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北海道大学 Collection of Scholarly and Academic Papers: HUSCAP
Study on invasiveness
of irradiation-tolerant lung adenocarcinoma cells
by three dimensional collagen matrices

Seiichiro ISHIHARA

2013
Doctoral Dissertation
Transdisciplinary Life Science Course,
Graduate school of Life Science,
Hokkaido University
## Contents

Chapter 1

General introduction ........................................................................................................... 5
1-1 Emergence and progression of cancer ........................................................................... 6
1-2 Invasion of cancer cells .................................................................................................. 8
1-3 Cancer therapies ............................................................................................................. 9
1-4 Radiotherapy and irradiation-induced cancer malignancy ............................................ 10
1-5 Key molecules .............................................................................................................. 11
   1-5-1 Integrin β1 ............................................................................................................. 11
   1-5-2 Myosin regulatory light chain (MRLC) ................................................................. 13
   1-5-3 Activating transcription factor 5 (ATF5) ............................................................... 15
1-6 Invasion assay by three dimensional collagen matrices ................................................. 17
1-7 The aim of this study ..................................................................................................... 18
Figures .............................................................................................................................. 20

Chapter 2

Establishment of experimental systems .............................................................................. 29
2-1 Establishment of subclonal A549 cells ........................................................................... 30
2-2 Establishment of IR cells ............................................................................................... 30
2-3 Phenotypic differences of the cells on 2D substrates and in 3D matrices ....................... 31
2-4 NF-κB activation of the cells on 2D substrate ............................................................... 31
2-5 An improved method of extracting proteins from the cells in 3D collagen matrices ....... 33
2-6 Materials and methods ............................................................................................... 34
Figures .............................................................................................................................. 40

Chapter 3

Integrin β1 dependent invasive activity in irradiation-tolerant lung adenocarcinoma cells ................................................................................................................................. 47
3-1 Abstract ....................................................................................................................... 48
3-2 Introduction ............................................................................................................... 49
3-3 Materials and methods ............................................................................................. 51
3-4 Results ....................................................................................................................... 56
Chapter 4
The role of myosin regulatory light chain for invasiveness in irradiation-tolerant lung adenocarcinoma cells ................................................................. 65
4-1 Abstract .................................................................................. 66
4-2 Introduction .............................................................................. 67
4-3 Materials and methods ............................................................. 69
4-4 Results ..................................................................................... 73
4-5 Discussion ................................................................................. 76
Figures ......................................................................................... 79

Chapter 5
Invasiveness regulated by activating transcription factor 5 in irradiation-tolerant lung adenocarcinoma cells ................................................................. 87
5-1 Abstract .................................................................................. 88
5-2 Introduction .............................................................................. 89
5-3 Materials and methods ............................................................. 91
5-4 Results ..................................................................................... 97
5-5 Discussion ................................................................................. 101
Figures ......................................................................................... 103

Chapter 6
Summary and remaining questions .................................................. 111
Figures ......................................................................................... 114
References ................................................................................... 116
Acknowledgements ....................................................................... 128
Abbreviations

2D: two dimension
3D: three dimension
ATF: activating transcription factor
CA: calyculin A
CRE: cAMP response element
ECM: extracellular matrix
FAK: focal adhesion kinase
FBS: fetal bovine serum
ILK: integrin linked kinase
IR: irradiation-tolerant subclonal P-3 A549 human lung adenocarcinoma cell line
KD: IR cells transfected with ATF5 siRNA
MLCK: myosin light chain kinase
MLCP: myosin light chain phosphatase
MMP: matrix metalloproteinase
MRLC: myosin regulatory light chain
MT1-MMP: membrane type 1- matrix metalloproteinase
NC: IR cells transfected with negative control RNA
NT: non-treated
P-1, P-2, P-3, P-4, P-5, P-6: subclonal A549 human lung adenocarcinoma cell lines
P-MRLC: monophosphorylated myosin regulatory light chain
PP-MRLC: diphosphorylated myosin regulatory light chain
RI: roundness index
ROCK: Rho kinase
SD: standard deviation
Chapter 1

General introduction
1-1 Emergence and progression of cancer

Cancer is one of the major causes of death worldwide. According to the World Health Organization, cancer accounted for 7.9 million death in 2007, and is estimated to jump to 11.5 million death in 2030 (Shieh, 2011). In Japan, cancer ranks the first in the cause of death from 1981 to 2011 (Cancer Statics in Japan, 2012). Furthermore, it is suggested that 54% of Japanese men and 41% of Japanese women will be diagnosed with cancer during their lifetime (Inoue et al., 2012). Thus, cancer is unignorable disease all over the world, including Japan.

Cancer is a malignant colony of unlimited proliferative cells (Weinberg, 2006). These abnormal cells are called cancer cells, which arise due to the mutations in cancer-associated genes (Hanahan and Weinberg, 2000). There are two types of cancer-associated gene. One is oncogene, such as src (Hanafusa et al., 1977; Hunter and Sefton, 1980), genes of epidermal growth factor receptors (Fiore et al., 1987; Slamon et al., 1987), and ras (Medema and Bos, 1993). Oncogenes are activated by the mutations in the genes and as a result act as triggers of cancer cell emergence. The other is tumor suppressor gene, such as Rb (Weinberg, 1995) and p53 (Symonds et al., 1994; Harris, 1996). Tumor suppressor genes normally repress cancerous phenotypes. However, when tumor suppressor genes are inactivated or lost by the gene mutations, the cells can achieve cancerous phenotypes. Cancer cells can contain multiple mutations of cancer-associated genes (Loeb et al., 2003). It is suggested that these multiple
mutations enable to maintain over-proliferative activity in cancer cells.

