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学位論文

Study of novel adjuvant treatments to improve radiotherapy by
targeting radiation-induced invasiveness in cancer cells

(放射線照射後に生き残ったがん細胞の浸潤能亢進を
抑制するための新規アジュバント療法に関する研究)

2020 年 3 月

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1. Ping-Hsiu Wu, Yasuhito Onodera, Yuki Ichikawa, Erinn B Rankin, Amato J Giaccia, Yuko Watanabe, Wei Qian, Takayuki Hashimoto, Hiroki Shirato, Jin-Min Nam
Targeting integrins with RGD-conjugated gold nanoparticles in radiotherapy decreases the invasive activity of breast cancer cells
International Journal of Nanomedicine, 12, 5069–5085, 2017
2. Ping-Hsiu Wu, Yasuhito Onodera, Frances C. Recuenco, Amato J. Giaccia, Quynh-Thu Le, Shinichi Shimizu, Hiroki Shirato, Jin-Min Nam
Lambda-carrageenan enhances the effects of radiation therapy in cancer treatment by suppressing cancer cell invasion and metastasis through Racgap1 inhibition
Cancers, 11, 1192, 2019

List of Presentations

1. Ping-Hsiu Wu, Yasuhito Onodera, Yuki Ichikawa, Yuko Watanabe, Wei Qian, Takayuki Hashimoto, Hiroki Shirato, Jin-Min Nam
Effects of RGD peptide conjugated gold nanoparticles in Breast Cancer Cells with ionization radiation.
2015 American Society for Cell Biology (ASCB) Annual Meeting. Dec 15, 2015. San Diego, USA
2. Ping-Hsiu Wu, Yasuhito Onodera, Yuki Ichikawa, Yuko Watanabe, Wei Qian, Takayuki Hashimoto, Hiroki Shirato, Jin-Min Nam
RGD-conjugated Gold Nanoparticles in Radiotherapy Decreases the Invasion Activity in Breast Cancer Cells
3rd Global Institution for Collaborative Research and Education (GI-CoRE) Medical Science and Engineering Symposium. March 3, 2016. Sapporo, Japan
3. Ping-Hsiu Wu, Yasuhito Onodera, Yuki Ichikawa, Yuko Watanabe, Wei Qian, Takayuki Hashimoto, Hiroki Shirato, Jin-Min Nam
Gold nanoparticles with RGD peptide in radiotherapy suppress the invasion activity of breast cancer cells
2016 Annual Meeting Program Committee of the American Society for Radiation Oncology (ASTRO). Sep 25, 2016. Boston, USA

4. 呉秉修、小野寺康仁、市川雄貴、渡辺ゆうこ、橋本孝之、白土博樹、南ジンミン
放射線照射後の乳癌細胞の浸潤能に与える RGD 修飾金ナノ粒子の効果
第 75 回日本癌学会学術総会・2016 年 10 月 8 日・横浜・日本
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放射線増感剤の探索～金ナノ粒子の応用
第 11 回北海道大学医学研究科連携研究センターシンポジウム・2016 年 11 月 1 日・札幌・日本
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Targeting integrins with RGD-conjugated gold nanoparticles in radiation therapy
日本放射線腫瘍学会第 30 回学術大会・2017 年 11 月 17 日・大阪・日本
7. 呉秉修、小野寺康仁、市川雄貴、Rankin Erinn、Giaccia Amato、渡辺ゆうこ、Qian Wei、橋本孝之、白土博樹、南ジンミン
インテグリン標的 RGD 修飾金ナノ粒子による放射線治療効果の検証
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8. 呉秉修、小野寺康仁、Recuenco Frances、Giaccia Amato、Le Quynh-Thu、清水伸一、白土博樹、南ジンミン
λ-カラギーナンの併用による放射線効果の向上：in vitro および in vivo
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Abstract

Radiotherapy is a standard and efficacious treatment that uses ionizing radiation (IR) to treat various cancers. Although the therapeutic efficacy of radiotherapy has significantly improved, several patients suffer from local recurrence and distant metastasis after treatment. IR-induced invasiveness in IR-surviving cells is believed to be a factor that contributes to distant metastasis in radiotherapy patients. To overcome this problem, finding new adjuvant therapies that could suppress IR-induced invasiveness while increasing radiosensitization is a promising strategy. To further improve the therapeutic efficacy of radiotherapy by enhancing cancer cell death and suppressing IR-induced invasiveness, we studied two adjuvants, RGD peptide-conjugated gold nanoparticles (AuNPs) and λ -carrageenan (CGN), to improve radiotherapy.

Chapter 1. RGD peptide-conjugated gold nanoparticles suppress IR-induced invasiveness in cancer cells

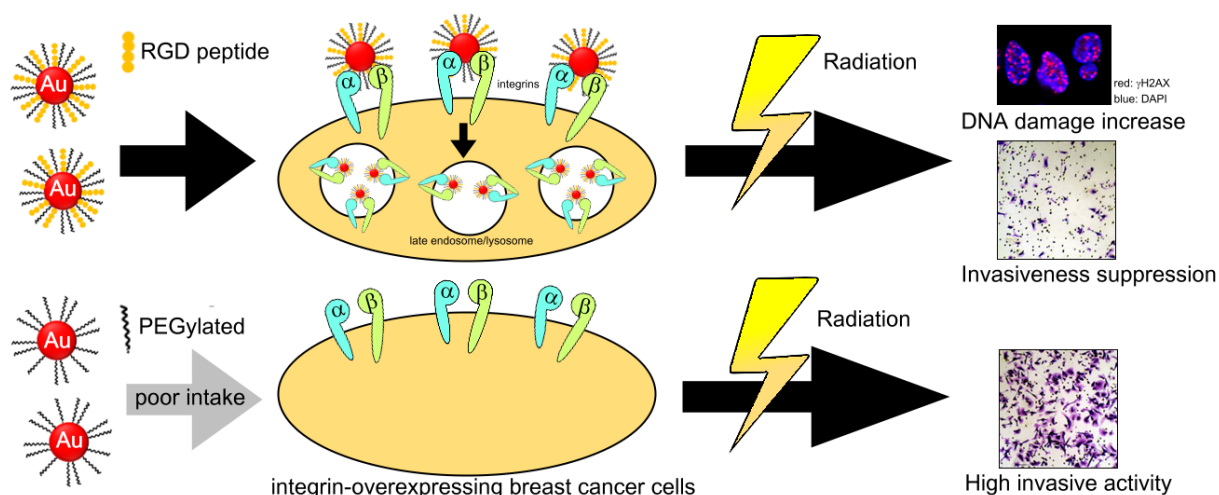


Fig. 1 Schematic representation of chapter 1.

[Background]

Gold nanoparticles have recently attracted attention as radiosensitizers that can increase the therapeutic efficacy of radiotherapy. We therefore produced arginine–glycine–aspartic acid (RGD)-conjugated AuNPs to specifically target integrin-expressing cancer cells. RGD is a peptide that specifically binds to integrin, a cell surface receptor that is overexpressed in highly invasive cancer cells. In this chapter, we describe how we validated the therapeutic efficacy of RGD-conjugated AuNPs by determining the extent of radiosensitization and invasiveness after RGD-conjugated AuNPs treatment in the targeted cancer cells.

[Methods & Results]

- (1) We produced polyethylene-glycolylated AuNPs (P-AuNPs) to stabilize the AuNPs and then conjugated them with RGD peptides to produce RGD/P-AuNPs. RGD/P-AuNPs were then characterized in terms of size variations, hydrodynamic diameter, and zeta potential.
- (2) The expression levels of RGD-binding integrins in different breast cancer cell lines were measured. RGD/P-AuNPs were efficiently internalized by integrin-overexpressing cell lines but not by low integrin-overexpressing cell lines.
- (3) By immunofluorescent imaging, RGD/P-AuNPs were found to colocalize with integrins in the late endosomes and lysosomes in these cells.
- (4) Cell viability was measured by Cell Counting Kit-8 (CCK-8). DNA damage was measured by counting γ -H2AX foci. A combination of RGD/P-AuNPs and IR treatment reduced cell viability and increased DNA damage compared to IR alone in the targeted cells.
- (5) The invasiveness of cancer cells was measured by the Matrigel chemoinvasion assay. The IR-induced invasiveness of the breast cancer cell lines was significantly inhibited by RGD/P-AuNP treatment.
- (6) We performed gene expression microarray analysis to elucidate molecular mechanism underlying the abovementioned effects. Our analysis revealed that the expression levels of fibronectin (FN) and its downstream signaling, the extracellular signal-regulated kinase (ERK) pathway, in irradiated cells were suppressed by RGD/P-AuNPs.

[Discussion]

In this chapter, we reveal that RGD/P-AuNPs could improve the therapeutic efficacy of radiotherapy by increasing the cytotoxic effect of IR and suppressing IR-induced invasiveness in integrin-overexpressing cancer cells.

- (1) RGD/P-AuNPs showed specific targeting to integrin-overexpressing cancer cells. Further evaluation of the therapeutic efficacy of RGD/P-AuNPs and IR in animal models should be performed.
- (2) We found that RGD/P-AuNPs suppressed IR-induced invasiveness, which may be caused by the suppression of the FN-ERK pathway. Further research on the relationship between this pathway and IR-induced invasiveness is recommended.

Chapter 2. Adjuvant of Lambda-carrageenan suppresses radiation-induced invasiveness in cancer cells through Racgap1

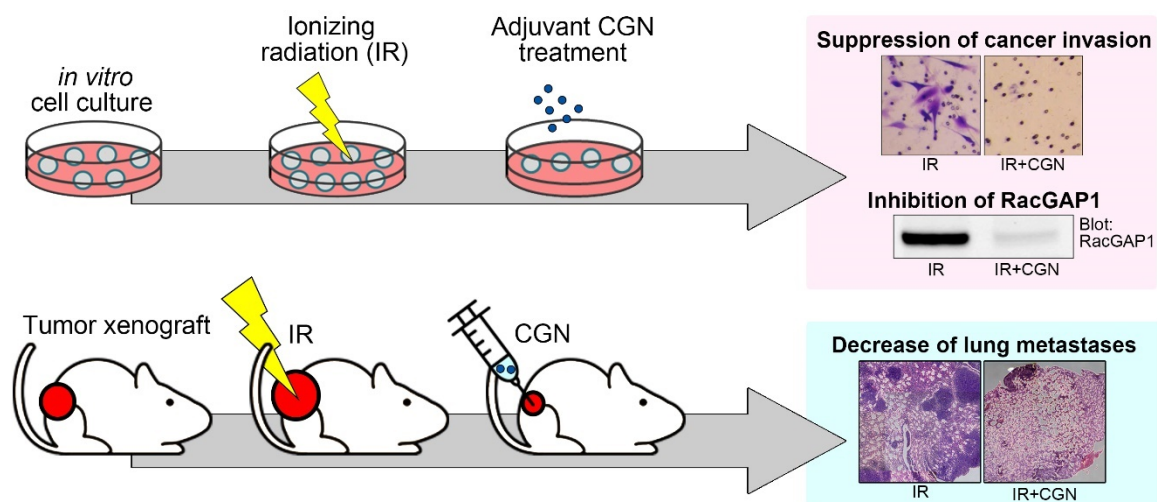


Fig. 2 Schematic representation of chapter 2.

[Background]

Lambda-carrageenan, a sulfated polysaccharide used as a daily food additive, has been recently found to exhibit anti-tumorigenic activities. In this chapter, we describe the evaluation of the therapeutic benefit of CGN in enhancing the efficacy of IR treatment and investigate its underlying molecular mechanism.

[Methods & Results]

- (1) We determined the therapeutic efficacy of IR coupled with CGN treatment in human breast cancer cell line, head and neck cancer cell line, and pancreatic cancer cell line. We found that CGN treatment increases cytotoxic efficacy and apoptosis in irradiated cancer cells.
- (2) We measured the change in several biological responses to further evaluate the mechanism behind the cytotoxic effect of this treatment. CGN treatment after IR significantly increased reactive oxygen species (ROS) accumulation, caspase activities, and polyploid formation in the cancer cells.
- (3) To measure cell invasiveness, Matrigel chemoinvasion assay was performed. CGN treatment was found to suppress IR-induced invasiveness.
- (4) CGN treatment after IR suppressed invasive growth, as observed by the 3D IrECM culture method.

- (5) We screened target molecules by performing microarray analysis and focused on Rac GTPase-activating protein 1 (RacGAP1). We found that RacGAP1 was upregulated in several cancer cell lines after IR treatment. Knockdown of RacGAP1 increased the cytotoxic effect of IR.
- (6) CGN treatment significantly suppressed RacGAP1 expression in breast cancer cells. RacGAP1 overexpression partially rescued CGN cytotoxicity.
- (7) In a mouse xenograft model, IR followed by CGN treatment significantly decreased tumor growth and lung metastasis compared to either treatment alone.

[Discussion]

In this chapter, we describe how CGN enhances IR treatment by significantly increasing cytotoxicity, suppressing IR-induced invasiveness, and distant metastasis by downregulating RacGAP1 expression.

- (1) CGN and IR have been reported to induce specific immune responses in cells. Further research is needed to determine the immune response elicited after the combination treatment of CGN and IR.
- (2) CGN was found to suppress RacGAP1 expression significantly. Apart from RacGAP1, other genes were also found to be suppressed by CGN treatment. These genes may be involved in radioresistance or IR-induced invasiveness. The roles of these molecules and related mechanisms could be investigated in the future.

Conclusion

In the first chapter, we describe how we produced integrin-targeting RGD/P-AuNPs to increase the therapeutic efficacy of IR treatment. Our results showed that RGD/P-AuNPs effectively target integrin-overexpressing cancer cells. Furthermore, RGD/P-AuNPs suppress IR-induced invasiveness in addition to increasing radiosensitization. With regard to the molecular mechanism underlying these observations, the expression of FN and activation of ERK, key modulators of cancer cell invasion, were found to be suppressed by RGD/P-AuNPs used in combination with IR. In the second chapter, we describe how we used CGN as an adjuvant to improve radiotherapy. CGN treatment increased the therapeutic efficacy of IR, suppressing both IR-induced invasiveness and distal metastasis by downregulating RacGAP1 expression.

These results have provided us novel strategies for improving radiotherapy by targeting IR-induced invasiveness in cancer cells. By understanding the molecular mechanisms of these treatments, we can also obtain new knowledge of how cancer cells respond to IR.

List of Abbreviations

3D ECM	Three-dimensional extracellular matrix
AuNPs	Gold nanoparticles
ATCC	American Type Culture Collection
AuNPs	Gold nanoparticles
CCK-8	Cell counting Kit-8
cDNA	Complementary DNA
CGN	λ -carrageenan
DCFDA	Dichloro-fluorescein diacetate
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EEA 1	Early endosome antigen 1
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FDA	Food and Drug Administration (United States)
FITC-PEG-SH5k	Fluorescein isothiocyanate-PEG-thiol, MW 5k
FN	Fibronectin
GAP	GTPase activation protein
GRB2	Growth-factor-receptor-bound-2
GTPase	Guanine triphosphatase
Gy	Gray
IgG	Immunoglobulin G
IR	Ionizing radiation
LAMP1	Lysosomal-associated membrane protein
lrECM	Laminin-rich ECM
MAPK	Mitogen-activated protein kinase
mPEG-SH5k	Methoxy-PEG-thiol, MW 5k
mRNA	Messenger RNA
MW	Molecular weight

P-AuNPs	Polyethylene glycolylated AuNPs
PBS	Phosphate-buffered saline
PDI	Polydispersity index
PI	Propidium iodide
PVDF	Polyvinylidene fluoride
RacGAP1	Rac GTPase-activating protein 1
RGD	Arginine (R)–glycine (G)–aspartic (D) acid
RIPA buffer	Radioimmunoprecipitation assay buffer
ROS	Reactive oxygen species
RT-PCR	Quantitative reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulfate
SE	Standard error
SFKs	Src-family kinases
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SOS	Son-of-sevenless
TBST	Tris-buffered saline Tween-20
TEM	Transmission electron microscopy

Introduction

Radiotherapy

Radiotherapy (also called radiation therapy) uses high energy ionizing radiation (IR) such as X-rays to treat cancer by eliminating cancer cells exposed to the radiation field. With technological advancements, radiotherapy has significantly improved the treatment outcomes of cancer patients (Bhide and Nutting, 2010). In clinical practice, radiotherapy is used as a postoperative treatment after surgery and major treatment for inoperable tumors; it may even replace surgery where organ preservation is desired (Forastiere et al., 2003; Mirza and Choudhury, 2016). Nowadays, radiotherapy contributes to approximately 50% of all cancer cures worldwide (Delaney and Barton, 2015), indicating its indispensable status in cancer treatment.

Current limitations of radiotherapy

Although the therapeutic efficacy of radiotherapy has significantly improved, local recurrences and distant metastases that occur after treatment remain as a major therapeutic challenge (Garcia-Aguilar et al., 2001; Holleczeck et al., 2019; van den Berg et al., 2015). With regard to the cause of local recurrence after radiotherapy, two major reasons are considered: (i) the IR dose is insufficient to eradicate radiation-resistant cancer cells; (ii) the treatment field of IR is not sufficiently large and some cancer cells escape the treatment site. Therefore, the complete eradication of cancer cells by using higher IR doses and larger treatment fields has been considered to increase the cancer control rate. However, higher doses and larger fields of radiotherapy also increase the risk of intolerable side effects to normal tissues. To improve the efficacy of radiotherapy without increasing IR-induced toxicity, several types of chemotherapeutic drugs were used to improve IR efficacy in the clinical settings; this treatment method is known as chemoradiotherapy (Lawrence et al., 2003). Although chemoradiotherapy significantly increases the cancer control rate compared to single treatment modality (Brunner and Seufferlein, 2016; Deng et al., 2017; Goodman, 2016), the treatment outcome is still not good enough to completely cure all cancers. Numerous studies have documented that toxicity is still a major limitation of chemoradiotherapy (Morgan et al., 2014; Todo and Watari, 2016). Chemotherapeutic drugs are known to be cytotoxic agents that cause severe adverse effects (Livshits et al., 2014). Although the patterns of adverse effects induced by IR and chemotherapy are usually different, these adverse effects caused together still cause great suffering in patients.

These adverse effects may even lead to treatment-related death in serious situations (Ohe, 2002). Chemoradiotherapy also has limitations when it comes to treating patients with poor performance. (Bowden et al., 2017; Higgins et al., 2015). Another critical problem is therapeutic efficacy. While numerous chemoradiotherapy combinations have been tested in clinical trials, there are still numerous patients who have shown high resistance to this treatment (Costa et al., 2018). Hence, finding new adjuvants to improve the therapeutic efficacy of radiotherapy with lower adverse effects is an important and essential research issue.

Radiation-induced invasiveness

Distant metastasis occurring after radiotherapy is another critical issue because metastasis is the leading cause of disease-related death in cancer patients (Siegel et al., 2019). The reasons why distant metastasis occurs after local treatment are generally considered to be the following: (i) the existence of occult or micrometastasis that was not found during diagnosis and (ii) novel metastasis from tumor cells that were not eliminated after IR treatment. Novel metastasis is believed to be caused by the enhancement of distant metastasis in cancer cells after IR treatment. For decades, enhancement of distant metastases after IR treatment has been observed in experimental animal models (Kaplan and Murphy, 1949; Sheldon and Fowler, 1976). This phenomenon was later confirmed by retrospective studies and clinical trials (Anderson and Dische, 1981; Fagundes et al., 1992). These studies reported an unexpected correlation between IR treatment and a higher incidence of metastasis. At the cellular level, *in vitro* studies revealed that the invasiveness of some cancer cells increased after IR treatment; this phenomenon is known as IR-induced invasiveness (Moncharmont et al., 2014). Because invasion of cancer cells is a prerequisite for distant metastasis (e.g., without invasion, no metastasis occurred) (Wittekind and Neid, 2005), IR-induced invasiveness is considered to be a mechanism that contributes to IR-enhanced metastasis. Therefore, to suppress the occurrence of metastasis after radiotherapy, targeting IR-induced invasiveness is a reasonable strategy.

As described above, the development of adjuvants that provide the following features would be a promising approach to improve radiotherapy: (i) increase the therapeutic efficacy of IR, (ii) suppress IR-induced invasiveness, and (iii) the adjuvants cause lower adverse effects themselves. In this study, we introduced two novel adjuvant therapies that meet these conditions, and investigated the potential molecular mechanisms by which they act to suppress IR-induced invasiveness. Chapter 1 describes how we used integrin-targeted gold nanoparticles (AuNPs) as an adjuvant to enhance the IR effect on highly invasive breast cancer cell lines. In the second

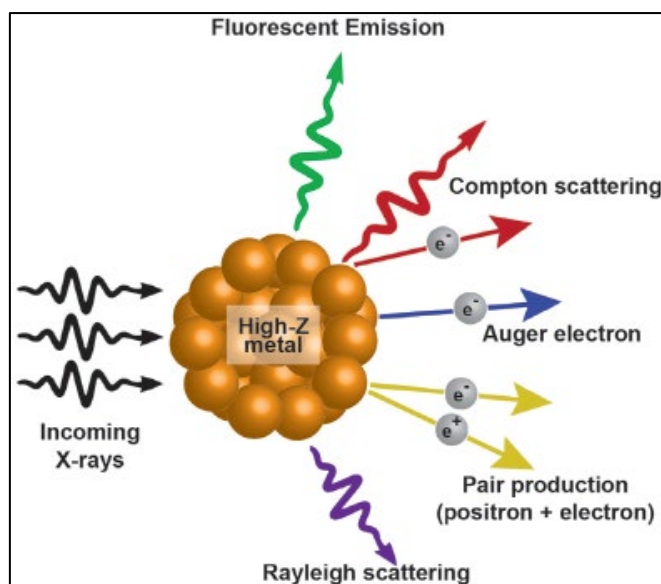
chapter, we describe how we used λ -carrageenan (CGN), a sulfated polysaccharide used as a daily food additive, to improve radiotherapy by suppressing IR-induced invasiveness and metastasis.

Chapter 1. RGD peptide-conjugated gold nanoparticles suppress IR-induced invasiveness in cancer cells

Background

Gold nanoparticles and radiotherapy

In recent years, AuNP has been widely studied for use in cancer nanomedicine (Elahi et al., 2018). The AuNP is a good nanoparticle platform for researchers to produce ideal nanoparticle-based therapy because of several features of gold, such as (i) excellent biocompatibility that allow AuNPs to enter the human body without harmful effect (Shukla et al., 2005), (ii) straightforward synthesis to produce different size of AuNPs (Akamatsu et al., 2010), and (iii) accessible to functionalization the AuNP by conjugated ligands on the surface (Zong et al., 2017; Hume et al., 2016). As a high atomic number (Z) material, AuNP itself can also serve as a sensitizer to enhance the IR effect (Refer to figure below). After IR hit the high Z material such as AuNP, several IR-related emissions occur, including scattered X-rays/photons, photoelectrons, Compton electrons, Auger electrons, and fluorescence photons (Haume et al., 2016). These emissions generate a higher ionization density in the area near the high Z material, which lead to an increase of the primary IR effect.



Interaction of X-rays with high-Z material nanoparticles
Reference: (Kwatra et al., 2013)

Modification of AuNPs with RGD peptide to target integrins

The delivery of NPs to cancer cells is a critical issue in cancer nanomedicine. In order to specifically transport NPs to cancer cells, the decoration of NPs with targeting ligands that recognize specific receptors on the cancer cell surface is the dominant strategy (Shi et al., 2017). In this study, we conjugated arginine (R)–glycine (G)–aspartic acid (D) (RGD) peptide, an integrin-targeted ligand, on the surface of AuNPs. Integrins are heterodimeric cell surface receptors that mediate the adhesion of cells to the extracellular matrix (ECM) (Hynes, 2002). There are 24 integrin heterodimers, and several types of integrins (e.g. $\alpha 5 \beta 1$ -, $\alpha v \beta 3$ - and $\alpha v \beta 5$ -integrins) are highly expressed in tumor cells. Signaling mediated by these integrins is essential for cancer invasion, metastasis and radioresistance of breast cancer cells (Hamidi and Ivaska, 2018; Nam et al., 2009). The RGD peptide is included in an ECM protein known as fibronectin (FN) (Pierschbacher and Ruoslahti, 1984), and is also the binding motif to cancer-related integrins, such as $\alpha 5 \beta 1$ -, $\alpha v \beta 3$ - and $\alpha v \beta 5$ -integrins.

In this chapter, we conjugated RGD peptides on polyethylene glycolylated (PEGylated) AuNPs (P-AuNPs) to enable AuNPs to bind and internalize into integrin-overexpressing cancer cells. We evaluated whether targeting integrins with RGD-conjugated P-AuNPs (RGD/P-AuNPs) could enhance the therapeutic efficacy of IR in cancer cells. Because high integrin-expressing cancer cells usually show high cancer invasiveness, we also focused on the invasive activity of cancer cells after combined treatment with AuNPs and IR.

Materials and methods

1. AuNPs

The following materials were used to produce RGD/P-AuNPs.

Material	Maker
i-colloid™ Au 15 nm (optical density 1 [OD1]) and 20 nm (OD1)	IMRA America, Inc.
Methoxy-PEG-thiol, MW 5k (mPEG-SH5k, MW 5,345, substitution purity: 90%)	Creative PEG Works
Fluorescein isothiocyanate-PEG-thiol, MW 5k (FITC-PEG-SH5k, substitution purity: 80%)	Nanocs, Inc.
Cysteine-modified (RGD) ₄ peptide (RGDRGDRGDRGDPGC, MW: 1,845.98, purity: 95%, abbreviated as “RGD peptide”)	RS Synthesis LLC

mPEG-SH5k, FITC-PEG-SH5k, and RGD peptide were in powder form and dissolved in deionized water, having an electric conductivity of 0.1 μ S/cm without further purification.

