) and visualized with streptavidin conjugated to Fluorescein or Texas red (Vector, Burlingame, CA).

### Immunohistochemistry

About 5 $\mu$m sections of tumor tissues embedded in paraffin were used for immunostaining with the ABC technique (Vector Elite Kit, Vector, Burlingame, CA). Sections were incubated over two nights at 4°C with monoclonal mouse anti-CD38 and CD57 (1:500, Chemicon, CA) and rinsed with 0.05M phosphate buffer saline (PBS) and 3% normal goat serum. Then, sections were incubated with the secondary biotinylated antibody (1:200, Vector, Burlingame, CA) and reacted with 0.5 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (DAB, Vector, Burlingame, CA). Reactions showed under the light microscope, and the number of stained cells was quantified by stereological analysis using Image-J software (freeware; rsbweb. NIH. Gov/ij).

### Cell migration assay

About 8 $\mu$m pore FluoBlok™-inserts (Corning Life Science, Tewksbury, MA) and 24-well plates were assembled as upper and lower chambers. About $5 \times 10^5$ IL-12 expressing mesenchymal stem cells (MSCs/IL-12) were placed in the upper chamber in the presence of 1 and 6 ng/ml recombinant MCP-1/CCL2 (Prospec, Ness Ziona, Israel) for 48 hr. At the end of incubation times, cells in the upper chamber were removed with cotton swabs and cells that traversed the membrane to the lower surface of the insert were fixed with 4% paraformaldehyde. Fixed cells were stained with Hoechst33342 (Thermo scientific, Pittsburgh, PA) and evaluated spectrophotometrically using a microplate reader (Synergy H4 hybrid reader, BioTek, Winooski, VT).

### Immunocytochemistry

MSCs/IL-12 were cultured for 24 hr on bio-coated round cover slip (BD bioscience, Bedford, MA). MSCs/IL-12 were fixed on cover slip using 4% paraformaldehyde. MSCs/IL-12-fixed cover slips were incubated for 15 hr with the primary antibodies: polyclonal rabbit anti-MCP-1/CCL2 and -CCR2 (1:1000, Abcam, Cambridge, MA). Then, cover slips were incubated with anti-rabbit secondary biotinylated antibody and visualized with streptavidin conjugated to Fluorescein or Texas red (Vector, Burlingame, CA).

### $CD8^+$ T-lymphocyte proliferation

Single cells were isolated from tumors as previously described and washed in phosphate-buffered saline (PBS) before

determining total cell numbers. Cells were resuspended to approximately $1 \times 10^6$ cells/ml in ice cold PBS, 10% FCS and 1% sodium azide, and 1 $\mu$g/ml of FITC-labeled anti-mouse CD8 antibody (clone 53-6.72, e-Bioscience, San Diego, CA) was added for 30 min at 4°C. Cells were washed twice, suspended in PBS and subjected to flow cytometry on a BD FACSVerse™ flow cytometer (BD Bioscience, Franklin Lakes, NJ). Data were analyzed using the FlowJo software (Tree Star, Ashland, OR).

### TUNEL assay

About 5 $\mu$m sections of tumor tissues embedded in paraffin were stained with the DeadEnd™ Colorimetric TUNEL System (Promega, Madison, WI) according to the manufacturer's protocol. Ten areas were randomly selected for each tumor section.

### Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). The one-way analysis of variance (ANOVA) is used to determine whether there are any significant differences between the means of groups, depending on the normality of the data. A difference of $p < 0.05$ was considered to be statistically significant. All statistical analyses were carried out using Sigma Stat (ver. 3.5, Systat Software Inc., Chicago, IL).

## Results

### Irradiation-induced expression of monocyte chemoattractant protein-1 in hepatoma cell lines and tissues

Expression of MCP-1/CCL2 was confirmed in HCa-I and Hepa 1-6 hepatoma cells irradiated with 10 Gy of irradiation. In the cultured medium, MCP-1/CCL2 levels began to increase 7 hr after and to decrease on Day 5 after irradiation. MCP-1/CCL2 expression was maximal on Day 1 (Fig. 1*a*). Endogenous expression of the MCP-1/CCL2 gene was also increased in the irradiated hepatoma cells. Analyses of MCP-1/CCL2 expression over time indicated that MCP-1/CCL2 levels began to increase 1 hr after and to decrease on day 5 following irradiation. The maximum expression of endogenous MCP-1/CCL2 was observed on Day 1, similar to its expression profile in the cultured medium (Fig. 1*b*). Using hematoxylin and eosin (H&E) staining, we evaluated tumor density to determine an optimal site for immunofluorescence analysis (Fig. 1*c*). A 10-Gy irradiation was separated into a single irradiation dose (SIR) or a five-way fractional dose (FIR) in the *in vivo* study. The expression of MCP-1/CCL2 was investigated in tumor tissues on Day 5 after the first irradiation. In hepatoma tissues, irradiation increased the expression of MCP-1/CCL2 (Fig. 1*c*). Western blot analysis showed the increased expression of MCP-1/CCL2 in the SIR and FIR groups (irradiated groups), compared to that in the tumor controls (Fig. 1*d*).

