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**Investigation of irradiation-induced increased
invasiveness of lung cancer cells in three-dimensional
collagen matrix and the involvement of integrin $\alpha 2\beta 1$
and epidermal growth factor receptor**

放射線照射が誘引する肺がん細胞の 3 次元コラーゲン
基質中における浸潤性の亢進とインテグリン $\alpha 2\beta 1$
および上皮成長因子受容体の関与に関する研究

Xue LI

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Abbreviation

2D: two-dimensional

3D: three-dimensional

DSB: DNA double-strand breaks

ECM: extracellular matrix

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

Erk: extracellular signal-regulated kinase

FAK: focal adhesion kinase

FBS: fetal bovine serum

HB-EGF: heparin-binding EGF

IR: ionizing radiation

MAPK: Raf-mitogen-activated protein kinase

MEK: mitogen-activated protein kinase kinase

NSCLC: Non-small cell lung cancer

PI3K: phosphatidylinositol 3-kinase

RT-PCR: reverse transcription polymerase chain reaction

siRNA: small interfere RNA

Stat3: signal transducer and activator of transcription 3

TCA: Trichloroacetic acid

TGF- α : transforming growth factor- α

qRT-PCR: quantitative real-time PCR

HR: homologous recombination

NHEJ: nonhomologous endjoining

DNA-PK: DNA-dependent protein kinase

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Chapter 1

General introduction

1.1 Background

1.1.1 Cancer invasion and metastasis

Cancer, known medically as a malignant neoplasm, is a broad group of various diseases. There are over 100 different types of cancer, and each is classified by the type of cell that is initially affected. Lung cancer is one of the most common types of cancer in men and women worldwide and is the leading cause of cancer-related deaths (American Cancer Society, 2013) largely as a result of its highly metastatic nature. Non-small cell lung cancer (NSCLC) accounts for 80% of all lung cancer and has an epithelial origin (Brambilla *et al.*, 2001).

Cancer is malignant because cancer cells invade into neighboring tissues and survive in this ectopic site. The term invasion indicates penetration into neighboring territories and their occupation. Cancer cells invade beyond the constraints of the normal tissue from which they originate; this invasion permits them to enter into the circulation from where they can reach distant organs and eventually form secondary tumors, called metastases (Mareel and Leroy, 2003)

Cancer invasion and metastasis are landmark events that transform a locally growing tumor into a systemic, metastatic, and life-threatening disease (Friedl and Alexander, 2011). The initial steps of local invasion include the activation of signaling pathways that control cytoskeletal dynamics in tumor cells and the turnover of cell-matrix and cell-cell junctions, followed by active tumor cell migration into the adjacent tissue (Chambers *et al.*, 2002; Friedl and Wolf, 2003; Sahai, 2007).

1.1.2 Radiotherapy

Treatment options for cancer mainly include surgery, chemotherapy, radiotherapy, and sequential or concurrent combination therapy.

Radiotherapy is the medical use of ionizing radiation (IR), and is considered a non-invasive local treatment, affecting mainly the cells and tissues that are situated inside the beam of IR. More than half of all cancer patients receive radiation therapy during their course of treatment (Owen *et al.*, 1992)

Radiotherapy holds the potential to provoke killing of cancer cells by transferring sufficient quantities of energy that interact with the biological material and break down chemical bonds (Hartlerode and Scully, 2009). Among the consequences of energy transfer of ionizing radiation, direct DNA damage in the form of DNA double-strand breaks (DSBs) possesses a central role in tumor cell viability. If the activated DNA repair mechanisms fail to correct damage, the resultant chromosomal aberrations lead to cell death or to mutations that might result in carcinogenesis (Prise and O'Sullivan, 2009).

Considerable efforts have been made to understand the mechanism of radioresistance of cancer cells. Basically, two major DSB repair pathways, homologous recombination (HR) and nonhomologous endjoining (NHEJ) rescue cells from DNA damage. DNA-dependent protein kinase (DNA-PK) plays a major role in DSB repair via NHEJ. It is known that cells defective in any of the DNA-PK subunits (i.e., DNA-PKcs, Ku70 and Ku80) are highly sensitive to

radiation because they cannot repair DSBs efficiently (Hashimoto *et al.*, 2003). Increased radiation survival in a 3D microenvironment results from an elevated amount of heterochromatin, a differential dissemination of euchromatin to heterochromatin-associated DSBs in 3D and *in vivo* versus 2D and a lower number of lethal chromosomal aberrations (Storch *et al.*, 2010).

In addition, many targets were found to be able to promote the radioresistance or survival of cancer cells. For example, TGF- β 1 inhibition increases the radiosensitivity of breast cancer cells *in vitro* and promotes tumor control by radiation *in vivo* (Bouquet *et al.*, 2011). Overexpression of hyperactive integrin-linked kinase leads to increased cellular radiosensitivity (Nils, 2004).

1.1.3 Ionizing radiation (IR)-induced malignancy

Although radiotherapy has many advantages and is being used for many cancer treatment, it may also lead to the progression to a more aggressive and malignant post-radiation tumor, due to the development of resistant cancer cell sub-populations with enhanced proliferative ability as well as invasive and angiogenic properties (Prise *et al.*, 2005), which may explain that why better local control of radiation fails to translate into longer survival duration, free of distant metastases. Furthermore, Induction of an acute erythema and fibrosis by radiation are common adverse effects observed in patients who under-going radiotherapy treatment (Nystrom *et al.*, 2004)

IR-induced invasion has been reported for many cancer types. For example, an enhancement of cancer cell invasion after their irradiation has been reported for pancreatic cancer cells (Qian *et al.*, 2002; Ohuchida *et al.*, 2004), glioma cells (Wild-Bode *et al.*, 2001; Park *et al.*, 2006), melanoma cells (Rofstad *et al.*, 2004; Kaliski *et al.*, 2005), rectal carcinoma cells (Speake *et al.*, 2005), colon carcinoma cells (Wang *et al.*, 2000) and non-small cell lung cancer (Jung *et al.*, 2007). These studies were designed to evaluate the invasiveness of irradiated cancer cells, which would survive after radiation treatment. Meanwhile, other studies suggested that irradiated stroma cells also play an important role in increased invasiveness of cancer cells. For example, the irradiated fibroblasts promoted the invasion of breast cancer cells (Paquette *et al.*, 2011).

Therefore, the identification of molecules and the mechanism of IR-induced metastatic cancer progression are required for improving the efficacy of radiotherapy and patient survival rate. Many studies have demonstrated that irradiation can promote invasion and/or metastasis by upregulating the expression of genes and activation of signaling pathways that are involved in the metastatic process. Among them, cell surface receptors, such as integrins and growth factor receptors, are often altered by IR and are capable of activating many different signaling pathways with multiple cellular responses, which will be introduced in details in the following chapters.

1.2 Purpose

Lung cancer is a common lethal cancer that is attributed with a high risk of metastatic dissemination. As a basic and important treatment for lung cancer, radiotherapy sometimes causes increased malignancy in the repopulated cancer cells. We initiated this study by aiming to identify the important molecules required for the increased invasiveness of IR-treated lung cancer cells to discover potential candidates that could be targeted in combination with radiotherapy.

1.3 Materials and methods

1.3.1 Cell culture

Human lung adenocarcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC; Manassas, VA). P cells and IR cells were generated as previously published (Ishihara, *et al.*, 2010). Both cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Equitech-Bio, Kerrville, TX) and 1% antibiotic mixture of penicillin/streptomycin (Sigma). Cells were maintained in a humidified incubator at 37°C and 5% CO₂.

1.3.2 Reagents

EGFR kinase inhibitor PD168393 (Calbiochem, Merck KGaA, Darmstadt), PI3K inhibitor LY294002 (Sigma-Aldrich, St. Louis, MO), and mitogen-activated protein kinase kinase (MEK) inhibitor U0126 (Cell Signaling Technology, Beverly, MA) were used at the indicated concentration in DMSO. A function-blocking antibody against integrin $\alpha 2\beta 1$ (BHA2.1) was purchased from Millipore (Billerica, MA). Western blotting antibodies specific for the integrin $\alpha 2$ and $\beta 1$ subunits were purchased from BD BioScience (San Jose, CA). The p-EGFR antibody (Tyr1068) was purchased from Signalway Antibody (College Park, Maryland). Antibodies specific to EGFR, Akt, p-Akt (Ser473), p44/42 Raf-mitogen-activated protein kinase MAPK (Erk1/2), p-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), signal transducer and activator of transcription 3 (Stat3), p-Stat3 (Ser727), p38 MAPK, and p-p38 MAPK (Thr180/Tyr182) were purchased from Cell Signaling Technology (Beverly, MA). GAPDH antibody was purchased from Ambion (Austin, TX). MFP488 phalloidin was purchased from Mo Bi Tec (Molecular Biologische Technologie, Göttingen).

1.3.3 Three-dimensional (3D) collagen culture

A 1.6 mg/mL collagen solution was prepared by mixing 3 mg/mL pig collagen type I-P solution (Nitta Gelatin, Osaka, Japan), 2.6× DMEM medium (Sigma), and buffer (Nitta Gelatin) at a ratio of 7:5:1 on ice. A 30-mm dish was first coated with 150 μ L of collagen solution and allowed to polymerize at 37°C for 30 min, then

rinsed with medium. Then, 10 μL of 2×10^5 cells in suspension was mixed thoroughly with 150 μL of collagen solution and plated on the lower layer of collagen gel. After collagen polymerization at 37°C for 30 min, the cell-collagen mixture was covered with 2 mL of FBS-containing medium and cultured at 37°C and 5% CO_2 for further analysis, including RT-PCR and western blotting. For morphology analysis and time-lapse observation, a glass dish was substituted for the plastic dish. For easier observation of cell movement in the same plane, gel-sand culture was used. Cells were first plated and allowed to adhere onto the lower gel and, after 16 h, the upper gel was overlaid and polymerized at 37°C for 30 min. Cells were maintained in 2 mL of FBS-containing medium at 37°C and 5% CO_2 .



Figure 1-1. 3D collagen culture.

Left: scheme of 3D collagen culture. Right: staining of collagen matrix by specific antibody.

1.3.4 Cell morphology analysis

Cell morphology was analyzed after being in the 3D collagen gel for 24 h. When indicated, inhibitors were added to the medium. Phase contrast images were taken randomly from 4 fields per sample, and the percentage of elongated cells was determined from at least 3 independent experiments including over 100 individual cells. A cell was considered elongated when its longest dimension was twice the shortest dimension, and when it showed at least one protrusion, as previously reported (Sanz-Moreno *et al.*, 2008).

1.3.5 Time-lapse microscopy and quantification of cell invasion speed

2×10^4 cells were cultured by 3D gel-sand assay for 24 h, and observed in a chamber at 37°C. Images of randomly chosen cells were taken every 5 min for 6 h. For inhibition experiments, inhibitors or antibodies were added into the culture medium after gel-overlay when indicated. To quantify the speed of cells, we tracked the movements of individual cells by Image-Pro software (Media Cybernetics Inc., Silver Spring, MD). The cell invasion speed was calculated as distance (µm) per minute from at least 3 independent experiments including 50 individual cells.

1.3.6 3D spheroid invasion assay

Spheroids were produced using the Gravity Plus system (InSphero, Zurich,

Switzerland) according to the manufacturer's instructions. Briefly, 40 μ L of cell suspension containing 10^3 cells was seeded into each well of the plate for 4 d, and spheroids were transferred to collagen gel and overlaid soon thereafter. After being on gelatin at 37°C for 30 min, medium with FBS was added, and cells were cultured for 24 h. When indicated, inhibitors were added during culture. Then, cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100 in PBS, and stained with MFP488 phalloidin. Fluorescence images were obtained by confocal laser scanning microscopy (C1 confocal imaging system; Nikon Instech., Tokyo). The perimeter and the area of spheroids were determined by ImageJ software (National Institutes of Health, Bethesda, Maryland) as previously reported (De Wever *et al.*, 2010). In brief, change the image to 8-bit type, and use the threshold function to convert areas of interest to saturated black areas in a uniform manner to have a binary (black and white) image. Then exclude all particles less than 3 pixels in size and remove any artifacts by comparing the binary image to the phase-contrast fluorescence pictures. Use the set measurements dialog box to specify area and perimeter. Use the analyze particle dialog box to measure all particles and to generate a "particle report" for each image in which the area and perimeter of individual particles and the area of the sum of individual particles is documented. The aspect ratio was calculated from $\text{perimeter}^2/[4\pi(\text{area})]$. A higher aspect ratio means a more irregular, infiltrating spheroid structure. Results were determined from 3 independent experiments carried out in triplicate.

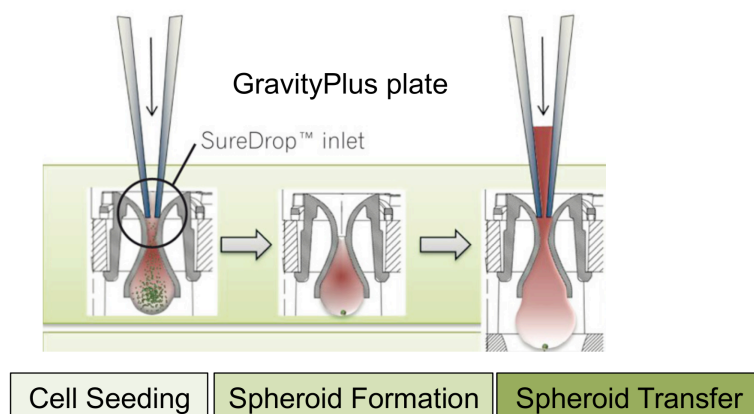


Figure 1-2. Scheme of cell spheroids preparation by GravityPlus plate.