Once a cancer cell arises, the cell proliferates uncontrollably and forms the mass of the cells, called a tumor. There are two categories of tumor: one is the benign tumor and the other is the malignant tumor (cancer). In benign tumors, the colony of cancer cells exists as one lump and does not spread across nearby tissues. In many cases, benign tumors do not threaten the life of hosts because benign tumors only grow locally and usually do not disrupt the vital organs. In contrast, cancers frequently cause lethal situations with a progression of it.

Progression of cancers is associated with invasion and metastasis (Figure 1-1). With the progression of it, cancer cells acquire the abilities to break in surrounding normal tissues across the border of normal and cancer tissues. This phenomenon is called invasion. Furthermore, progressive cancer cells often form secondary tumors at distant sites from an originally formed tumor (primary tumor) by traveling through blood or lymph vessels. That is called metastasis. Several types of cancer tend to develop metastases at specific sites. For example, brain metastases are common events in lung cancer patients (Postmus and Smit, 1999) whereas breast cancer cells often metastasize to lung, liver and bones (Weigelt et al., 2005). By invasion and / or metastasis, cancers finally disrupt vital tissues or organs, and as a result, bring a crisis of life in a host. Thus, invasion and metastasis is critical phenomena of cancers but the molecular mechanisms of them are not fully understood.
1-2 Invasion of cancer cells

In invasion, which is one of the crucial phenomena of cancer progression, cancer cells break in surrounding tissues with cell migration. The major tissues nearby tumors are connective tissues, mainly filled with extracellular matrices (ECMs) such as collagens, fibronectins, and laminins (Frantz et al., 2010). The most abundant component of connective tissues is collagen, which constructs a three-dimensional network of their fibers and functions as structural supports of the tissues (Nishida et al., 1988). To invade connective tissues, cancer cells must migrate into three-dimensional networks of collagen fibers.

Invasive cancer cells are able to migrate in collagen networks by using several invasion manners, such as mesenchymal, amoeboid, and collective invasion (Friedl and Wolf, 2010). In each type of invasion, the mechanisms of migrating in collagen networks and the morphology of the cells are different (Figure 1-2). In mesenchymal invasion, the cells degrade collagen fibers by proteases such as matrix metalloproteinases (MMPs) and invade the open area of the collagen network (Wolf et al., 2003). These cells show spindle morphologies because the cells extend invasive protrusions, which express proteases for degradation of collagens. In amoeboid type invasion, cancer cells pass through the gaps of collagen fibers by forcing open the collagen networks, independently of proteases (Sahai and Marshall, 2003; Wolf et al., 2003).
The cells indicate round, shrunken shapes because the cells squeeze the cell body to open collagen gaps. In collective invasion, cancer cells adhere to each other and invade into collagen networks with keeping a lump colony of the cells (Friedl et al., 2004). In this manner, the cells on the front of the colony degrade collagen fibers by expressing proteases and migrate into the collagen networks with leading the other cells.

The manners of cancer cell invasion are dependent on cancer types. For example, some melanoma cells show collective invasion (Hegerfeldt et al., 2002) whereas some fibrosarcoma cells indicate mesenchymal invasion (Friedl and Wolf, 2010). For cancer therapies, inhibition of cancer cell invasion that is specific to cancer types may be effective. However, the detail mechanisms of each manners of invasion are not understood.

1-3 Cancer therapies

Surgery, chemotherapy, and radiotherapy are major therapies for cancer treatment. There are noted advantages and disadvantages for each therapy. Surgery is the method to remove tumors by operations. This therapy is a major treatment for solid tumors like lung cancers (Sartori et al., 1999) and gastric cancers (Saka et al., 2011). Surgery can cure cancer completely when the cancer cells are removed completely, whereas this way may do a lot of damages on a patient body. Chemotherapy is a therapeutic way to treat cancer with drugs, which target
cancer-associated molecules (DeVita and Chu, 2008). For example, trastuzumab is a functional-blocking monoclonal antibody against HER-2 protein, which is essential component for specific breast cancer cells (Boekhout et al., 2011). This reagent functions as a good medicine for several breast cancers. However, some drugs can increase the tumor invasiveness and spread. For example, it is reported that bevacizumab, which is VEGF-neutralizing antibody and expected as an effective treatment against cancer, increased detectable spreading of glioblastoma (Ellis and Reardon, 2009). For radiotherapy, I discuss in next section.