### Gene-modified MSCs/IL-12 were able to localize to the region corresponding to the MCP-1/CCL2 expression

A method of coxsackievirus-adenovirus receptor (CAR)-independent internalization was used for transduction of recombinant adenovirus (rAd) into MSCs (Fig. 2*a*). The chemotactic effect of MCP-1/CCL2 was investigated in cultured MSCs/IL-12. The migratory ability of MSCs/IL-12 was enhanced by treatment with recombinant MCP-1/CCL2, but there was no significant difference in the migration of cell treated with doses between 1 and 6 ng/ml of MCP-1/CCL2 (Fig. 2*c*). C-C chemokine receptor type 2 (CCR2), the MCP-1/CCL2 receptor, was expressed in easily detectable amounts on MSCs/IL-12 treated with 1 ng/ml MCP-1CCL2 (Fig. 2*b*; non-merged images are shown in Supporting Information Fig. 1A). MSCs/IL-12 were intratumorally injected to investigate whether MSC/IL-12 localization reflected the changes in MCP-1/CCL2 expression in irradiated hepatoma tissues. Hepatoma tissues were immunostained for both cluster of differentiation (CD)73 and CD90, two well-known markers for identifying MSCs,[19] to confirm the presence of MSCs/IL-12 in the irradiated hepatoma tissues. Although the MSCs/IL-12 were detectable within the tumor 30 min after injection of MSCs/IL-12, the localization of MSCs/IL-12 was well-maintained in the tumor microenvironment of the irradiated hepatoma up to Day 5 of injection, compared to the non-irradiated hepatomas (Figs. 2*d* and 2*e*). Quantitative analysis indicated the significant enhancement of MSCs/IL-12 localization into the hepatoma treated with the combination of irradiation and MSCs/IL-12 (Fig. 2*f*).

### Establishment of therapeutic strategies for combination treatment with irradiation and MSCs/IL-12

Novel therapeutic strategies were devised targeting murine metastatic hepatoma based on the data described in the Figures 1 and 2. HCa-I and Hepa 1–6 ($1 \times 10^6$) cells were injected into the right thighs of mice, and tumors were grown until they were 8 mm in size. The mice were irradiated with a single dose of 10 Gy or with five doses of 2 Gy (total of 10 Gy). MSCs/IL-12 were sub-cultured for 24 hr and injected intratumorally, for the first time 7 hr after irradiation. As a MCP-1/CCL2 expression was reduced starting on Day 5 post-irradiation (Figs. 1*a* and 1*b*), MSCs/IL-12 cells were injected once again on post-irradiation Day 7 to boost the efficacy of the treatment. The tumor volume and survival rate were measured from the first day of the experiment, and the lungs were harvested on experimental Day 14 or 20 for analysis of metastasis (Fig. 3*a*).

### Combination treatment with irradiation and MSCs/IL-12 had increased antitumor and anti-metastatic effects

The tumor growth and metastasis were significantly inhibited by the combined treatment, compared to treatment with irradiation or MSCs/IL-12 alone. Tumor volume was significantly decreased in the FIR + MSCs/IL-12 group between post-irradiation Days 15 and 17 in HCa-I tumors and