2. Synthesis of P-AuNPs and RGD/P-AuNPs

i-colloid™ Au was mixed with a solution of mPEG-SH5k or FITC-PEG-SH5k and kept undisturbed for longer than 12 h. After the reaction, centrifugal purification ($12,000 \times g$, 15 min for Au 15 nm; $5,000 \times g$, 20 min for Au 20 nm) was performed for 2 times. Final optical density (OD) was adjusted to ~ 30 by adding deionized water to the pellet after removing the supernatant. To produce RGD/P-AuNPs, P-AuNPs (OD30) and 0.2 M borate buffer (pH 8.2) were mixed with RGD peptide solution to achieve a ratio of RGD to AuNPs of 1,200 in 4 mM borate buffer (pH 8.2). The solution was kept undisturbed for longer than 24 h. After the reaction, two runs of centrifugal purification as above were performed in 4 mM borate buffer (pH 8.2). Finally, the OD was adjusted to ~ 30 by adding 4 mM borate buffer (pH 8.2) to the pellet after discarding the supernatant. (The synthesis of P-AuNPs and RGD/P-AuNPs was performed by the co-author Dr. Yuki Ichikawa)

3. Characterization of AuNPs

The size distribution of AuNPs was analyzed by the method of differential centrifugal sedimentation using the CPS Disc Centrifuge instrument (model DC24000 UHR; CPS Instruments Inc.). The Hydrodynamic diameter and zeta potential of RGD/P-AuNPs were obtained from DLS measurements using a Zetasizer Nano ZS90 (Malvern Instruments). Transmission electron microscope (TEM) analysis was performed by using JEOL JEM-2100F

transmission electron microscope (JEOL Ltd., Tokyo, Japan).

3. Cell culture and AuNP treatment

MDA-MB-231, Hs578T, and SK-BR-3 human breast cancer cell lines were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; HyClone, GE Healthcare Life Sciences). For the AuNPs treatment, Cells were cultured in the dish for 24 h and then replaced with medium containing 0.3 nM (1:90 dilution) or 1 nM (1:30 dilution) of 20 nm AuNPs.

4. Irradiation

Cells were irradiated with 130 kV X-rays using a Faxitron CellRad X-ray generator (Faxitron) after incubation of AuNPs for 48 h.

5. Lysis and Western blotting

Cells were lysed in 1% radioimmunoprecipitation assay (RIPA) buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 5 mM ethylenediaminetetraacetic acid [EDTA], 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1 mM Na₃VO₄, 1 mM NaF and protease inhibitor cocktail; Merck Millipore). Proteins were transferred onto polyvinylidene fluoride (PVDF) membrane (Merck Millipore). The PVDF membranes were blocked with Odyssey® blocking buffer (LI-CORE Biosciences) and were probed with primary antibodies overnight at 4°C. After washing the PVDF membranes with Tris-buffered saline Tween-20 (TBST; 25 mM [pH 7.4], 120 mM NaCl, 3 mM KCl and 0.1% Tween-20), membranes were incubated with secondary antibody for 1 h at room temperature and then washed with TBST. The signals were detected with Odyssey CLx Imager (LI-CORE Biosciences).

6. Antibodies

The following primary antibodies were used for immunoblotting:

Antibody	Maker
anti- α 5-integrin	Merck Millipore
anti- α v-integrin	Abcam
anti- β 1-integrin	BD Transduction Laboratories
anti- β 3-integrin	Merck Millipore
anti- β 5-integrin	Abcam

anti-pERK	Cell Signaling Technology
anti-ERK	Cell Signaling Technology
anti- β -actin	Sigma-Aldrich

The following primary antibodies were used for immunofluorescence staining

Antibody	Maker
anti- α 5-integrin	Cell Signaling Technology
anti- α v-integrin	Cell Signaling Technology
anti-LAMP1	Cell Signaling Technology
anti-EEA1	Cell Signaling Technology
anti-Rab5	Cell Signaling Technology
anti-Rab7	Cell Signaling Technology
anti-Rab9	Cell Signaling Technology
anti- γ -H2AX	Merck Millipore

7. Cell viability assay

To measure the cell viability after treatment, Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) or cell counting were performed. Cell Counting Kit-8 (CCK-8) is a sensitive colorimetric assay for the determination of cell viability in cell proliferation and cytotoxicity assays. Cells were cultured in 96-well plates and treated with P-AuNPs or RGD/P-AuNPs for 12 to 48 h and then incubated with CCK-8 solution. For radiosensitivity, cells treated with P-AuNPs or RGD/P-AuNPs received IR 48 h later and then measured the cell viability with cell counting or CCK-8 solution. The OD at 450 nm was determined using Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific) to measure cell viability.

8. Immunostaining and image acquisition

After treatment, cells were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100/phosphate-buffered saline (PBS), and then incubated with primary antibodies and coupled with Alexa Fluor secondary antibody. Images were acquired by a Leica True Confocal Scanning (TCS) SP8 microscope system (Leica Microsystems) or by Fluorescence Microscope BZ-9000 (Keyence). Colocalization analysis with endosome markers was performed with Coloc 2, a Fiji's plugin for ImageJ (Schneider et al., 2012). For DNA damage assessment, cells incubated with AuNPs were fixed and stained with anti- γ -H2AX 12 h after IR. The number of γ -H2AX foci per nucleus was counted in ImageJ. At least 50 nuclei were analyzed in each

independent experiment.

9. Matrigel invasion assay

One $\times 10^5$ of MDA-MB-231 or 10^4 of Hs578T cells were seeded on to the upper wells of chambers in the absence of serum, and the lower wells were filled with culture medium. After 8 h for the MDA-MB-231 cells and 5 h for the Hs578T cells, the cells that migrated out on to the lower surface of the membranes were fixed with 4% paraformaldehyde. The number of cells was scored by staining with 1% crystal violet.

10. Microarray analysis

Total cellular RNA was isolated by NucleoSpin® RNA (MACHEREY-NAGEL). For the microarray analysis, the highly sensitive 3D-Gene® Human oligo chip 25k ver 2.10 (Toray Industries) was used. The data were normalized and analyzed by Toray Industries.

11. Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA of the MDA-MB-231 cells after RGD/treatment was isolated by NucleoSpin® RNA II (MACHEREY-NAGEL). Complementary DNA (cDNA) was synthesized from total RNA by the SuperScript™ First-Strand Synthesis System (Thermo Fisher Scientific) using random primers. Quantitative real-time PCR was performed with the LightCycler® nanosystem (Hoffman-La Roche Ltd.) using the FastStart Essential DNA Green Master kit (Hoffman-La Roche Ltd.). The following primers were used to amplify FN and 18S rRNA:

primer	forward	reverse
18S	TCGGAAGTGGAGCCATGATT	CCTCCGACTTTCGTTCTTGATT
Total FN	CCAAGCTCAAGTGGTCCTGT	CACTTCTTGGTGGCCGTACT

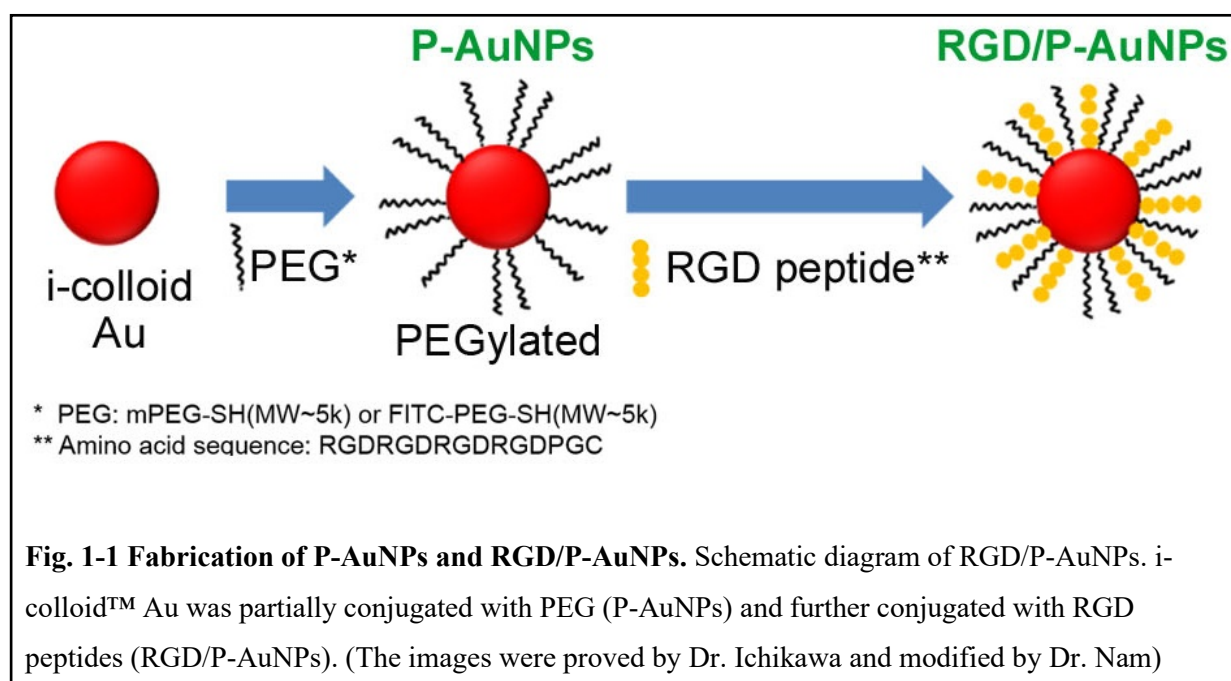
12. Statistical analysis

Graphs are expressed as the mean $\pm$ standard error (SE). Data were analyzed by Student's *t*-test. Significant differences are indicated by *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ and ns for not significant.

Results

1-1 Fabrication of P-AuNPs and RGD/P-AuNPs

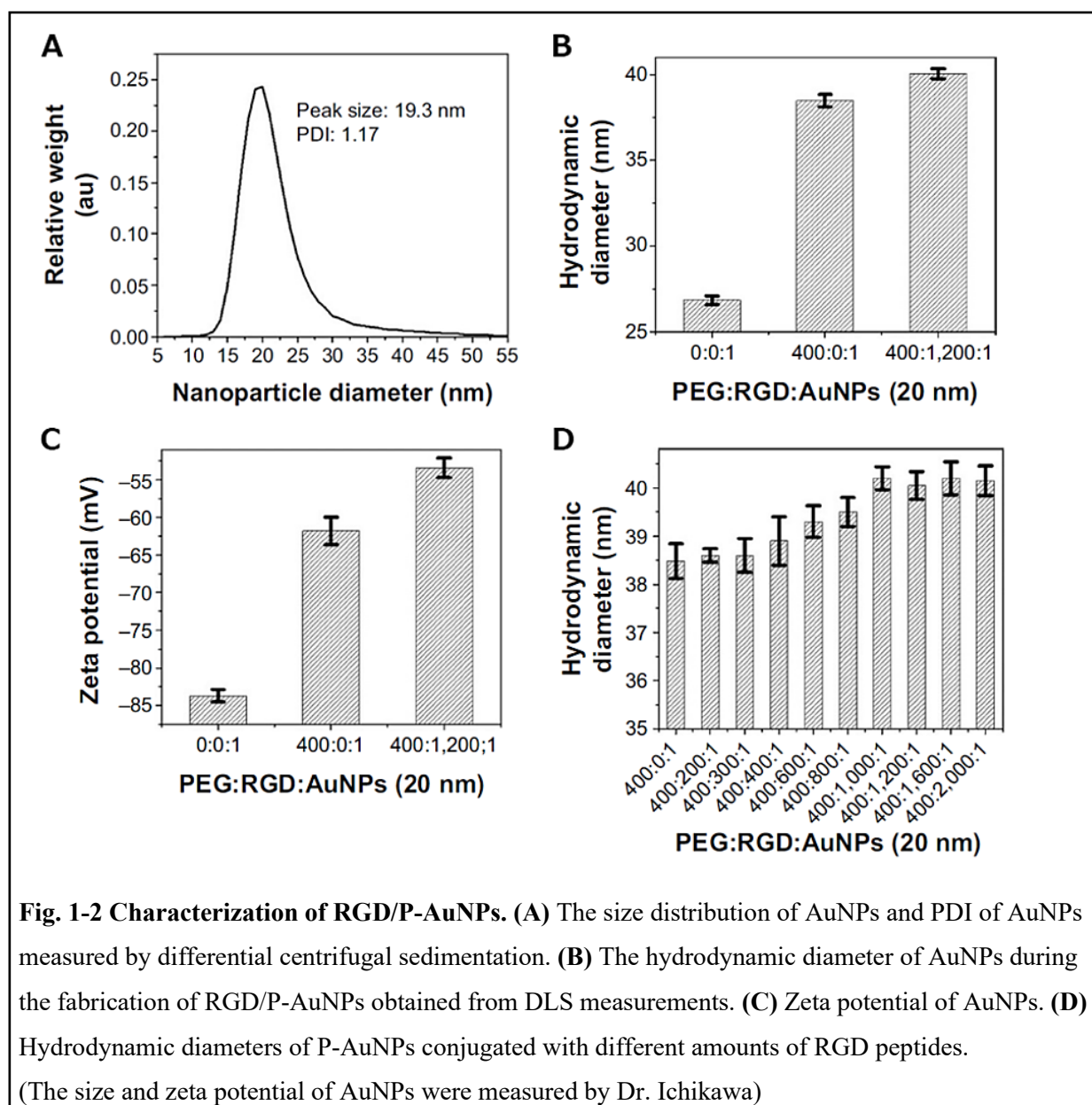
To produce RGD peptide conjugated AuNPs, AuNP was partially PEGylated (P-AuNPs) and subsequently conjugated with RGD peptides (RGD/P-AuNPs) (Fig. 1-1). i-colloid™ Au (IMRA America, Inc.) was used to take advantage of its novel surface properties allowing sequential surface modification (Chen et al., 2013; Qian et al., 2011; Shao et al., 2015). PEGylated on the surface of AuNPs has been reported to enhance stability under physiological conditions by providing a steric barrier (Qian et al., 2011).



1-2 Characterization of P-AuNPs and RGD/P-AuNPs

The size distribution of AuNPs was analyzed (Fig. 1-2A), and the peak size was 19.3 nm in the weight size distribution graph (weight of nanoparticles plotted against diameter). The polydispersity index (PDI, the distribution of molecular mass in polymer samples) was 1.17 defined as the ratio between weight average diameter (Dw) and number average diameter (Dn). To prepare a perfectly uniform sample consisting of exactly one size, Dw and Dn should be the same value and PDI should be 1.0. The hydrodynamic diameter (Fig. 1-2B) and zeta potential (a scientific term for electrokinetic potential in colloidal dispersions.) (Fig. 1-2C) of AuNPs were obtained by DLS measurements during the fabrication of RGD/P-AuNPs. These results showed that the hydrodynamic diameter of AuNPs increased and the absolute zeta potential of AuNPs decreased along with the sequential conjugation of PEG molecules and RGD peptides,

confirming the result of AuNPs binding with PEG molecules and RGD peptides on to the surface. Fig. 1-2D shows the hydrodynamic diameters of the P-AuNPs (input molar ratio of PEG to AuNP used was 400:1) conjugated with different amounts of RGD peptides. The increase in hydrodynamic size was plotted against the increased amounts of RGD peptides added to the P-AuNPs. The results showed that the diameter of the AuNPs first increased with an increasing molar ratio of RGD peptides to P-AuNPs (RGD/P-AuNPs) from 0 to 1,000 and then plateaued for RGD peptides >1,000, representing saturation of RGD peptides on the surface of P-AuNPs. Based on this result, we conjugated ~1,000 RGD peptides to each P-AuNP with an initial input molar ratio of RGD peptides to one P-AuNP of 1,200 in the fabrication of RGD/P-AuNPs.



1-3 Images of RGD/P-AuNPs

To observe the morphology of RGD/P-AuNPs, transmission electron microscope (TEM) was used. Dark-field TEM images of AuNPs are shown in Fig. 1-3A. Images of synthesized P-AuNPs and RGD/P-AuNPs are shown in Fig. 1-3B.

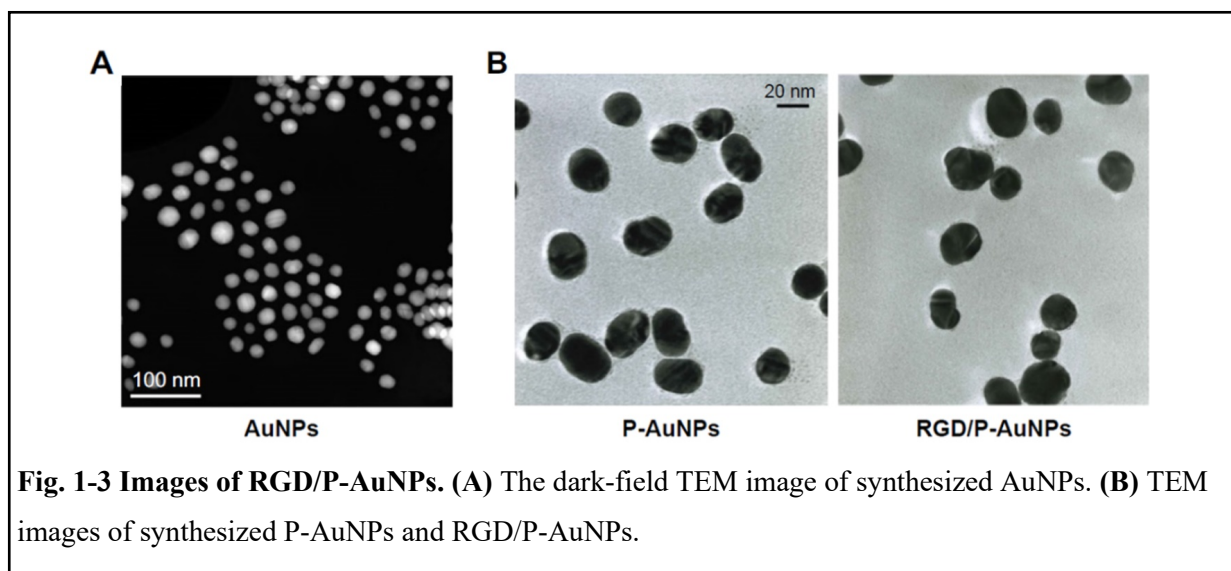


Fig. 1-3 Images of RGD/P-AuNPs. (A) The dark-field TEM image of synthesized AuNPs. **(B)** TEM images of synthesized P-AuNPs and RGD/P-AuNPs.

1-4 Expression of integrins in breast cancer cells

Various expression patterns of integrins are exhibited in several types of cancer cells (Desgrosellier and Cheresh, 2010). Our previous study showed that $\alpha 5 \beta 1$ -integrin is upregulated in highly invasive breast cancer cells in three-dimensional laminin-rich extracellular matrix (3D-lrECM) cultures (Nam et al., 2010). To determine which breast cancer cell lines can be targeted by RGD peptides, we compared the protein expression level of the $\alpha 5 \beta 1$ -, $\alpha v \beta 5$ - and $\alpha v \beta 3$ -integrins in several breast cancer cell lines. We found that the highly invasive breast cancer cell lines MDA-MB-231 and Hs578T significantly increased the expression of $\alpha 5$ - and αv -integrin heterodimers, but that the minimally invasive breast cancer cell line SK-BR-3 showed low integrin expression (Fig. 1-4).

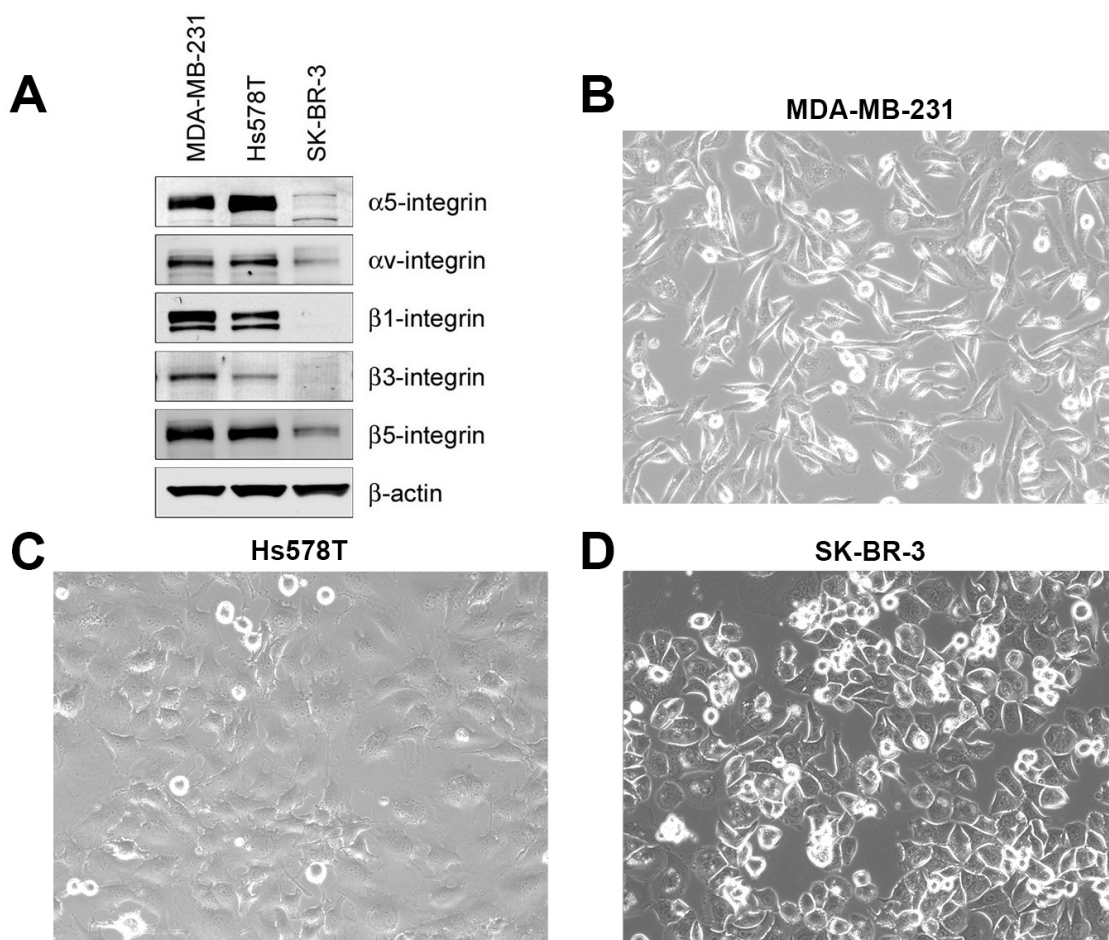


Fig. 1-4 Expression of integrins in breast cancer cells. (A) Protein expression of $\alpha 5\beta 1$ -, $\alpha v\beta 5$ - and $\alpha v\beta 3$ -integrins in breast cancer cell lines. Total cell lysates were subjected to Western blotting using the indicated antibodies. (The experiments were performed by Dr. Nam) (B) Cell morphology of MDA-MB-231 cells by phase-contrast microscopy. (C) Cell morphology of Hs578T cells by phase-contrast microscopy. (D) Cell morphology of SK-BR-3 cells by phase-contrast microscopy.

1-5 Internalization of different size of RGD/P-AuNPs in integrin-overexpressing breast cancer cells

The efficacy of AuNPs uptake in cancer cells has been reported to be related to its size (Chithrani et al., 2010). Therefore, we generated RGD/P-AuNPs using different sizes of i-colloid Au and confirmed their internalization in integrin-overexpressing breast cancer cell lines (Fig. 1-5). We found that 20 nm RGD/P-AuNPs internalized into breast cancer cells and accumulated significantly compared to P-AuNPs and 15 nm RGD/P-AuNPs in MDA-MB-231 and Hs578T cells (Fig. 1-5A). The diameter of 20 nm Au has increased to ~40 nm after PEGylation (Fig. 1-5B). Hence, we used 20 nm RGD/P-AuNPs for the following experiments.