1-4 Radiotherapy and irradiation-induced cancer malignancy

Radiotherapy is effective therapies for treating various types of cancer. In this method, X-rays are often used for killing cancer cells. Most fractions of irradiated cancer cells with X-rays result in apoptosis because of irradiation-induced DNA damages (Dormand et al., 2005; Figure 1-3). An advantage of this way is that well-controlled X-rays effectively attack cancer cells while it give less damage on a patient body (Ahmad et al., 2012). However, a part of irradiated cancer cells survives and regenerates tumors, which result in poor prognoses (Von Essen, 1991). Furthermore, cancer cells surviving after irradiation often indicate higher malignant phenotypes than non-irradiated cancer cells. For example, irradiated QRsP mouse fibrosarcoma cells indicate higher ability of tumor formation in vivo experimental model (Nishioka et al., 2011). In
addition, irradiation-tolerant H1299 human lung carcinoma cells show higher migrating activity
in vitro experiments (Tsutsumi et al., 2009). These findings suggest that irradiation to tumors
can trigger malignant transformation in cancer cells and result in poor prognosis. However, the
irradiation-induced malignant phenotypes and mechanisms of it are not well understood,
including cancer cell invasion.

1-5 Key molecules

Here, the key molecules in this study are summarized. I hypothesized that these molecules
regulate malignant phenotypes in irradiated cancer cells. Integrin β1, which contributes
cell-matrix adhesion; myosin regulatory light chain (MRLC), which regulates actomyosin
contractions; and activating transcription factor 5 (ATF5), which induces irradiation resistance,
are introduced in this section.

1-5-1 Integrin β1

Integrins are a family of heterodimeric transmembrane proteins, contributing to the adhesion of
cells to ECMs (Hynes, 2002). The heterodimer of integrins consists of α and β chains. To date,
25 types of integrin heterodimer involving 19 α chains and 8 β chains have been reported
(Caswell et al., 2009). Each types of the heterodimer display distinguish affinity to specific
ECMs (Humphries et al., 2006). Therefore, the expressions of the heterodimer determine adhesion properties of the cells to specific ECMs.

Integrins do not only contribute to the cell-ECM adhesion but also regulate cellular behaviors by transmitting biochemical signalings. Integrins consist of cytoplasmic domain, transmembrane domain, and extracellular domain. The extracellular domain of integrins directly binds to ECMs whereas the cytoplasmic domain associates with signal transducing proteins and regulates cellular phenotypes. For example, scaffold proteins such as focal adhesion kinase, Src, and p130Cas are recruited at cytoplasmic domain of integrins when its extracellular domain makes a connection with ECMs (Giancotti and Ruoslahti, 1999; Figure 1-4A). These recruitments promote cell motility and invasiveness via association of scaffold proteins with accumulated actin fibers (Hood and Cheresh, 2002). These signal transductions are called “outside-in signaling”. In addition, “inside-out signaling” transductions are reported. For instance, talin binds to cytoplasmic domain of integrin β chains, and as a result, increases ECM binding activity of the integrins (Tadokoro et al., 2003; Figure 1-4B). Thus, integrins transduce biochemical signalings between cytoplasmic and extracellular proteins, and as a result, regulate cellular behaviors.

Integrin β1 plays important roles for invasiveness in cancer cells. In invasion, cancer cells bind to collagen fibers via collagen-specific integrin heterodimers, such as α1β1, α2β1,
\( \alpha_{10}\beta_1, \) and \( \alpha_{11}\beta_1 \) integrins (Hynes, 2002). Integrin \( \beta_1 \) is common component of these heterodimers and widely expresses in invasive cancer cells. Indeed, malignant colon and kidney cancer cells up-regulate integrin \( \alpha_2\beta_1 \) expressions (Mizejewski, 1999). Additionally, in cancer cells that displays mesenchymal, amoeboid, and collective types of invasion, integrin \( \beta_1 \) expresses at collagen adhering sites of the cell surface (Sahai and Marshall, 2003; Wolf et al., 2003; Friedl et al., 2004; Figure 1-5). Furthermore, inhibition of integrin \( \beta_1 \) function decreases mesenchymal, amoeboid, and collective invasive abilities in cancer cells (Hegerfeldt et al., 2002; Sahai and Marshall, 2003; Wolf et al., 2003). Therefore, integrin \( \beta_1 \) is suggested to play crucial roles for wide types of cancer invasion.

In addition to cancer cell invasion, it is reported that integrin \( \beta_1 \) is affected by irradiation in cancer cells. For instance, X-ray irradiation increases expression of functional integrin \( \beta_1 \) in lung cancer cell lines (Cordes et al., 2002) and glioma cell lines (Cordes et al., 2003). Moreover, H1299 irradiation-tolerant human lung adenocarcinoma cells showed strongly localization of integrin \( \beta_1 \) at the adhesion sites than non-irradiated cells (Tsutsumi, et al., 2009). Thus, integrin \( \beta_1 \) may contribute to malignant phenotype of irradiated cancer cells.

**1-5-2 Myosin regulatory light chain (MRLC)**

Cells generate contractile force for cell migration, division, and so on. Cellular contractile force
is generated by the contractility of actomyosin, composed of actin filaments and myosin II (Cooke, 1993; Figure 1-6A). This contractility is regulated by phosphorylation of MRLC. MRLC has two residues to be phosphorylated: Thr18 and Ser19. Di-phosphorylated MRLC of these residues provokes much stronger contractility in actomyosins than mono- or non-phosphorylated MRLC (Mizutani et al., 2006a; Figure 1-6B). The phosphorylation of MRLC is up-regulated by MRLC specific kinases such as myosin light chain kinase (MLCK; Somlyo and Somlyo, 2003), ZIP kinase (Murata-Hori et al., 1999), and Rho kinase (ROCK; Ueda et al., 2002). On the other hand, phosphorylation of MRLC is reported to be down-regulated by myosin light chain phosphatase (MLCP; Hirano et al., 2003). Thus, MRLC regulates cellular contractile force generated by actomyosins via its phosphorylation manner.