1.3.7 RT-PCR

Cells were lysed with Tripure (Roche Applied Science, Indianapolis, IN) for RNA extraction, and the reverse transcription reaction was performed by ReverTra Ace qPCR RT Kit (Toyobo). For cells cultured on/in 3D collagen gel, extraction was performed twice. PCR was performed with Taq Polymerase in ThermoPol Buffer using primers showed in Table 1-1 (NEB, Ipswich, MA).

Table 1-1 Primers for RT-PCR.

Target	Sequence
Integrin α 1	5'-GCCTCCTTTCTTGCTGTGTC-3' (F)
	5'-TGGGTGCTTATTGGTTCTCC-3' (R)
Integrin α 2	5'-GAGCACCAGCAACAAAGTGA-3' (F)
	5'-CGGGTGTGTGTTCTGACATC-3' (R)
Integrin α 4	5'-GAGATTTTCCCCTTGCATGA-3' (F)
	5'-GAGTGCAATGCAGACCTTGA-3' (R)
Integrin α 5	5'-CACAGAGTTGCCCCGAGCACA-3' (F)
	5'-GCAGGGCTAGTGCCAGGGTTT-3' (R)
Integrin β 1	5'-AATGAAGGGCGTGTTGGTAG-3' (F)
	5'-CCTCGTTGTTCCCATTCACT-3' (R)
GAPDH	5'-ACCACAGTCCATGCCATCAC-3' (F)
	5'-TCCACCACCCTGTTGCTGTA-3' (R)

F, Forward; R, Reverse.

1.3.8 Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed by PikoReal (Thermo Scientific, Waltham, MA) as described in the manuscript. Briefly, total RNA (1 μ g) was reverse transcribed using the specific primers showed in table 1-2. β -actin was used as a reference gene for normalization.

Table 1-2 Primers for qRT-PCR.

Target	Sequence
Integrin α 2	5'-CACAGAGTTGCCCCGAGCACA-3' (F)
	5'-GCAGGGCTAGTGCCAGGGTTT-3' (R)
Integrin β 1	5'-GACGCCGCGCGGAAAAGATG-3' (F)
	5'-GCACCACCCACAATTTGGCCC-3' (R)
EGFR	5'-CGCAGATAGTCGCCCAAAG-3' (F)
	5'-CCATCAGGGCACGGTAGAA-3' (R)
β -actin	5'-GAGCCTCGCCTTTGCCGATCC-3' (F)
	5'-ACATGCCGGAGCCGTTGTCG-3' (R)

F, Forward; R, Reverse.

1.3.9 Western blotting

Cells in 3D collagen culture were fixed in 500 μ L ice-cold Trichloroacetic acid (TCA) for 3 min, and digested with 200 μ L 0.1% collagenase at 37°C for 1 h. Cell pellets were collected by centrifugation at 14000 rpm for 2 min, resuspended in 100 μ L Laemmli buffer, and heated at 95°C for 5 min, prior to lysates being sonicated for 30 sec and stored at -20°C until use. To perform western blotting, cell lysates were separated on a 12% SDS-polyacrylamide gel, and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was blocked with 5% reconstituted skim milk powder in TBST solution (10 mM Tris-HCl pH 7.5 containing 150 mM NaCl and 0.05% Tween 20). The blots were incubated with primary antibodies diluted in TBST or Can Get Signal Immunoreaction Enhancer Solution 1 (Toyobo, Osaka) at 4°C overnight. After washing with TBST, horseradish peroxidase-conjugated secondary antibodies

diluted in TBST or Can Get Signal Immunoreaction Enhancer Solution 2 were applied and the blots were developed by the Enhanced Chemiluminescence Detection System (Perkin Elmer, Waltham, MA). Levels of GAPDH immunocomplexes were used as an internal standard for equal loading. Quantification of signal intensity was performed using the ImageJ software and normalized to the control value.

1.3.10 Small interfere RNA (siRNA) transfection

Cells were transfected with siRNA against the integrin $\alpha 2$ target sequence 5'-AACCAAAGAAGAAATGATTGTAG-3' (sense sequence, si α 2-1) or 5'-AACAAGAATGCTCAGATAATTCT-3' (sense sequence, si α 2-2) using Lipofectamine RNAiMAX Reagent (Invitrogen, Carlsbad, CA). A siRNA against the Azami Green target sequence 5'-AGCAGATATTCAGGACTATTTC-3' (sense sequence) was used as a negative control.

1.3.11 Statistical analysis

Each experimental condition was repeated at least 3 times. The data are expressed as mean $\pm$ S.D. Statistical analysis was performed using the Student's *t*-test, and a P value ≤ 0.05 was considered significant.

Chapter 2

IR-induced cancer cell invasion

2.1 Introduction

2.1.1 Mechanisms of IR-induced cancer malignancy

Irradiation can cause stress responses to tumor cells and, in combination with hypoxia, low glucose concentration, high lactate concentration and low extracellular pH, conditions that exist within the tumor mass and are accelerated by irradiation, up-regulating gene products important for cell migration (Rofstad, 2000). Irradiation of human hepatoma cells enhanced secretion of vascular endothelial growth factor (VEGF) and promoted cell growth in vivo, as well as intratumoral angiogenesis. Thus, radiotherapy might result in the outgrowth of previously dormant microtumors, and up-regulation of VEGF could be a result of a paracrine proliferative stimulus (Chung *et al.*, 2006).

Promotion of invasion and migration is associated with increased cell motility through morphological changes consistent with epithelial-mesenchymal transdifferentiation (EMT). Irradiation of cancer cells induced the phenotypic changes observed during EMT, such as spindle-shaped cells, loss of polarity, intercellular separation and pseudopodia formation, and subsequently increased tumor cell migration (Jung *et al.*, 2007). The expression of Twist, an organizer of EMT, was induced by ionizing irradiation, whereas irradiation-induced cell invasion was inhibited by down-regulation of Twist via siRNA (Tsukamoto *et al.*, 2007). IR enhances the migratory and invasive abilities of several types of cancer cells through transforming growth factor (TGF- β)–mediated EMT (Zhou *et al.*, 2011).

2.2 Results

As described in Chapter 1, cancer cell malignancy could be elevated upon IR. So we first confirmed that whether IR-promoted cancer cell invasion. Cell phenotype was compared between P and IR cells. For general cell culture on a two-dimensional (2D) stiff substrate, such as a plastic culture dish or glass coverslip, morphologies of P and IR cells presented no obvious difference (Fig. 2-1A, upper panel). To mimic the microenvironment *in vivo*, a 3D type I collagen gel which forms a dense matrix full of collagen fibers, was used to culture cells. Interestingly, cell morphologies differed significantly when embedded in this collagen gel (Fig. 2-1A, lower panel). P cells are spherical, whereas IR cells are elongated. Morphology analysis showed an approximately eight-fold elevation of elongated phenotype in IR cells, compared with that in P cells (Fig. 2-1B).

To visualize and quantify the cell invasiveness, two kinds of invasion assays were performed. For evaluating individual cancer cell invasion, we took advantage of the time-lapse observation of cells in 3D collagen gel-sand. The results showed that P cells could barely move, whereas IR cells invaded the surrounding collagen gel by their long protrusions (Fig. 2-2A), and moved faster by about two-fold (Fig. 2-2B). Moreover, trajectories of IR cells were longer and more directed than those of P cells were, with cells often turning around (Fig. 2-2C). Increased invasiveness of IR cells was further confirmed by 3D spheroid invasion assay to mimic the characteristic of tumors *in vivo* (Fig. 2-3A). The results show that, after embedded in collagen gel for 24 h, both P and IR spheroids increased in volume by about 20–40% (Fig. 2-3B), whereas IR spheroids extended massive protrusions, with

some cells already escaped from the body, and presented as a higher aspect ratio than that of P cells (Fig. 2-3C).

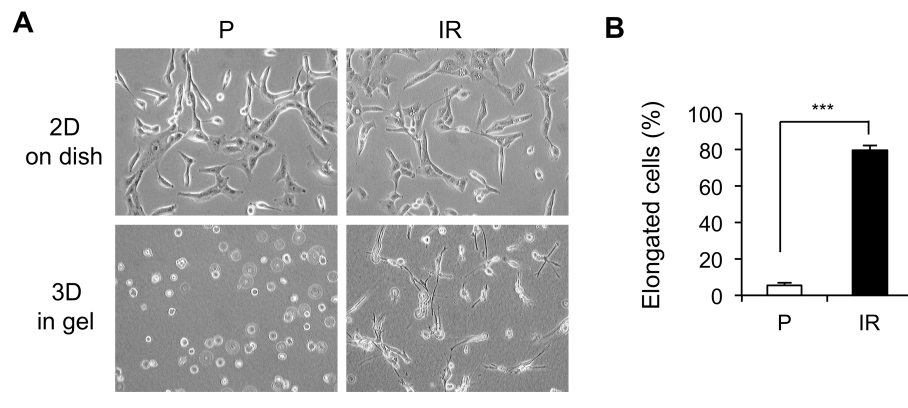


Figure 2-1. IR cells present aggressive morphology in 3D collagen gel.

(A) Morphologies of P and IR cells on 2D plastic dish (upper panel) or in 3D collagen gel (lower panel) after culturing for 24 h. Scale bar, 100 μ m. (B) Cell morphology analysis of P and IR cells in 3D collagen gel by quantifying the percentage of elongated cells in total, expressed as mean values $\pm$ S.D (***) $p < 0.001$) from three independent experiments including about 100 cells.

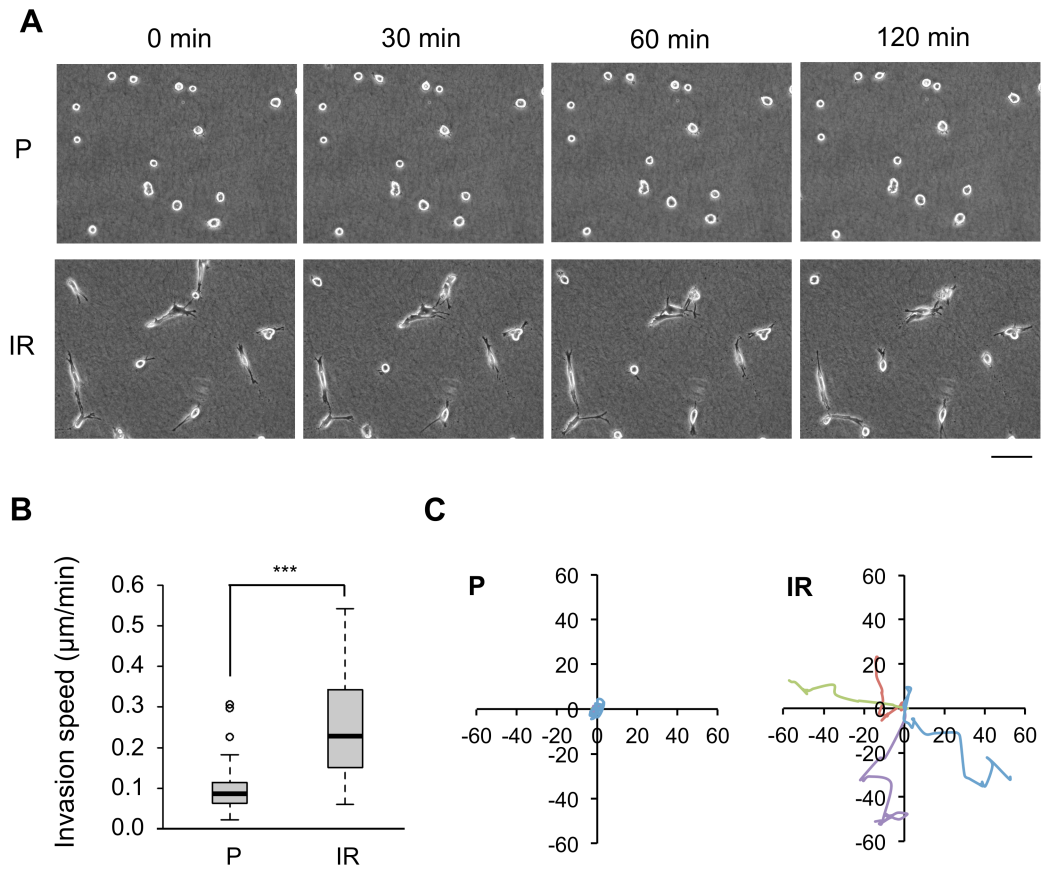


Figure 2-2. Individual IR cells are highly invasive in 3D collagen gel.

(A) Time-lapse phase contrast images of P and IR cells in 3D collagen gel-sand at 0 min, 30 min, 60 min and 120 min after observation. Scale bar, 100 μm . (B) Quantification of invasion speed in P and IR cells presented as mean values $\pm$ S.D, *** $p < 0.001$. (C) Diagrams representing the invasion trajectories of four representative cells from P and IR cells in 3D collagen gel-sand covered for 6 h. Cell origins were set as (0, 0), and scale unit is μm .