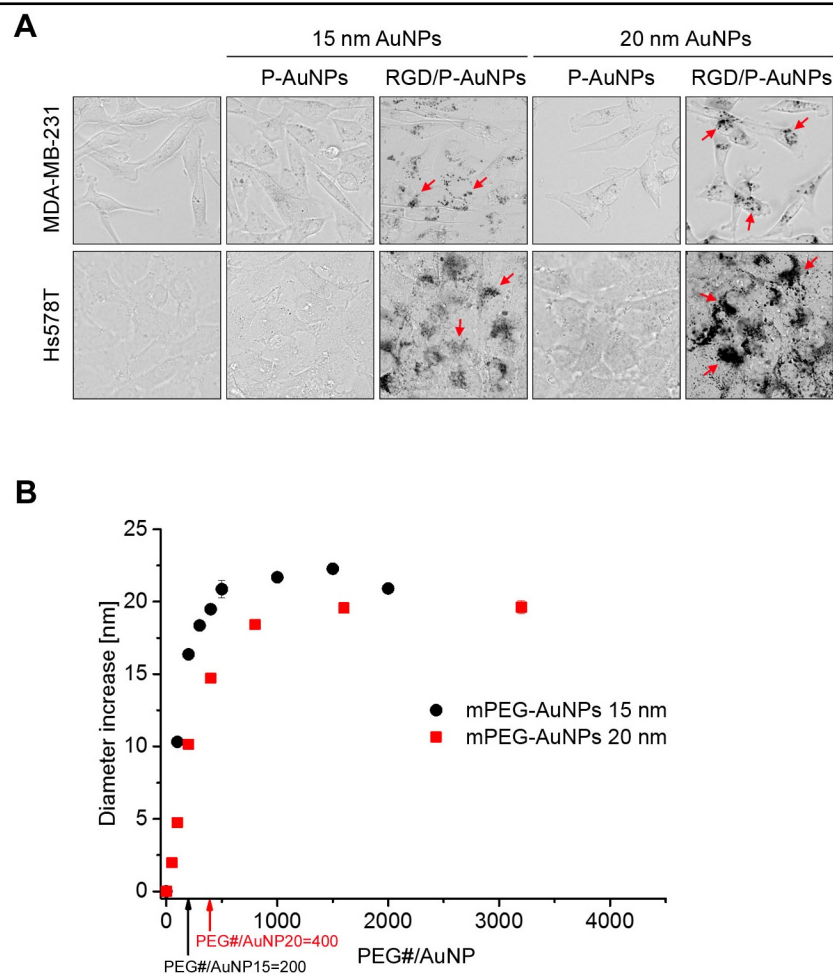


Fig. 1-5 Internalization of different sizes of RGD/P-AuNPs in integrin-overexpressing breast cancer cells. (A) Phase-contrast microscopic images of different-sized AuNPs in MDA-MB-231 and Hs578T cells. Red arrow: localization of RGD/P-AuNPs. Bar, 20 μ m. (The experiments were performed by Dr. Nam) (B) The hydrodynamic diameter of AuNPs was measured by DLS. Black arrow: saturation of RGD peptides on the surface of 15 nm-sized P-AuNPs; red arrow: saturation of RGD peptides on the surface of 20 nm-sized P-AuNPs. (The experiments were performed by Dr. Ichikawa)

1-6 Internalization of RGD/P-AuNPs in breast cancer cells with different integrin-expressing level

To determine the internalization of RGD/P-AuNPs in different cancer cells, we treated MDA-MB-231, Hs578T, and SK-BR-3 cells with P-AuNPs or RGD/P-AuNPs. Compared to integrin-overexpressing cancer cells, low integrin-expressing SK-BR-3 cells did not uptake the RGD/P-AuNPs as efficiently as the invasive cell lines (Fig. 1-6). These results indicate that the RGD/P-AuNPs can specifically target integrin-overexpressing breast cancer cells.

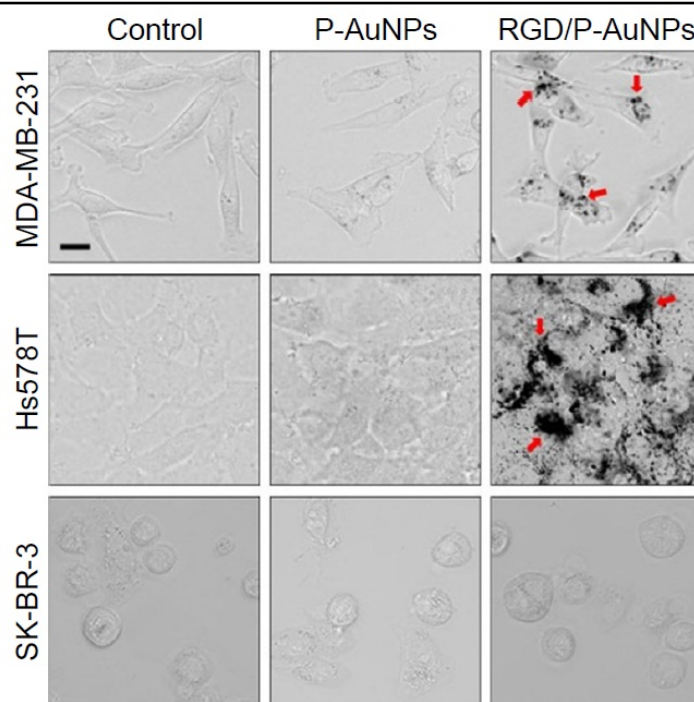


Fig. 1-6 Internalization of RGD/P-AuNPs in breast cancer cells with different integrin-expressing level. Phase-contrast microscopic images of MDA-MB-231, Hs578T, and SK-BR-3 cells cultured with or without AuNPs. Bar, 20 μ m.

1-7 Internalization of RGD/P-AuNPs with integrins in cancer cells

We generated fluorescent RGD/P-AuNPs (FITC/RGD/P-AuNPs) using FITC-PEG-SH5k instead of mPEG-SH5k and observed the uptake by fluorescence microscopy to determine the localization of RGD/P-AuNPs in cells. Immunofluorescent images obtained by confocal microscopy showed that FITC/RGD/P-AuNPs were colocalized with $\alpha 5$ - or αv -integrins in endosomal structures in MDA-MB-231 cells (Fig. 1-7), suggesting that RGD/P-AuNPs were uptaken into cells by integrin-mediated endocytosis.

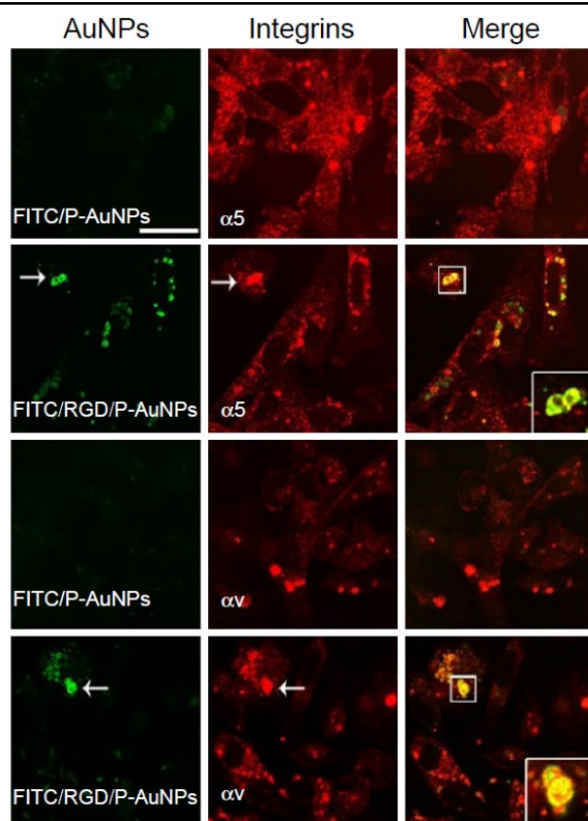


Fig. 1-7 Internalization of RGD/P-AuNPs with integrins in cells. Confocal immunofluorescence images of FITC-conjugated AuNPs (green) and integrins (red) in cells. MDA-MB-231 cells were cultured with FITC-conjugated AuNPs, and then integrins were labeled with an anti- $\alpha 5$ -integrin or αv -integrin antibody coupled to Alexa 568-labeled anti-mouse IgG antibody. White arrows: colocalized FITC/RGD/P-AuNPs or integrins. Bar, 25 μm . (The experiments were performed by Dr. Nam)

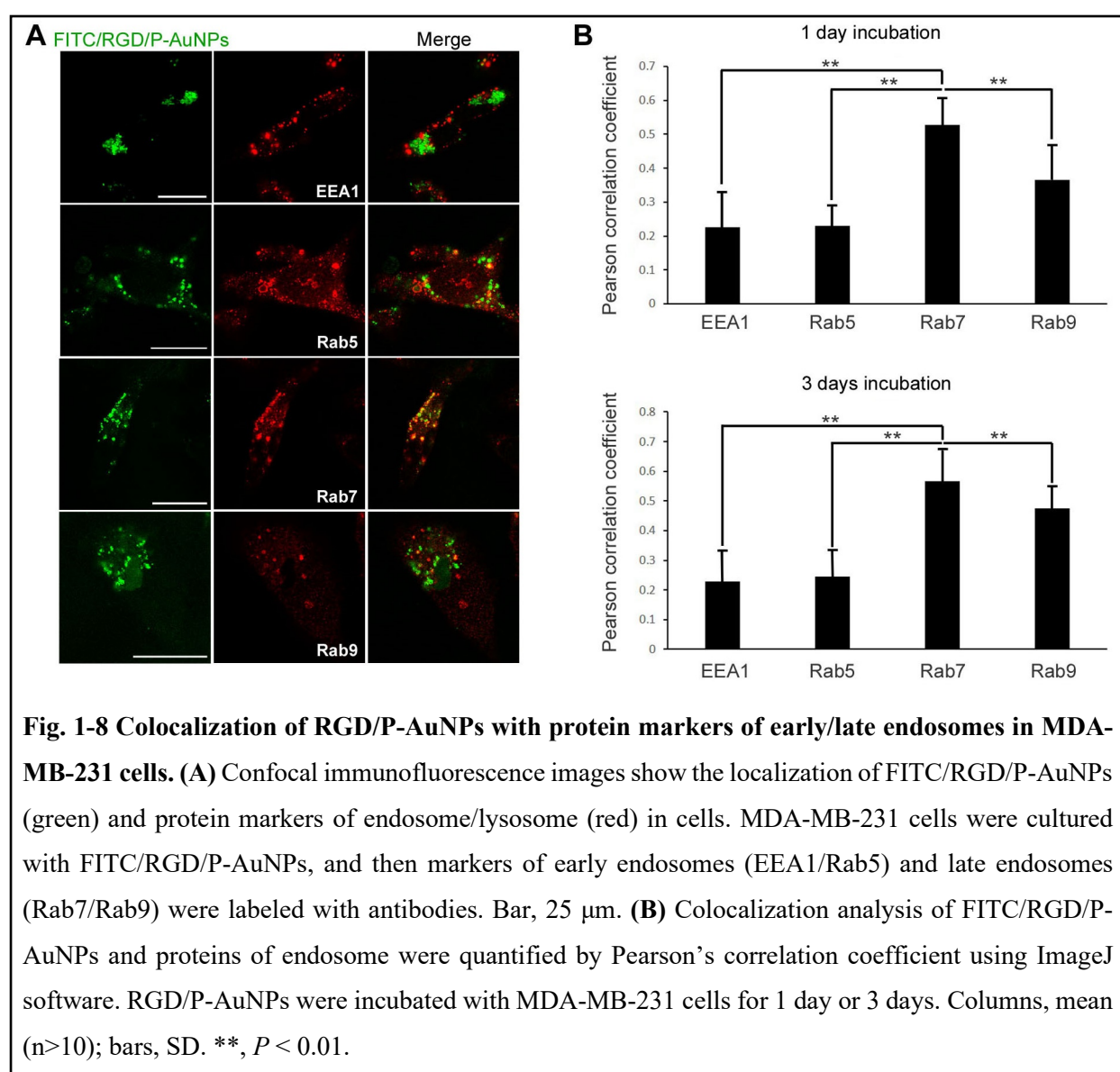
1-8 Intracellular trafficking of RGD/P-AuNPs

To evaluate the intracellular localization of RGD/P-AuNPs after uptake in cells by endocytosis, following protein markers were immunostained after the treatment of FITC/RGD/P-AuNPs in MDA-MB-231 cells (Fig. 1-8A).

Makers	Localization
Rab5	Early endosomes (Christoforidis et al., 1999)
Early endosome antigen 1 (EEA1)	Early endosomes (Zerial and McBride, 2001)
Rab7	Late endosomes (Feng et al., 1995)
Rab9	Late endosomes and the trans-Golgi network (Lombardi et al., 1993)

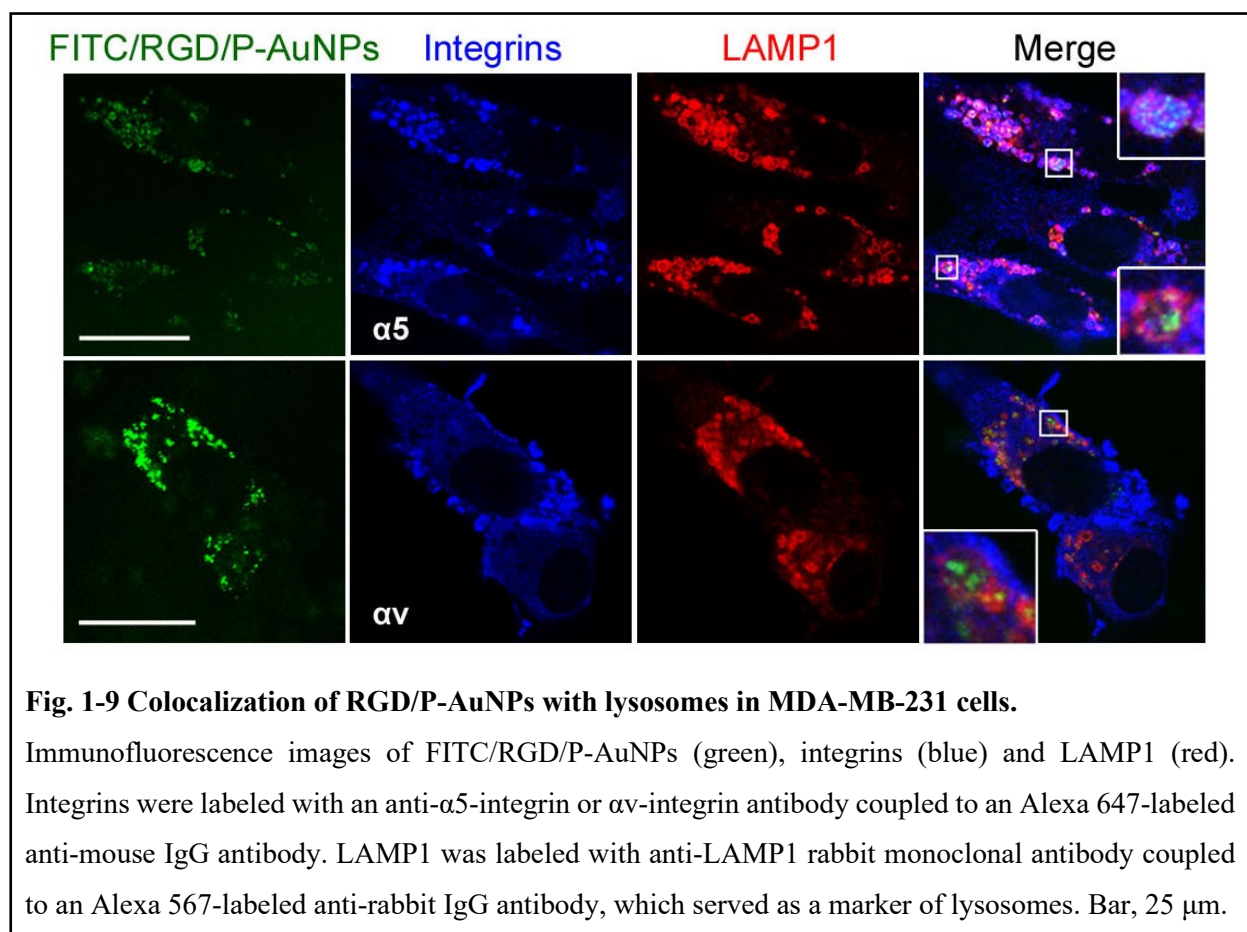
Lysosomal-associated membrane protein 1 (LAMP1)	Lysosomes
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The colocalization of FITC/RGD/P-AuNPs and the endosome markers was analyzed by determining the Pearson product-moment correlation coefficient using software Coloc 2, the Fiji's plugin of ImageJ. FITC/RGD/P-AuNPs were significantly colocalized with Rab7 compared to other markers (Fig. 1-8A and B). There were no significant differences between 1 day and 3 days of incubation of FITC/RGD/P-AuNPs (Fig. 1-8B). These data suggest that RGD/P-AuNPs are internalized by binding to integrins and trafficked to late endosomes in a similar manner to FN by integrin-mediated endocytosis.



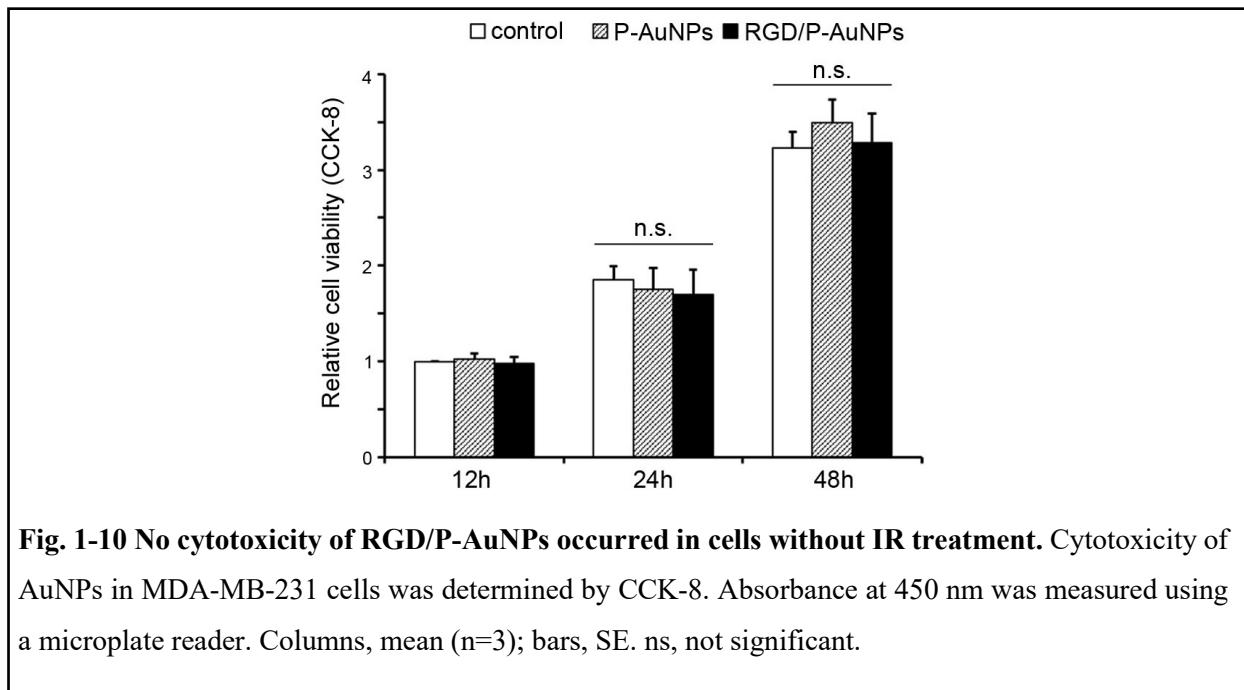
1-9 Colocalization of RGD/P-AuNPs with lysosomes in MDA-MB-231 cells

As FN and integrins were trafficked to lysosomes via late endosomes after ligand-receptor interactions (Rainero and Norman, 2013), we considered that integrin-bound RGD/P-AuNPs also localized to lysosomes as a transient destination. Confocal microscopy showed that FITC/RGD/P-AuNPs colocalized with or covered by LAMP1 (Fig. 1-9). These data suggest that RGD/P-AuNPs are internalized by binding to integrins and are trafficked to late endosomes and then to lysosomes.



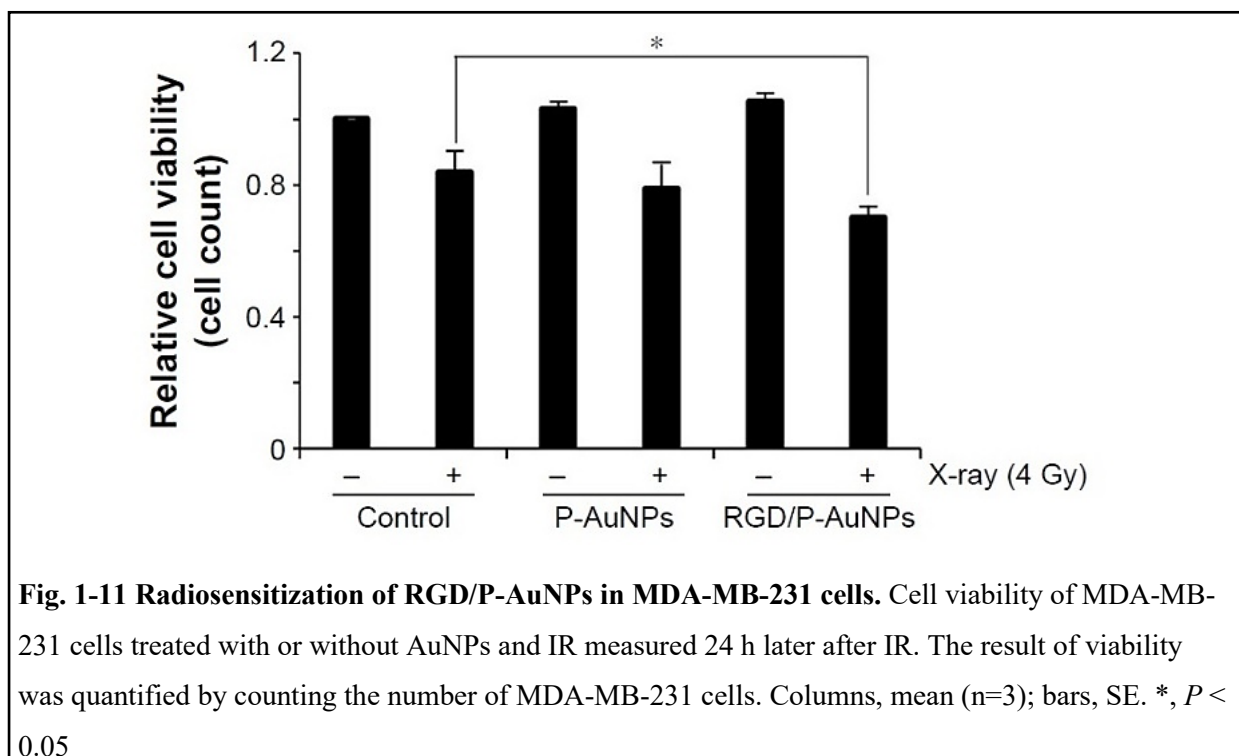
1-10 No cytotoxicity of RGD/P-AuNPs occurred in cells without IR treatment

To measure if RGD/P-AuNPs cause cytotoxicity to cells without IR, we measured cell viability after P-AuNPs or RGD/P-AuNPs treatments in MDA-MB-231 cells. Individually, P-AuNPs and RGD/P-AuNPs did not show significant cytotoxicity in MDA-MB-231 cells after 12 h, 24 h and 48 h of incubation (Fig. 1-10), suggesting the safety of AuNPs accumulation in cells without IR treatment.



1-11 Radiosensitization of RGD/P-AuNPs in MDA-MB-231 cells

AuNPs were reported to enhance the efficacy of IR in reducing cell viability (Chattopadhyay et al., 2013). To compare the radiosensitization of P-AuNPs and RGD/P-AuNPs, we measured cell viability after RGD/P-AuNPs followed by IR treatments. Importantly, the combination of 4 Gy IR following RGD/P-AuNP treatments significantly reduced cell viability compared with IR alone in MDA-MB-231 cells (Fig. 1-11).



1-12 RGD/P-AuNPs show no effect of radiosensitization in low integrin-overexpressing cells

Compared to integrin-overexpressing cells MDA-MB-231, the treatment of RGD/P-AuNPs followed by IR was less effective in the SK-BR-3 cell line (Fig. 1-12), which expresses low levels of integrins and exhibits low cellular uptake of RGD/P-AuNPs as shown above.