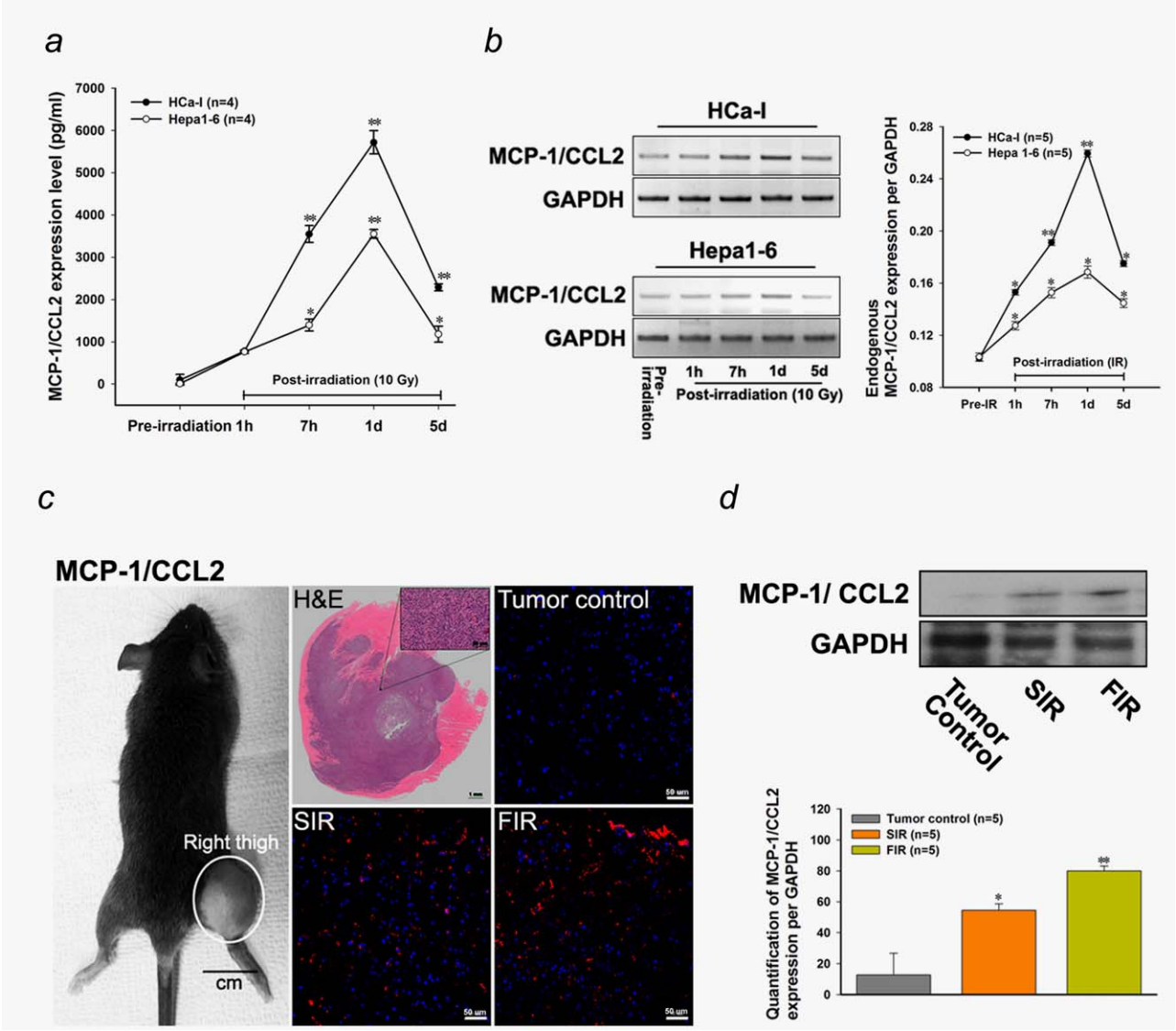


**Figure 1.** Irradiation enhanced MCP-1/CCL2 expression. (*a*, *b*) Chronological changes of MCP-1/CCL2 expression in cultured medium (*a*) and mRNAs (*b*) of irradiated hepatoma cell lines. (*c*) Increased expression of MCP-1/CCL2 in irradiated hepatoma tissues. (*d*) Western blot for MCP-1/CCL2 expression (11 kDa) in protein lysates of hepatoma and quantification analyses. $*p < 0.05$; $**p < 0.001$ (*vs*. tumor control).

between post-irradiation Days 11 and 17 in Hepa 1–6 tumors, compared to the other treatment groups (Figs. 3*b* and 3*d*). Representative images of tumor growth in mice that received the different treatments are shown in Supporting Information Figure 1B. The metastatic nodules significantly decreased in the FIR + MSCs/IL-12 group, although there was some suppression of metastasis in the MSCs/IL-12 and SIR + MSCs/IL-12 groups as well (Figs. 3*f* and 3*g*). The survival rate was 30–40% in the single treatment groups and 70–90% in the combination treatment group, based on the decreased tumor volume and pulmonary metastasis analyses, respectively (Figs. 3*c* and 3*e*). The antitumor effects of MSCs/IL-12 were better in the combination treatment group that received the fractionated irradiation dose, compared to those in a group that received the single dose.

### Irradiation-mediated increase in localization of MSCs/IL-12 enhanced IL-12 expression in murine metastatic hepatoma

The IL-12 expression level was assessed in the cultured media of MSCs/IL-12. The expression of IL-12 in MSCs/IL-12 increased gradually in proportion to the rAd concentration, and IL-12 started to increase significantly when the multiplicity of infection (MOI) $\geq$50 (Fig. 4*a*). As the titer of rAd increased, the expression levels of IL-12 also increased. However, the viability of MSCs was negatively affected by infection with viral doses $\geq$100 MOI (data not shown). Therefore, an MOI of 50 of IL-12-expressing rAd was determined to be the optimal condition for transduction. As MSCs/IL-12 became more localized within the irradiated tumor tissues due to the chemotactic effect of MCP-1/CCL2, the expression