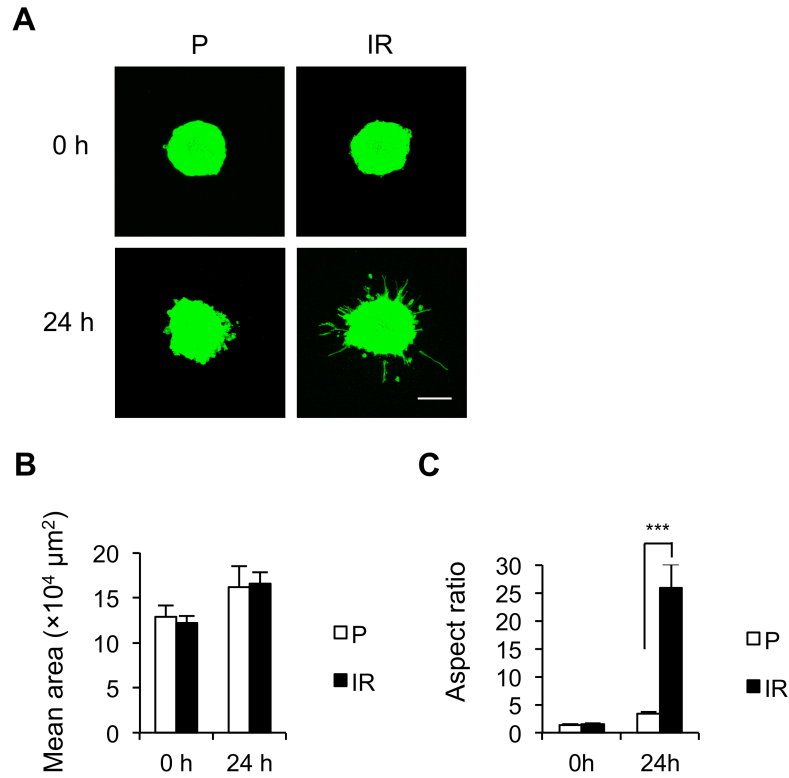


Figure 2-3. Spheroid IR cells are highly invasive in 3D collagen gel.
 (A) Confocal images of representative MFP488 phalloidin-stained P and IR spheroids in collagen gel at 0 h or 24 h. Scale bar, 200 μm . (B) Quantification of the area of spheroids by ImageJ software. (C) The aspect ratio of spheroids was calculated from $\text{perimeter}^2/[4\pi(\text{area})]$. Results are presented as mean values $\pm$ S.D. (***) $p < 0.001$) from three independent experiments in triplicate.

Chapter 3

Integrin in IR-induced cancer cell invasion

3.1 Introduction

As cancer cells become metastatic, they develop altered affinity and avidity for their extracellular matrix (ECM). The phenotypic change is initially mediated by alterations in the expression of cell-surface molecules known as integrins, release of proteases that remodel the ECM and the deposition of new ECM molecules (Hood and Cheresch, 2002).

3.1.1 Integrin family

Integrins are a diverse family of glycoproteins that form $\alpha\beta$ heterodimeric receptors for ECM molecules. The family can form at least 24 distinct pairings of its 18 α subunits and 8 β subunits (Fig. 3-1), with each pairing being specific for a unique set of ligands (van der Flier *et al.*, 2001). Together, these chains mediate adhesion and mechanotransduction to extracellular ligands, including integrin $\alpha2\beta1$ predominantly binding to fibrillar collagen; $\alpha4\beta1$ and $\alpha5\beta1$ interacting with fibronectin; and $\alpha3\beta1$ and $\alpha6\beta1$ engaging with laminin (Hynes, 2002).

Integrins are bi-directional signaling receptors involved in outside-in and inside-out signaling (Fig. 3-2). The inside-out signaling mainly acts to bring the integrin into the active conformation. Upon ligand binding, integrins undergo conformation changes leading to outside-in signaling. The combination of these two events leads to intracellular signals that control cell polarity, cytoskeletal structure, gene expression and cell survival and proliferation (Shattil *et al.*, 2010). The scheme of integrin initiated signaling network that lead to cell invasion and

migration was presented in Fig. 3-3.

3.1.2 Integrin in cancer cell invasion

Numerous studies have documented marked differences in surface expression and distribution of integrins in malignant tumors compared with pre-neoplastic tumor of the same type (Mizejewski *et al.*, 1999). Changes in the integrin pattern during malignant transformation are highly dependent on the type of the cancer. An altered integrin pattern allows the cancer cells to recognize variable matrices, but it may also lead to altered signalling and changes in gene expression.

The expression of $\alpha 2\beta 1$ and $\alpha 3\beta 1$ was increased in metastatic cells when compared to cells in the primary tumor (Klein *et al.*, 1991; Melchiori *et al.*, 1995). Integrin $\alpha 5\beta 1$ facilitates cancer cell invasion through enhanced contractile forces. Blocking anti- $\alpha 5$ or anti- $\beta 1$ integrin antibodies to $\alpha 5\beta 1^{\text{high}}$ cells as well as $\alpha 5$ -integrin-specific knockdown dramatically decreased the invasiveness into 3D ECMs. Cell invasiveness was also reduced after addition of the myosin light chain kinase inhibitor ML-7 in $\alpha 5\beta 1^{\text{high}}$ cells (Mierke *et al.*, 2010).

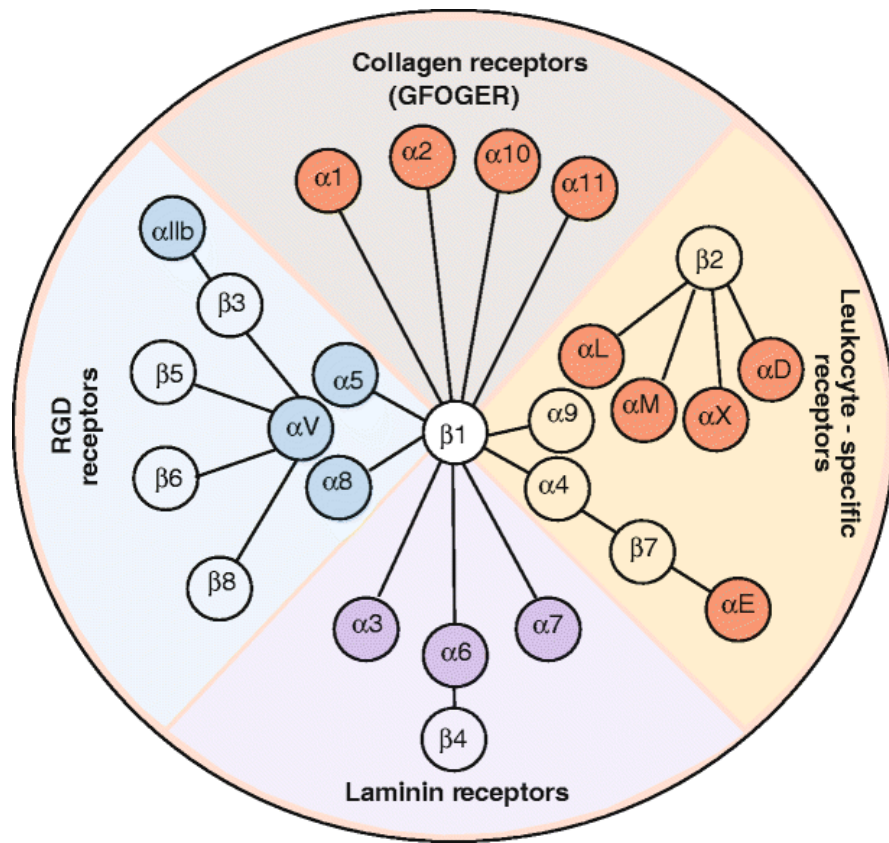


Figure 3-1. Classification of integrin family heterodimers (Barczyk *et al.*, 2010).

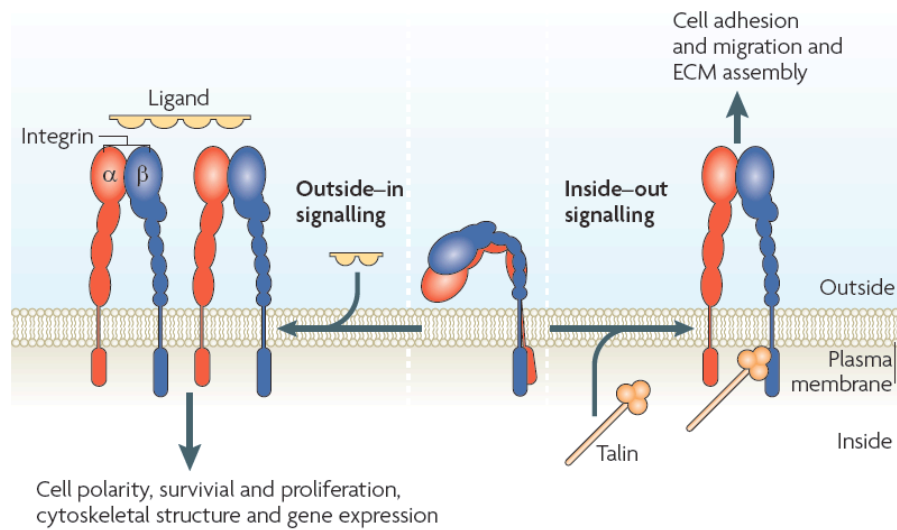


Figure 3-2. Two directions of integrin signalling (Shattil *et al.*, 2010)

During ‘inside-out’ signalling, an intracellular activator, such as talin or kindlins, binds to the β -integrin tail, leading to conformational changes that result in increased affinity for extracellular ligands (integrin ‘activation’). Inside-out signalling controls adhesion strength and enables sufficiently strong interactions between integrins and extracellular matrix (ECM) proteins to allow integrins to transmit the forces required for cell migration and ECM remodelling and assembly. Integrins also behave like traditional signalling receptors in transmitting information into cells by ‘outside-in’ signalling. Binding of integrins to their extracellular ligands changes the conformation of the integrin and, because many of the ligands are multivalent, contributes to integrin clustering.

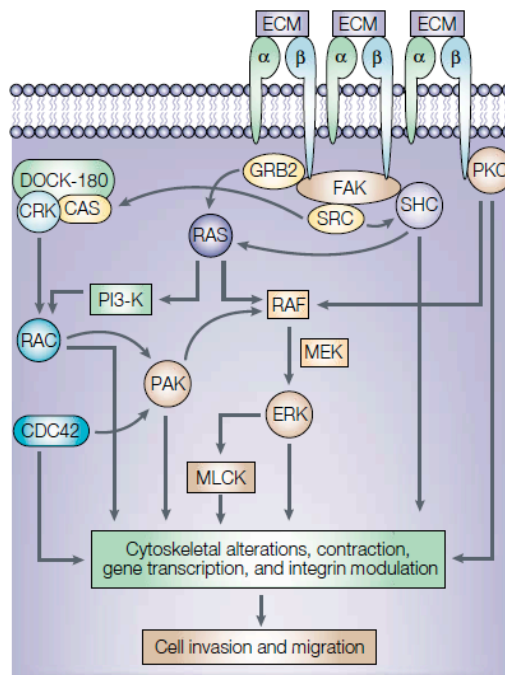


Figure 3-3. Regulation of intracellular signalling by integrins (Hood and Cheresh, 2002).

Integrin ligation induces a complex network of signalling pathways to control cell migration. Integrin binding to ligands in the extracellular matrix (ECM) activates focal adhesion kinase (FAK), which binds and activates multiple signalling proteins. Activated FAK can bind to growth-factor-receptor-bound protein 2 (GRB2) and activate another small G protein, RAS. Activated RAS recruits RAF to the cytoplasmic membrane, where it can be activated by protein kinases such as SRC125, thereby leading to mitogen-activated protein kinase kinase (MEK) and extracellular-signal-regulated kinase (ERK) activation. Once activated by FAK or SHC, RAS can activate phosphatidylinositol 3-kinase (PI3K) and RAF. Activated SRC can also phosphorylate CRK-associated substrate (CAS), enabling it to bind CRK and dedicator of cytokinesis 180 (DOCK-180), leading to RAC activation. Activated RAC, in conjunction with activated CDC42, can regulate numerous biochemical pathways, including activation of p21-activated kinase (PAK). MEK can phosphorylate and activate ERK, which leads to transcriptional activity, alterations in integrin affinity for ligand, and myosin-light-chain kinase (MLCK) activity.

3.1.3 Altered integrins in irradiated cancer cells

Given that the crucial role of integrins in cancer cell invasion, it is reasonable that integrins are also important in IR-induced invasion. In deed, integrins are often altered by IR and are capable of activating many different signaling pathways with multiple cellular responses. For instance, expression of integrin $\alpha v \beta 3$ in glioma cells (Wild-Bode *et al.*, 2001) and $\alpha 5 \beta 1$ in pancreatic cancer (Yao *et al.*, 2011) are upregulated by IR, facilitating both cell migration and invasion. Integrin $\alpha 3 \beta 1$ is overexpressed after IR, promoting the migration of meningioma cells via focal adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK) (Gogineni *et al.*, 2011). Our group (Ishihara *et al.*, 2010) and others (Nalla *et al.*, 2010) showed a pivotal role of integrin $\beta 1$ in IR-induced invasiveness in lung cancer and medulloblastoma, respectively.

3.2 Results

3.2.1 Expression pattern of integrin subunits in IR cells

Integrin-mediated adhesion to the ECM triggers intracellular signaling pathways to modulate cell morphology, migration, invasion, proliferation, and survival (Hood and Cheresch, 2002). The dramatic morphological change of IR cells compared to P cells when surrounded by a collagen matrix encouraged us to investigate the integrin expression pattern. In our previous study, we showed that knockdown of integrin $\beta 1$ by siRNA or treatment with its inhibitory antibody

AiIB2 induced spherical morphology of IR cells in 3D collagen gel, similar to P cells (Ishihara *et al.*, 2010). Given that collagen type I and fibronectin (sequestered from the FBS in the medium and secreted from the cells) are the main ECM components in our model, the expression pattern of integrins, including $\alpha1\beta1$, $\alpha2\beta1$, $\alpha4\beta1$, and $\alpha5\beta1$, was investigated by RT-PCR. Among them, $\alpha1\beta1$ and $\alpha2\beta1$ were reported as collagen receptors, while $\alpha4\beta1$ and $\alpha5\beta1$ were reported as fibronectin receptors (Humphries *et al.*, 2006).

The results of RT-PCR indicate that, in IR cells, the transcription levels of $\alpha2$ and $\beta1$ increased, the level of $\alpha1$ decreased, and there was no obvious change in levels of $\alpha4$ and $\alpha5$ (Fig. 3-4A). qRT-PCR further confirmed that the transcription level of $\alpha2$ was enhanced by 4.8-fold, and that of $\beta1$ was enhanced by 2.2-fold (Fig. 3-4B). Western blotting was carried out to detect their protein levels, which showed similar elevation (Fig. 3-4C). These results suggest that integrin $\alpha2\beta1$ might play an important role in the altered interaction between IR cells and the ECM.