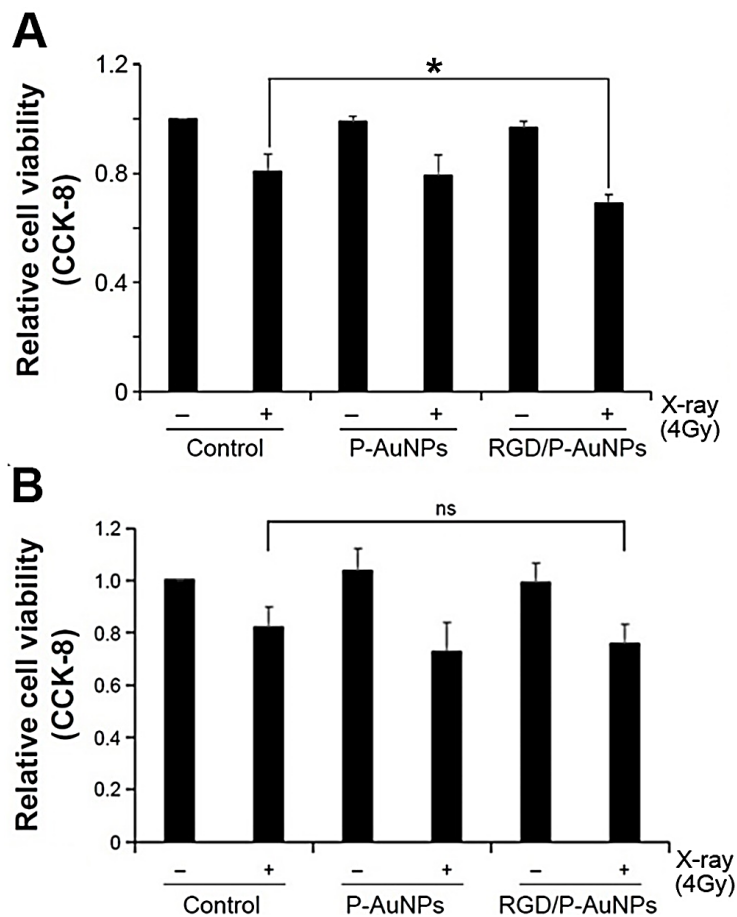
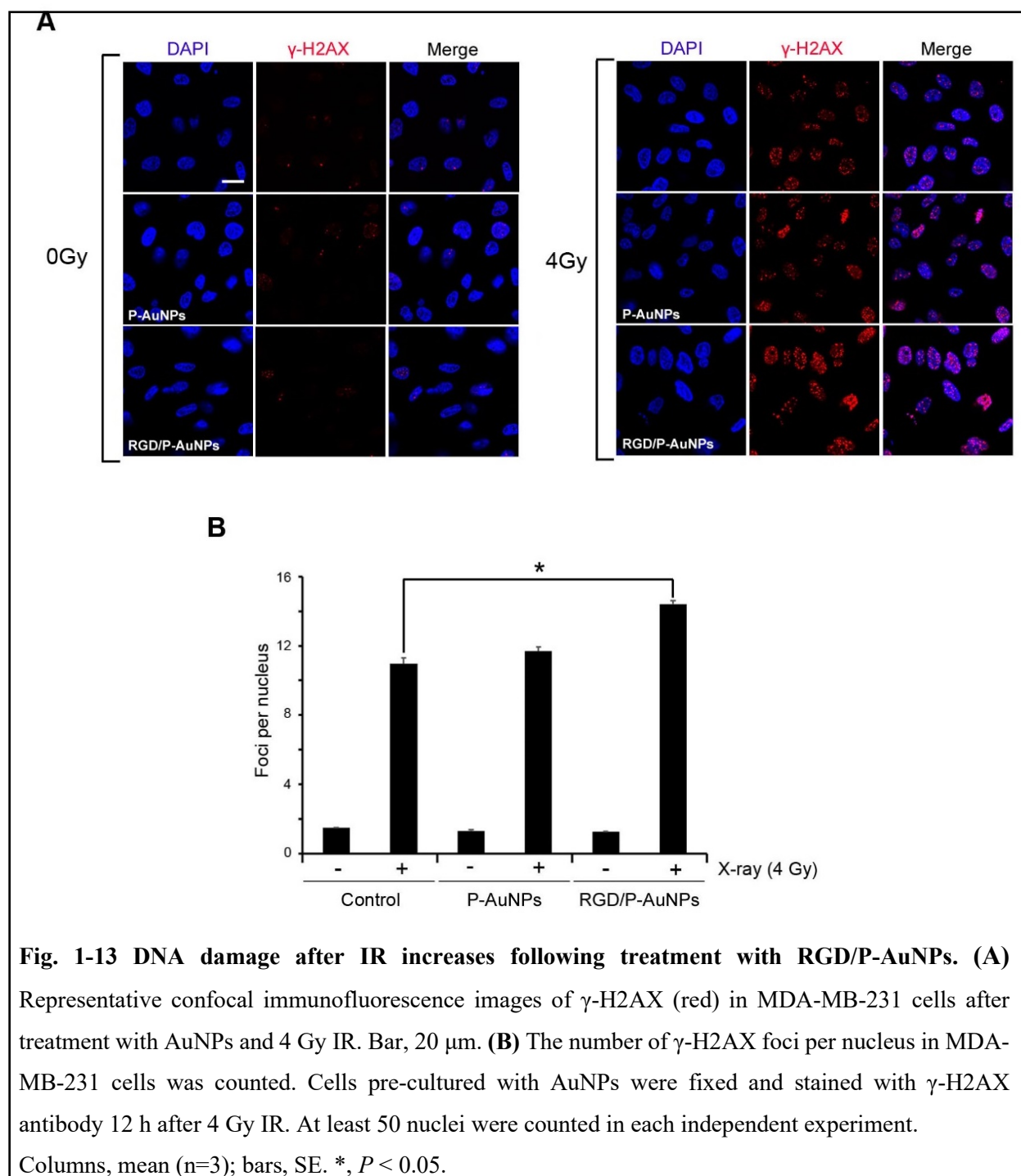


Fig. 1-12 RGD/P-AuNPs show no effect of radiosensitization in low integrin-overexpressing cells
(A) Viability of MDA-MB-231 cells treated with or without AuNPs and IR measured at 6 days after IR. Viability was determined using CCK-8. **(B)** Viability of SK-BR-3 cells treated with or without AuNPs and IR measured at 6 days after IR. Viability was determined using CCK-8. Columns, mean (n=3); bars, SE. *, $P < 0.05$, ns, not significant.

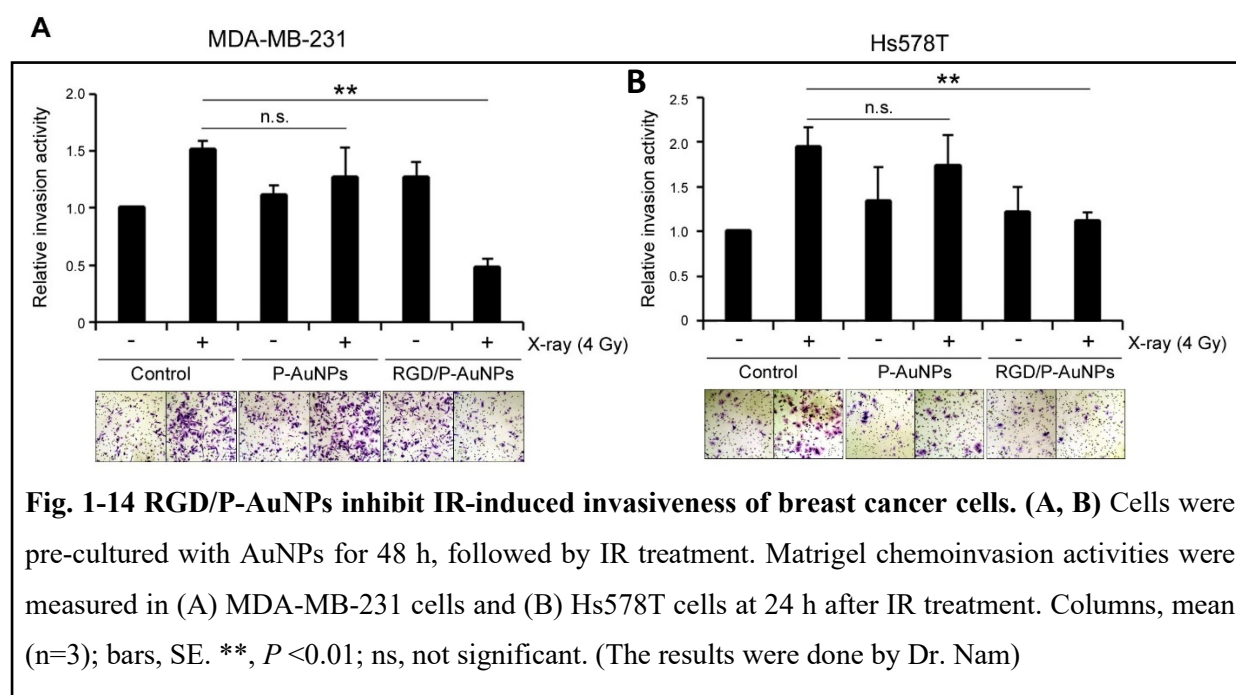
1-13 DNA damage of RGD/P-AuNPs and IR in MDA-MB-231 cells

To assess DNA damage after IR treatment, MDA-MB-231 cells after RGD/P-AuNP and IR treatment were fixed and stained with γ -H2AX, a marker of DNA damage and repair (Fig. 1-13A). The number of residual γ -H2AX foci 12 h after IR was significantly higher in the RGD/P-AuNPs and IR treatment group, compared to the group with treatment of IR alone (Fig. 1-13B). These data suggest that RGD/P-AuNPs can increase the cytotoxic effects of radiation on cancer cells by enhancing DNA damage.



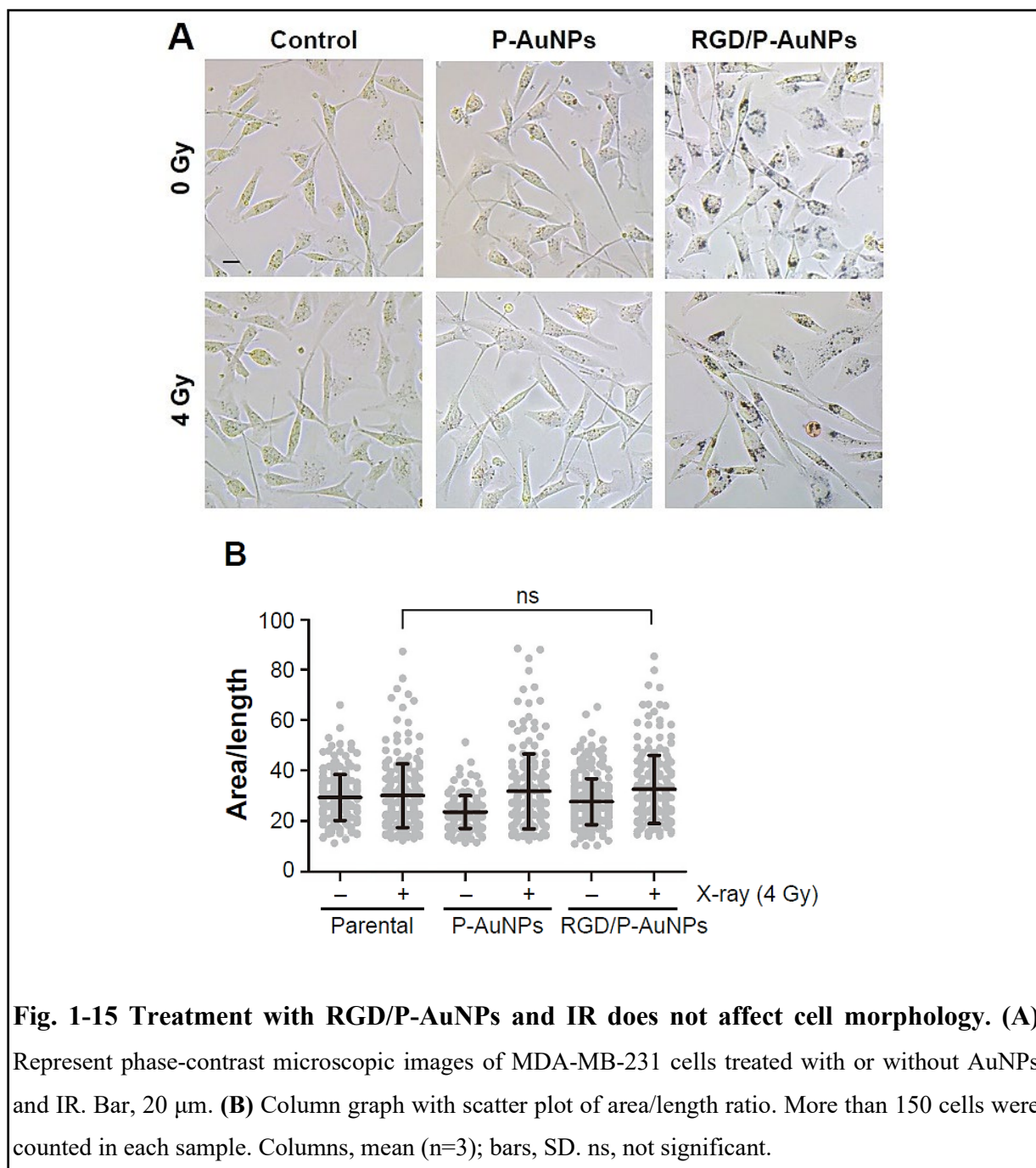
1-14 RGD/P-AuNPs inhibit IR-induced invasiveness of breast cancer cells

As described above, invasive recurrent disease after radiotherapy is a leading cause of mortality in breast cancer patients. Thus, we investigated the effect of RGD/P-AuNPs on cancer invasion after IR treatment. To evaluate the effect of invasive activity, we performed a Matrigel chemoinvasion assay. We found that invasion was increased in MDA-MB-231 or Hs578T cells 24 h after 4 Gy radiation treatment, known as IR-induced invasiveness. Strikingly, cells treated with RGD/P-AuNPs showed significantly decreased cell invasive activity compared to IR alone in MDA-MB-231 cells (Fig. 1-14A) or Hs578T cells (Fig. 1-14B). These results suggest that RGD/AuNPs suppress the IR-induced invasiveness of breast cancer cells.



1-15 Treatment with RGD/P-AuNPs and IR did not affect cell morphology

To determine whether there were changes in cell morphology that might have affected the result of cancer invasion after RGD/P-AuNP and IR treatments, we measured the cell morphology by calculating the area/length ratio. We found that treatment with RGD/P-AuNPs and IR did not affect cell morphology (Fig. 1-15).



1-16 Reduction of FN mRNA expression by combination treatment with RGD/P-AuNPs and IR

To understand the molecular mechanism of RGD/P-AuNPs on IR-related invasiveness, differential gene expression between the control group and RGD/P-AuNP-treated group was assessed by microarray. Within invasion-related genes (Tracey A. Martin, 2013), we found that FN expression was increased in the cells after 4 Gy IR, but this increase was negated by RGD/P-AuNP treatment (Table 1). We confirmed that the messenger RNA (mRNA) level of total FN was downregulated by RGD/P-AuNP and IR treatments based on using quantitative real-time PCR (Fig. 1-16).

Table 1 Expression of invasion-related genes in MDA-MB-231 cells

Gene symbol	Gene name	Global normalization				Fold-change of control and RGD/P-AuNPs groups (4 Gy)
		Control		RGD/P-AuNPs		
		0 Gy	4 Gy	0 Gy	4 Gy	
FN1	Fibronectin Precursor	81	428	88	215	0.50 *
COL6A1	Collagen alpha-1(VI) chain Precursor	22	20	17	15	0.75
COL1A2	Collagen alpha-2(VI) chain Precursor	6	5	4	4	0.80
C9orf126	Protein SCAI	10	12	10	10	0.83
CD44	CD44 antigen Precursor	262	269	281	245	0.91
CLDN7	Claudin-7	287	296	252	272	0.92
CLDN1	Claudin-1	22	23	22	22	0.96
MET	Hepatocyte growth factor receptor Precursor (HGF receptor)	140	146	106	141	0.97
ICAM1	Intercellular adhesion molecule 1 Precursor	105	89	83	87	0.98
BMP7	Bone morphogenetic protein 7 Precursor	2	2	1	2	1.00
CADM1	Cell adhesion molecule 1 Precursor	2	2	1	2	1.00
COL1A1	Collagen alpha-1(I) chain Precursor	2	2	1	2	1.00
COL3A1	Collagen alpha-1(III) chain Precursor	2	2	2	2	1.00
MMP9	Matrix metalloproteinase-9 Precursor	2	2	1	2	1.00
PCDH1	Protocadherin-15 Precursor	2	2	2	2	1.00
CDH3	Cadherin-3 Precursor	9	8	7	8	1.00
OCLN	Occludin	32	34	32	37	1.09
VTN	Vitronectin Precursor	31	27	29	31	1.15

Note: *, >1.5-fold increased (up) or <0.67-fold decreased (down) expression from the 4 Gy RGD/P-AuNPs group to the control group

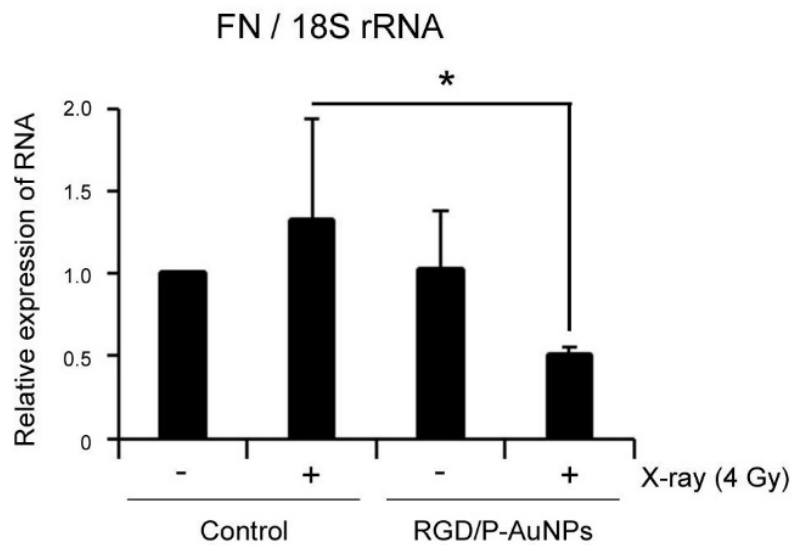
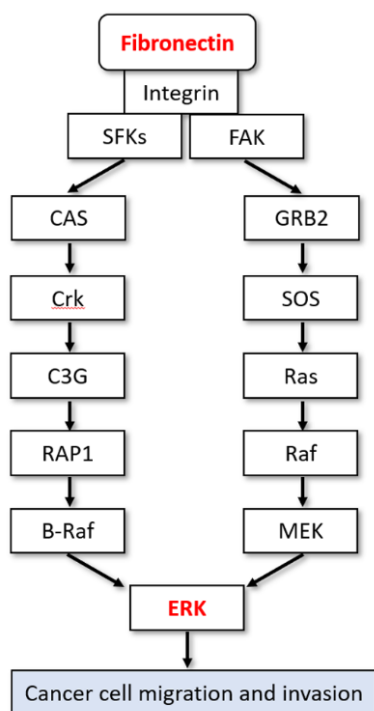
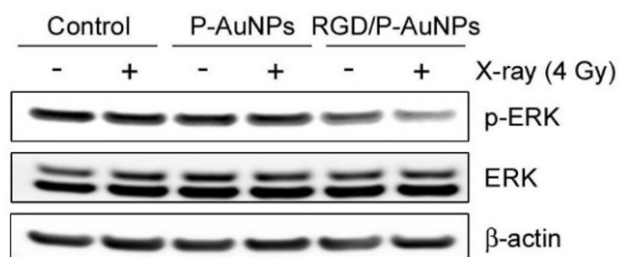


Fig. 1-16 Reduction of FN mRNA expression by combination treatment with RGD/P-AuNPs and IR. Quantification of mRNA levels of FN in MDA-MB-231 cells assessed by quantitative real-time PCR. The levels of FN are relative to 18S rRNA. Columns, mean (n=5); bars, SE. *, $P < 0.05$).

1-17 Suppression of extracellular signal-regulated kinase (ERK) signaling by combination treatment with RGD/P-AuNPs and IR

As a possible molecular mechanism that functions downstream of FN and integrins, we evaluated the potential role of ERK, a downstream protein of integrins implicated in cell invasion (Guo and Giancotti, 2004; Matsuo et al., 2006) (Fig. 1-17A). We hypothesized that the downregulation of FN and ERK signaling may be involved in suppressing invasive activity after treatment with RGD/P-AuNPs and IR. Indeed, ERK phosphorylation was downregulated after IR treatment in the presence of RGD/P-AuNPs (Fig. 1-17B). Taken together, these data suggest that the reduction of ERK signaling downstream of FN by RGD/P-AuNPs is involved in suppressing invasiveness post-IR, which may be a promising strategy for improving the efficacy of radiotherapy.

A**B**

Abbreviation	Proteins
C3G	Guanine nucleotide-exchange factor (GEF) of RAP1 protein
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
GRB2	Growth-factor-receptor-bound-2
MAPK	Mitogen-activated protein kinase
SFKs	Src-family kinases
SOS complex	Son-of-sevenless

Fig. 1-17 Suppression of ERK signaling by combination treatment with RGD/P-AuNPs and IR. (A) Signal pathway of FN and ERK. Modified from following reference (Guo and Giancotti, 2004). (B) Total cell lysates after AuNP and IR treatments were subjected to Western blot analysis using the indicated antibodies (n=3). (The result of Western blotting were done by Dr. Nam)

Discussion

In this chapter, we developed RGD/P-AuNPs to enhance the therapeutic effect of radiotherapy. RGD/P-AuNPs internalized effectively into integrin-overexpressing breast cancer cells, followed by increased DNA damage after IR treatment and suppressed IR-induced invasiveness.

Active targeting of RGD/P-AuNPs

The delivery of NPs to cancer cells is a major issue for NPs-based cancer treatment. To enhance the accumulation of NPs in cancer cells, scientists decorate NPs with targeting ligands that recognize specific receptors on the tumor cell surface, which is called active targeting (Bertrand et al., 2014). To optimize the efficiency of active targeting, choosing the appropriate targeting ligand is critical. Representative ligands used for active targeting in NPs contain antibodies, peptides, nucleic acids, sugars, and/or other small molecules (Muhamad et al., 2018). In this chapter, we used RGD peptides to modify AuNPs. Our result showed that RGD/P-AuNPs were stable with good efficacy in targeting integrin-overexpressing cancer cells *in vitro* experiments. For clinical application of RGD/P-AuNPs, the targeting efficacy in animal models should be further confirmed.

Radiosensitization of RGD/P-AuNPs

The localization of NPs in the cell may affect their effect (Anja Ostrowski, Beilstein J Nanotechnol. 2015). AuNPs after special functionalization can localize in specific cell organelles. Chang *et al.* reported that AuNPs localize in the endoplasmic reticulum (ER) and Golgi apparatus in murine melanoma cells (Chang et al., 2008). They hypothesized that continuous ER stress induced by accumulation of AuNPs results in apoptotic cell death. Mackey *et al.* conjugated AuNPs with a nuclear localization sequence peptide to induce internalization of AuNPs into the nucleus (Mackey et al., 2013). In contrast, other studies have revealed that the majority of AuNPs accumulated in endosomes or lysosomes. Ma *et al.* showed that AuNPs accumulate in the lysosomes in a size-dependent manner and impair the degradation capacity of lysosomes related to autophagy (Ma et al., 2011). Our results indicate that the surface modification of AuNPs with RGD peptide plays an important role in efficient cellular internalization by the endocytic pathway through integrins and the accumulation in late endosomes and lysosomes without significant degradation.

Our results showed that RGD/P-AuNP alone did not harm cells without IR treatment. However, DNA damage increased, and cell viability decreased following IR treatment in the

presence of RGD/P-AuNPs in MDA-MB-231 cells. A previous study shows that large AuNPs of 15 nm in diameter did not cause cytotoxicity in several cell lines (Pan et al., 2007). AuNPs show nearly no or very low cytotoxicity in cells and better biocompatibility compared to other high atomic number (Z) materials (Connor et al., 2005; Shukla et al., 2005). These results suggest that even RGD/P-AuNPs accumulated in unexpectable normal tissue outside the IR treatment field of the human body, they are safe and may not cause adverse effects without IR exposure.

Radiation kills cancer cells by ionizing water to form free radicals and causes double-strand DNA breaks. Regarding the radiosensitive effect of AuNPs, Zheng *et al.* treated plasmid DNA with 60 keV electrons alone or in the presence of AuNPs (Zheng et al., 2008). In their study, the AuNPs increased the number of DNA double-strand breaks by ~2.5-fold. To evaluate DNA damage, we used immunofluorescence study of γ -H2AX. Mirjolet *et al.* used titanate nanotubes (TiONts) to treat the SNB-19 and U87MG cell lines with 2 Gy IR (Mirjolet et al., 2013). The percentage of cells with high γ -H2AX foci (>8 per nucleus) increased in the TiONts and IR group compared to IR alone in SNB-19 human glioma cell line. Joh *et al.* used PEGylated AuNPs to treat the human U251 glioblastoma cell line with 4 Gy IR (Joh et al., 2013). In their study, a custom macro in ImageJ software was used to quantify γ -H2AX density, and AuNPs with IR led to a 1.7-fold increase in γ -H2AX density in U251 cells compared with the IR-alone group. Although previous studies have reported that AuNPs enhance DNA damage by IR treatment, Gilles *et al.* showed that AuNPs after functionalization conversely protect cells from DNA damage (Gilles et al., 2014). Their results showed that functionalized AuNPs decreased hydroxyl radical productions and lower DNA damage compared to non-functionalized AuNPs. Taking the results, they suggested that the functionalization of AuNPs should be designed well for the most efficiency in radiotherapy applications. Our results show that the RGD peptide-conjugated AuNPs increase DNA damage after radiation, which suggests that RGD/P-AuNPs do not reduce hydroxyl radical production and may not decrease the DNA damage after IR. In fact, compared to the IR-alone group, the average number of γ -H2AX foci per nucleus measured 12 h later increased in the combination group of RGD/P-AuNP and 4 Gy IR treatments. Importantly, the mechanism that increased the radiosensitivity of AuNPs may be attributed not only to DNA damage but also to the relationship between apoptosis induction, ROS production and mitochondrial alternation (Taggart et al., 2014), which should be measured in further research.