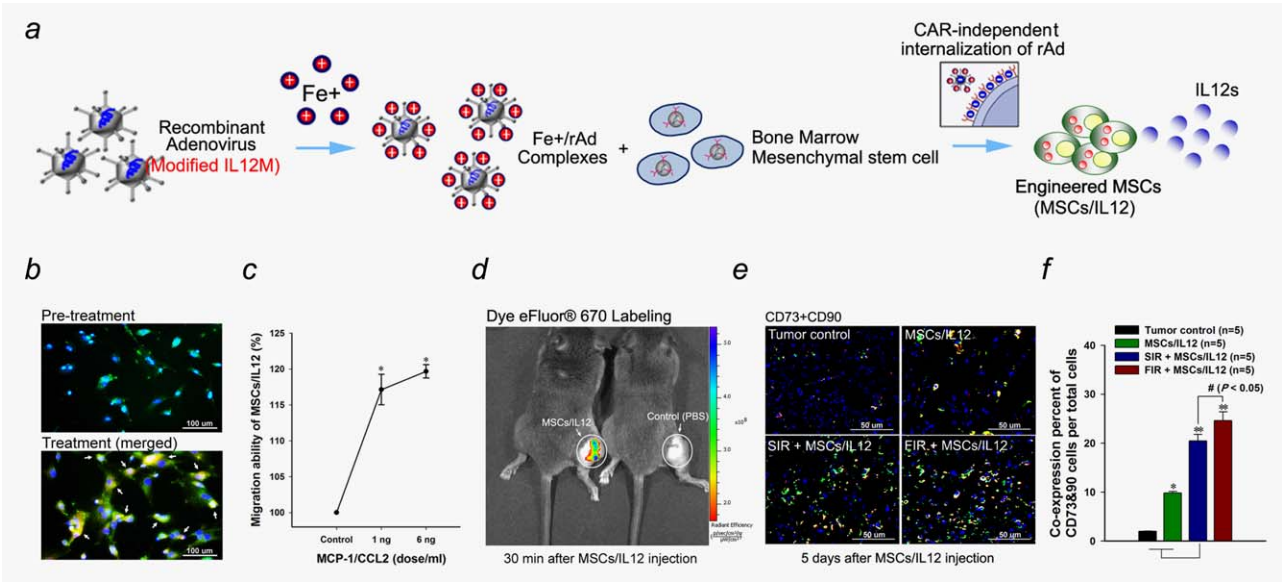


**Figure 2.** Irradiation induced localization of MSCs/IL-12 in murine hepatoma. (*a*) Illustration of MSCs/IL-12 manufacture. (*b*) CCR2 expression in MSCs/IL-12 by the treatment of 1 ng MCP-1/CCL2. (*c*) The migration ability of MSCs/IL-12 with recognition to different dose of MCP-1/CCL2. (*d*) Noninvasive imaging of MSCs/IL-12. (*e*) Co-localization of CD73 and CD90 as merged images at 20× magnification. Scale bars, 50 $\mu$m. (*f*) Quantitative analyses of co-locally expressed CD73 and CD90 cells per total cells in five randomly selected tumor sections. $^{*}p < 0.05$ (*vs*. tumor control); $^{**}p < 0.001$. Results are mean ± S.E.M.