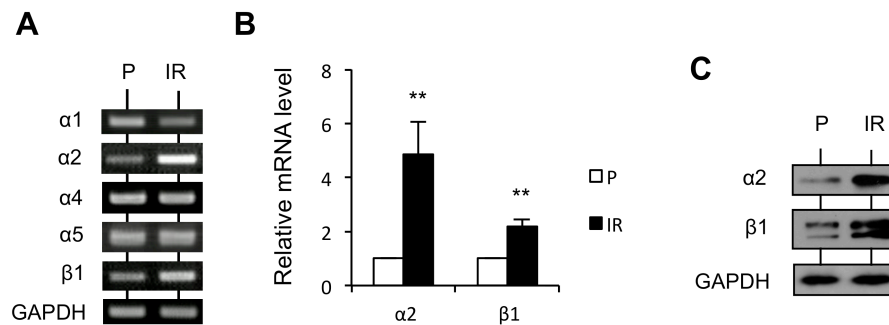


Figure 3-4. Integrin $\alpha 2$ and $\beta 1$ subunits were specifically upregulated in IR cells. (A) Semi-quantitative analysis of the mRNA levels of integrin subunits, including $\alpha 1$, $\alpha 2$, $\alpha 4$, $\alpha 5$, and $\beta 1$, from P and IR cells by RT-PCR. Intensity of signals was quantified by densitometry and normalized with GAPDH. Representation is mean value $\pm$ S.D of relative mRNA level (* $p < 0.05$, ** $p < 0.01$) from three independent experiments, indicated as fold change relative to P cells. (B) Quantitative analysis of the mRNA levels of integrin $\alpha 2$ and $\beta 1$ subunits from P and IR cells by qRT-PCR. Intensity of signals was quantified by densitometry and normalized with β -actin. Representation is mean value $\pm$ S.D of relative mRNA level (** $p < 0.01$) from three independent experiments, indicated as fold change relative to P cells. (C) Analysis of the protein levels of integrin $\alpha 2$ and $\beta 1$ subunits from P and IR cells cultured in 3D collagen gel by western blotting. GAPDH was used as a loading control.

3.2.2 Inhibition of integrin $\alpha 2\beta 1$ on IR cell invasion

To confirm whether the elevated expression of integrin $\alpha 2\beta 1$ is essential for IR cell invasiveness, knockdown of $\alpha 2\beta 1$ expression in IR cells by two kinds of siRNA specific to integrin $\alpha 2$ was carried out, and the effect was verified by RT-PCR (Fig. 3-5A). Indeed, knockdown of $\alpha 2$ impaired IR cell elongation (Fig. 3-5B) and invasion in collagen gel (data not shown).

Since integrins directly bind components of the ECM and provide the traction necessary for cell motility and invasion, we considered whether the interaction between integrin $\alpha 2\beta 1$ and the ECM was important for IR cell invasion. The function-blocking antibody BHA2.1 (400 ng/mL) that recognizes the I domain of $\alpha 2$, the binding site for collagens, was used to treat IR cells in the gel. Time-lapse observation showed that blocking the activation of integrin $\alpha 2\beta 1$ induced contraction of cell protrusions and low invasiveness soon after treatment, and removing the antibody by the addition of fresh medium restored invasion (Fig. 3-6A). BHA2.1 treatment significantly decreased the ratio of elongated phenotype (Fig. 3-6B) and invasion speed in IR cells (Fig. 3-6C), and abolished spheroid invasion (Fig. 3-6D, E, F), which suggests that functional integrin $\alpha 2\beta 1$ is required for IR cell invasion.

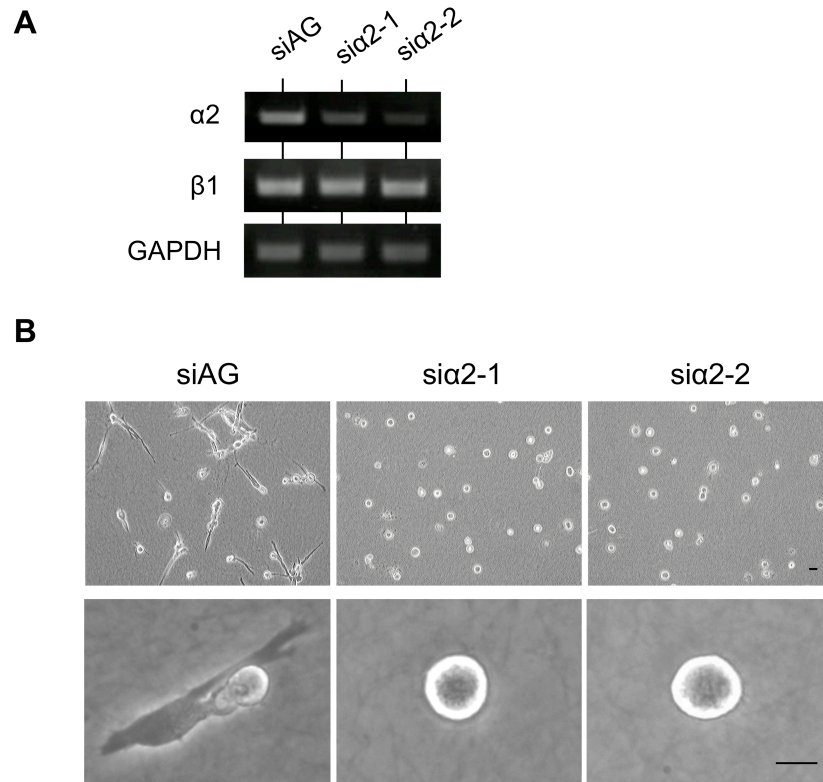


Figure 3-5. Integrin $\alpha 2$ expression is required for IR cell invasion.

(A) Knockdown of the integrin $\alpha 2$ subunit in IR cells resulted in a round morphology in 3D collagen gel. IR cells that were transfected with two different siRNAs specific to integrin $\alpha 2$, indicated as si $\alpha 2$ -1 and si $\alpha 2$ -2. Specific siRNA targeted to Azami-Green, indicated as siAG, was transfected as a control. Two days after transfection, cells were transferred to 3D collagen gel and cultured for 24 h. Left: RT-PCR results to verify the effect of specific knockdown of integrin $\alpha 2$. Integrin $\beta 1$ and GAPDH were also detected as controls. Both siRNA transfections resulted in a decreased level of integrin $\alpha 2$ mRNA, without affecting the level of integrin $\beta 1$ mRNA. Right: representative images of cell morphologies. Scale bar, 100 μ m.

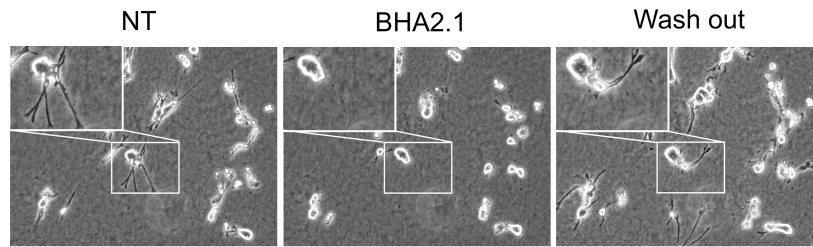
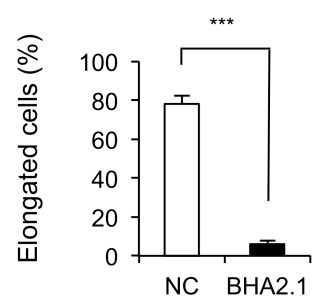
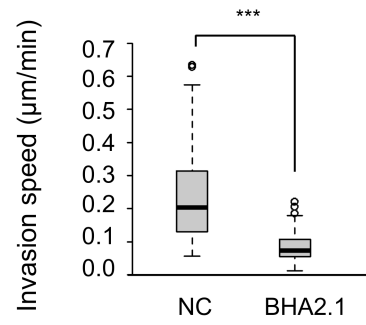
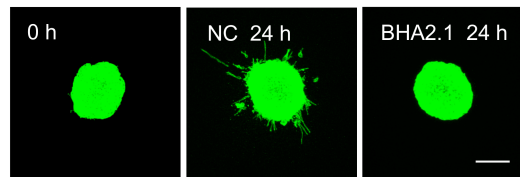
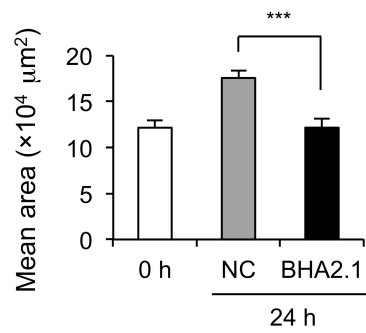
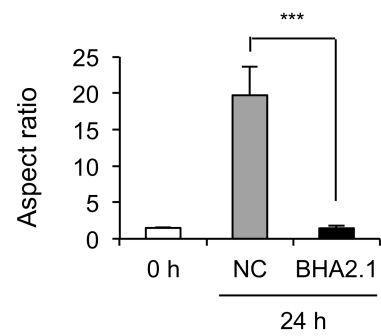
A**B****C****D****E****F**

Figure 3-6. Integrin $\alpha 2\beta 1$ activation is required for IR cell invasion.

(A) Function blocking of integrin $\alpha 2\beta 1$ induced a reversible retraction of protrusions and invasiveness of IR cells. Time-lapse observation of IR cells treated with an integrin $\alpha 2\beta 1$ -blocking antibody BHA2.1 in 3D collagen. Cells were first non-treated (NT) for 12 h, then treated with BHA2.1 (BHA) for 12 h, and washed out (Wash out) with fresh medium three times, followed by observation for another 24 h. The images taken from each step are shown. Scale bar, 100 μm . (B) Cell morphology analysis of BHA2.1-treated IR cells versus controls in 3D collagen gel by quantifying the percentage of elongated cells in total, expressed as mean values $\pm$ S.D ($***p < 0.001$) from three independent experiments including about 100 cells. (C) Quantification of speed in P and IR cells in 3D collagen gel-sand for 6 h, expressed as mean values $\pm$ S.D ($***p < 0.001$) from three independent experiments including about 100 cells. (D) Confocal images of representative of MFP488 phalloidin-stained P and IR spheroids in collagen gel at 0 h or 24 h. Scale bar, 200 μm . (F) Quantification of the area of spheroids by ImageJ software. (E) The aspect ratio of spheroids was calculated from $\text{perimeter}^2/[4\pi(\text{area})]$. Results are presented as mean values $\pm$ S.D ($***p < 0.001$) from three independent experiments in triplicate.

Chapter 4

Epidermal growth factor receptor (EGFR) in IR-induced cancer invasion

4.1 Introduction

4.1.1 EGFR

The epidermal growth factor receptor (EGFR; ErbB-1; HER1) is a member of ErbB family of receptor tyrosine kinase (Yarden and Sliwkowski, 2001). Upon activation by their ligands, such as epidermal growth factor (EGF), transforming growth factor- α (TGF- α) and heparin-binding EGF (HB-EGF), EGFR forms homodimer or heterodimer with other members of ErbB. Dimerization consequently stimulates the intrinsic tyrosine kinase activity of the receptors and triggers autophosphorylation of specific tyrosine residues within the cytoplasmic domain which serve as docking sites for signaling molecules involved in the regulation of intracellular signaling cascades (Fig. 4-1) (Olayioye *et al.*, 2000). There is also evidence that EGFR can translocate to the nucleus where it acts as a transcription factor (Lin *et al.*, 2001; Waugh and Hsuan, 2001).

Frequent mutations in the kinase domain of the EGFR gene result in overexpression, decreased turnover of receptor internalization and constitutive tyrosine kinase activation. These aberrant regulations render an advantage in survival and proliferation mediated by PI3K/Akt and p42/p44 MAPK signaling and promote tumor-associated angiogenesis and matrix metalloproteinase activation (Schlessinger, 2004; Hynes and Lane, 2005).

Owing to the fact that both EGFR-Akt and ILK-Akt signaling are PI3K-dependent and that the EGFR-Akt pathway is involved in the repair of radiation-induced DNA-double strand break (DNA-DSB) lesions, ILK-EGFR

interactions may be critical for cellular radiation survival (Baumann *et al.*, 2003, Toulany *et al.*, 2005).

4.1.2 EGFR in cancer cell invasion

EGFR activation can stimulate proliferation, angiogenesis, protection from apoptosis, loss of differentiation, migration and invasion — all hallmarks of cancer. (West *et al.*, 2008). Especially in NSCLC, EGFR is frequently overexpressed or harbors constitutively active mutations in NSCLC (Gazdar and Minna, 2008).

Increased EGFR signaling in cancer cells can occur through several mechanisms. The receptor itself can be modified either by activating deletions or mutations in receptor gene (Pines *et al.*, 2010) or by increased receptor expression, often caused by gene amplification (Bellone *et al.*, 2007).

4.1.3 EGFR in cell response to radiotherapy

There is evidence that ionizing radiation has the ability to activate EGFR signalling, and other receptor tyrosine kinases, independent of ligand binding (Bowers *et al.*, 2001). It is already known that anti-EGFR strategies potentiate the effects of radiotherapy and inhibition of DNA repair appears to be important amongst a wide range of mechanisms, which include effects on angiogenesis, differentiation and immunological response (West *et al.*, 2008). Recently, several studies suggested that EGFR also involved IR-induced invasion. For example, Src/EGFR-mediated p38/Akt and PI3K/Akt pathways regulate IR-induced

invasion of glioma cells (Park *et al.*, 2006). Blocking of EGFR pathway inhibited IR-induced migration of human head and neck squamous cell carcinoma (Pickhard *et al.*, 2011).