In cancer cell invasion, the role of cellular contractile force is not well understood. On a two dimensional substrate (2D substrate), the major contribution of contractile force to cell migration is identified. Cell migration on 2D substrates consists of the four processes: protrusion, adhesion, translocation, and retraction (Figure 1-7). First, cells polymerize actin filaments to the migrating direction and form membrane protrusions such as filopodia and lamellipodia (protrusion; Yamazaki et al., 2005). Next, protruded membranes contact the substrate and construct new integrin-dependent cell-substrate adhesions (adhesion). After the construction of new adhesion sites, the cell body is translocated by actomyosin contractions.
(translocation). Finally, the trailing edge of migrating cells retracts (retraction). The sequence of processes is strongly regulated by Rho small GTPases (Hall, 1998). Formations of filopodia, lamellipodia, and contractile actomyosin are induced by Cdc42, Rac, and Rho, respectively. Like this, when cells migrate on a 2D substrate, contractile actomyosin regulated by Rho pulls the rear site of the cell body toward the leading direction. On the other hand, in a three dimensional substrate such as collagen networks, the relationship between cell migration and cellular contractile force is not well understood, although previous study indicated that invasive cancer cells generate traction force in a 3D collagen matrix (Koch et al., 2012). Thus, the contributions of cellular contractile force to invading cancer cells in a three dimensional substrate are to be investigated.

1-5-3 activating transcription factor 5 (ATF5)

Activating transcription factor (ATF) family proteins are identified as proteins to bind to a specific site of DNA (the sequence: GTGACGT(A/C)(A/G); Hai et al., 1989). This protein family represents a group of basic-region leucine zipper transcription factors with wide ranging functions (Persengiev and Green, 2003). For example, ATF1 maintains cellular viability (Bleckmann et al., 2002). ATF2 indicates oncogenic activities in melanoma whereas it displays tumor suppressor activities in non-malignant skin tumors and breast cancer (Lau and Ronai,
2012). ATF3 and ATF6 are involved in stress responses (Hai et al., 1999; Wang et al., 2000). ATF4 regulates development (Hai and Hartman, 2001). ATF family proteins also regulate cancerous phenotype, such as cell survival and cell growth (Persengiev and Green, 2003; Figure 1-8A). In this study, ATF5 is focused because this protein is involved in cancerous phenotypes and irradiation resistance as mentioned below.

ATF5, also referred to as ATF7 or ATFx, binds to cAMP response element (CRE, the sequence: TGACGTCA) and suppresses apoptosis (Nishizawa et al., 1991; Pati et al., 1999; Peters et al., 2000; Persengiev et al., 2002). ATF5 plays different roles between non-cancerous and cancerous cells. In non-cancerous cells, ATF5 is reported to be a regulator of cell differentiations (Figure 1-8B). For example, ATF5 represses the differentiation of neural progenitor cells (Angelastro et al., 2003), oligodendrocytes (Mason et al., 2005), adipose-derived stem cells (Leong et al., 2012), and olfactory sensory neurons (Wang et al., 2012). In addition, ATF5 is highly expressed in liver and it is suggested that ATF5 regulates drug metabolism in the liver (Pascual et al., 2008). On the other hand, in cancerous cells, ATF5 plays an important role for cell survival. It is reported that ATF5 expression in cancerous cells is higher than non-cancerous cells in glioblastoma (Angelastro et al., 2006) and breast carcinoma (Monaco et al., 2007). Furthermore, the repression of ATF5 in these cancerous cells induces apoptosis whereas in the non-cancerous cells does not. Therefore, ATF5 may be a possible target
molecule for cancer therapy.

ATF5 is associated with irradiation resistance and cell motility. QRsP mouse fibrosarcoma cells expressing higher ATF5 gene show much irradiation tolerance and cell motility than the cells expressing lower ATF5 gene (Nishioka et al., 2009). These results suggest that ATF5 plays an important role for irradiation resistance and cancer cell motility. However, the relationship between the expression of ATF5 and invasion of cancer cells has not been reported.

1-6 Invasion assay by three dimensional collagen matrices

Three dimensional collagen matrices (3D matrices) is a suitable substrate for assay of cancer cell invasion in vitro. As a canonical way to investigate cell migration, migration assays on 2D substrate have been often performed (Yamazaki et al., 2005). However, the environment of 2D substrate is far different from in vivo situation for cells because connective tissues in vivo are mainly constructed from three dimensional networks of collagen fibers, as mentioned above. Thus, to investigate invading ability in cancer cells in vitro, much suitable environment to mimic in vivo situation are required. Because 3D matrices allow cells to be embedded in a three dimensional networks of collagen fibers, culturing cancer cells in 3D matrices is more proper way to investigate cancer cell invasion.
In this study, collagen gel overlay assay was used for culturing cells in 3D matrices (Figure 1-9). First, a dish was filled with collagen gel. After seeding the cells onto the collagen gel, collagen sol solution was poured onto the cells and incubated for gelation. Finally, the media were added onto the gel to culture cells. This way enables us to observe multiple cells on one focus by microscope because cells seeded on the same surface of the gel.