There are several different methods for evaluating the treatment results of IR in the

presence of AuNPs. Some studies used clonogenic assays with multiple radiation dosage to construct a survival curve (Butterworth et al., 2012; Liu et al., 2010; Rima et al., 2013; Roa et al., 2009), while others conducted viable cell counting, metabolic assays (enzymatic reduction of tetrazolium dye including MTT, MTS or WST-1) or resazurin sodium salt reduction (Alamar blue test) at a single radiation dose (Khoshgard et al., 2014; Kong et al., 2008; Xiao et al., 2011). Thus, it is difficult to compare our results from other studies because different cell lines, different types of AuNPs, radiation energy, radiation dosage, evaluation methods, and measurement time of cell viability after IR were used. In our study, the viability of MDA-MB-231 cells treated with RGD/P-AuNPs and 4 Gy IR decreased by ~17% according to cell counting assay results and decreased by ~16% based on CCK-8 metabolic assays compared to IR alone. Compared to other single-dose studies, Roa *et al.* used glucose-capped AuNPs to treat the prostate cancer cell line DU-145 with 2 Gy IR (Roa et al., 2009). The cell viability of DU-145 cells decreased, with a 26.8% inhibition rate measured by MTT assay. Butterworth *et al.* used 1.9 nm AuNPs to treat different cancer cell lines with 3 Gy IR (Butterworth et al., 2010). A significant cytotoxic effect of AuNPs was observed in MDA-MB-231 cells in a clonogenic assay, showing a 21% reduction in the surviving fraction; however, no reduction was observed in DU145 or L132 cells. Xiao *et al.* evaluated the viability of HeLa cells after exposure to multifunctional core/satellite nano theranostics followed by 6 Gy IR (Xiao et al., 2013). Their result showed that cell viability decreased by ~21% following treatment with nano theranostics compared to IR alone. According to these *in vitro* studies, cell viability after combination of AuNP and IR treatments was decreased by around 20%. Our data show similar results with recent studies, although actually it is difficult to compare them because of the different experimental conditions and evaluation methods.

RGD/P-AuNP and IR-induced invasiveness

In this study, we focused on not only the sensitizing effect of IR but also the invasive activity by RGD/P-AuNPs in cancer cells. The invasion of cancer cells is related to the regulation of several molecular factors, including cell adhesion molecules such as integrins, cadherins, desmosomes, selectins, CD44 and epithelial-mesenchymal transition (EMT)-related factors such as matrix metalloproteinases (MMPs), bone morphogenetic protein (BMP7) and epithelial protein lost in neoplasm (EPLIN) (Tracey A. Martin, 2013). Binding of integrins to their ligands, such as FN, activates focal adhesion kinase (FAK), which further activates multiple signaling proteins (Fig. 1-17). The phosphorylated FAK binds to growth factor receptor-bound protein 2 (GRB2) and activates the small GTPase Ras. Activated Ras recruits Raf, leading to mitogen-

activated protein kinase (MEK) and ERK activation (Hood and Cheresh, 2002). Several studies have suggested that targets downstream of ERK play key roles in angiogenesis, cell migration, invasion, and metastasis (Giehl, 2005; Reddy et al., 2003).

In our study, the expression of FN mRNA was suppressed by combination treatment with RGD/P-AuNPs and IR, which may lead to a decrease in FN. Liu *et al.* used AuNPs to treat lung cancer cells and investigated the cytotoxicity and the effect on the invasive activity of cells (Liu et al., 2014). Their study showed that invasive activity increased after AuNP treatment, and the mechanism may be associated with the increase of MMP9 and intercellular adhesion molecule 1 (ICAM1) expression. Based on our microarray analysis results, however, the expression of MMP9, ICAM1, and other invasion-related molecules did not clearly change. Further studies are needed to determine the detailed molecular mechanisms.

Chapter 2. Adjuvant of Lambda-carrageenan suppresses the radiation-induced invasive activity in cancer cells through Racgap1

Background

Recently, experimental and epidemiological studies have supported the concept that many different bioactive components of food (e.g. polyphenols, mono- and polyunsaturated fatty acids, methyl-group donors, etc.) may be implicated in the prevention or treatment to cancer (Crowe and Allison, 2015; Redondo-Blanco et al., 2017). At the cellular level, such compounds can have an impact on biological processes such as growth and differentiation, DNA repair, programmed cell death, and oxidative stress. In addition, compelling evidence suggests the existence of primary epigenetic targets of dietary compounds, which in turn impair the expression and function of target genes. In this chapter, we introduce a novel application of using a daily food additive, λ -carrageenan (CGN), as an adjuvant therapy to improve radiotherapy by the inhibition of specific protein expression.

CGN belongs to a family of linear sulfated polysaccharides that are extracted from red edible seaweeds and are widely used in the food industry for their gelling, thickening, and stabilizing properties. CGNs as food additives are safe for the human body and are approved under FDA regulations in the United States. Moreover, CGN has also been reported to exhibit diverse biological activities that include anticoagulants (Shanmugam and Mody, 2000), antiviral (Carlucci et al., 2004), and antitumorigenic effects (Luo et al., 2015). Recently, several studies have reported the antitumorigenic effects of CGN by inducing programmed cell death or stimulating cytotoxic immune responses (Li et al., 2017; Luo et al., 2015). However, the effect of CGN in combination with IR on cancer treatment has not been studied yet. Considering the safety profile of this agent, we evaluated the effect of CGN as an adjuvant treatment to radiotherapy.

In this chapter, we describe how we investigated the therapeutic efficacy of CGN combined with IR treatment on different cancer cell lines, and determined the underlying molecular mechanisms. We found that CGN treatment significantly suppressed the expression of RacGAP1, which correlated with the results of IR and CGN combined treatment in our study. RacGAP1 (also known as MgcRacGAP or hCYK-4) is a member of the guanine triphosphatase (GTPase) activation protein (GAP) family that has been identified as playing an important role

in promoting tumor progression in several types of cancer (Imaoka et al., 2015; Mi et al., 2016; Stone et al., 2010). RacGAP1 regulates the activation of Rho GTPase, which has been reported to drive tumor growth (Toure et al., 1998) and to act as an oncogene in basal-like breast cancers (Lawson and Der, 2018). Moreover, RacGAP1 is required for integrin-related invasive cell migration in the three-dimensional extracellular matrix (3D ECM) (Lawson and Ridley, 2018). In clinical studies, RacGAP1 has attracted increasing attention as a predictive biomarker for metastasis and prognosis in several types of cancers (Imaoka et al., 2015; Wang et al., 2018). In this study, we investigated the effect of CGN combined with IR on cancer treatment and determined its underlying molecular mechanisms. Our results indicate that CGN is a potential therapeutic adjuvant to radiotherapy, and can improve its therapeutic effect by suppressing RacGAP1 expression.

Materials and Methods

(The methods described in Chapter 1 are omitted)

1. Cell culture

The MDA-MD-231 human breast cancer cell line, PANC-1 human pancreatic cancer cell line, FaDu human head, and neck cancer cell line, and 4T1 mouse mammary carcinoma cell line were purchased from ATCC. MDA-MB-231 and PANC-1 cells were cultured in DMEM containing 10% FBS. FaDu cells were cultured in Minimum Essential Medium Eagle (Sigma-Aldrich) containing 10% FBS. 4T1 cells were cultured in RPMI-1640 (Sigma-Aldrich) containing 10% FBS.

2. Carrageenan

CGN plant mucopolysaccharide (Sigma-Aldrich, Lot number BCBP8978V) was dissolved in water at a concentration of 10 mg/mL. The typical molecular weight of λ -carrageenan was reported as 1,054 kDa (McKim, 2014). The purity of the carrageenan used in this study was $88.72\% \pm 2.21$, which was determined by EDTA/2-propanol recovery method (McKim et al., 2015). The amount of glucose/dextrose dissolved in the wash solution was also determined by Picoprobe Glucose Assay Kit (Abcam), and the results suggested that $4.23 \pm 0.62\%$ of the initial weight is accounted by glucose. To dissolve CGN in water, the solution of CGN was gently shaken for 24 h at 37°C. Then, the CGN was filtered through 0.45 μ m filters (Advantec). Cells were treated with 2.5 mg/mL CGN or Milli-Q water for 24 h after 4 Gy IR treatment.

3. Irradiations

Cells were irradiated with 4 Gy 130 kV X-rays using a CellRad X-ray generator (CellRad; Faxitron). Mice were irradiated with a daily fraction of 2 Gy 125 kV X-rays for 4 days (HITACHI).

4. Cell viability assay

After seeding in a dish for 24 h, the cells were treated with 4 Gy IR. Twenty-four hours after IR, the cells were then treated with 2.5 mg/mL CGN (approximately 2.2 mg/mL CGN was contained considering the purity mentioned above) or pure water for 48 h and then subjected to each experiment. Cell viability and cytotoxicity were examined by cell counting using the trypan blue exclusion method and flow cytometry after PI staining. The proportion of PI-positive cells was quantified by placing a polygon gate.

5. Apoptosis analysis

To determine apoptosis, Annexin V staining was used by Annexin V-FITC Apoptosis Detection Kit (Abcam). Cells more than 1×10^5 were harvested, resuspended in 500 μ L Annexin V binding buffer, and incubated with Annexin V-FITC and PI for 5 min at room temperature in the dark. Fluorescence was analyzed using a FACS Aria III flow cytometer (BD Biosciences). The percentage of FITC-positive cells was calculated and normalized from the untreated group. To determine caspase activity, Caspase-3, Caspase-8, and Caspase-9 Multiplex Activity Assay Kit (Abcam) was used. Briefly, cells were treated and seeded in 96-well plates at 2×10^4 cells/100 μ L FACS buffer (2% FBS in PBS). After incubation at 37°C, 5% CO₂ for 1 h, fluorescence was monitored using a microplate reader (CLARIOstar) with the following wavelengths: caspase-3 excitation (Ex)/emission (Em) = 535/620 nm; caspase-8 Ex/Em = 490/525 nm; caspase-9 Ex/Em = 370/450 nm.

6. ROS detection assay

To measure ROS levels in the cells, the treated cells were stained with 20 μ M dichlorofluorescein diacetate (DCFDA) for 30 min at 37°C using a Cellular ROS Detection Assay Kit (Abcam). The cells were then analyzed using a FACS Aria III flow cytometer.

7. DNA content and polyploidy analysis

For polyploidy analysis, 70% of ethanol was added slowly to cell pellets. The cells were stored at -80°C overnight, and then the cells were centrifuged and washed with cold PBS twice. The cells were then resuspended in 300 μ L staining solution (0.1% (v/v) Triton X-100, 2 mg RNase A (NIPPON GENE) and 400 μ L of 500 μ g/mL PI (Setareh Biotech) in 10 mL PBS. After incubation at 37°C for 15 min, samples were analyzed by a FACS Aria III flow cytometer.

8. Immunofluorescence

The cells were fixed in 4% paraformaldehyde (PFA), permeabilized with 0.2% Triton X-100/PBS, and then washed with PBS. For examination of polyploidy, cells were incubated with an α -tubulin (Cell Signaling Technology) antibody after blocking, and then washed with PBS, followed by incubation with an Alexa Fluor secondary antibody. Cell nuclei were counterstained with PI. For images of RacGAP1 localization, cells were stained with an anti-RacGAP1 antibody (Abcam), an α -tubulin antibody and DAPI. Images were acquired by Leica True Confocal Scanning (TCS) SP8 microscope system (Leica Microsystems).

9. Microarray analysis

After treatment with CGN and IR, total RNA of the MDA-MB-231 cells was isolated using a NucleoSpin® RNA kit (MACHEREY-NAGEL). For the microarray analysis, the high sensitivity 3D-Gene® Human oligo chip 25k ver 2.10 (Toray Industries) was used. The data were normalized and analyzed by Toray Industries. Gene expression values lower than 100 after global normalization were excluded. To focus on the effects of CGN, genes were selected in the following order (Fig. 2-12). First, genes increased 1.25-fold or higher in the IR group compared to the untreated group were selected. Second, genes reduced to one-eighth or less in the IR and CGN treatment groups, compared to untreated, were selected. The genes that meet both of these criteria were selected as candidates for further analyses.

10. siRNA and transfection

To knockdown RacGAP1, siRNAs with the following sequences were used:

siRNA	sense (5'-3')	antisense (5'-3')
scramble	GUUUAUUGACAAGUUAAGAd TdT	UCUUAACUUGUCAAAUAAACdT dT
RacGAP1 #1	CAGGUGGAUGUAGAGAUCAA AdTdT	UUUGAUCUCUACAUCCACCUG dTdT
RacGAP1 #2	CUAGGACGACAAGGCAACUU UdTdT	AAAGUUGCCUUGUCGUCCUAG dTdT

The siRNA duplexes were synthesized by Hokkaido System Science. The Cells were transfected with siRNA duplexes using Lipofectamine RNAiMAX for 2 days (Thermo Fisher Scientific).

11. Overexpression

For the RacGAP1 overexpression experiment, complementary DNA (cDNA) of RacGAP1 was obtained by PCR from the first-strand cDNA of the MDA-MB-231 cells. The RacGAP1 cDNA was subcloned into the mVenus N1 vector, and followed by subcloning into a PiggyBac transposon-based doxycycline-inducible vector, pPB-TRE3G-MCS-CEH-rtTA3-IP (Onodera et al., 2018). Transfection of the resulting RacGAP1-mVenus plasmid, together with a hyperactive PiggyBac transposase vector (Onodera et al., 2018) to MDA-MB-231 cells was performed by ViaFect Transfection Reagent (Promega), and cells were selected by puromycin. The expression of RacGAP1-mVenus was induced by the addition of 200 ng/mL doxycycline.

All procedures followed the Hokkaido University Safety Management Regulations on Genetic Recombination.

12. In vivo study

One million 4T1 cells in 100 μ L PBS were injected into the left thighs of 6-week-old Balb/c mice subcutaneously. Eleven days after inoculation, the mouse tumors were treated with a daily fraction of 2 Gy of X-rays for 4 days under anesthesia. Tumors were treated with 50 mg/kg CGN or PBS 3 times by intratumoral injection 1 week after the first IR fraction. Tumor size was measured after IR, and was normalized to the tumor size measured on the first day after radiation. All animal procedures were approved by the Institutional Animal Care and Use Committee of Hokkaido University (#16-0137).

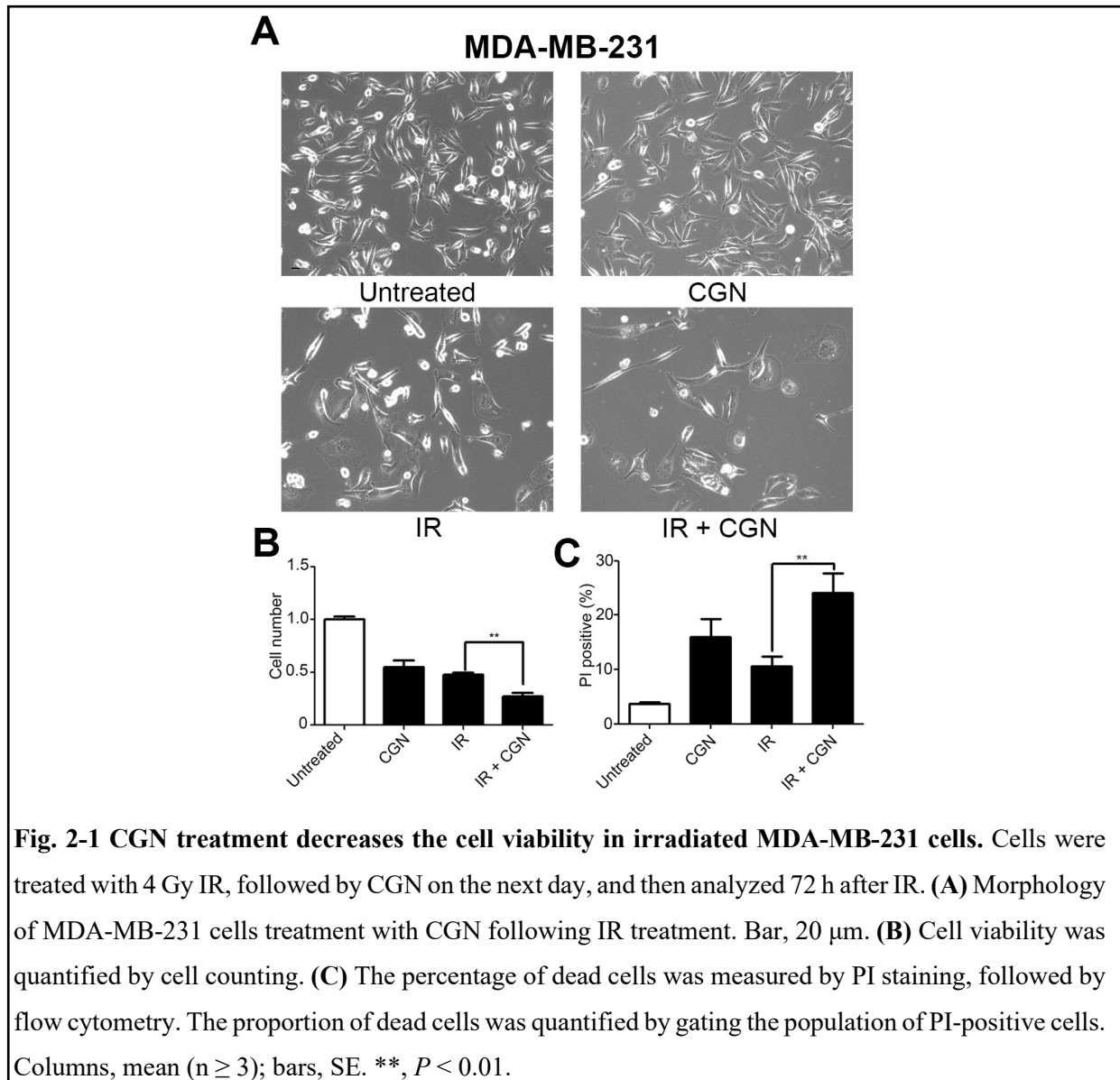
13. Immunohistochemistry

The tumor tissues were collected and placed in 4% PFA solution, fixed for 24 h, dehydrated through a gradient of ethanol, and embedded into paraffin blocks for immunohistochemistry. The paraffin blocks were cut to 4 μ m sections and mounted onto microscope slides for analysis. For antigen retrieval, the slides of tumor sections were incubated with antigen unmasking solution (Vector Laboratories) at 80°C for 1 h. Endogenous peroxidase activity was quenched using 3% H₂O₂ in 10% methanol. Each slide was incubated in 2% blocking buffer (Roche) for 1 h, and then incubated with a RacGAP1 primary antibody (Proteintech) overnight. Super Sensitive IHC Detection Systems (BioGenex) were used to amplify the signal. Sections were stained with horseradish peroxidase (HRP) secondary antibodies. After two washes, the slides were counterstained with hematoxylin (Muto Pure Chemicals). Positive staining was scored using the following formula: $(r3/t) \times 3 + (r2/t) \times 2 + (r1/t) \times 1$. Where t is the total area of tumor tissue for a whole tumor section, r3 is the total area of high-intensity staining (intensity 3), r2 is the total area of medium intensity staining (intensity 2), and r1 is the total area of weak intensity staining (intensity 1).

Results

2-1 CGN treatment decreases the cell viability in irradiated cancer cell lines

Several studies have reported using polysaccharides as an adjuvant to improve the anti-tumor efficacy of traditional chemotherapy (Zhang et al., 2016; Zhao et al., 2013). Here, we determined the anti-tumor effects of CGN combined with IR in the MDA-MB-231 human breast cancer cell lines (Fig. 2-1), FaDu human head and neck cancer cell lines (Fig. 2-2), and PANC-1 human pancreatic cancer cell lines (Fig. 2-3). In each case, when compared to IR alone, the cell number decreased and propidium iodide (PI) positive cells increased significantly when IR followed by CGN treatment. The result also showed that CGN alone caused damage to cancer cells (Fig. 2-1 to 2-3).



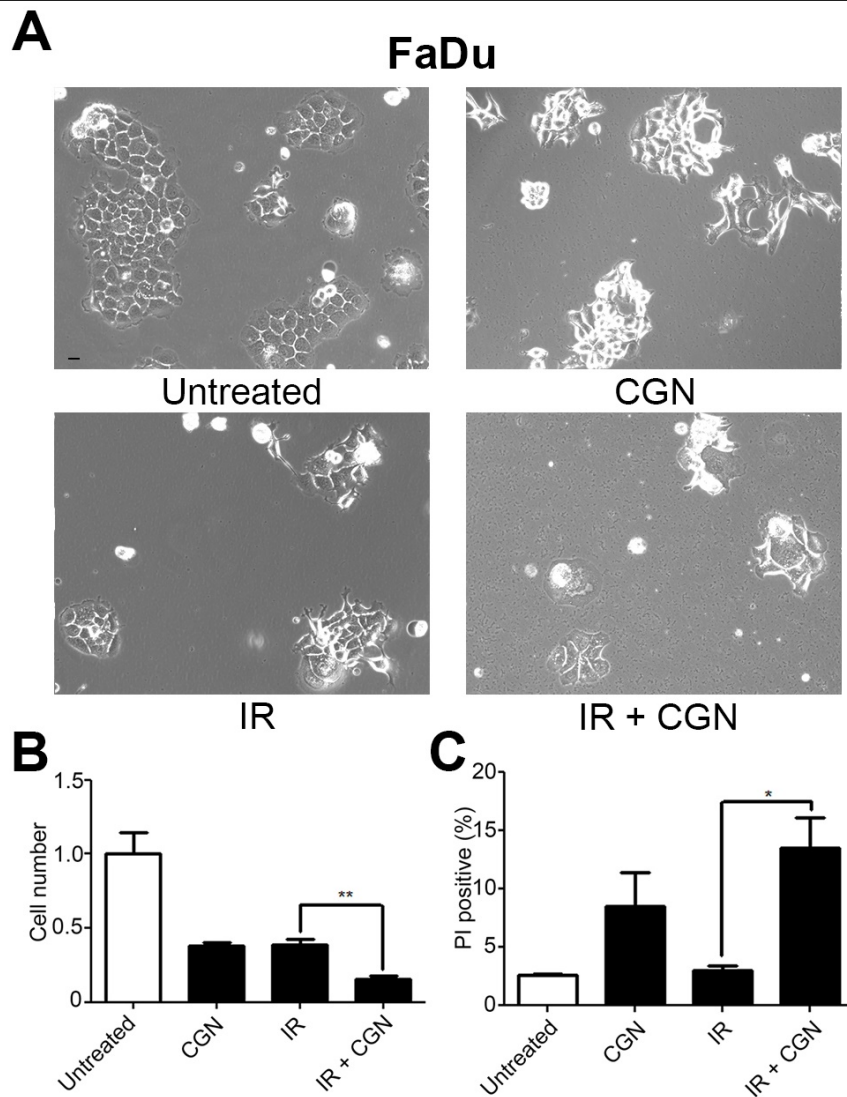


Fig. 2-2 CGN treatment decreases the cell viability in irradiated FaDu cells. Cells were treated with 4 Gy IR, followed by CGN on the next day, and then analyzed 72 h after IR. **(A)** Morphology of FaDu cells treatment with CGN following IR treatment. Bar, 20 μ m. **(B)** Cell viability was quantified by cell counting. **(C)** The percentage of dead cells was measured by PI staining, followed by flow cytometry. The proportion of dead cells was quantified by gating the population of PI-positive cells. Columns, mean ($n \geq 3$); bars, SE. *, $P < 0.05$; **, $P < 0.01$.