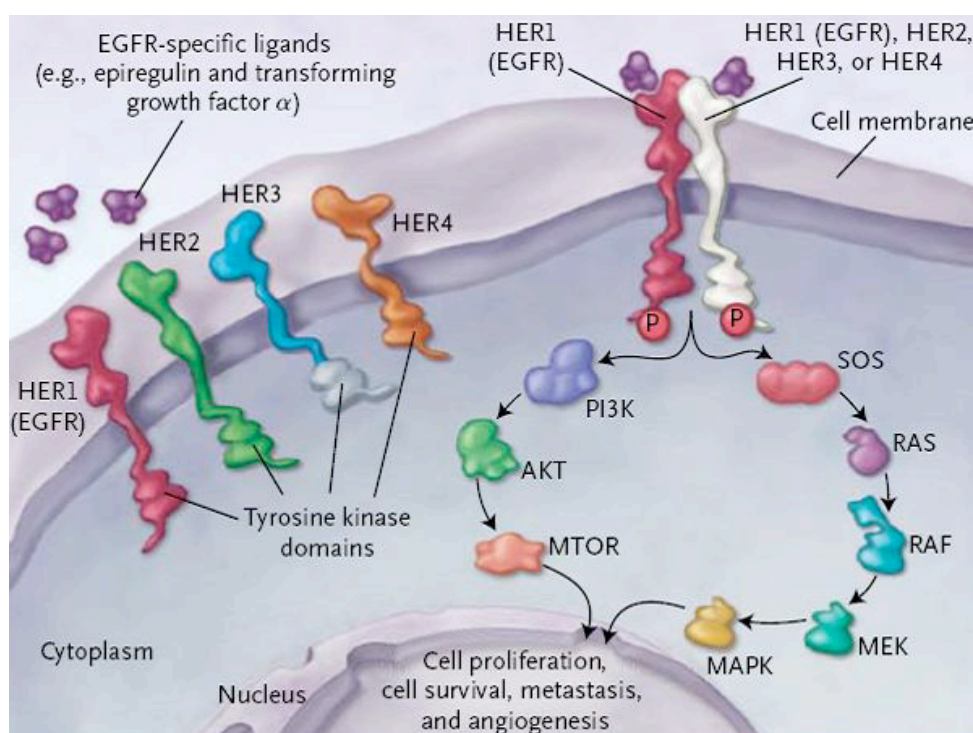


Figure 4-1. Scheme of EGFR dimerization and downstream signaling pathways. (Cataldo *et al.*, 2011)

4.2 Results

4.2.1 Expression and activation of EGFR in IR cells

Considered the important role of EGFR in invasion and radioresistance, we checked whether any alterations of EGFR occurred in IR cells. Surprisingly, both EGFR transcriptional level and protein level were much elevated in IR cells, compared with those in P cells (Fig. 4-2A, B). A consistently high level of EGFR activation on the signaling-related residue Tyr1068 was also observed in IR cells without any stimulation by EGFR ligand (Fig. 4-2C).

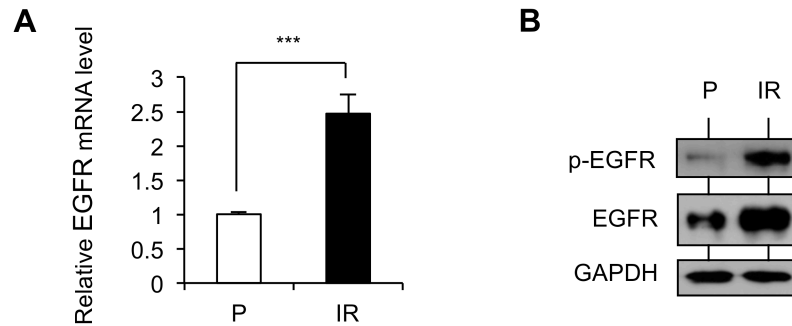


Figure 4-2. EGFR is overexpressed and activated in IR cells

(A) Quantitative analysis of EGFR mRNA levels from P and IR cells by qRT-PCR, represented as mean values $\pm$ S.D of relative mRNA level (** $p < 0.001$) from three independent experiments, indicated as fold change relative to P cells. (B) Analysis of protein levels of total EGFR and phosphorylated EGFR (Tyr1068) by western blotting. Intensity of signals were quantified by densitometry and normalized with GAPDH. Results are represented as mean values $\pm$ S.D of relative protein level (** $p < 0.01$) from three independent experiments, indicated as fold change relative to P cells.

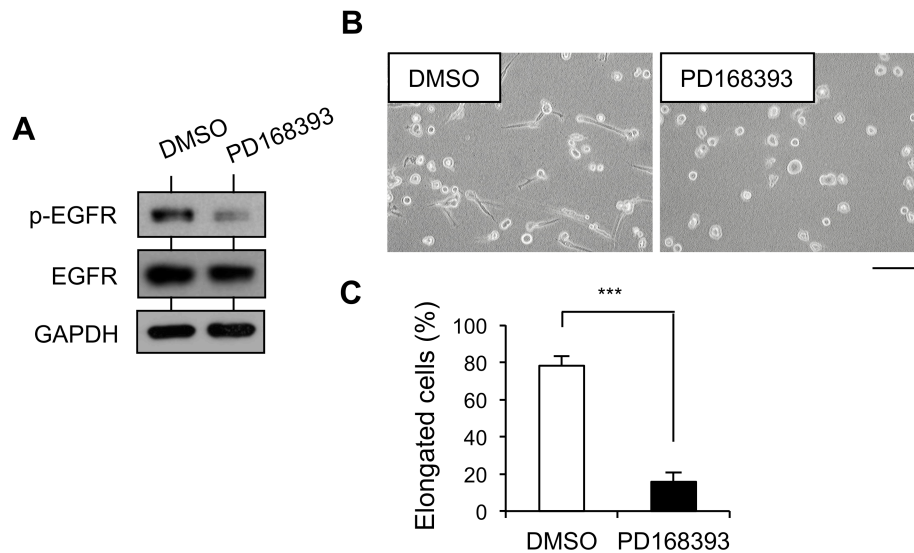


Figure 4-3. Inhibition of EGFR activation induced a round morphology of IR cells.

IR cells in 3D collagen gel were treated with 10 μ M EGFR inhibitor PD168393 or DMSO as a control for 24 h. (A) Expression analysis of phosphorylated EGFR (Tyr1068) and total EGFR from PD168393- and DMSO-treated samples by western blotting. GAPDH was used as a control. (B) Representative images of cell morphologies of PD168393-treated samples versus the DMSO-treated control. (C) The percentage of elongated cells was quantified from three independent experiments including about 100 cells, *** $p < 0.001$.

4.22 Inhibition of EGFR on IR cell invasion

Therefore, a specific inhibitor targeting the tyrosine kinase of EGFR, PD168393 (10 μ M), was used to treat IR cells, and was shown to decrease the phosphorylation of EGFR (Fig. 4-3A), the ratio of elongated IR cells (Fig. 4-3B, C), and the invasion speed (Fig. 4-4A). Like integrin $\alpha 2\beta 1$ inhibition, PD168393-treated IR spheroids remained regular spheroids, embedded without volume expansion (Fig. 4-4C) or protrusion (Fig. 4-4D). These results support the hypothesis that the EGFR signaling pathway is involved in the increased invasiveness of IR cells.

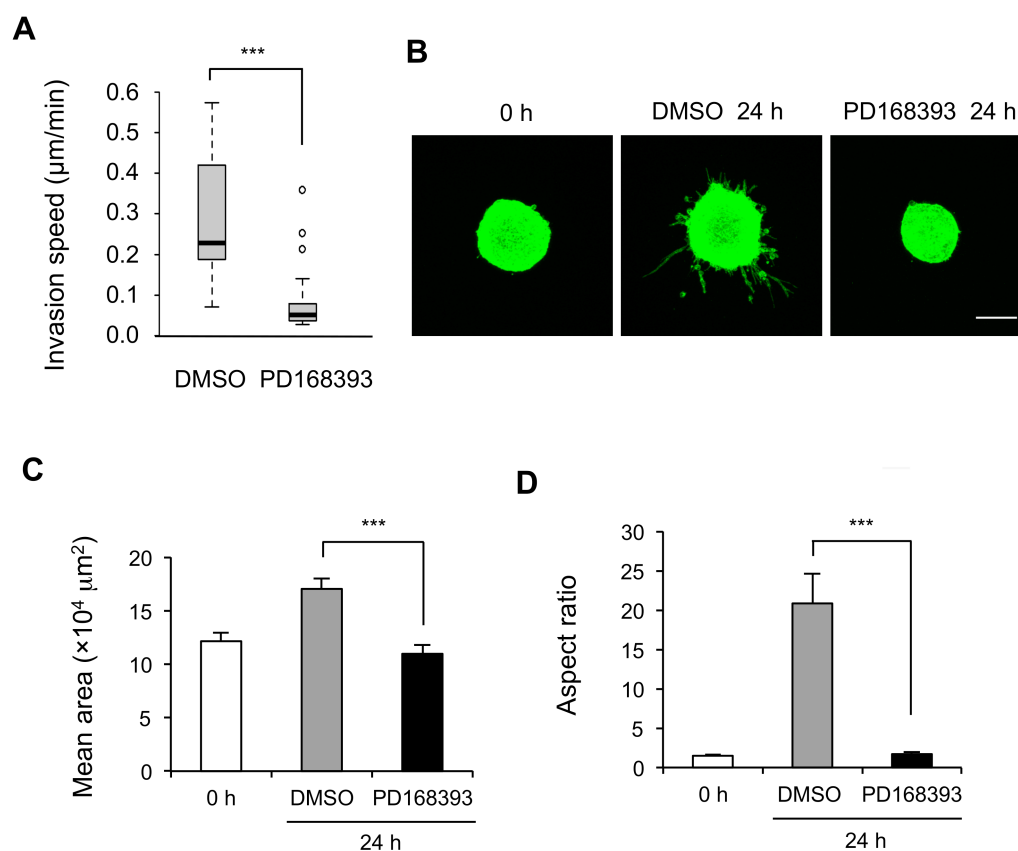


Figure 4-4. Inhibition of EGFR activation induced abrogated IR cells invasiveness.

(A) Quantification of speed in DMSO- or PD168393-treated IR cells in 3D collagen gel-sand for 6 h are represented as mean values $\pm$ S.D (** $p < 0.001$) from three independent experiments including about 50 cells. (B) Confocal images of representative MFP488 phalloidin-stained IR spheroids in collagen gel for 0 h and IR spheroids treated with DMSO or PD168393 for 24 h. Scale bar, 200 μm . (C) Quantification of the area of spheroids by ImageJ software. (D) The aspect ratio of spheroids was calculated from $\text{perimeter}^2/[4\pi(\text{area})]$. Results are represented as mean values $\pm$ S.D (** $p < 0.001$) from 3 independent experiments in triplicate.

Chapter 5

IR-induced signaling pathways

5.1 Introduction

Well-known pathways that display enhanced activity upon irradiation include the mitogen-activated protein kinase (MAPK) superfamily and the phosphatidylinositol 3-kinase (PI3K) pathway. The role of PI3K as a radioprotective molecule is widely distributed among different tumor cells. PI3K signaling pathway is associated with major radioresistance mechanisms, such as intrinsic radiosensitivity, tumor cell proliferation and hypoxia (Schuurbiers *et al.*, 2009). ERK1/2 signaling protects irradiated tumor cells by enhancing pro-survival and DNA-repair mechanisms. Radiation-induced ERK has been associated with induction of DNA repair proteins, such as ERCC1, XRCC1, XPC and also interacts with ATM signaling to regulate homologous recombination repair (Yacoub *et al.*, 2001; Golding *et al.*, 2007).

5.2 Results

5.2.1 Expression and activation of downstream signaling molecules in IR cells

To further identify the mechanism of the integrin $\alpha 2\beta 1$ - and EGFR-promoted IR cell invasion, we surveyed several important downstream signaling molecules that were regulated by integrin $\alpha 2\beta 1$ and/or EGFR, including MEK/Erk1/2 (Sawhney

et al., 2003; Ramis *et al.*, 2012), PI3K/Akt (Ramis *et al.*, 2012; Cabodi *et al.*, 2009, Stat3 (Wang *et al.*, 2006), and p38 MAPK (Klekotka *et al.*, 2001; Junga *et al.*, 2007). Among them, western blotting showed only Erk1/2 and Akt activation to be significantly upregulated in IR cells, with the formers' total and phosphorylated protein levels on the residues necessary for signal transduction (Fig. 5-1).

5.2.2 Inhibition of PI3K/Akt or MEK/Erk1/2 on IR cell invasion

To confirm whether their activation is related to IR cell invasiveness, specific inhibitors targeting their upstream kinases were used, including MEK inhibitor U0126 (10 μ M) for Erk1/2 and PI3K inhibitor LY294002 (50 μ M) for Akt. The activation of Akt and Erk1/2 was abrogated by decreased phosphorylation upon inhibition of their upstream molecules (Fig. 5-2B). Morphology analysis showed that LY294002 treatment increased the percentage of round cells (Fig. 5-2A) and, thus, invasion speed (Fig. 5-3A), while U0126 treatment did not. Consistently, 3D spheroid invasion assay showed that IR cell protrusion into collagen gel was suppressed only after treatment with LY294002, whereas U0126 had little effect (Fig. 5-3B, D), even though spheroid expansion was inhibited slightly (Fig. 5-3C). These results suggest the involvement of PI3K/Akt, but not MEK/Erk1/2, in invasive signal transduction in IR cells.