The most commonly used in vitro invasion assay is a modified Boyden chamber assay (Shaw, 2005). The results obtained by this assay have shown a strong correlation between the ability of tumor cells to invade in vitro and the invasive behavior in vivo. However, this method is not suitable for observing the morphological changes in cancer cells during the invasion because the results of this method show only the percentage of invasive cells. On the other hand, collagen gel overlay assay has applicability to time-lapse observation, which enables to observe the morphological changes in living cells. Thus, collagen gel overlay assay is performed in this study to observe the morphological changes of invading cells in a 3D matrix.

1-7 The aim of this study

Previous studies reported that irradiation treatment to cancer cells can induce malignant phenotypes and poor prognosis. However, the detail mechanisms of irradiation-inducing malignancy, including invasiveness, are not well understood. The aim of this study is (1) to
elucidate the invasive phenotypes of irradiation-tolerant cancer cells (2) to identify the key molecules that regulate invasiveness in irradiation-tolerant cancer cells. First, using collagen gel overlay assay, the invasive ability in irradiation-tolerant cancer cells was investigated (Chapter 2). Second, integrin β1 (Chapter 3), MRLC (Chapter 4), ATF5 (Chapter 5) were identified as key molecules to regulate the invasiveness of irradiation-tolerant cancer cells. Finally, a model of malignant transformation after irradiation and the remaining questions were described (Chapter 6).
Figures

Figure 1-1. The schematic view of cancer progression.
Figure 1-2. The mechanisms of cell migration in a network of collagen fibers.
Figure 1-3. The schematic view of irradiation effects on tumors.
Figure 1-4. Biochemical signaling pathways via integrins.

(A) The connection between integrins and ECMs induces the accumulation of cytoplasmic proteins such as Src, focal adhesion kinase (FAK), and p130Cas on cytoplasmic domain of the integrin, and as a result, promotes cell motility and invasion via recruitment of actin fibers. (B) The connection between talin and integrin enhances binding activity of integrins to ECMs.
Figure 1-5. Integrin β1 expression in invasive cancer cells.
Figure 1-6. Cellular contractions generated by actomyosin.

(A) The schematic view of actomyosin. Actomyosin consists of actin fibers and myosin II, including myosin regulatory light chain (MRLC). Contractions are generated by sliding myosin II on actin fibers. (B) The regulation of actomyosin contraction by MRLC phosphorylation. Di-phosphorylated MRLC on Thr18 and Ser19 residues triggers higher contractions than mono- or non-phosphorylated MRLC.
Figure 1-7. The schematic view of 2D cell migration.
Figure 1-8. Roles of activating transcription factor (ATF) family proteins.

(A) Roles of ATF family proteins in cancerous phenotypes. (B) Roles of ATF5 for cellular functions.
Figure 1-9. The overview of collagen gel overlay assay.
Chapter 2

Establishment of experimental systems
2-1 Establishment of subclonal A549 cells

In this study, the A549 continuous tumor cell line derived from a human lung adenocarcinoma was used (Lieber et al., 1976). To obtain the cell lines that show uniform phenotypes such as morphology and motility, subclones of A549 lung adenocarcinoma cell line were established. Six subclonal cell lines from the A549 cell line were obtained by limited dilution cloning method. On a 2D substrate, three of six subclones (P-2, P-3, and P-5) displayed lump colonies, which kept tight cell-cell adhesion (Figure 2-1). On the other hand, the other subclones (P-1, P-4, and P-6) showed scattered colonies, which indicated loose cell-cell adhesion (Figure 2-1). Because P-3 cells showed stable growth and uniform morphology, the cells were used in this study.

2-2 Establishment of IR cells

Next, irradiation-tolerant P-3 cells were established. P-3 cells were irradiated with X-ray at a dose of 10 Gy. 20 days after irradiation, lump colonies with high cell density were observed (Figure 2-2). This suggests that irradiation-tolerant P-3 cells start to growth about 20 days after irradiation. 30 days after irradiation, irradiation-tolerant P-3 cells were harvested and designated as IR cells.
### 2-3 Phenotypic differences of the cells on 2D substrates and in 3D matrices

Cell morphology and motility on a 2D substrate were observed. For a 2D substrate, a collagen-coated glass was used. There were no notable phenotypic differences between P-3 and IR cells on a 2D substrate (Figure 2-3A). There also were no significant differences of cell motility between these two cell lines (Figure 2-3B). Then, collagen gel overlay method was used because this method better mimics the *in vivo* environment than collagen coated glass. This method allows cells to be cultured in a 3D collagen gel matrix. Under the collagen gel overlay condition, time-lapse observation was performed to investigate cell invasion. P-3 cells displayed a round morphology and low invasive activity in the 3D collagen gel (Figure 2-4A). In contrast, IR cells showed a spindle morphology and rapid changes in cell surface, alternating phases of elongation and retraction of protrusions (Figure 2-4A), and significant increase in invasion speed (Figure 2-4B). These results indicate that IR cells show higher invasiveness than P-3 cells in a 3D collagen matrix whereas there are no significant differences between P-3 and IR cells on a 2D substrate.