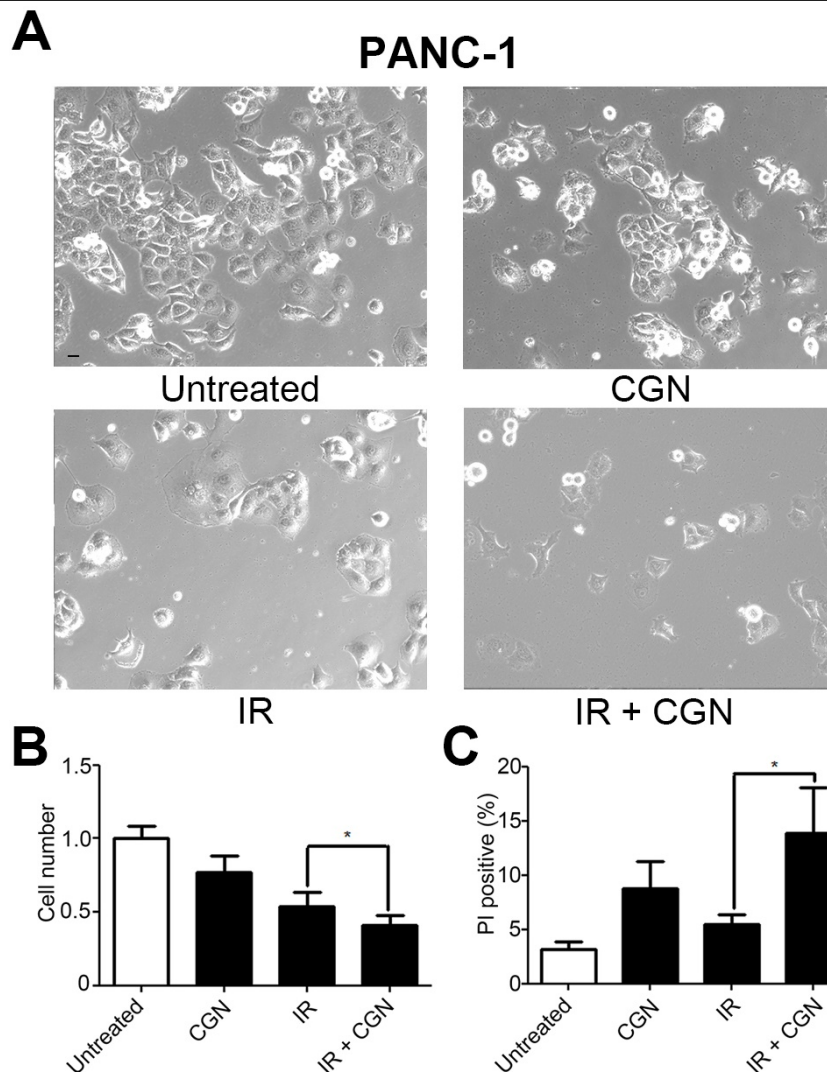
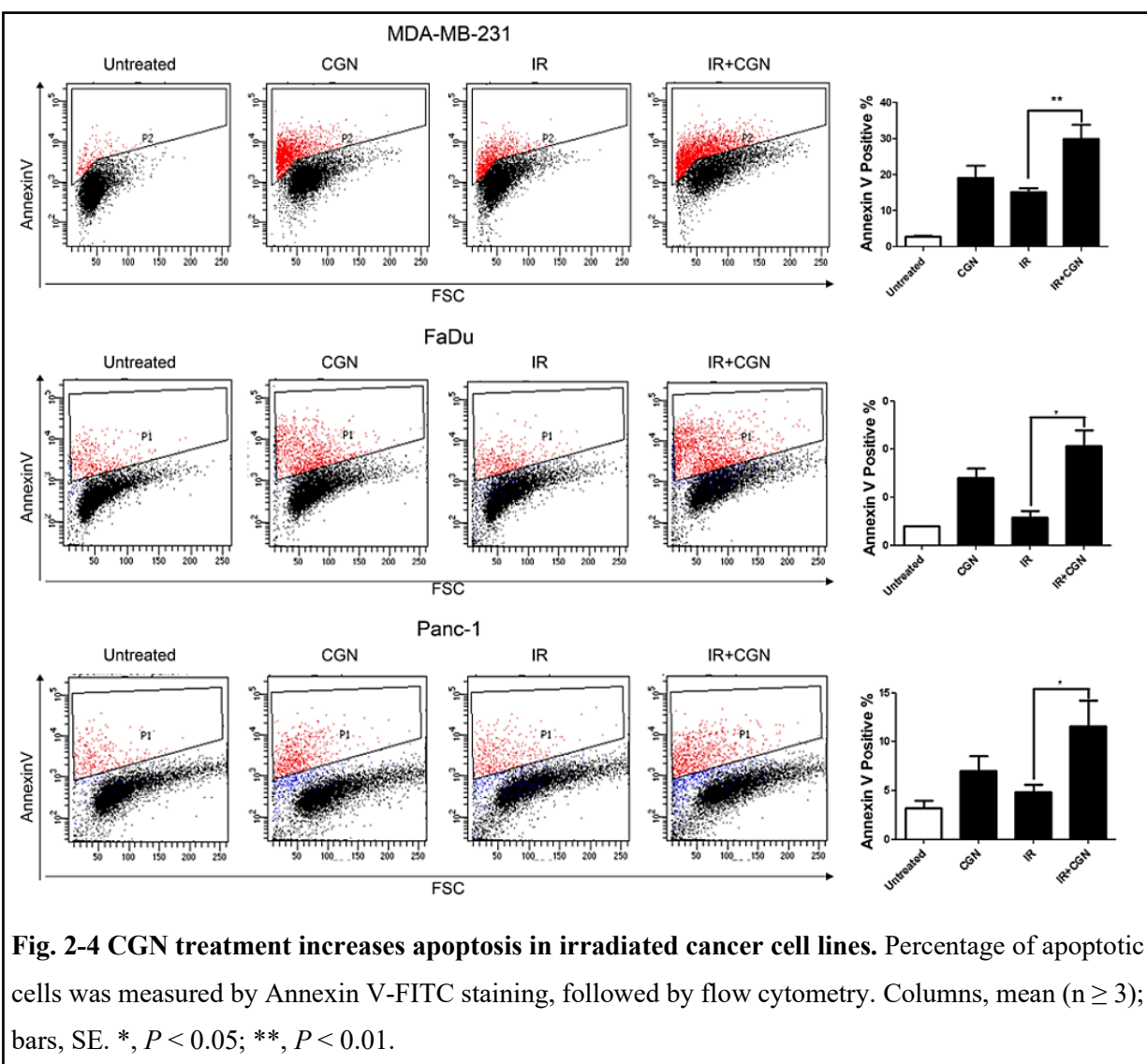


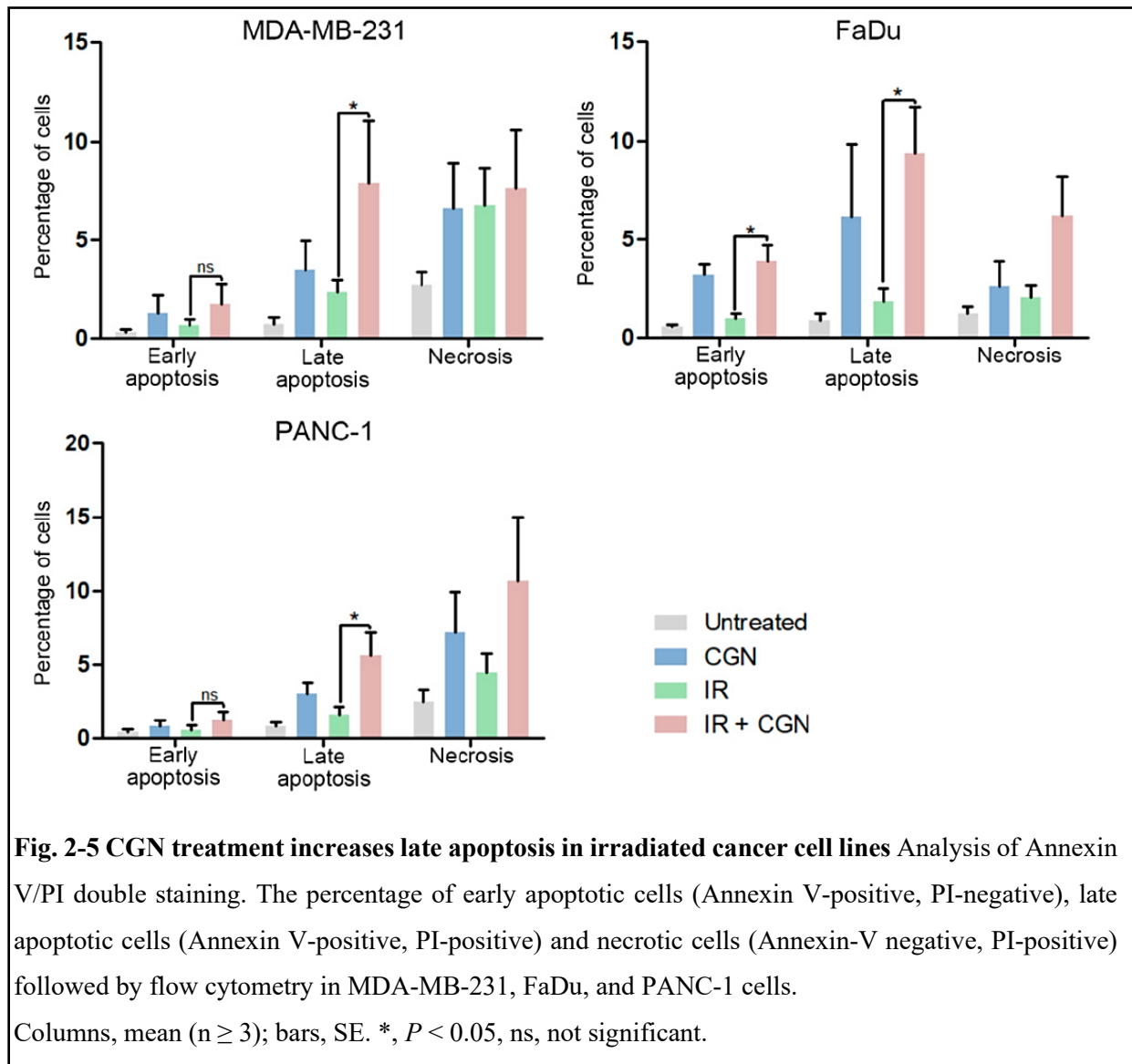
Fig. 2-3 CGN treatment decreases the cell viability in irradiated PANC-1 cells. Cells were treated with 4 Gy IR, followed by CGN on the next day, and then analyzed 72 h after IR. **(A)** Morphology of PANC-1 cells treatment with CGN following IR treatment. Bar, 20 μ m. **(B)** Cell viability was quantified by cell counting. **(C)** The percentage of dead cells was measured by PI staining, followed by flow cytometry. The proportion of dead cells was quantified by gating the population of PI-positive cells. Columns, mean ($n \geq 3$); bars, SE. *, $P < 0.05$.

2-2 CGN treatment increased apoptosis in irradiated cancer cell lines

To further analyze the mechanism of cell death after CGN following IR treatment, we next measured the cell apoptosis by staining the cells with Annexin V. CGN treatment following IR led to a significant increase in Annexin V-positive cells compared to the IR alone group (Fig. 2-4). Annexin V/PI double staining analysis showed that late apoptosis was increased in cells treated with IR and CGN compared to those treated with IR alone (Fig. 2-5). These results

suggest that CGN combined with IR decreases viability in several cancer cell lines by inducing apoptotic cell death.





2-3 Effect of CGN and IR treatment in non-malignant cells

We evaluated the effect of CGN and IR treatment in non-malignant epithelial cell line MCF10A (Fig. 2-6). The CGN-induced toxicity following IR treatment in MCF10A cells was not higher than that in other malignant cells.

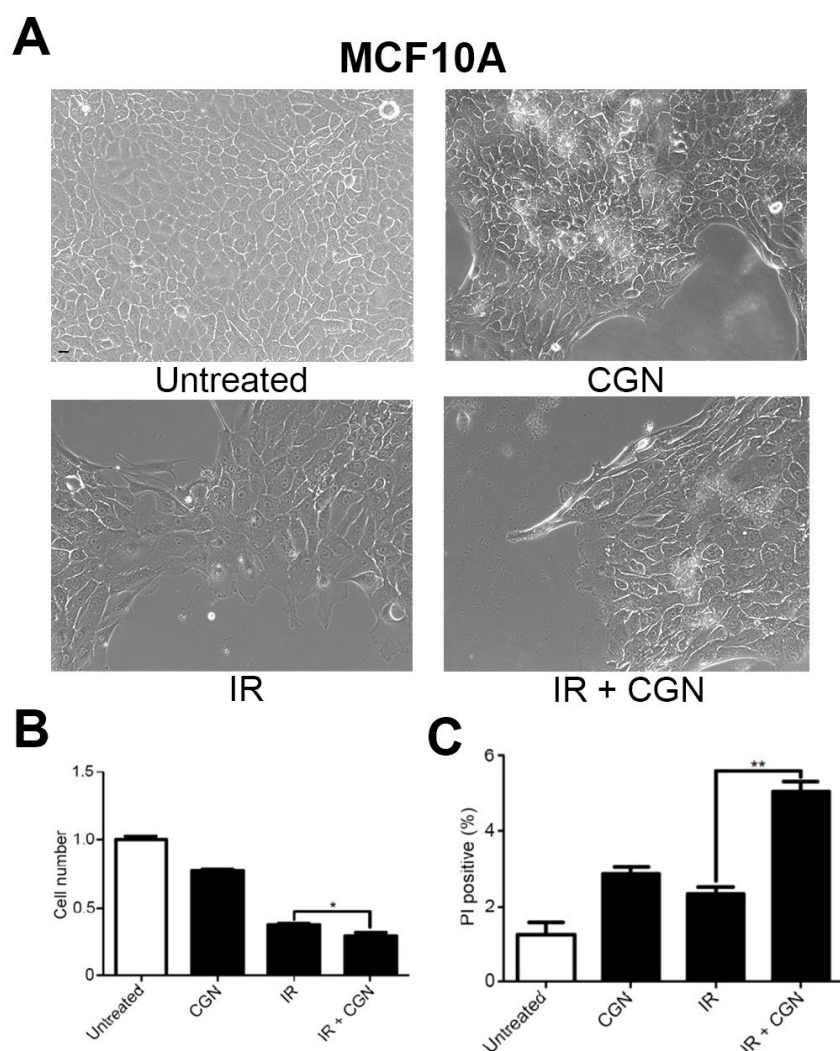
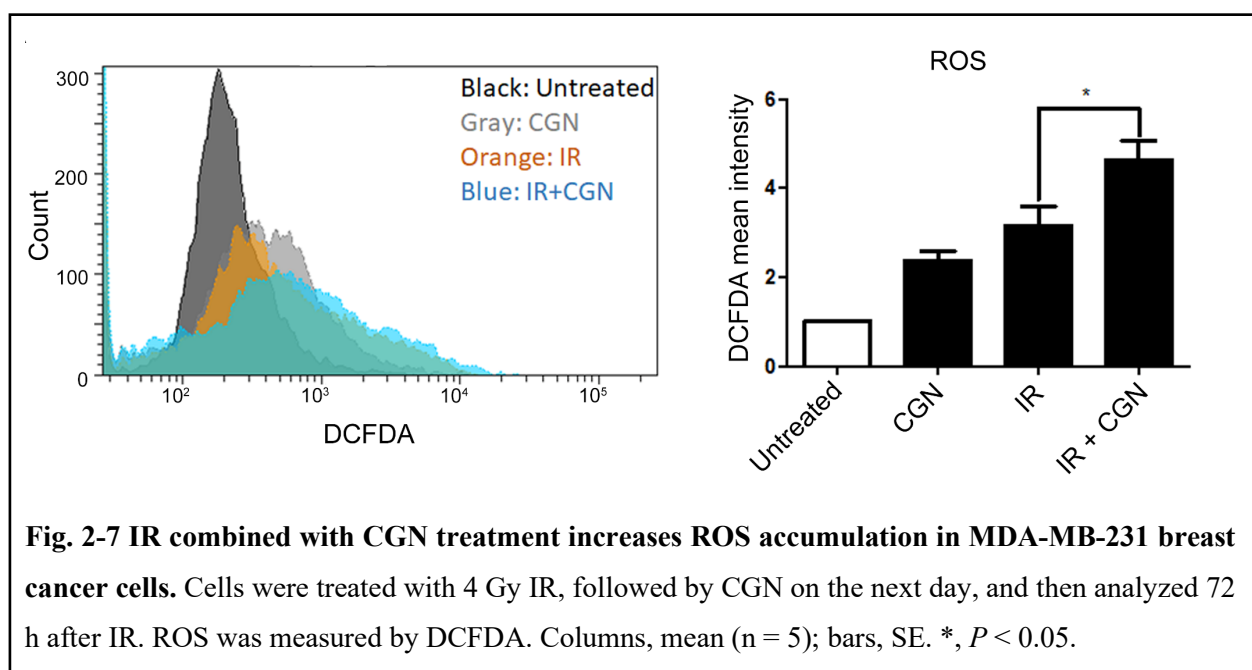


Fig. 2-6 Effect of CGN and IR treatment in non-malignant cells. Normal breast cell line MCF10A was treated with 4 Gy IR, followed by CGN on the next day, and then analyzed 72 h after IR. Bar, 20 μ m. **(A)** Morphology of MCF10A cells treatment with CGN following IR treatment **(B)** Cell viability was quantified by cell counting. **(C)** The percentage of dead cells was measured by PI staining, followed by flow cytometry. Columns, mean (n = 3); bars, SE. *, $P < 0.05$; **, $P < 0.01$; ns, not significant.

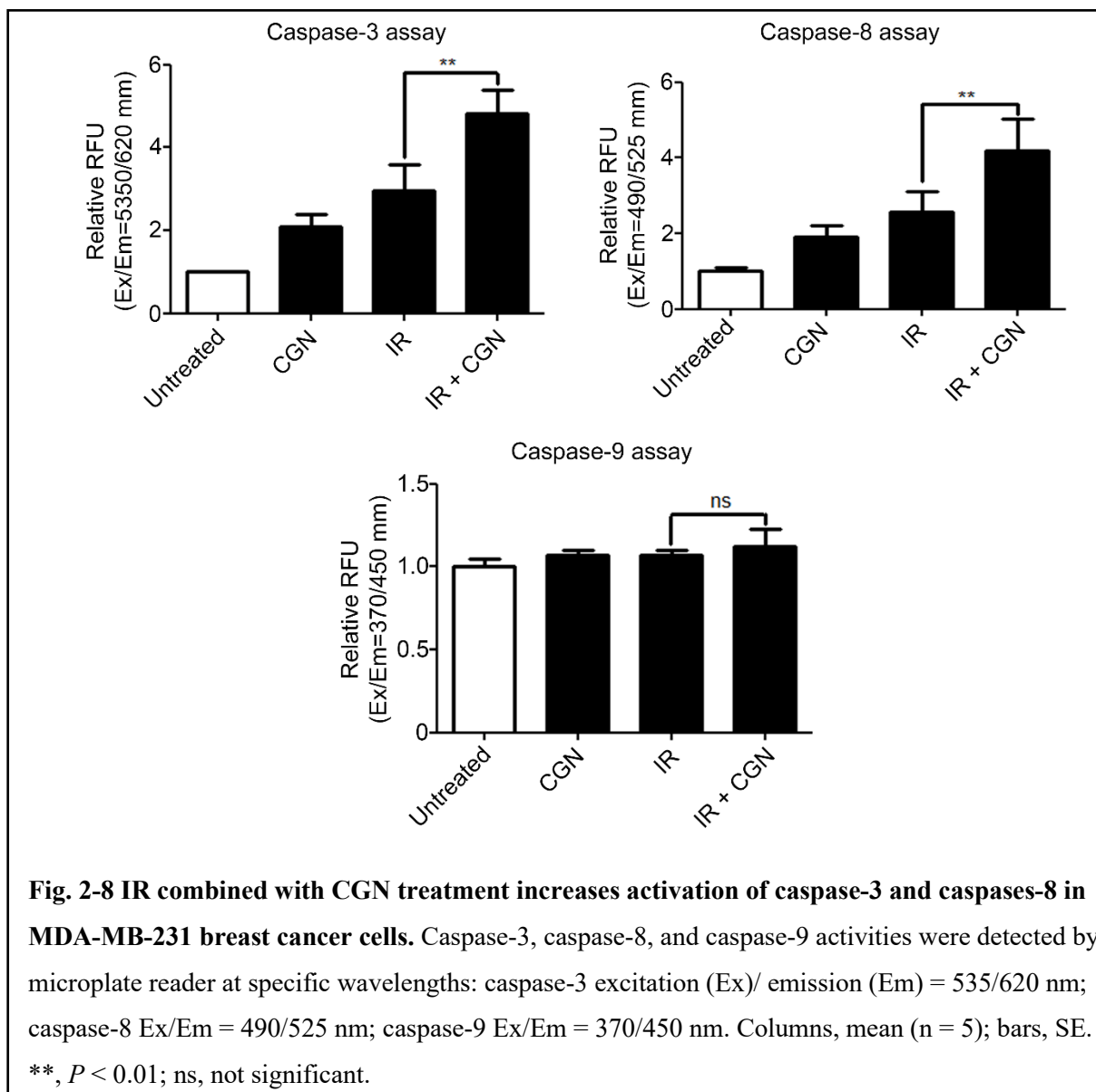
2-4 IR combined with CGN treatment increases ROS accumulation in MDA-MB-231 breast cancer cells

ROS is an important factor related to IR-induced cancer cell death (Gupta et al., 2012). It is known that IR induces ROS, which mediates apoptotic cell death and mitotic failure. Besides, CGN has been reported to increase the production of ROS in human colonic epithelial cells (Bhattacharyya et al., 2010). Therefore, we analyzed cellular ROS levels using DCFDA, which fluoresces when oxidized by ROS. Increased ROS levels were observed in the IR and CGN treated cells, compared to IR alone (Fig. 2-7). CGN or IR alone also showed an increase in ROS accumulation.



2-5 IR combined with CGN treatment increases activation of caspase-3 and caspases-8 in MDA-MB-231 breast cancer cells

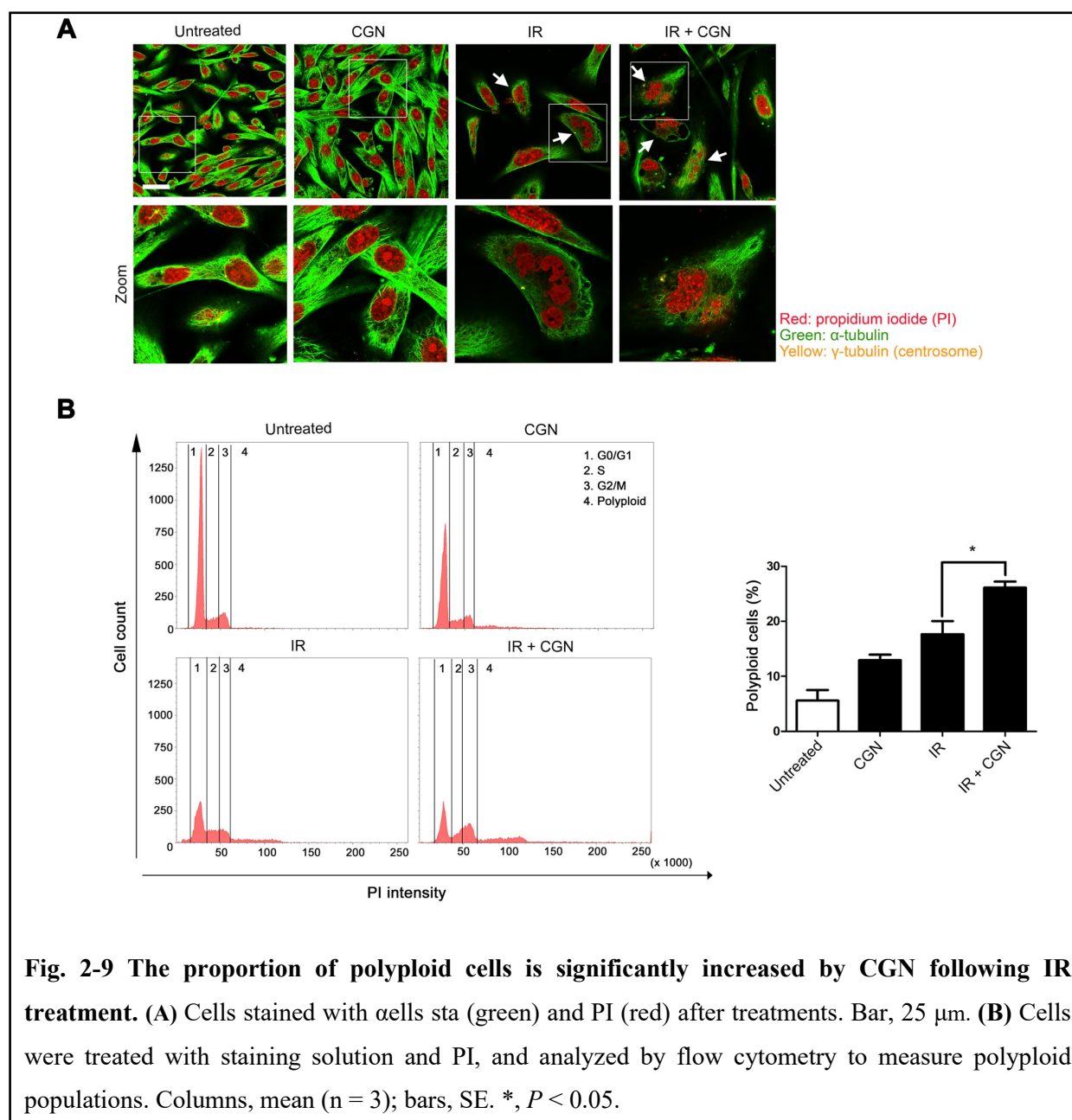
High levels of ROS are known to activate caspase-3 and caspases-8, which are key proteins of apoptosis (Redza-Dutordoir and Averill-Bates, 2016). To measure the activated level of caspase, we performed caspase assay kits. The activities of caspase-3 and caspase-8, but not caspase-9, were elevated after IR followed by CGN in comparison to IR alone in MDA-MB-231 cells (Fig. 2-8). These results indicate that apoptosis-related cell death is efficiently induced by CGN following IR, which is consistent with the PI and Annexin V staining (Fig. 2-4).