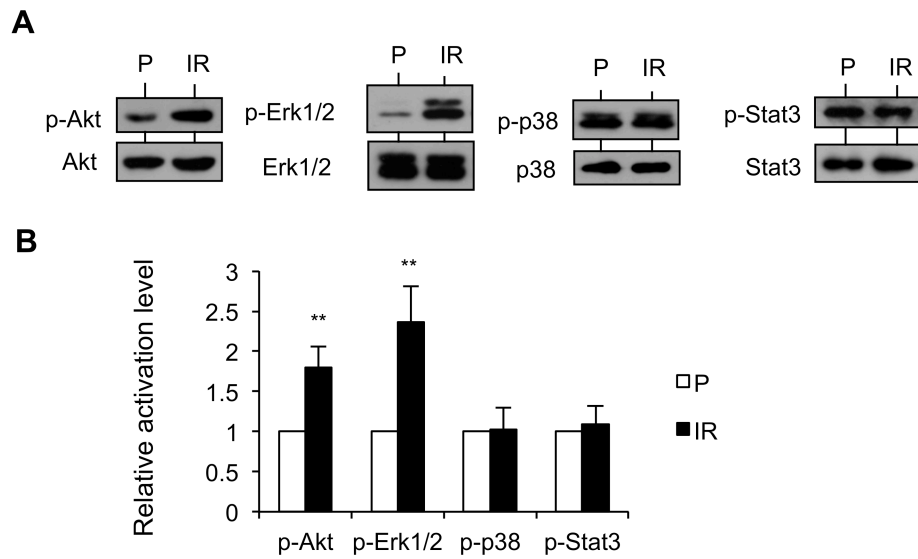


Figure 5-1. Activation of Akt and Erk1/2 in IR cells.

(A) Expression analysis of the total and phosphorylated forms of Akt (Ser473), Erk1/2 (Thr202/Tyr204), p38 (Thr180/Tyr182), and p-Stat3 (Ser727) by western blotting. (B) Intensity of signals was quantified by densitometry and normalized with GAPDH. Results are represented as mean values $\pm$ S.D of relative protein level (** $p < 0.01$) from three independent experiments, indicated as fold change relative to P cells.

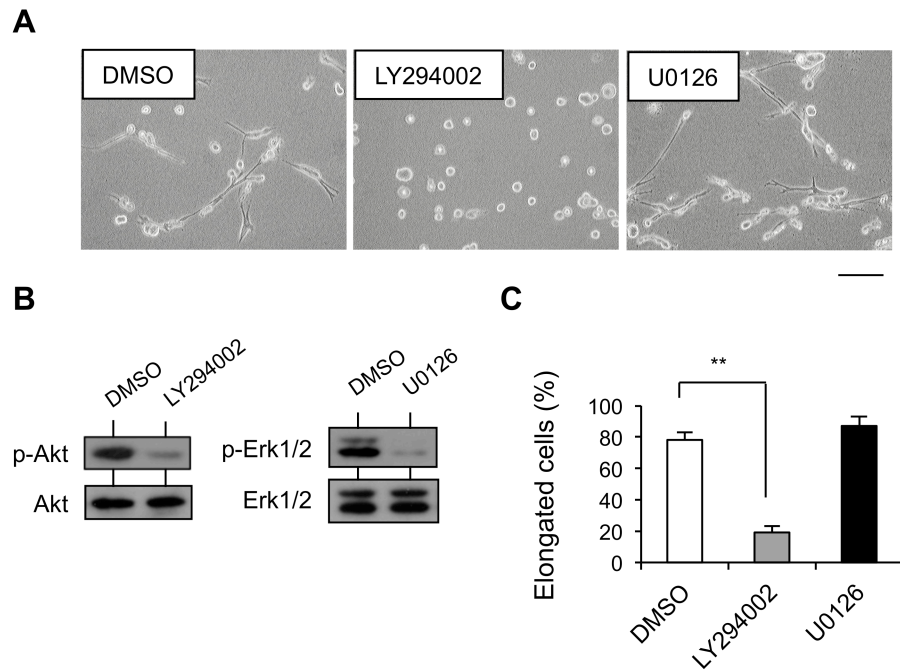


Figure 5-2. Inhibition of Akt and Erk1/2 activation on morphology of IR cells.
 (A) Phase contrast images of PI3K inhibitor LY294002 (50 μ M) or MEK inhibitor U0126 (10 μ M) treated IR cells versus DMSO-treated IR cells for 24 h in 3D collagen gel (B) The percentage of elongated cells was quantified from three independent experiments including about 100 cells, *** $p < 0.001$.

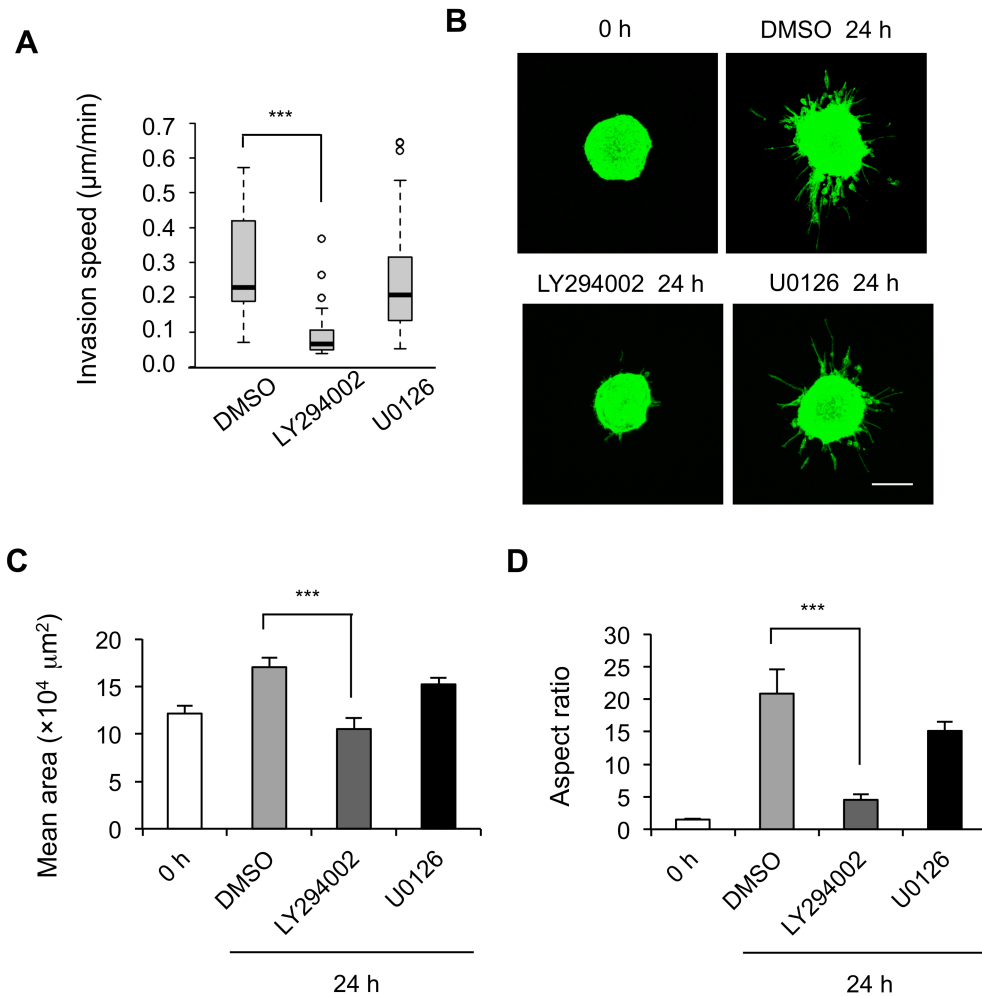


Figure 5-3. Inhibition of PI3K/Akt but not MEK/Erk1/2 reduces IR cell invasion. (A) Quantification of speed in DMSO-, LY294002-, or U0126-treated IR cells in 3D collagen gel-sand for 6 h, represented as mean values $\pm$ S.D. (***) $p < 0.001$) from three independent experiments including about 50 cells. (B) Confocal images of representative MFP488 phalloidin-stained IR spheroids in collagen gel for 0 h and IR spheroids treated with DMSO, LY294002, or U0126 for 24 h. Scale bar, 200 μm . (C) Quantification of the area of spheroids by ImageJ software. (D) The aspect ratio of spheroids was calculated from $\text{perimeter}^2/[4\pi(\text{area})]$, and results are presented as mean values $\pm$ S.D. (***) $p < 0.001$) from three independent experiments in triplicate.

Chapter 6

Interaction between integrin $\alpha 2\beta 1$ and EGFR

6.1 Introduction

Integrin signalling coordinates with signalling originating from growth factor receptors in the cooperative control of cell proliferation, survival and migration. The role of integrins as the primary motors controlling GFR signals has been demonstrated in many systems. Several mechanisms by which integrins control GFR activity have been proposed (Fig. 6-1), including the recruitment of specific adaptors to the plasma membrane and concentrate them proximal to GFRs (Goel *et al.*, 2005), changing the localization of GFR to focal contacts (Burrige *et al.*, 1997), and alteration of the internalization and degradation of GFR (Baron and Schwartz, 2000).

Specifically, EGFR has been reported to interact with $\alpha v \beta 3$ (Moro *et al.*, 2002; Cabodi *et al.*, 2004), $\beta 1$ (Moro *et al.*, 1998; Wang *et al.*, 1998), $\alpha 2 \beta 1$ (Yu *et al.*, 2000), and $\alpha 6 \beta 4$ (Mariotti *et al.*, 2001). Reginato *et al.*, (2003) showed that EGFR expression is regulated by the $\beta 1$ integrin and is reduced when the cells are detached from the ECM. Bill *et al.*, (2004) proposed that integrin-mediated activation of EGFR is required for multiple signaling events induced by integrins.

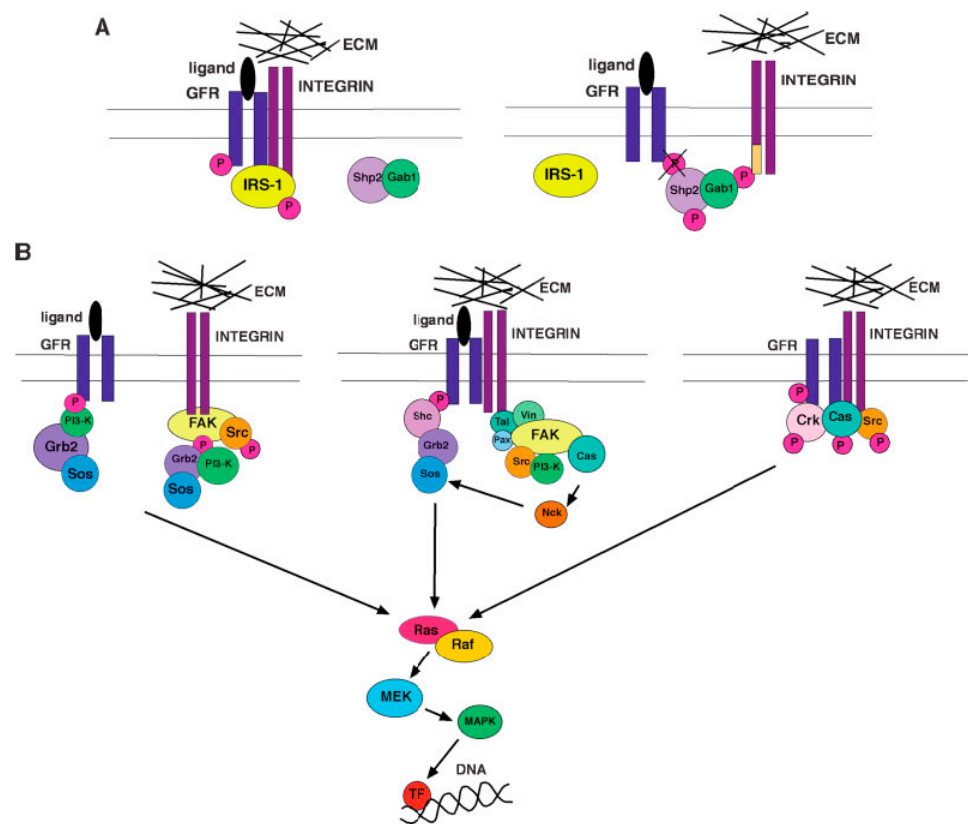


Figure 6-1. Regulation of growth factor receptor (GFR) signaling by integrins (Alam *et al.*, 2007).

6.2 Results

Since both PI3K/Akt and MEK/Erk1/2 signaling pathways could be activated by EGFR and integrin, we investigated which is responsible for their activation in IR cells. We found that Akt activation was downregulated by either inhibiting EGFR or blocking integrin $\alpha 2$ expression or $\alpha 2\beta 1$ function (Fig. 6-2). Although Erk1/2 is regarded as being regulated by EGFR (Edick *et al.*, 2007; Gan *et al.*, 2010), decreased Erk1/2 activation was only observed when silencing the integrin $\alpha 2$ expression or blocking the integrin $\alpha 2\beta 1$ function (Fig. 6-2).

The similar effect of integrin $\alpha 2\beta 1$ and EGFR on Akt activation and IR cell invasiveness prompted us to study whether their overexpression and/or activation are dependent on each other. Knock down integrin $\alpha 2$ or function blocking of integrin $\alpha 2\beta 1$ suppressed activation of EGFR (Fig. 6-2). On the other hand, inhibition of EGFR tyrosine kinase activity did not affect expression of $\alpha 2$ or $\beta 1$ (data not shown), but attenuated cell protrusion into the collagen gel (Fig. 6-3). These results suggest that EGFR activation might be necessary for integrin $\alpha 2\beta 1$ function in mediating cell invasion into the collagen matrix, moreover, the switch to the invasive morphology of IR cells not only depends on the presence of collagen substrate for interaction with integrin $\alpha 2\beta 1$ extracellular domain, but also depends on the intracellular signaling activation by integrin $\alpha 2\beta 1$ cytoplasmic domain. Taken together, based on the results, it appears that integrin $\alpha 2\beta 1$ and EGFR coordinately promote invasion of IR-surviving cells, partially through activation of the PI3K/Akt signaling pathway.