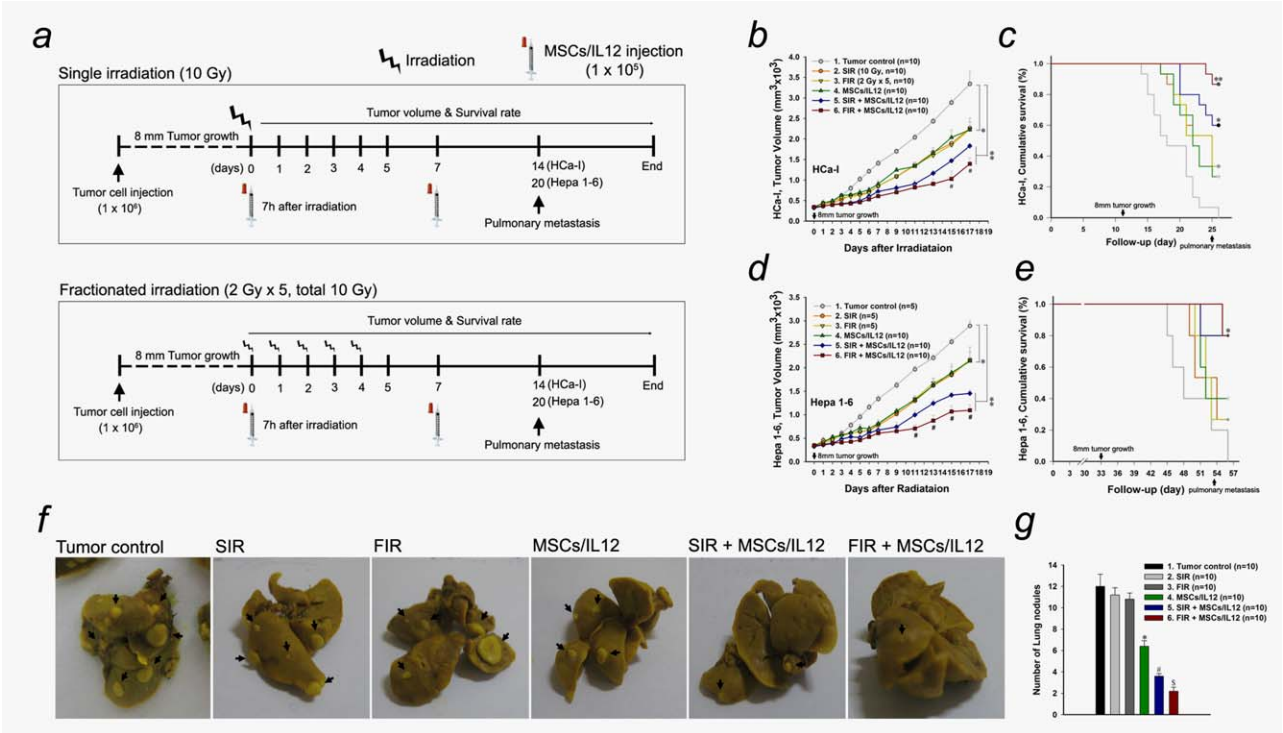


**Figure 3.** Investigation of antitumor effects to the murine hepatoma with establishment of treatment strategies using the combined treatments. (*a*) Treatment strategies for combination treatment with irradiation and MSCs/IL-12. (*b* and *d*) Comparison of tumor growth. $^{*}p < 0.05$; $^{**}p < 0.001$; $^{\#}p < 0.05$ (*vs*. Group 5). (*c* and *e*) Survival rate was analyzed by a log-rank test based on the Kaplan–Meier method. $^{*}p < 0.05$ (*vs*. Group 1–4), $^{**}p < 0.001$ (*vs*. Group 1–5). (*f*) Representative images about pulmonary metastasis (black arrows). (*g*) Quantitative analysis of the metastatic nodule number of pulmonary. $^{*}p < 0.05$ (*vs*. Group 1–3); $^{\#}p < 0.001$ (*vs*. Group 1–4); $^{\$}p < 0.001$ (Group 1–5). Results are mean ± S.E.M.

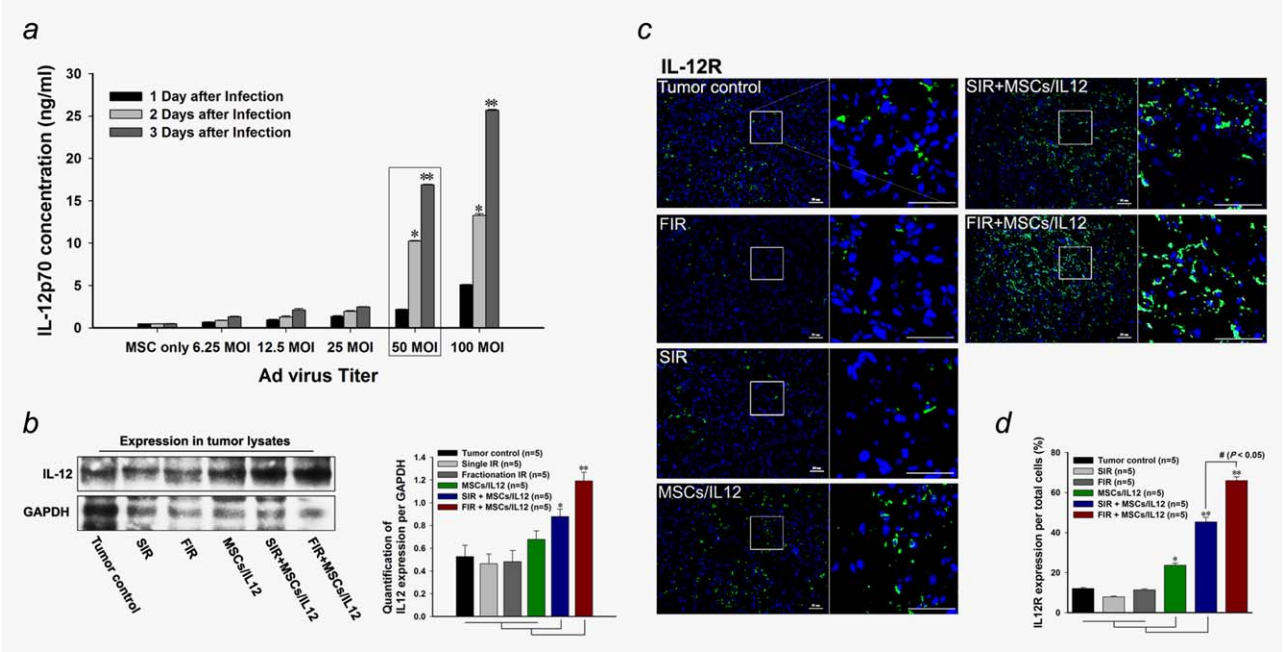


**Figure 4.** Measurement of IL-12 expression induced by MSCs/IL-12. (*a*) Comparison of IL-12 expression by different concentration of rAd/IL-12. The gray-line box indicates the optimal concentration of rAd/IL-12 for transduction into the MSCs. (*b*) Western blot for IL-12 expression (25 kDa) in protein lysates of hepatoma and quantification analyses. (*c*) Immunofluorescence shows an expression of IL-12R. Scale bars, 50 $\mu$m. (*d*) Quantitative analyses of the IL-12R expression cells per total cells in five randomly selected tumor sections. The statistical significance of all data was shown by $*p < 0.05$ and $**p < 0.001$. Results are mean ± S.E.M.

of IL-12 in the tumor microenvironment was investigated on the last day on which the tumor growth was measured. IL-12 expression in tumor lysates was significantly increased in the combination treatment group (Fig. 4*b*). Moreover, the expression of the IL-12 receptor (IL-12R) was significantly increased in many cells within the tumor microenvironment in the combination treatment group (Figs. 4*c* and 4*d*).