### 2-4 NF-κB activation of the cells on 2D substrate

Next, I investigate the biochemical signalings, which cause the phenotypic differences between
the cells on a 2D substrate and the cells in a 3D matrix. Substrate stiffness is one of the remarkable differences between these culturing environments. In this study, a collagen-coated glass and a collagen gel were used for a 2D substrate and a 3D matrix, respectively. Glass indicates 2-4 GPa stiffness (Butcher et al., 2009) whereas collagen gel shows 400-800 Pa stiffness (Mizutani et al., 2006b). Previous studies reported that substrate stiffness affects cellular behaviors such as motility (Pelham et al., 1997; Lo et al., 2000), morphology (Yeung et al., 2005), collectiveness (Haga et al., 2005), and differentiation (Engler et al., 2006). Furthermore, substrate stiffness changes the expressions of proteins such as adhesion molecules (Wang et al., 2003) and the activities of transcription factor YAP/TAZ (Dupont et al., 2011). Thus, substrate stiffness regulates cellular phenotypes and biochemical signalings in the cells.

Transcription factor NF-κB is a major regulator of inflammatory reaction and cancer progression. NF-κB is activated by various stimuli such as chemical stimuli and DNA damages. NF-κB consists of 5 subunits (RelA/p65, p105/p50, p100/p52, RelB, and c-Rel), which form homo- or hetero-dimers (Hayden 2004). Once the stimuli are received, NF-κB dimers are exported from the cytoplasm to the nucleus, where they function as transcriptional activators. Activated NF-κB induces the expression of cytokines (IL-1β, IL-8, etc.) and cancer regulatory genes (MMP9, TNFα, etc.) (Karin et al., 2002; Li et al., 2002; Sliva et al., 2002; Saitoh et al., 2010).
Based on these previous studies, I hypothesize that substrate stiffness regulates NF-κB activity and changes the expressions of NF-κB related genes. To investigate the activity of NF-κB, the localization of NF-κB was observed. For the stiff substrates, collagen-coated glasses or plastics were used. For the soft substrates, 1.6 mg/mL type-I collagen gels were used. The cells showed a higher rate of NF-κB localization to the nucleus 2–6 h after seeding on the glass substrate than on the collagen gel substrate (Figure 2-5). This result indicates that stiff substrates induce NF-κB activation. Furthermore, NF-κB related genes, such as IL-1β, IL-8, and MMP9, were up-regulated to a greater extent in the cells on a plastic substrate than in cells on a collagen gel substrate (Figure 2-6). These results suggest that stiff substrates induce biochemical signalings involving NF-κB activation and related gene expressions. These signalings may cause phenotypic differences between the cells on a 2D substrate and the cells in a 3D matrix in this study.

2-5 An improved method of extracting proteins from the cells in 3D collagen matrices

Western blotting is one of the well-established methods for detecting specific proteins and determining the quantity of each protein (Laemmli, 1970; Towbin et al., 1979). For detection of specific proteins in the cells cultured in collagen gels by western blotting, an improved method
of extracting proteins was established. In this method, the sample was treated with trichloroacetic acid and collagenase. Trichloroacetic acid is used for protein fixation (Cafruny, 1957). Collagenase is used for degradation of collagen molecules to remove collagen gel from the protein extractions (Bond et al., 1984). By this method, the greater signal of NF-κB protein was detected (Figure 2-7). In this study, the improved method to extract proteins was used for western blotting analysis.

2-6 Materials and methods

Subcloning and cell culture

The lung carcinoma A549 and H1299 cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA). A549 cells were cloned by limiting dilution, and subclonal cell lines were established. Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; JRH Biosciences Inc., Lenexa, KS) and 1% antibiotic (Sigma). Cells were incubated at 37°C in a humidified incubator with 5% CO₂. Phase-contrast images were captured by a phase-contrast microscope (TE300, Nikon Instech.), equipped with a 10× objective.
**Irradiation of P-3 cells**

Semi-confluent P-3 cells were irradiated with X-rays at room temperature at a dose of 10 Gy. The cells were immediately dispersed with trypsin-EDTA and $5 \times 10^6$ cells were seeded into a culture flask (75 cm$^2$-culture area). Cells were cultured for 30 days, and the surviving cells were harvested and designated as IR cells.

**Time-lapse observation**

For 2D culture, a glass dish of 12.5 mm radius was coated with 1000 μl of 0.3 mg/mL Cellmatrix Type I-C (Nitta Gelatin Inc., Osaka, Japan) and $1 \times 10^4$ cells were seeded. For 3D culture, a glass dish of 8.0 mm radius was filled with 400 μl of collagen gel, and $4 \times 10^3$ cells were seeded onto the collagen gel. After 24 h, 200 μl of collagen sol solution was poured onto the cells, and incubated for 60 min at 37°C to complete gelation. This method allows the cells to be cultured in a 3D environment. Then, the dish was filled with culture medium, and sealed with silicone grease to avoid changes in pH of the medium. A phase-contrast microscope (TE300, Nikon Instech., Tokyo, Japan), equipped with a 10x objective and kept at 37°C in an acrylic resin box, was used for time-lapse observations. Image-Pro software (Media Cybernetics Inc., Silver Spring, MD) was used for time-lapse observation by capturing images every 5 min. Cell
migration speed was calculated by measuring the displacement of the cell center every 30 min.