2-6 The proportion of polyploid cells was significantly increased by CGN following IR treatment

Besides apoptotic cell death, IR is known to cause mitotic catastrophe (Ianzini and Mackey, 1997, 1998), a mechanism of mitosis-linked cell death resulting in polyploid cell formation (Eriksson et al., 2007). The generation of ROS is also reported to permit inappropriate entry into mitosis and to induce mitotic catastrophe (Hung et al., 2013). To determine whether mitotic catastrophe was induced by CGN combined with IR, we analyzed polyploid formation in the cells by immunofluorescence. Under confocal fluorescence microscopy, abnormal polyploid giant cells were observed in both the IR alone and the combined treatment groups (Fig. 2-9A). The proportion of polyploid cells was significantly increased by combined treatment with CGN and IR compared to IR alone by cell cycle analysis (Fig. 2-9B). These data suggest that CGN

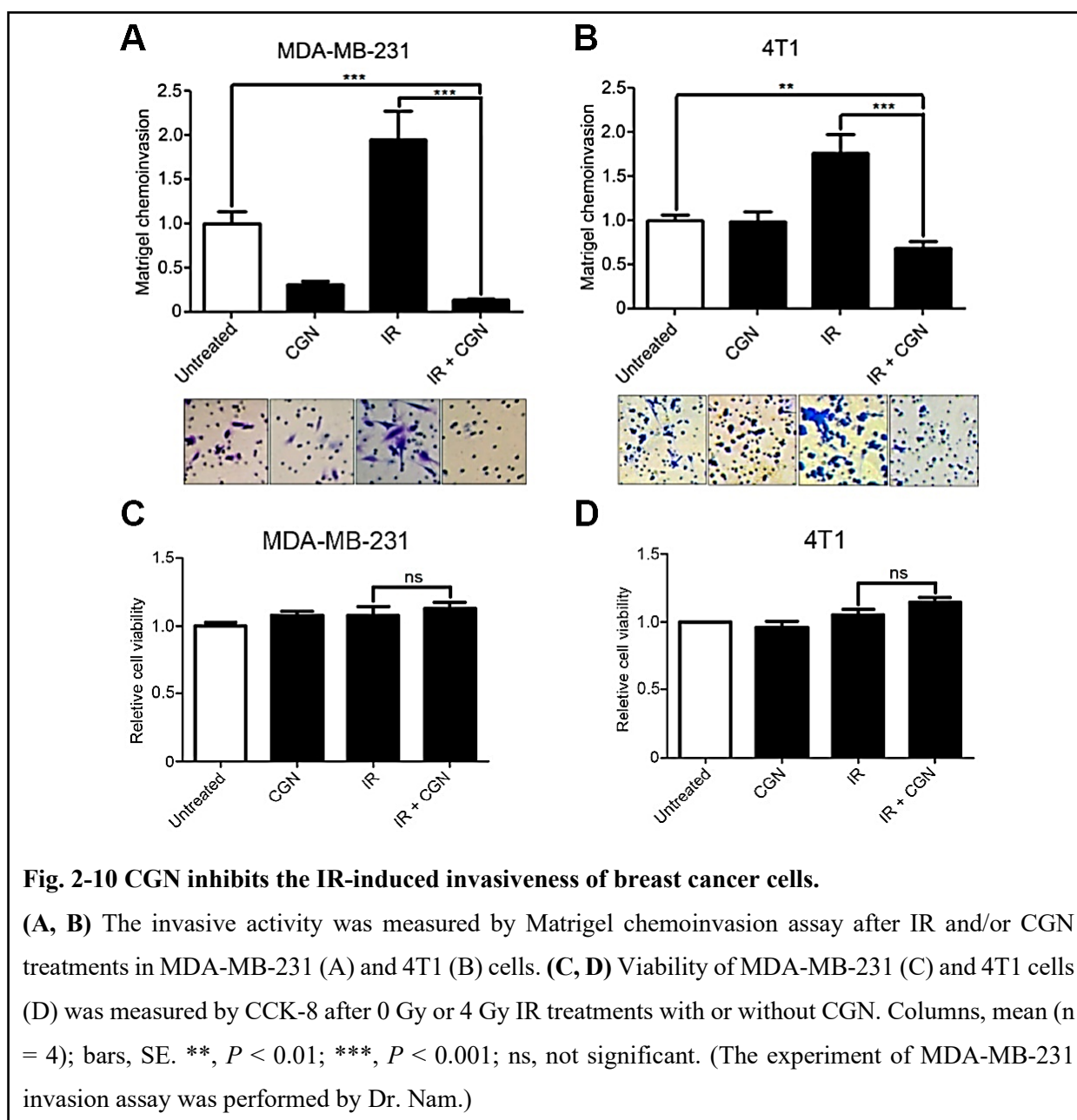
can increase ROS accumulation in irradiated cells, which may further enhance caspase-mediated apoptosis and mitosis-related cell death.



2-7 CGN inhibits the IR-induced invasiveness of breast cancer cell lines

As shown in Chapter 1, cancer cell invasiveness could be increased in the surviving population after IR treatment through integrin-mediated pathways. Therefore, we investigated the CGN's effect on the invasiveness of cancer cells after IR. The invasive activity was increased in the breast cancer cell lines after IR treatment, as the results in Chapter 1. Interestingly, the invasive ability of the MDA-MB-231 (Fig. 2-10A) and 4T1 (Fig. 2-10B) breast cancer cell lines was

significantly lower in the combined treatment with IR and CGN compared to IR alone. Cell viability was not affected during the invasion assay (Fig. 2-10C and D). These data indicate that CGN suppresses the IR-related invasiveness of these cells. Compared to the results of cytotoxicity depicted in Fig. 2-1 to 2-3, CGN showed a higher anti-invasive effect which is specific in post-IR cells. This effect was more obvious in MDA-MB-231 cells, which leads to a more significant reduction in invasive ability than that of CGN alone, suggesting the possibility that CGN suppresses specific mechanisms, which induced the increase in invasiveness in post-IR cancer cells.



2-8 CGN inhibits the invasive growth of MDA-MB-231 cells in 3D culture

To further confirm the effect of CGN in the invasive growth of cancer cells, we performed 3D laminin-rich ECM (lrECM) culture. Culturing cells in 3D lrECM is a common method for assessing the physiologically relevant morphogenesis and oncogenic properties of non-malignant or cancerous mammary epithelial cells (Lee et al., 2007). As shown in Fig. 2-11, untreated MDA-MB-231 cells under 3D culture displayed aggressive invasive growth with stellate protrusions extending into the lrECM. The formation of protrusions was reduced in cancer cells that received the combination of CGN and IR treatment, indicating suppression of their invasive capacity in the 3D lrECM culture.

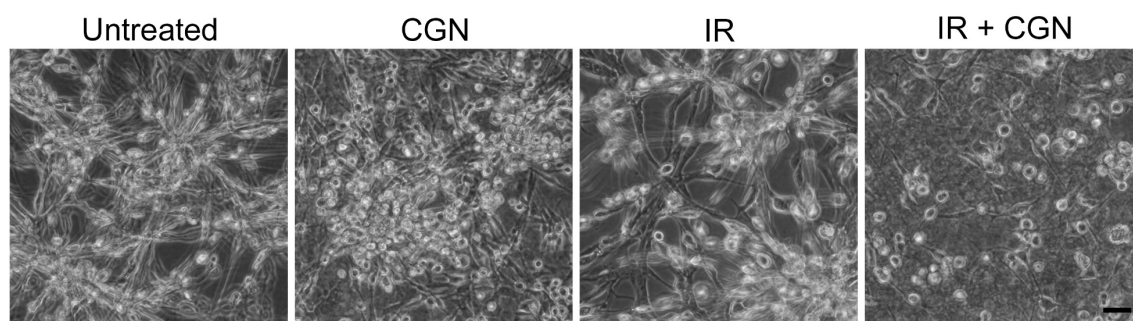
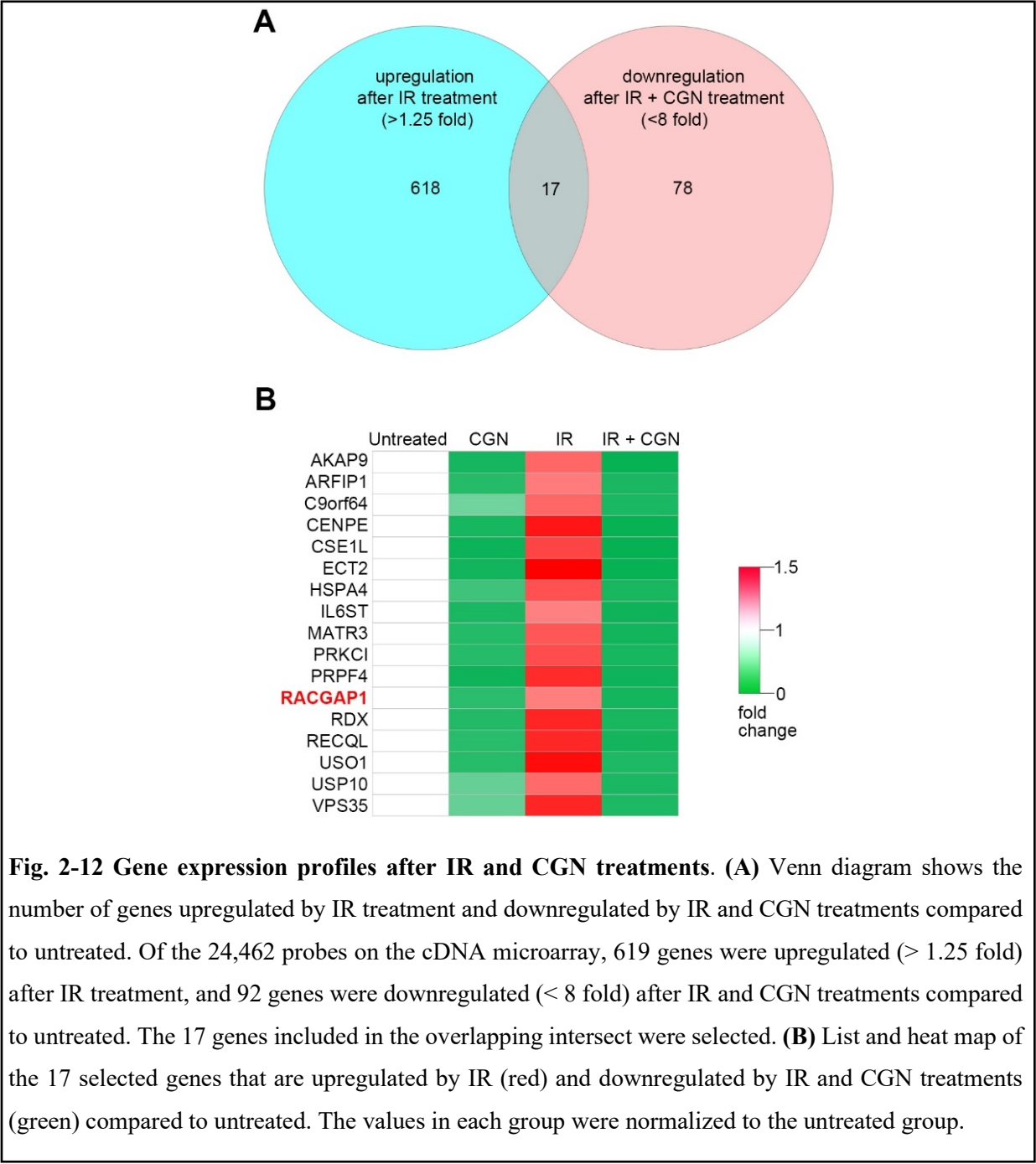


Fig. 2-11 CGN inhibits the invasive growth of MDA-MB-231 cells in 3D culture. MDA-MB-231 cells were cultured in 3D lrECM. Bar, 50 μ m. (The experiment was performed by Dr.Nam.)

2-9 Searching for genes which are involved in cancer cell survival and invasion after IR and CGN

To determine the effects on molecular pathways, the differential gene expression in each treatment group was assessed by cDNA microarray. We selected the genes that were both upregulated by IR treatment and suppressed by the following CGN treatment (Fig. 2-12). Seventeen genes were selected according to the method mentioned in the figure legend of Fig. 2-12.



2-10 Upregulation of RacGAP1 is involved in cancer cell survival and invasion after IR

From these genes, we focused on RacGAP1 as a potential target (Fig. 2-13A) because this protein is intimately connected with integrin signaling, which has been mainly working on by our group (Nam et al., 2013; Onodera et al., 2013; Wu et al., 2017). Moreover, RacGAP1 is recently reported to have important roles in oncogenic activity (Lawson and Ridley, 2018), tumor progression (Imaoka et al., 2015; Mi et al., 2016), and cancer invasion (Jacquemet et al., 2013; Saigusa et al., 2015).

To investigate its role in irradiated cells, protein levels of RacGAP1 were determined after IR treatment in different types of cancer cell lines (Fig. 2-13B). RacGAP1 was found to be upregulated after IR treatment, suggesting that it plays a role in the cellular response to radiation.

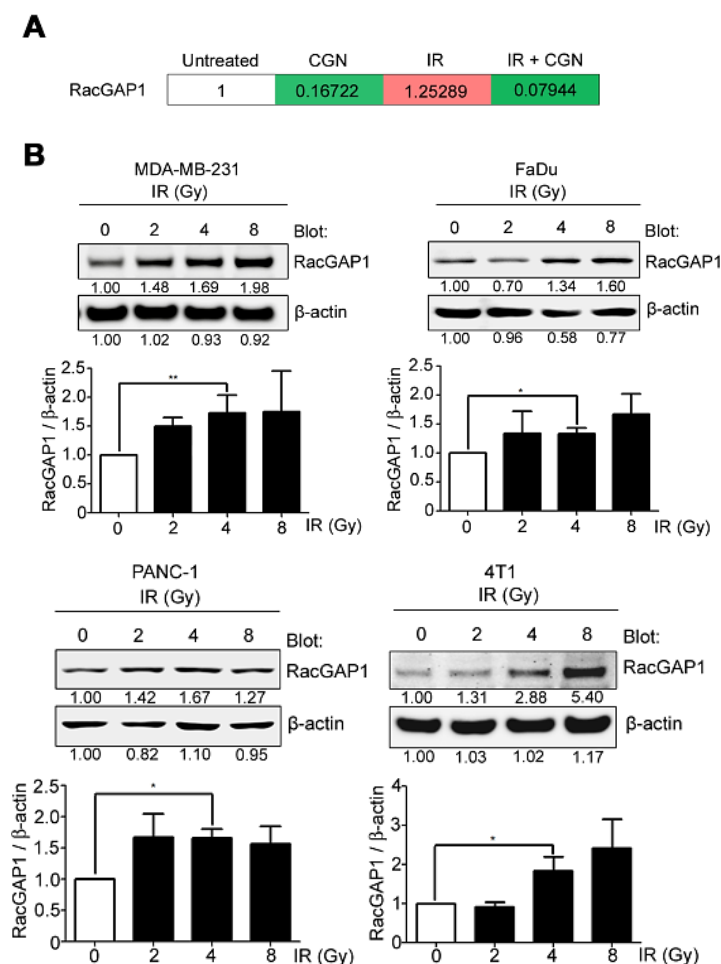
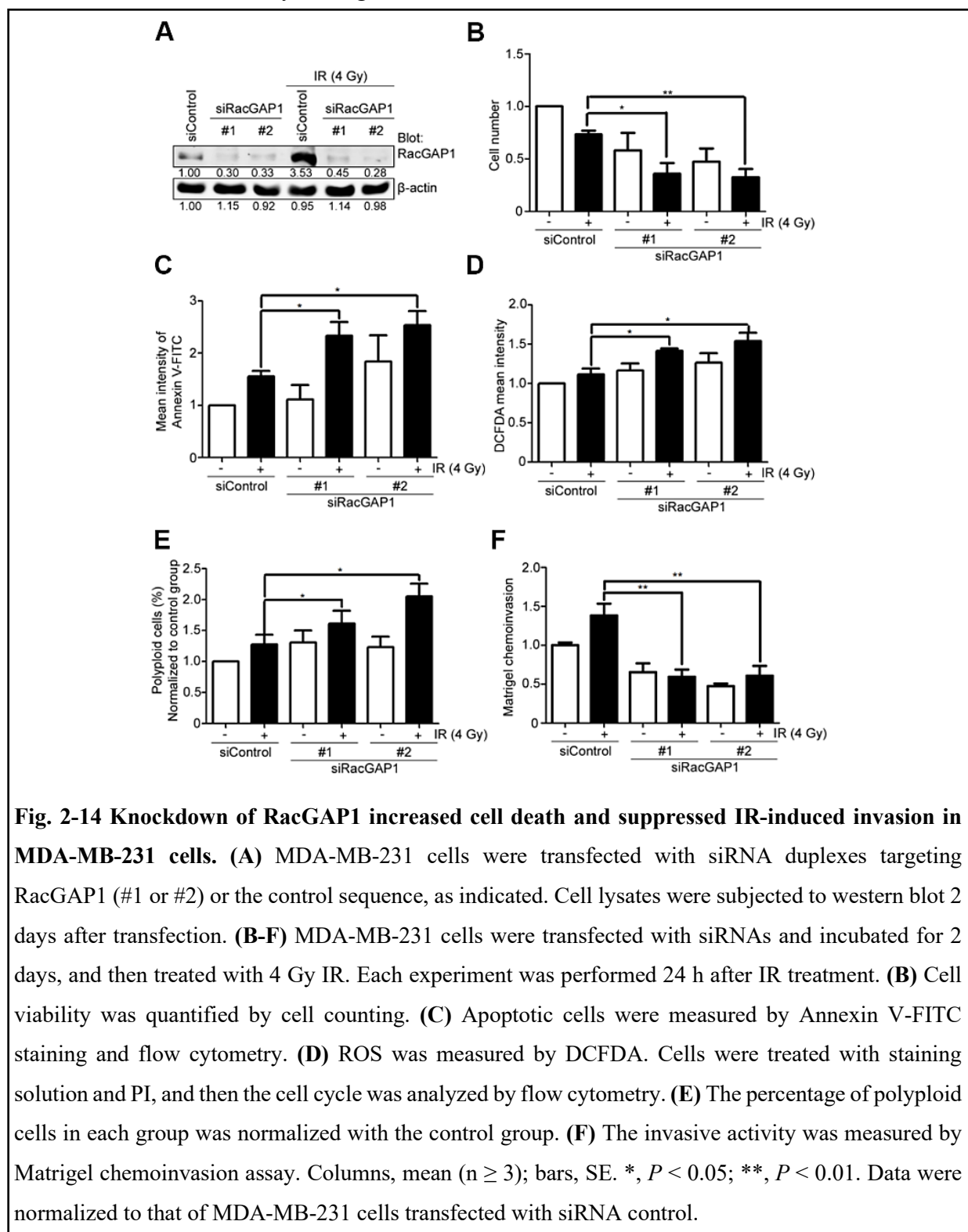


Fig. 2-13 Upregulation of RacGAP1 is involved in cancer cell survival and invasion after IR. (A) Gene expression was analyzed by cDNA microarray. RacGAP1 expression level is shown by the heat map. The values were normalized to the untreated group. **(B)** RacGAP1 protein expressions with different doses of IR were analyzed in MDA-MB-231, FaDu, PANC-1, and 4T1 cell lines. Columns, mean ($n \geq 3$); bars, SE. *, $P < 0.05$; **, $P < 0.01$.

2-11 Knockdown of RacGAP1 increased cell death and suppressed IR-induced invasiveness in MDA-MB-231 cells

To measure the role of RacGAP1 in IR treatment, we knocked down RacGAP1 in MDA-MB-231 cells by siRNA and treated these cells with IR (Fig. 2-14A). Compared to the control group, The knockdown of RacGAP1 resulted in a significant decrease in cell viability following IR treatment (Fig. 2-14B). The Knockdown of RacGAP1 also increased apoptosis, ROS

accumulation, and polyploid formation after IR treatment in MDA-MB-231 cells (Fig. 2-14C to E). Furthermore, the knockdown of RacGAP1 effectively suppressed the IR-induced invasiveness of cancer cells (Fig. 2-14F). These results indicate that RacGAP1 inhibition could increase the effectiveness of IR by reducing both cell viability and IR-induced invasiveness, which was also achieved by adding CGN after IR.



2-12 RacGAP1 expression was suppressed by CGN in MDA-MB-231 cells

The cDNA microarray data suggest that CGN treatment suppresses RacGAP1 (Fig. 2-12). Consistent with the gene expression data, we also confirmed that the protein expression of RacGAP1 was significantly downregulated by CGN (Fig. 2-15A). Moreover, the elevation of RacGAP1 protein levels following IR, and its suppression after CGN treatment were also confirmed by western blot (Fig. 2-15B). Immunofluorescent staining showed that RacGAP1 mainly localized in the nucleus and that its nuclear level was increased after IR and suppressed by CGN treatment (Fig. 2-15C).

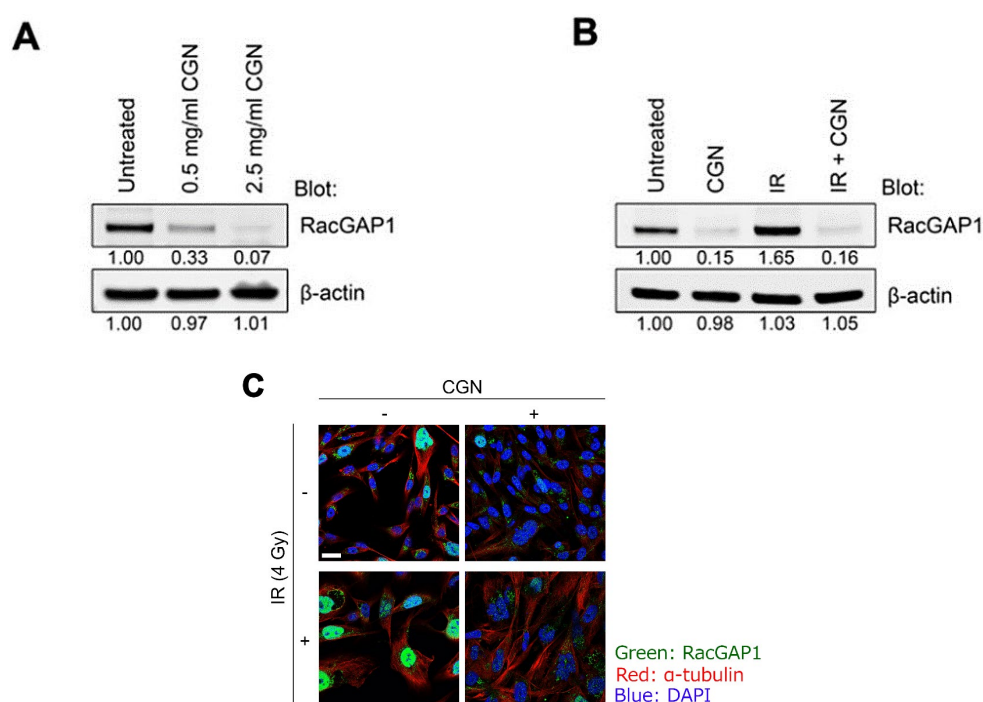
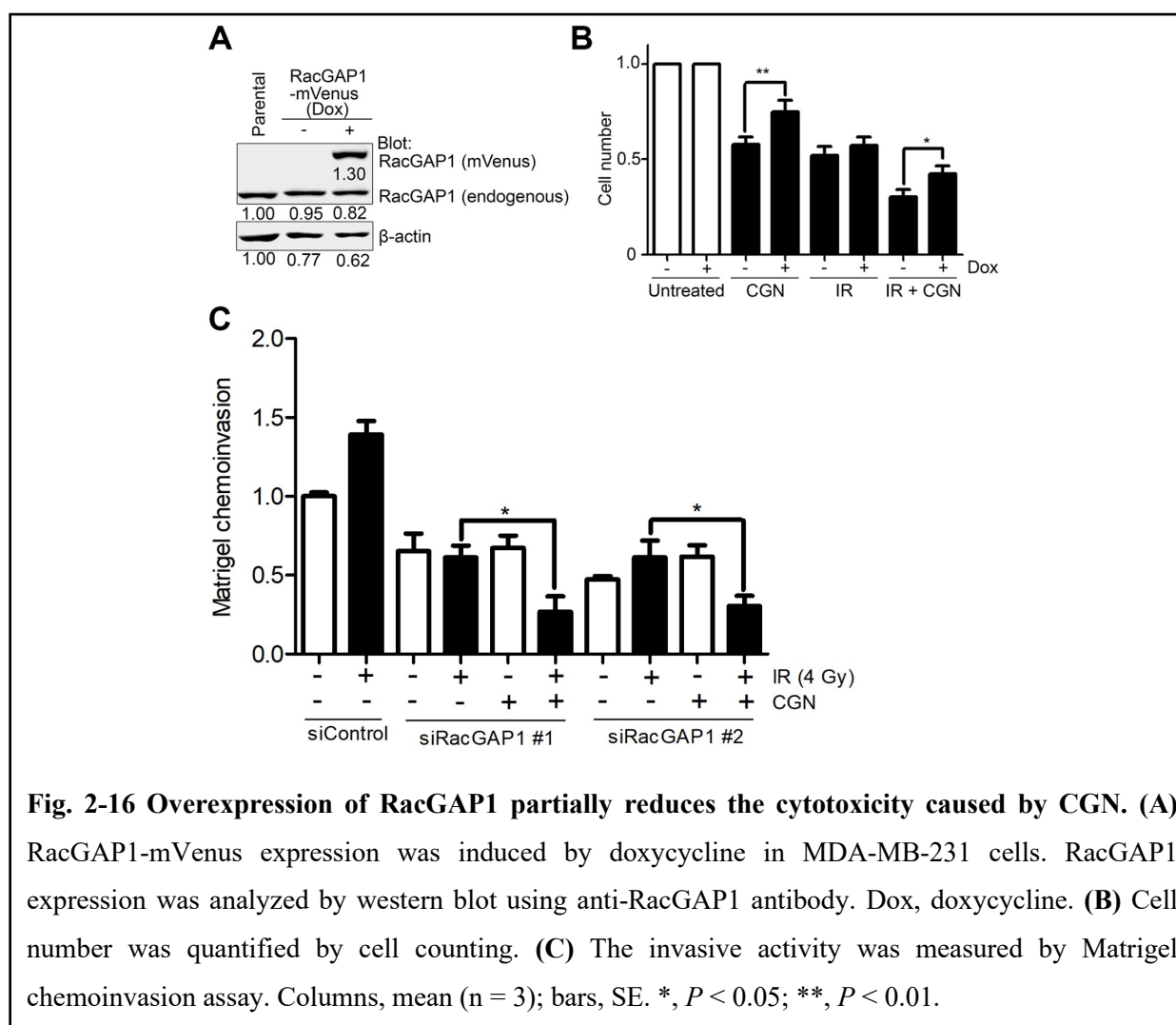


Fig. 2-15 RacGAP1 expression is suppressed by CGN in MDA-MB-231 cells. (A) Protein expression of RacGAP1 was analyzed after treatment with different concentrations of CGN in MDA-MB-231 cells. Total cell lysates were subjected to western blot. **(B)** RacGAP1 protein expression was analyzed using cell lysates after IR and/or CGN treatment in MDA-MB-231 cells. **(C)** Immunofluorescence images show RacGAP1 (green), α-tubulin (red) and nuclei (blue). Bar, 25 μm. (The results of Western blotting were done by Dr. Nam.)