### Induction of interferon-γ expression by IL-12-induced CD8$^+$ T-lymphocytes and natural killer cells

Proliferation of CD8$^+$ T-lymphocytes and natural killer (NK) cells in the tumor microenvironment was investigated to delineate the mechanism underlying the antitumor effects of enhanced IL-12 expression (Figs. 5*a* and 5*b*). Proliferation of CD8$^+$ T-lymphocytes was significantly increased to 29.1 ± 3.4% in the SIR + MSCs/IL-12 group and to 38 ± 2.8% in the FIR + MSCs/IL-12 group (Fig. 5*a*). The combination treatment groups also showed increases in the number of activated NK cells, which was confirmed by staining for the CD57 marker in the hepatoma tissues (Fig. 5*b*). The results of the quantitative analysis of marker expression are shown in Supporting Information Figure 1C. Next, the IFN-γ expression level in the tumor tissue, splenocytes and blood serum was assessed. In tumor tissues and splenocytes, IFN-γ expression was significantly increased in the combination treatment group. IFN-γ expression in the MSCs/IL-12 group was increased only in the protein lysates of hepatoma tissues. The results of IFN-γ expression analysis in sera showed IFN-γ in the FIR + MSCs/IL-12 group alone (Fig. 5*c*).

### Apoptotic activity increased in the murine metastatic hepatoma

The tumor cells in mice that received the combination treatment exhibited increased apoptosis (to 43.6 ± 5.3%) in the SIR + MSCs/IL-12 and FIR + MSCs/IL-12 groups (to 52.3 ± 3.6%), compared to those that received the single treatment (Fig. 6). Although this increased apoptosis was lower than that observed in the combination treatment group, the proportion of apoptotic cells was also increased in the single treatment groups, compared to corresponding untreated tumor controls (Fig. 6; to 35.2 ± 4.1% in the SIR, to 32.1 ± 2.8% in the FIR, and to 25.6 ± 2.4% in the MSCs/IL-12 groups).

## Discussion

Curative treatments for HCC include radical resection of tumor and liver transplantation has strict criteria for candidate selection or finding organ donors. Although percutaneous ethanol injection and radio frequency ablation have helped improve the prognosis of HCC patients in recent years, the narrow range of patient selection, non-specific tumor killing and the impact on remaining liver function are some of the disadvantages of these additional therapies. To address these and other disadvantages in current treatment options for HCC, new strategies that can be applied more readily to a wider patient population and that specifically