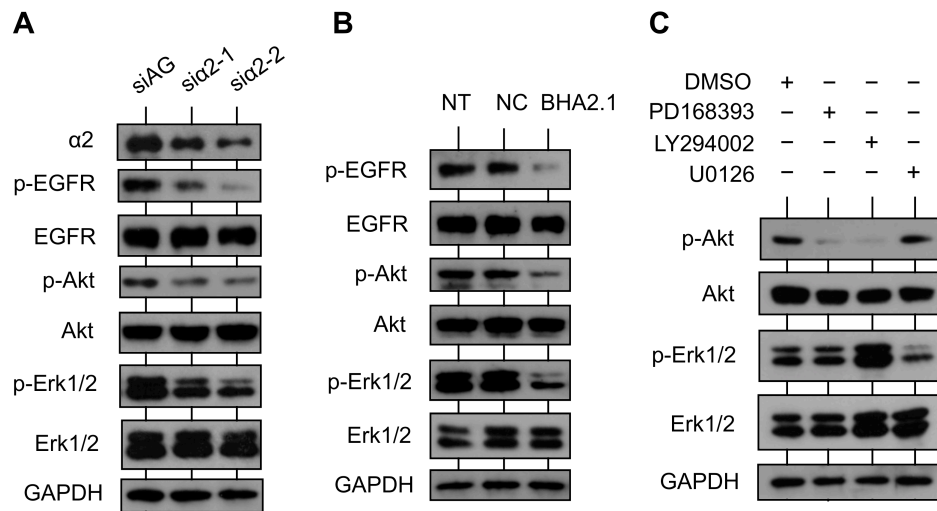


Figure 6-2. Integrin $\alpha 2\beta 1$ regulate activation of EGFR and downstream signaling pathways.

(A, B) Regulation of integrin $\alpha 2\beta 1$ on EGFR, PI3K/Akt, and MEK/Erk1/2 signaling pathways. (A) IR cells in 3D collagen gel were non-treated (NT), treated with non-function blocking antibody for integrin $\alpha 2\beta 1$ (NC), or treated with function blocking antibody for integrin $\alpha 2\beta 1$ (BHA2.1) for 24 h. (B) IR cells that transfected with siRNA specific to AzamiGreen (siAG) as a negative control or a siRNA specific to integrin $\alpha 2$ (si $\alpha 2$ -1 or si $\alpha 2$ -2) were transferred to 3D collagen gel and cultured for 24 h. Cells were harvested, and the amounts of integrin $\alpha 2$, phosphorylated and total EGFR (Tyr1068), Akt (Ser473), and Erk1/2 (Tyr202/Tyr204) were analyzed by western blotting. (C) Regulation of EGFR in PI3K/Akt and MEK/Erk1/2 signaling pathways. IR cells in 3D collagen gel were treated with DMSO (control), EGFR inhibitor PD168393 (10 μ M), PI3K inhibitor LY294002 (50 μ M), or MEK inhibitor U0126 (10 μ M) for 24 h. Cells were harvested, and the amounts of phosphorylated and total Akt (Ser473) and Erk1/2 (Tyr202/Tyr204) were analyzed by western blotting.

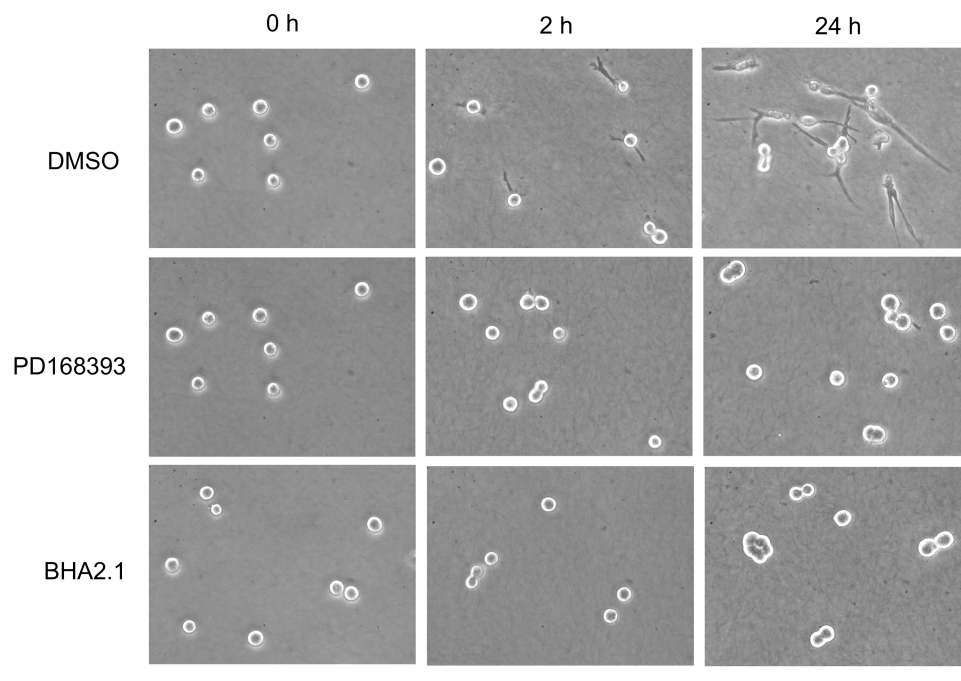


Figure 6-3. Inhibition of EGFR or function blocking of integrin $\alpha 2\beta 1$ in cells invading collagen.

IR cell suspension in medium with DMSO, PD168393, or BHA2.1 was seeded on collagen gel. Phase contrast images for each sample at 0 h, 2 h, and 24 h are shown. DMSO-treated IR cells have already protruded into collagen at 2 h and present long protrusions at 24 h. In contrast, PD168393- or BHA2.1-treated IR cells simply adhere to the collagen with a spherical morphology.

Chapter 7

Discussion

In this study, we confirmed that ionizing radiation could promote the invasiveness of lung cancer cells *in vitro*. To decrease the possibility that cancer stem cells induce radioresistance (Baumann *et al.*, 2008), and for better analysis of IR-induced invasiveness, heterogeneous A549 cells were first screened as a relatively less invasive subclone to be parent cells. Then, P cells were subjected to a therapeutic dose of IR to mimic the clinical observation in which most of the cancer cells undergo apoptosis after IR exposure. The small fraction of cancer cells that survived was harvested as IR cells.

Invasive behavior was compared between P cells and IR cells in a fibrillar collagen matrix, the most abundant ECM component in the lung connective tissue (Suki *et al.*, 2005), to mimic the *in vivo* environment. The different invasive potential of P and IR cells were first reflected in cell morphology on/in the collagen matrix but not on 2D stiff substrate. We observed that P cells were spherical, whereas IR cells were elongated with protrusions. The time-lapse observation of the migration of cells in collagen gel showed that this elongated morphology of IR cells favor their directional invasion, cell migrated fast along the direction of the protrusions. However, the round morphology of P cells hindered this mesenchymal type movement, resulted in the half less speed of cell movement. More important, P cells often turning around, so they barely leave their starting point.

In addition to the real-time observation of individual cell movement in collagen matrix, we also performed the 3D spheroid invasion assay. There are growing evidences that tumor cell aggregates or spheroids provide a more representative

model of tumors in vivo than can be achieved with monolayers. Spheroids exhibit several relevant physiological traits including similar morphology, formation of cell-cell bonds, decreased proliferation rates, increased cell survival, tumor dormancy and a hypoxic core. Applying this model in a 3D culture invasion assay provides a more physiological approach for assessing tumor invasion and providing a visual component that can be quantitated through image analysis. Especially in our study, all the experiments were carried out using cells in a 3D collagen gel. The morphology and invasive behavior of P and IR cells, and IR cells treated with inhibitors can be easily observed and compared in the gel, but not on the stiff substrate like a plastic dish, or even those coated with a thin layer of collagen.

Transwell assay is a common assay to evaluate invasiveness, however, it is thought inappropriate in our study. Because, firstly cells are normally need to be serum starved to drive cell invading towards the higher concentration of chemoattractant (normally medium contains serum) in the lower chamber. But we found IR cell could not spread and elongate without serum. Secondly, transwell inserts with polycarbonate filter coated with a matrix substrate, which maybe more close to the collagen coated condition rather than 3D collagen matrix used in our laboratory. P and IR cells showed similar spreading morphology on this stiff substrate possibly due to the difference of stiffness and structure. And migration through this thin membrane is thought different from the observation in 3D collagen matrix in our study. Thirdly, when invaded cells attach to the underside of the polycarbonate filter, the possibility to perform morphologic and morphometric

analysis is greatly compromised.

The results of 3D spheroid invasion assay and the individual cancer cell invasion assay showed the similar results that IR cells were much invasive than P cells. The correlation of cell morphology and the invasiveness in the collagen matrix prompt us to study the integrin family, the main cell membrane receptors mediate cell and ECM interaction. The RT-PCR and qRT-PCR showed that the mRNA level of $\alpha 2$ and $\beta 1$ subunit upregulated in IR cells, the protein level increased in IR cells as well. Many studies have observed an upregulation of integrin $\beta 1$ subunit in irradiated cancer cells, including meningioma cells (Gogineni *et al.*, 2011), colorectal tumor cell (Meineke *et al.*, 2002), medulloblastoma (Nalla *et al.*, 2010), head and neck cancer cells (Eke *et al.*, 2012), pancreatic cancer cells (Yao *et al.*, 2011) and also in NSCLC (Ishihara *et al.*, 2010), which is essential to either radioresistance or cell migration and invasion. In our study, among several collagen and fibronectin integrin receptors, only the level of $\alpha 2$ and $\beta 1$ subunits showed obvious elevation in IR cells. Knockdown or inhibition of either of them induced the round morphology of IR cells and prevented their invasion, which suggested the essential role of integrin $\alpha 2\beta 1$. The overexpression and enhanced activity of integrin $\alpha 2\beta 1$ were required for the long protrusion and invasion of IR cells.

Recent work has underlined the implication of integrin $\alpha 2\beta 1$ in cancer cell invasion and metastasis. For example, the expression of integrin $\alpha 2\beta 1$ is upregulated in highly aggressive melanoma cells, mediating the reorganization of collagen I fibrils (Klein *et al.*, 1991). $\alpha 2\beta 1$ integrin affects the metastatic potential

of ovarian carcinoma spheroids by supporting disaggregation and proteolysis (Shield *et al.*, 2007). Reorganization of the integrin $\alpha 2$ subunit was suggested to control adhesion and invasion in prostate cancer (Slambrouck *et al.*, 2009). It is worth noting that the integrin $\alpha 2$ subunit was identified as a human lung tumor-associated antigen, and its overexpression is considered directly involved in the pathogenesis of non-small cell tumors through its effects on invasion and/or metastasis (Chen *et al.*, 1991).

In our study, we first time showed a pivotal role of integrin $\alpha 2\beta 1$ in the increased invasiveness of lung cancer cells after IR treatment. In addition to IR cells generated from A549 cells, IR cells generated H1299 cells (HIR cells) showed similar elevation of integrin $\alpha 2$ subunit compared to H1299-P cells (HP cells) confirmed by western blotting (Fig. S-1). Knock down of integrin $\alpha 2$ also induced the round morphology from spindle one (Fig. S-2) and lower invasiveness (data not shown). Thus, the elevation of integrin $\alpha 2$ subunit seemed to be necessary for invasive phenotype of irradiated NSCLC in collagen matrix.

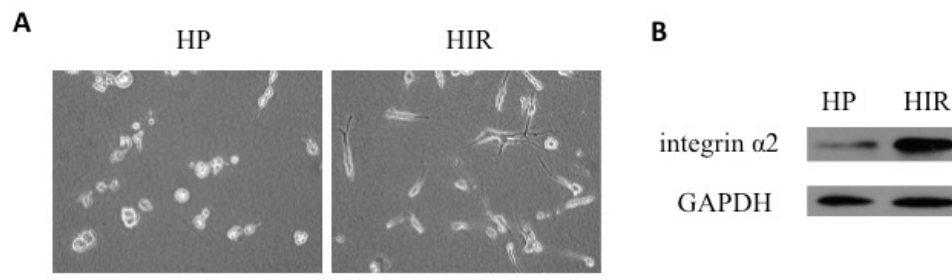


Figure S-1. Morphology of H1299-P cells and IR cells and expression level of integrin $\alpha 2$

(A) Morphologies of P and IR cells in 3D collagen gel after culturing for 24 h. Scale bar, 100 μm . (B) Analysis of the protein levels of integrin $\alpha 2$ from HP and HIR cells cultured in 3D collagen gel by western blotting. GAPDH was used as a loading control.

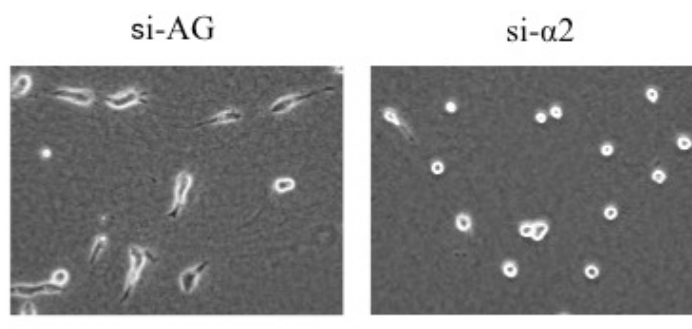


Figure S-2. Integrin $\alpha 2$ expression is required for HIR cell invasion.

Representative images of cell morphologies after knockdown of the integrin $\alpha 2$ subunit in HIR cells, which resulted in a round morphology in 3D collagen gel. HIR cells that were transfected with siRNAs specific to integrin $\alpha 2$. Specific siRNA targeted to Azami-Green, indicated as siAG, was transfected as a control. Two days after transfection, cells were transferred to 3D collagen gel and cultured for 24 h. Scale bar, 100 μm .