**Immunofluorescence staining**

NLS-venus expressing H1299 cells (1–2 × 10⁴) were seeded on collagen-coated cover glasses or collagen gels and cultured for 0.5, 2, 4, or 6 h. After culturing, the cells were fixed with paraformaldehyde (4% in PBS, room temperature, 10 min) and washed 3 times. Next, Triton-X100 (0.5% in PBS, room temperature, 10 min) permeabilization was performed. The samples were washed 3 times. Blocking treatment with 0.5% BSA in PBS was performed for 1 h at room temperature. Primary antibody solution (Anti-NF-κB p65 (IBL, Fujioka, Japan, 18667), 1:200 in PBS) was added and incubated overnight at 4°C. After washing 3 times, secondary antibody solution (Alexa Fluor 594 goat anti rabbit IgG (Invitrogen, Carlsbad, CA, A-11012), 1:200 in PBS for p65 staining) was added and incubated for 1 h at room temperature. After washing 3 times, anti-fading solution (2.5% DABCO, 90% glycerol, 6% PBS; pH, 8.0) was added. The images were captured with Nikon C1 confocal imaging system (Nikon Instech., Tokyo, Japan). The proportion of NF-κB localization in the nucleus was calculated using Image J software as the p65 intensity in the nucleus divided by the p65 intensity in the cytoplasm. Statistical analysis was performed using the Student’s T-test.
**RT-PCR**

H1299 cells (3 × 10⁵) were seeded on each substrate and incubated at 37°C. RNAs were isolated 6 h later by using TriPure isolation Reagent (Roche, Indianapolis, IN). The isolating manipulation was performed twice to ensure removal of any contaminating DNA or protein. cDNAs were generated with RevTra Ace kit (TOYOBO, Osaka, Japan), and PCR was performed with Taq polymerase with ThermoPol Buffer (NEB, Ipswich, MA). The following primers were used:

GAPDH, 5′-ACCACAGTCCATGCCATCAC-3′ (upper) and 5′-TCCACCACCCTGTTGCTGTA-3′ (lower)

IL-1β, 5′-TGCACGCTCCGGGACTCACA-3′ (upper) and 5′-AGGCAGGCAGTTGGGCATTGG-3′ (lower)

IL-8, 5′-GTGGACCACACTGCACCATTGG-3′ (upper) and 5′-GGCCCTTGGCCTCAATTGTGTT-3′ (lower)

MMP9, 5′-GGCCCCTTGGCCTCAATTGTGTT-3′ (upper) and 5′-GACACCTCTGCCCTCACCATGAG-3′ (lower)

**Western blotting**

A plastic cell culture dish of 17.5 mm radius was filled with 500 μL of collagen gel. P-3 cells
were seeded onto the collagen gel. After 24 h, protein extractions were prepared. In a canonical method, the sample was lysed with SDS sample buffer (0.25 M Tris-HCl, 5% dithiothreitol, 2.3% sodium dodecyl sulfate, 10% glycerol, 0.01% bromophenol blue, pH 8.8) and collected into a micro tube. The sample was treated with supersonic wave and boiled for 5 min. In an improved method, the sample was fixed by 10% trichloroacetic acid in PBS and washed 3 times with PBS on ice. Then, the sample was incubated with 250 μL of 0.1% collagenase L (Nitta Gelatin Inc.) in PBS at 37°C for 1 h to digest the collagen gel. The sample was collected into a micro tube and centrifuged at 14,000 rpm for 3 min at 4°C. After removing the supernatant, the sample was lysed with SDS sample buffer. The sample was treated with supersonic wave and boiled for 5 min.

10% polyacrylamide gels were prepared for SDS-PAGE (20 mA per gel, 1 h). After SDS-PAGE, blotting to PVDF membranes was performed (92 mA per gel, 30 min). After blotting, the membranes were incubated in 5% skim milk in TBS-Tween solution for 1 h at room temperature. Membranes were incubated with primary antibodies (p65 (IBL, 18667), 1:1000 and GAPDH (Ambion, Foster City, CA, 1103016), 1:1000000) overnight at 4°C. After 3 washes, membranes were incubated in secondary antibodies (HRP anti rabbit IgG (Cell Signaling Tech, #7074), 1:10000 for p65 and HRP anti mouse IgG (Bio-Rad Hercules, CA, 70-6516), 1:200000 for GAPDH) for 1 h at room temperature. Signals were detected with
Immobilon Western Chemiluminescent HRP substrate (Millipore).
Figures

Figure 2-1. Phase-contrast images of subclonal A549 lung adenocarcinoma cell lines.

Cells were cultured on a 96 well plastic plate. Bar = 40 μm.
Figure 2-2. Phase-contrast images of P-3 cells after irradiation.

Control: non-irradiated P-3 cells. IR: 10 Gy irradiated P-3 cells. Images were captured at indicated days after irradiation. A red circle indicates a lump colony of the cells. Bar = 100 μm.
Figure 2-3. Cell morphology and motility on a 2D substrate.

(A) Phase-contrast images of P-3 and IR cells cultured on a 2D substrate (collagen-coated glass).