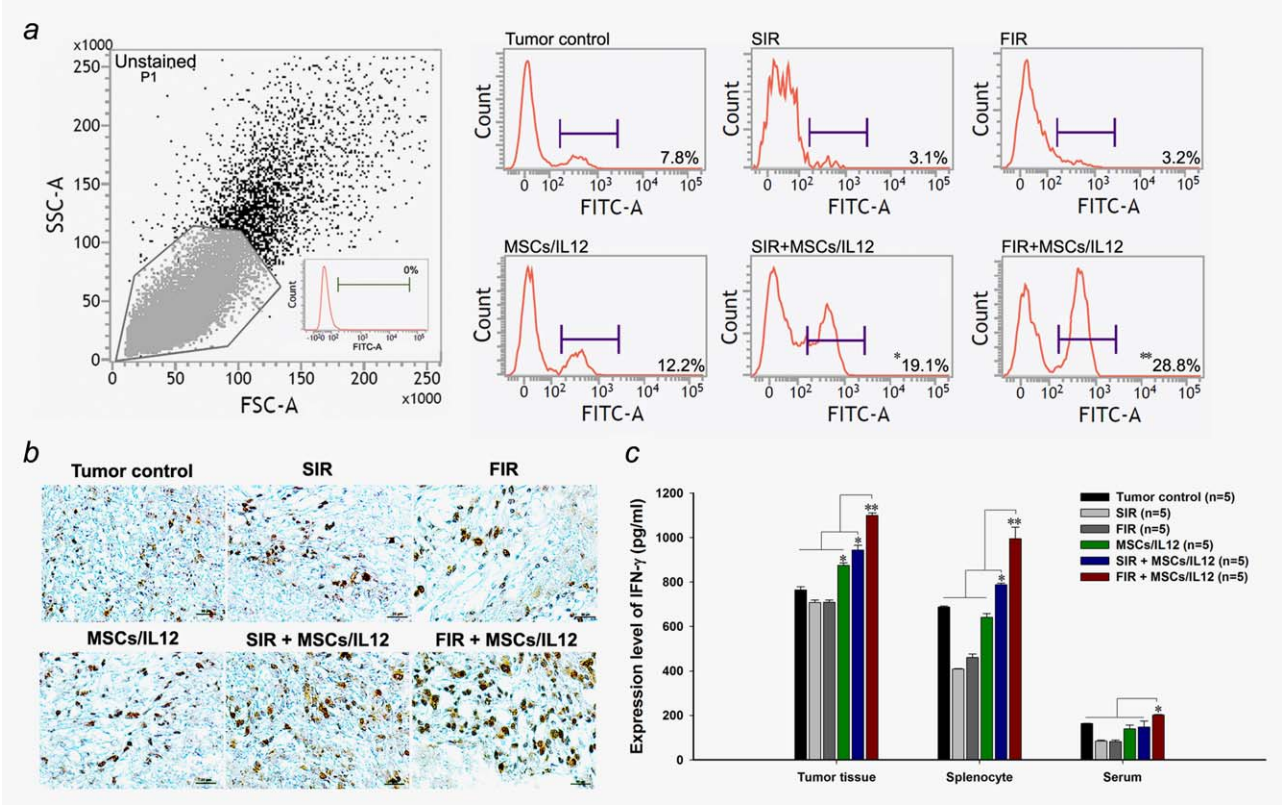


**Figure 5.** Proliferation of $CD8^+$ T-lymphocytes and NK cells induced IFN-$\gamma$ expression. (*a*) FACS analysis of $CD8^+$ T-lymphocytes proliferation among quantified total cells of the hepatoma. The horizontal axis represents the density range of FITC-A, and the vertical axis represents the count of $CD8^+$ T-lymphocytes. Percent values in the histogram represent the mean of $CD8^+$ T-lymphocytes per total cells. (*b*) Confirmation of activated NK cells in the hepatoma tissues. Scale bars, 50 $\mu$m. (*c*) Comparison of IFN-$\gamma$ expression in the protein extracts from tumor, splenocytes and blood. $*p < 0.05$ and $**p < 0.001$. Results are mean ± S.E.M.

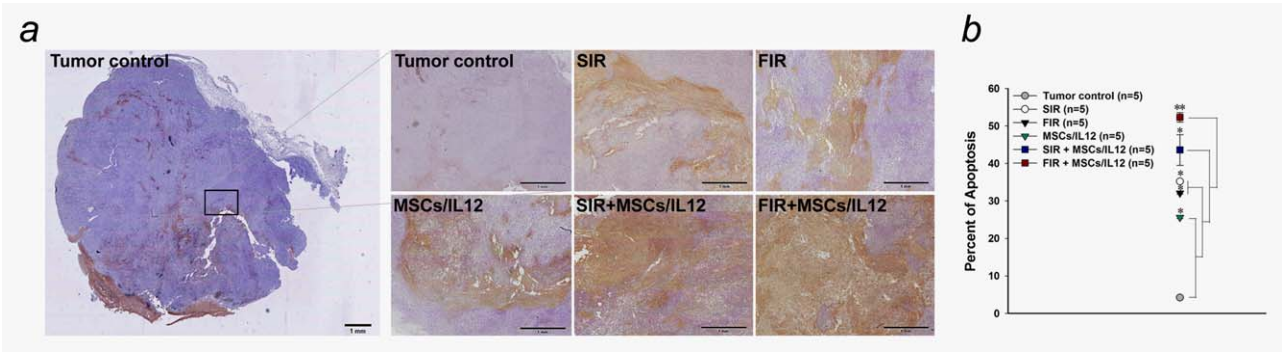


**Figure 6.** Apoptotic activities to the murine metastatic hepatoma. (*a*) Microscopic images show apoptotic activities in the tumor tissues. Scale bars, 1 mm. (*b*) Quantitative analysis of apoptotic activities was indicated as the percent of apoptotic bodies per 2,000 nuclei. The statistical significance of all data was shown by $*p < 0.05$ and $**p < 0.01$. Results are mean ± S.E.M.

target tumors with minimal influence on any normal liver tissues are needed urgently. A number of strategies are being evaluated currently, including gene- and immunotherapies based on suicide, cytokine and anti-angiogenic factors. Gene-modified MSCs have been investigated as a novel anticancer therapeutic option in a murine HCC model in recent years.[13] Nevertheless, there are few reports of their application and curative efficacy in HCC treatment.

We used the xenogeneic MSCs from rat bone marrow. When injected into mice, a total MSCs population will decrease to less than 20% by generations of MSC-specific antibodies (data not shown).[20] However, the use of syngeneic MSCs has safety concerns. Syngeneic MSCs can transform *in vivo* with repeated replication.[21] Generation of MSCs by repeated replication in the tumors might have the secondary effect of promoting tumorigenesis or metastasis.[22] Therefore,

we utilized xenogeneic MSCs to inhibit the excessive replication of MSCs. And, our treatment strategies suggested once more injection of the MSCs/IL-12 at 7days after the first injection than several times injection. No studies have reported abnormal transformation of xenogeneic MSCs *in vivo*.

rAd is one of the most potent vectors used in gene delivery *in vitro* and *in vivo* due to its broad tropism, ease of manufacture and low risk of insertional mutagenesis. However, it has some concerns by an immune response to viral proteins.[23] In our previous study, we confirmed the activity of immune cells using MSC transduced with Ad constructs lacking IL-12. Our previous reports had used MSCs which transfected with mock recombinant adenovirus as a control. Judging from our previous results, there were no effects on the immune cell activity in the treatment of the naïve MSCs or the mock rAd-transduced MSCs.[11,12,24] The neutralizing Ab assay also indicated that it could not hinder therapeutic effects mediated by MSCs/rAd (IL-12 lacking) and naïve MSCs (data not shown). Therefore, immune response against the rAd or the naïve MSCs was negligible in our system.