Interestingly, another collagen receptor we investigated, integrin $\alpha 1$ subunit, decreased in IR cells compared to P cells. Several studies reported that integrin $\alpha 1\beta 1$ and $\alpha 2\beta 1$ might play contrasting roles in many aspects, such as collagen and collagenase gene expression (Langholz *et al.*, 1995), and EGFR activation (Mattila *et al.*, 2005; Yu *et al.*, 2000), which suggests that decreased expression of $\alpha 1$ integrin might also favor the increased invasiveness of IR cells.

In addition to integrin $\alpha 2\beta 1$, a growth factor receptor that is often aberrant in NSCLC, EGFR, was found overexpressed and activated in IR cells. Although it has been demonstrated that advantages of EGFR inhibition on radiosensitization of cancer cells is mainly due to a reduction in cell proliferation and clonogenic survival (Wang *et al.*, 2011), our results provided new evidence for the importance of EGFR inhibition. We showed here that EGFR expression and activation were elevated in cancer cells that survived IR, and this elevation was required for increased invasiveness.

The roles of EGFR and integrin $\alpha 2\beta 1$ in the activation of Akt were noted through its impaired activation after inhibition of EGFR or function blocking of integrin $\alpha 2\beta 1$. On the other hand, inhibition of PI3K/Akt resulted in similar spherical morphology and partially blocked the EGFR- and integrin $\alpha 2\beta 1$ -mediated invasion in IR cells. In contrast, the elongated phenotype and invasiveness of IR cells were not dependent on MEK/Erk1/2, even though Erk1/2 was also showed activation in IR cells. Alternatively, increased Erk1/2 activation in the presence of the PI3K inhibitor suggests the existence of a compensatory mechanism between PI3K/Akt and MEK/Erk1/2 signaling pathways, which has

been implicated in other studies (Chakrabarty *et al.*, 2011; Serra *et al.*, 2011; Hu *et al.*, 2012). In addition, Erk1/2 activation was dependent on activation of integrin $\alpha 2\beta 1$, but not EGFR, which is possibly related to the survival of IR cells upon the stress of IR, as other studies have suggested (Marampon *et al.*, 2011; Carter *et al.*, 1998). However, direct inhibition of MEK/Erk1/2 may cause undesirable outcomes, such as augmenting EGFR-driven motility demonstrated in prostate cancer (Gan *et al.*, 2010).

Recent work showed crosstalk between signaling pathways involving integrins and EGFR in cancer progression (Alam *et al.*, 2007; Ricono *et al.*, 2009; Morello *et al.*, 2011). For example, physical association between integrin $\alpha 2\beta 1$ and EGFR at cell-cell contact sites was reported in A431 cells with unknown biological function (Yu *et al.*, 2000). Expression of the integrin $\alpha 2$ subunit was selectively increased upon EGF-mediated EGFR activation in both A431 cells and A549 cells (Krensel and Lichtner, 1999). $\beta 1$ integrin-silenced cells show defective activation of the EGFR signaling cascade, leading to decreased in vitro proliferation, enhanced sensitivity to cisplatin and gefitinib, impaired migration, and invasive behavior of A549 cells (Morello *et al.*, 2011). These observations support our hypothesis that integrin $\alpha 2\beta 1$ and EGFR may coordinately regulate signal transduction responsible for IR cell invasion.

Our results demonstrated that the elongated morphology of IR cells in collagen matrix is correlated to their invasiveness, which are regulated by both integrin $\alpha 2\beta 1$ and EGFR. It is well appreciated that alterations in ECM-integrin interactions cause changes in cell shape and behavior (Jones *et al.*, 1997; Folkman

and Moscona, 1978; Chen *et al.*, 1997). Some studies have demonstrated that ECM-dependent changes in cell shape and 3D tissue architecture determine cell function by modulating integrin signaling pathways. (Roskelley *et al.*, 1994; Re *et al.*, 1994). The ECM-directed cell morphology and associated cytoskeletal changes may spatially coordinate cell receptors and intracellular signaling molecules, thereby permitting upstream effectors, including focal adhesion proteins such as FAK, Ras and Raf, to couple with their downstream targets, including MAPKs and PI3K (Bouderau *et al.*, 1999). It is reported that the activation of Rho signaling via PI3K is required for radiation-induced invasion (Zhai *et al.*, 2006). Therefore, integrin $\alpha 2\beta 1$ and EGFR may regulate the elongated morphology of IR cells in 3D collagen matrix by Rho family of small GTPase in a spatial pattern. Further detail need to be study to reveal the mechanism.

Considered the effect of integrin $\alpha 2\beta 1$ and EGFR on the morphology of IR cells, we further check the level of them in non-irradiated A549 cells. Since the heterogenous A549 cells also contain elongated or spreading cells, the morphology of which are similar with IR cells, so we generated four subclones from heterogeneous A549 cells, qRT-PCR results suggested that the elongated subclones also showed higher mRNA level of integrin $\alpha 2$ and EGFR, but not the integrin $\beta 1$ (Fig. S-3, S-4). These suggested that IR-induced invasiveness might share similar mechanism to become invasive with non-irradiated cancer cells.

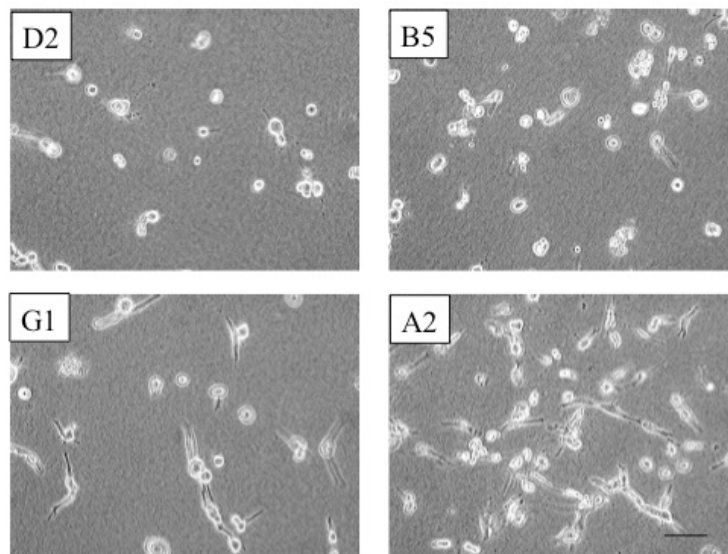


Figure S-3. A549 subclones in 3D collagen gel.

Morphologies of 4 subclones (D2, B5, G1, A2) generated from heterogeneous A549 cells in a 3D collagen gel after culturing for 24 h. Scale bar, 100 μm .

Quantitative analysis of the mRNA levels of integrin $\alpha 2$ and $\beta 1$ subunits from P and IR cells by qRT-PCR. Intensity of signals was quantified by densitometry and normalized with β -actin. Representation is mean value $\pm$ S.D of relative mRNA level (**p < 0.01) from three independent experiments, indicated as fold change relative to P cells.

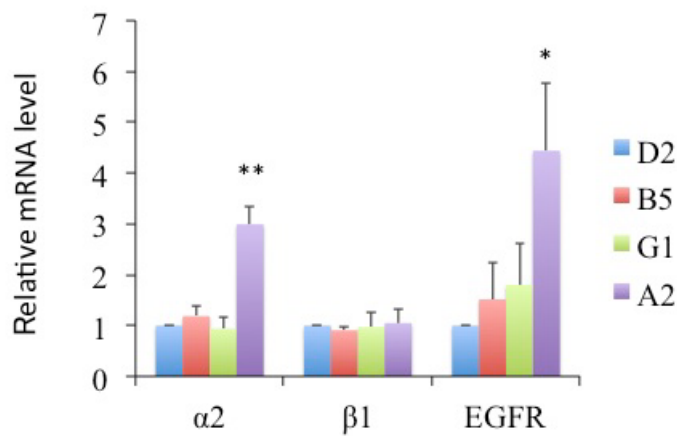


Figure S-4. A549 subclones in 3D collagen gel.

Quantitative analysis of the mRNA levels of integrin $\alpha 2$ and $\beta 1$ subunits and EGFR from the subclones by qRT-PCR. Intensity of signals was quantified by densitometry and normalized with β -actin. Representation is mean value $\pm$ S.D of relative mRNA level (* $p < 0.05$, ** $p < 0.01$) from three independent experiments, indicated as fold change relative to P cells.

EGFR is a promising target for combination with radiotherapy in many cancer types (Liang *et al.*, 2003; Thariat *et al.*, 2007). Specific antibodies or small molecule inhibitors against EGFR are already used for the treatment of NSCLC, and have improved progression-free and overall survival. However, despite initial response and long lasting remission, the development of secondary resistance inevitably leads to treatment failure (Brugger *et al.*, 2012).

In contrast to EGFR-targeting therapy, integrin inhibitors are not fully appreciated partially due to the lack of knowledge of the particular integrin that plays the dominant role in pathological microenvironments (Goodman and Picard, 2012). Integrin antagonists, including the $\alpha v\beta 3$ and $\alpha v\beta 5$ inhibitor cilengitide, have shown encouraging results in Phase II clinical trials, and cilengitide is currently being tested in a Phase III trial in patients with glioblastoma (Desgrosellier and Cheresch, 2010). Our results point out that the integrin $\alpha 2\beta 1$ is required for aggressive phenotype and increased invasiveness of repopulated lung cancer cells after irradiation, and its function blocking is sufficient to abrogate the IR cell invasion in 3D collagen matrix, supporting the rationale for combining integrin inhibitors with radiotherapy.

Summary of main results in this study about the mechanism underlying IR-induced invasion is demonstrated in figure S-5.

Integrin $\alpha 2\beta 1$ is overexpressed in IR cells, and bind with collagen I to mediate cell elongation and invasion. Which can be abrogated by integrin $\alpha 2\beta 1$ function blocking by BHA2.1. On the other hand, growth factor receptor EGFR is also overexpressed and much activated in IR cells. Inhibition of its tyrosine kinase activity by inhibitor PD168393 also abrogated IR cell invasion. Integrin $\alpha 2\beta 1$ and EGFR activate PI3K/Akt cascade, which inactivation by PI3K inhibitor LY294002 decreases IR cell invasion. MEK/ ERK cascade also activated in IR cells under integrin $\alpha 2\beta 1$ regulation, while inhibition of MEK/Erk by U0126 doesn't affect IR cells invasion. MEK/ERK probably plays a role in cell survival from IR as described before. Moreover, integrin $\alpha 2\beta 1$ activation is required for EGFR

activation. And EGFR activation is also involved in $\alpha 2\beta 1$ mediated IR cell protruding and invasion. On the basis of our data, we conclude that integrin $\alpha 2\beta 1$ and EGFR coordinately promote invasion of IR survived cells partially through the activation of PI3K/Akt signaling pathway

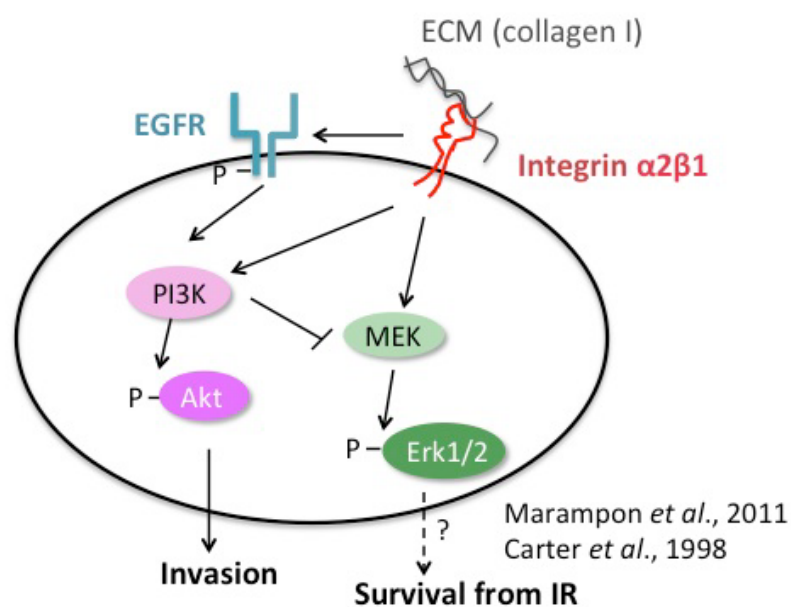


Figure S-5. Scheme of signaling pathways in IR cells.

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Appendix

1. Publication

X. Li, S. Ishihara, M. Yasuda, T. Mizutani, M. Ishikawa, K. Kawabata, H. Shirato and H. Haga: "Lung cancer cells that survive ionizing radiation show increased integrin $\alpha 2\beta 1$ - and EGFR-dependent invasiveness" PLoS ONE, 2013, e70905.

2. Presentation

- 1) X. Li, S. Ishihara, T. Yamada, M. Yasuda, T. Mizutani, K. Kawabata, T. Nishioka and H. Haga: "Integrin $\alpha 2$ -Dependent Increased Invasiveness of Lung Adenocarcinoma Cells that Survived 10 Gy Ionizing Radiation" IGP International Symposium (Sapporo, Japan, 2012)
- 2) X. Li, S. Ishihara, T. Yamada, M. Yasuda, T. Mizutani, K. Kawabata, T. Nishioka and H. Haga: "Integrin $\alpha 2$ -Dependent Increased Invasiveness of Lung Adenocarcinoma Cells that Survived 10 Gy Ionizing Radiation" Joint Meeting of The 45th Annual Meeting of the Japanese society of Developmental Biologists & The 64th Annual Meeting of the Japan Society for Cell Biology (Kobe, Japan, 2012)
- 3) X. Li, S. Ishihara, M. Yasuda, T. Mizutani, K. Kawabata, T. Nishioka and H. Haga: "Mechanism of Increased Invasiveness of Lung Adenocarcinoma Cells that Survived 10 Gy Ionizing Radiation" The American Society for Cell Biology 2012 Annual Meeting (San Francisco, USA, 2012)