2-13 Overexpression of RacGAP1 partially reduced the cytotoxicity caused by CGN

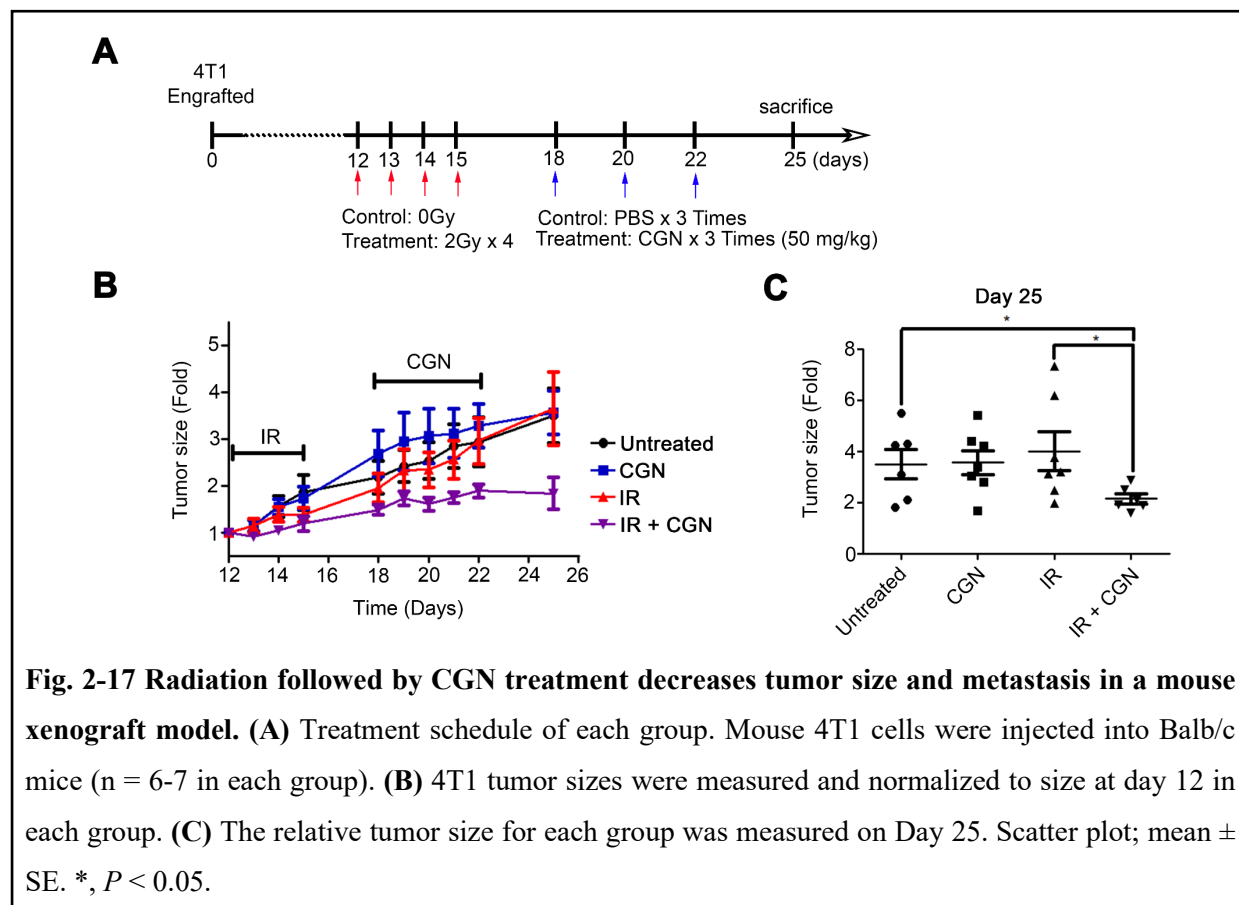
To further confirm that the downregulation of RacGAP1 is involved in the cytotoxic effect of CGN, we generated a doxycycline-inducible overexpression system of RacGAP1 (Fig. 2-16A). RacGAP1 overexpression partially reduced the cytotoxicity caused by CGN with or without IR treatment (Fig. 2-16B), which suggests that RacGAP1 is indeed an important molecular target in CGN treatment.

Given these results, RacGAP1 is one of the predominant genes in the radiation-induced invasion. However, as shown in Fig. 2-10, the inhibitory effect of CGN treatment on invasion activity is much stronger than that of siRacGAP1, suggesting that CGN also affects pathways other than RacGAP1. Therefore, we performed the invasion assay experiments to assess the CGN on the MDA-MB-231 cells with the knockdown of RacGAP1 (Fig. 2-16C). The results indeed support the notion that CGN treatment further suppressed the IR-induced invasiveness in siRacGAP cells.



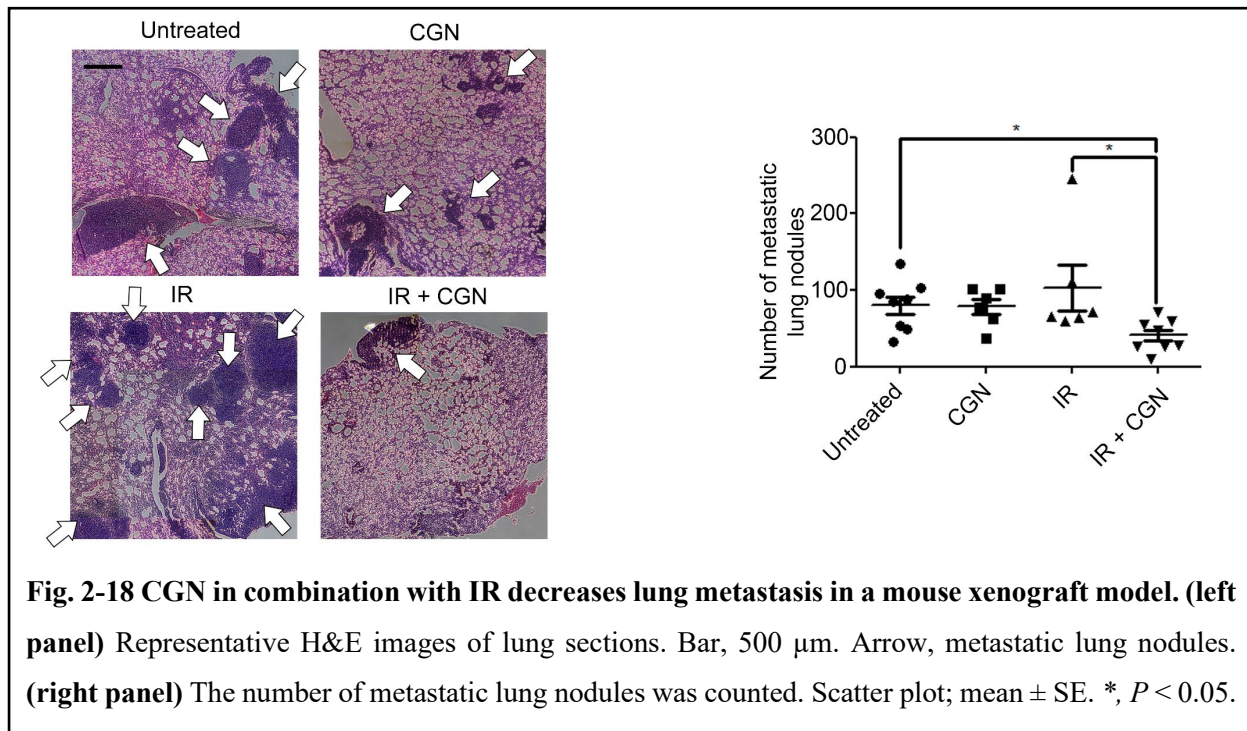
2-14 CGN in combination with IR decreases tumor size and metastasis in a mouse xenograft model

To determine the *in vivo* effect of adjuvant CGN treatment after IR, we used a 4T1 xenograft animal model with the experimental schedule shown in Fig. 2-17A. Tumor growth was significantly decreased in the group treated with CGN after IR compared to either CGN or IR treatment alone (Fig. 2-17B). Similarly, the terminal tumor size at day 25 was smallest in the CGN and IR group (Fig. 2-17C).



2-15 CGN in combination with IR decreases lung metastasis in a mouse xenograft model

Local cancer invasion is the initial step in the spread of cancer cells from a local site to distant metastasis sites (Eccles et al., 2005). Therefore, we determined the therapeutic effect of CGN in combination with IR on distant metastasis. Compared to the other groups, combined treatment with IR and CGN significantly inhibited lung metastasis (Fig. 2-18).



2-16 CGN in combination with IR decreases RacGAP1 expression in mouse xenograft model

To confirm whether RacGAP1 expression was suppressed by CGN treatment *in vivo*, we stained RacGAP1 in tumor samples by IHC. Consistent with the *in vitro* data shown in Fig. 2-15, RacGAP1 was suppressed in 4T1 tumors treated by either CGN alone or CGN in combination with IR (Fig. 2-19).

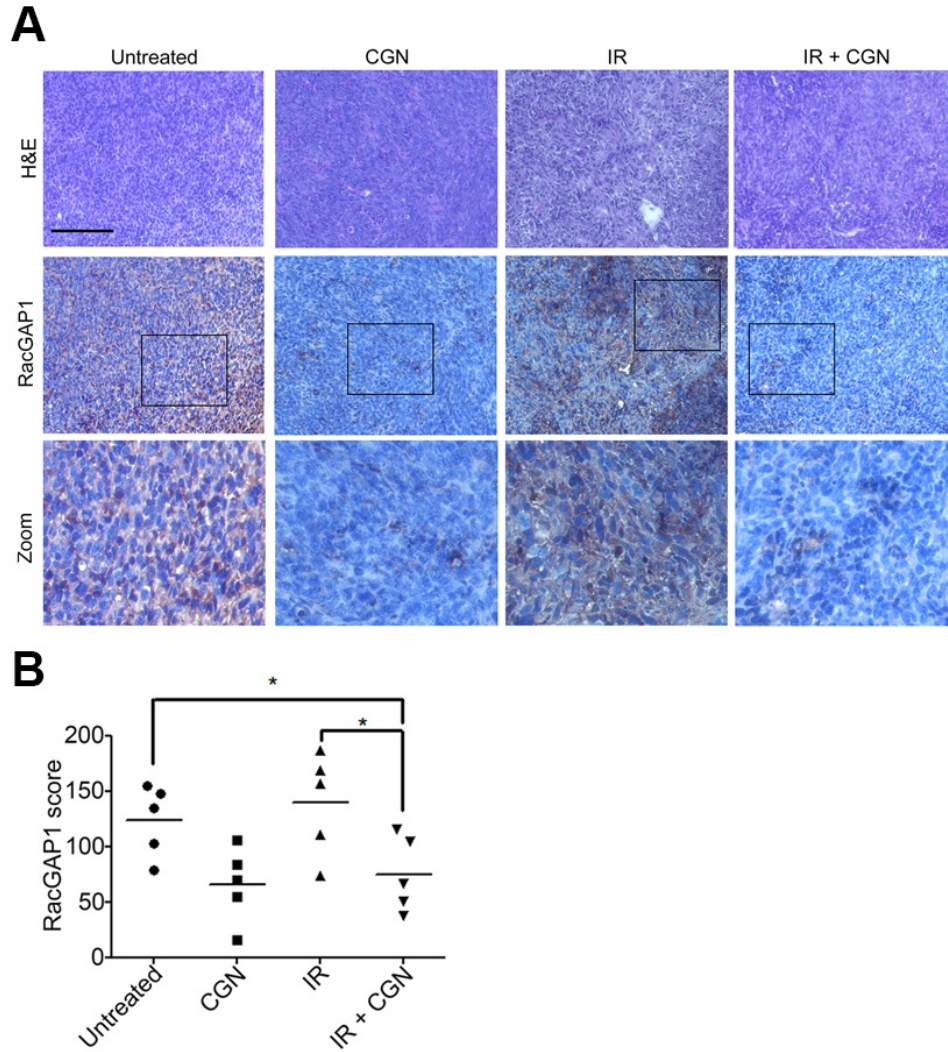


Fig. 2-8 CGN in combination with IR decreases RacGAP1 expression in a mouse xenograft model. (A) Sections from 4T1 tumors were subjected to IHC staining with antibodies against RacGAP1. Bar, 100 μ m. (B) RacGAP1 expression level was determined by scoring, as described in the methods. Scatter plot; bars, mean (n = 5). *, $P < 0.05$.

Discussion

In this chapter, we tested CGN as an adjuvant to improve the efficacy of radiotherapy. Administration of CGN in IR treatment increased cancer cell death. Furthermore, CGN treatment resulted in the notable suppression of IR-induced invasiveness. RacGAP1 signaling is a possible molecular mechanism of the CGN effect following IR treatment of cancer cells. In the mouse xenograft model, combined treatment of IR and CGN significantly suppressed the tumor growth and lung metastasis.

The cytotoxic effect of CGN following IR

In Fig. 2-9, we found that the proportion of polyploid cells was increased after CGN following IR treatment. Polyploid cells are considered the result of enhanced mitotic catastrophe (Erenpreisa et al., 2005; Ianzini and Mackey, 1997), which resulted from delayed DNA damage from IR (Ianzini and Mackey, 1998). Eriksson *et al.* revealed that the generation of morphological mitotic catastrophes and polyploid formation were in a dose-dependent manner of IR (Eriksson et al., 2007). In their study, the proportion of polyploid cells in HeLa Hep2 cells increased from $2.8 \pm 1.3\%$ to $17.6 \pm 2.1\%$ following treatment with 10 Gy (Eriksson et al., 2007). Our data show that the proportion of polyploid cells in MDA-MB-231 cells was $5.6 \pm 2.7\%$ in the untreated group, $12.9 \pm 1.7\%$ in the CGN group, $17.6 \pm 3.3\%$ in the 4 Gy IR group, and $26.1 \pm 1.9\%$ in the IR and CGN group. These results show that IR followed by CGN led to a higher proportion of polyploid cells, indicating that CGN could enhance the induction of cellular mitotic catastrophe following IR treatment. Besides CGN, various of anticancer drugs are also known to induce mitotic catastrophe by influencing the stability of microtubule spindles or defective cell cycle checkpoints (Castedo et al., 2004). Mansilla *et al.* reported that mitotic catastrophe could be caused by the chemotherapeutic agent including doxorubicin and the anthracycline antibiotic WP631 in MDA-MB-231 cells and MCF-7/VP cells (Mansilla et al., 2006). In their study, treating cells with both agents resulted in increased polyploid formation, followed by increased cell death. Besides, the activation of the caspase-3-related apoptotic pathway was observed only in MDA-MB-231 cells treated with doxorubicin, indicating that caspase-3-related pathway is not mandatory for all cell death induced by mitotic catastrophe. In our study, cells cultured with CGN had increased caspase-3 activity as well, suggesting that different patterns of cell death are also caused by CGN.

Although single IR or CGN alone induced cell death in *in vitro* experiment (Fig. 2-1 to 2-3), single IR or CGN alone failed to control the tumor growth and metastasis *in vivo* (Fig. 2-

17). In a mouse model, we chose a low dose of IR treatment for a big tumor (about 500 mm³) to elucidate the adjuvant effect of CGN with IR. IR was treated from day 12 after the injection of 4T1 cells. In fact, on day 12, the tumor size was already too big to be affected by 2 Gy × 4 doses. In our preliminary experiments, when we treated mice with higher doses (2 Gy × 5) on the smaller tumor (less than 100 mm³), we observed the effect of IR alone as compared with the untreated group. With regard to CGN alone, a previous study showed that intratumoral injection of CGN decreases tumor growth in the B16-F10 or 4T1 xenograft model by stimulating immune responses (Luo et al., 2015). They treated tumor-bearing mice with CGN every two days in the early stage of tumor implantation, which decreased significantly in 25 days after tumor inoculation compared to the control group. In contrast, our study showed that CGN alone did not inhibit *in vivo* 4T1 (Fig. 2-17), while it decreased cell viability *in vitro* (Fig. 2-1 to 2-3). We started CGN injection in the late stage of tumor growth and limited the treatment to three injections to better assess the adjuvant effect of CGN for radiation therapy. Therefore, the different *in vivo* results for CGN alone may be due to the differences in starting time and a total number of CGN injections.

Role of RacGAP1 in radiotherapy

The expression of RacGAP1 has been widely reported to be associated with more aggressive phenotypes in many cancers, including high-grade breast cancer, epithelial ovarian cancer, gastric cancer, colorectal cancer, and hepatocellular carcinoma in the transition from low- to high-invasive disease (Casado-Medrano et al., 2016; Imaoka et al., 2015; Saigusa et al., 2015; Wang et al., 2018; Yang et al., 2018). Besides, RacGAP1 is implicated in the resistance to chemotherapeutic agents such as doxorubicin treatment in squamous cell carcinoma (Hazar-Rethinam et al., 2015). These studies suggest that RacGAP1 is a potential therapeutic target for the treatment of highly aggressive cancers. In this chapter, we disclose the role of RacGAP1 in radiotherapy. To our knowledge, this is the first report to connect RacGAP1 to radiation resistance. In addition, we reveal a novel method for targeting RacGAP1 by administering CGN after radiation therapy, which may lead to future clinical applications.

Several studies have reported that $\alpha 5 \beta 1$ -integrin trafficking regulates RacGAP1 activation, which is essential to promote cancer cell invasion (Jacquemet et al., 2013; Lawson and Ridley, 2018). In this chapter, we show that the upregulation of RacGAP1 after IR is accompanied by increased cancer invasion, and the knockdown of RacGAP1 significantly suppresses IR-induced cancer invasiveness (Fig. 2-14F). Moreover, CGN suppresses IR-induced cancer invasiveness, which is partially restored by overexpression of RacGAP1 (Fig. 2-16B). These

findings suggest that the upregulation of RacGAP1 in cancer cells after IR treatment may be one of the pivotal mechanisms contributing to IR-induced invasiveness. The detailed regulatory mechanisms of RacGAP1 on IR-induced invasiveness should be investigated in future studies.

Further prospects

As shown in Fig. 2-15, CGN treatment was found to significantly suppress RacGAP1. Although the details of the mechanism remain unclear, we could find some possible clues from other studies. For example, signal transducer and activator of transcription 3 (STAT3) is a transcriptional factor that has been reported to activate the transcription of RacGAP1 in hepatocellular carcinoma cells (Yang et al., 2018). Besides, some other polysaccharides from plants have been reported to induce biological effects in cells via suppression of STAT3-related pathways (Liu et al., 2018; Tao et al., 2018). Therefore, it is reasonable under the hypothesis that CGN, as a natural polysaccharide from plants, would regulate the expression of RacGAP1 via the same STAT3-related pathway. Further investigation is worth to figure out how CGN regulates RacGAP1.

Besides *RacGAP1*, other genes were also found to be upregulated by IR and suppressed by the following CGN treatment (Fig. 2-12). Of these genes, *AKAP9*, *CENPE*, *PRKCI*, *RDX*, *RECQL*, and *USOI* have also been reported to be associated with cancer progression as described as below.

Gene	Protein name	Function
<i>AKAP9</i>	A-kinase anchor proteins-9	Development of metastasis in colorectal cancer (Hu et al., 2016), breast cancer (Frank et al., 2008), lung cancer (Truong et al., 2010), melanomas (Kabbarah et al., 2010), thyroid carcinomas (Caria and Vanni, 2010)
<i>CENPE</i>	Centromere-associated protein E	Cancer cell apoptosis and tumor progression (Wood et al., 2010)
<i>PRKCI</i>	Protein kinase C, iota	Overexpressed in ovarian cancer (Tsang et al., 2017) Promotion of immune suppression (Sarkar et al., 2017)
<i>RDX</i>	Radixin	Overexpressed in many tumor tissues and was suggested to enhance colon cancer cell invasion (Jiang et al., 2014)
<i>RECQL</i>	RecQ helicase-like protein	A DNA helicase which plays a vital role in the DNA damage response, a plausible candidate breast cancer susceptibility gene (Bowden and Tischkowitz, 2019)
<i>USOI</i>	General vesicular transport factor p115	Knockdown <i>USOI</i> inhibited cell proliferation and induced cell apoptosis in multiple myeloma cells (Jin and Dai, 2016) and colon cancer cells (Sui et al., 2015).

Although these molecules have various functions in cancer cells, they may be involved in radioresistance or radiation-induced invasion that can be targeted by CGN adjuvant treatment. The roles of these molecules and related mechanisms could be investigated in the future.

Conclusions

Summary of Chapter 1

- RGD/P-AuNPs successfully targeted integrins and internalized in integrin-overexpressing breast cancer cells.
- RGD/P-AuNPs were colocalized with the $\alpha 5$ - and αv -integrins in these cells and accumulated mainly in the late endosomes and lysosomes.
- A combined treatment with RGD/P-AuNPs and IR reduced cancer cell viability and accompanied by increasing of DNA damage compared to single modality treatment.
- RGD/P-AuNPs suppressed IR-induced invasiveness of breast cancer cells.
- The expression of FN and activation of ERK, a key modulator of cancer invasion, were suppressed by RGD/P-AuNPs in combination with IR.

Summary of Chapter 2

- CGN treatment decreased viability in irradiated cancer cells and enhanced ROS accumulation, apoptosis, and polyploid formation.
- CGN suppressed IR-induced invasiveness and invasive phenotype in 3D IrECM cultures.
- Protein expression of RacGAP1 was upregulated in several cancer cell lines after IR treatment, which was significantly suppressed by CGN treatment.
- Knockdown of RacGAP1 decreased cell viability and invasiveness after radiation, while overexpression of RacGAP1 partially rescued CGN cytotoxicity.
- In a mouse xenograft model, local irradiation followed by CGN treatment significantly decreased tumor growth and lung metastasis compared to either treatment alone.

In chapter 1, we have shown that RGD/P-AuNPs specifically accumulated in integrin-overexpressing cancer cells. RGD/P-AuNPs enhanced cell cytotoxicity and increased DNA damage after IR treatment. In contrast, RGD/P-AuNPs caused no effect on cell viability without IR treatment. These results indicate two significant advantages of RGD/P-AuNPs as an adjuvant treatment of radiotherapy: 1) The specific targeting ability to integrin-positive cancer cells not integrin negative cells. 2) No side effect of RGD/P-AuNPs itself to normal cells. Based on these results, RGD/P-AuNPs could be considered as a useful adjuvant for radiation to improve therapeutic outcomes in cancer treatment.

Although RGD/P-AuNPs show a high affinity to integrin-overexpressing cancer cells *in*

vitro experiments, the delivery efficacy of RGD/P-AuNPs to tumor is unclear *in vivo* and human body. Therefore, the targeting ability of RGD/P-AuNPs should be further confirmed *in vivo* study for application to clinical practice in the future. Besides, to enhance the cytotoxic effect of RGD/P-AuNPs with IR, we could further modify with other materials, such as anti-hypoxia agents or poly ADP ribose polymerase (PARP) inhibitors, in addition to RGD peptides on AuNPs. These applications could be a next strategy to further increase the therapeutic effect of RGD/P-AuNPs with radiotherapy.

In chapter 2, we discovered the novel role of RacGAP1 in IR and CGN treatment. After IR treatment, we found that RacGAP1 was significantly upregulated in different cancer cell lines. Furthermore, knockdown of RacGAP1 increased the IR-induced cell death and suppressed the IR-induced invasiveness. These results suggest that RacGAP1 is an important factor that decreases the efficacy of radiotherapy. To update the knowledge of radiobiology, molecular mechanisms on upregulation of RacGAP1 by IR should be investigated.

We also reported the significances of CGN treatment to suppress the expression of RacGAP1. As described above, RacGAP1 has been studied as an oncogenic enhancer, and its expression is related to the progression of several types of cancer disease. Therefore, it is reasonable to hypothesize that CGN treatment could be specifically used to treat high RacGAP1-expressing cancer cells, which is known as personalized cancer medicine. Besides RacGAP1, several genes were also found to be suppressed by CGN treatment. Further researches of these genes may provide us a deeper understanding of the biological effect of CGN treatment, and may widely extend its application in cancer therapy.

Taken together, I here report two adjuvants with molecular significance to improve radiotherapy by increasing IR-induced cell death and suppressing IR-induced invasiveness. These findings provide cues molecular mechanisms as to how cancer cells survive and acquire invasiveness in response to radiation.

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Conflict of Interest

Jin-Min Nam, Hiroki Shirato and Takayuki Hashimoto, the co-authors of the publication **“Targeting integrins with RGD-conjugated gold nanoparticles in radiotherapy decreases the invasive activity of breast cancer cells”**, disclose financial interests in Aisin Seiki Co., Ltd and IMRA America, Inc.

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