MSCs migrated *via* the chemotactic effects of the MCP-1/CCL2 that is secreted from tumor or immune cells.[25] Our data show that the expression of MCP-1/CCL2 was significantly increased in the irradiated hepatoma cell, compared to non-irradiated cells. This increased expression of MCP-1/CCL2 in the combination treatment with MSCs/IL-12 and irradiation could enhance the localization of MSCs/IL-12, improving the treatment efficacy in the murine HCC model. We also confirmed that the expression of CCR2, a receptor for the MCP-1/CCL2 ligand, was also increased in the tumor tissues in the combination treatment group (Supporting Information Fig. S2). These data indicate that MSCs/IL-12 cells were "honed" to tumor cells, *via* a mechanism involving their interaction with the enhanced levels of MCP-1/CCL2 in the irradiated hepatoma tissue. We also confirmed the expression of stromal-derived factor (SDF)-1$\alpha$, a known chemoattractant that promotes MSC growth.[26] However, the expression level of SDF-1$\alpha$ in irradiated hepatoma was not significantly different, compared to the non-irradiated controls (data not shown).

In this study, we sought to examine whether the therapeutic potential of genetically modified MSCs could be enhanced in a murine hepatoma model, based on injection of HCa-1 and Hepa 1-6 cells into mice. HCa-I cells are highly radioresistant and undergo spontaneous pulmonary metastasis.[18] We also evaluated the therapeutic efficacy of the combined treatment, using the Hepa 1-6 tumor model to rule out tumor specificity. Hepa 1-6 cells form tumors in C57/BL6 mice spontaneously.[27] It is possible to utilize these cell lines as orthotopic models, because they have the tumorigenic ability in liver (Supporting Information Fig. S3A). However, utilizing an orthotopic model in mice is difficult, since precision during irradiation at the tumor site in mice is not as facile as it is in humans. To circumvent this limitation, we confirmed the expression of alpha fetoprotein (AFP) in the tumor tissue and serum samples in the irradiated mice (Supporting Information Fig. S3B). AFP, which is measured in serum as a constituent of the screening test for a subset of developmental abnormalities, is elevated in a person with HCC. A recent study also indicated that the expression of tumor-associated glycoprotein (TAG)-72 is increased in HCC involving metastasis to the lymph node.[28] Therefore, we confirmed expression of TAG-72 to support the rationale of using the invasive HCa-I cell line (Supporting Information Fig. S3C).

Tumor recurrence after radiotherapy increases in HCC patients who have decreased IL-12 levels in serum (treatment failure in 18 patients in a total of 25 patients; $p < 0.043$, unpublished data). We chose to modify MSCs by increasing the expression of IL-12 in MSCs to overcome the immunological shortcoming brought about by reducing IL-12 expression in HCC. In addition, induction of IL-12 over-expression would facilitate the recognition of cancer cells by cytotoxic T-lymphocytes.[29] Our data showed increased proliferation of $CD8^+$ T-lymphocytes and NK cells in the tumor microenvironment, indicating a direct cytotoxic activity and induction of apoptosis, following enhancement of IFN-$\gamma$ expression. Therefore, our results provide a basis for a clinical strategy to induce a strong tumor-specific immune response using IL-12 gene-based immunotherapy in combination with irradiation. Moreover, the combined treatment is expected to compensate for the disadvantages of irradiation alone, by attenuating the tumor recurrence and metastasis promoted by radioresistant of cancer.[30]

We also investigated the influx of endogenous MSCs in the tumor microenvironment of irradiated mice to confirm recruitment of MSCs by irradiation (Supporting Information Fig. S4). Endogenous MSCs could also be recruited by the chemoattractant effect of MCP-1/CCL2. However, endogenous MSCs did not alter the antitumor effects of irradiation and/or the combined treatments. Although endogenous MSCs did not affect antitumor activity, recruitment of endogenous MSCs by irradiation may be considered for further development as a target for potential tumor treatment.[31,32]

We established two different irradiation plans using SIR and FIR. Many antitumor studies utilizing irradiation focus primarily on the effects of single-dose irradiation. However, we also utilized a fraction-dose irradiation protocol. Our data showed that the combination with fractionated irradiation and MSCs/IL-12 showed better efficacy, as indicated by its effect on tumor growth and metastasis. A hypofractionated radiation, such as is used for stereotactic body radiotherapy, and combination treatment with gene therapy have focused on hyperfractionated irradiation in low doses.[33,34] However, selecting optimal irradiation doses for gene therapeutics has been controversial. Our future studies will address the differences between the two treatments and we expect these studies to provide important information on the immune responses of the tumor microenvironment.

In conclusion, increased expression of MCP-1/CCL2 in irradiated hepatoma induced localization of MSCs/IL-12, and consequently, the IL-12 expression is increased in the tumor microenvironment. The inhibition of tumor growth and pulmonary metastasis results in increased cytotoxic activity in metastatic hepatoma, promoted by the IL-12-induced proliferation of $CD8^+$ T-lymphocytes and NK cells, ultimately resulting in significant improvements in the survival rate. Taken together, our results suggest that combination treatment with irradiation and MSCs/IL-12 is a potential strategy for effective treatment of metastatic hepatoma through immune modulation of the tumor microenvironment.

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