Bar = 250 μm. (B) Statistical analysis of the migrating speed in the cells on a 2D substrate. The mean values of more than 14 cells are shown with SD.
Figure 2-4. Cell morphology and invasiveness in a 3D matrix.

(A) Temporal sequence of phase contrast micrographs of P-3 and IR cells cultured in a 3D collagen gel. Numbers in the images denote relative time from the start of observation. The white arrow indicates the direction of cell movement. Bar = 50 µm. (B) Statistical analysis of the migrating speed in the cells cultured in a 3D matrix. The mean values of more than 12 cells are shown with SD. *P<0.01.
Figure 2-5. Activation of NF-κB by a stiff substrate.

(A) Immunofluorescent images of NF-κB (p65) in NLS-venus expressing H1299 cells on a stiff substrate (a cover glass coated with collagen) and on a soft substrate (a collagen gel) 2 h after seeding. The nucleus is shown together. and (B) The proportion of NF-κB localization in the nucleus of the cells 0.5, 2, 4, 6 h after seeding evaluated by immunofluorescent intensity. The mean values of more than 30 cells are shown with SD. *P < 0.05.
Figure 2-6. Stiff substrates induce the expression of NF-κB related genes.

MMP9, IL-1β, IL-8, and GAPDH mRNA expressions detected by RT-PCR in H1299 cells on a stiff substrate (a plastic dish coated with collagen) or on a soft substrate (a collagen gel) 6 h after seeding.
Figure 2-7. NF-κB and GAPDH protein signals from the protein extractions prepared by a canonical or an improved method detected by western blotting.

-: a protein extraction prepared by a canonical method. +: a protein extraction prepared by an improved method. The extractions were from P-3 cells on a collagen gel.
Chapter 3

Integrin β1 dependent invasive activity in irradiation-tolerant lung adenocarcinoma cells
**3-1 Abstract**

Radiotherapy is one of the effective therapies used for treating various malignant tumors. However, the emergence of tolerant cells after irradiation remains problematic due to their high metastatic ability, sometimes indicative of poor prognosis. In this study, I showed that subcloned human lung adenocarcinoma cells (P-3) that are irradiation-tolerant indicate high invasive activity *in vitro*, and exhibit an integrin β1 activity-dependent migratory pattern. In collagen gel overlay assay, majority of the P-3 cells displayed round morphology and low migration activity, whereas a considerable number of irradiation-tolerant P-3 cells surviving irradiation displayed a spindle morphology and high migration rate. Blocking integrin β1 activity reduced the migration rate of irradiation-tolerant P-3 cells and altered the cell morphology allowing them to assume a round shape. These results suggest that the P-3 cells surviving irradiation acquire a highly invasive integrin β1-dependent phenotype, and integrin β1 might be a potentially effective therapeutic target in combination with radiotherapy.
3-2 Introduction

Radiotherapy is commonly used for treating cancerous tumors. However, it has been reported that local tumor irradiation can lead to enhancement of metastases (Von Essen, 1991). Recent studies indicate that tumors surviving irradiation acquire high metastatic ability in vivo (Rofstad et al., 2004), and the irradiation-tolerant cancer cells enhance cellular physiological activities, such as invasion, migration, and adhesion in vitro (Tsutsumi et al., 2009). These results suggest that irradiation of tumors could induce malignant transformation and result in poor prognosis, although the detailed mechanism is not well understood.

Integrins are a family of heterodimeric transmembrane proteins, involved in the adhesion of cells to the extracellular matrices (ECMs), such as fibronectin, vitronectin, laminin, and collagen (Hynes, 2002). They also regulate cell proliferation, differentiation, and apoptosis (Giancotti and Ruoslahti, 1999), and mediate intracellular signals that control cytoskeletal organization (Hood and Cheresh, 2002). Especially, integrin β1 is one of the probable key players in regulating cell invasion. Human fibrosarcoma HT1080 cells overexpressing membrane type 1-matrix metalloproteinase (MT1-MMP) elongate a leading edge and invade into 3D collagen matrix by coclustering MT1-MMP and integrin β1 in the direction of cell migration (Wolf et al., 2003).
Several studies revealed that irradiation of cancer cells affects integrin expression. For instance, x-ray irradiation increases expression of functional integrins β1 and β3 in glioma cell lines in a dose-dependent manner (Cordes et al., 2003). In lung adenocarcinoma A549 and SKMES1 cells, X-ray irradiation also induces expression of functional integrin β1 (Cordes et al., 2002). Furthermore, a signal through the cytoplasmic domains of integrin β1 contributes to cell survival after irradiation (Seidler et al., 2005). Previous studies on irradiation effects in cancer cells mainly focused on phenomena just after irradiation, or within a few days after irradiation. In this study, a new cancer cell line that survived after irradiation was established and the properties of these irradiation-tolerant cells were investigated. This study also showed that the irradiation-tolerant cells exhibit a highly invasive integrin β1-dependent phenotype in a 3D collagen matrix.
3-3 Materials and methods

Cell culture

The lung adenocarcinoma A549 cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA). A subclonal A549 cell line (P-3) and irradiation-tolerant P-3 cell line (IR) were established, as shown in chapter 2 (Ishihara et al., 2010). Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; JRH Biosciences Inc., Lenexa, KS) and 1% antibiotic (Invitrogen, Carlsbad, CA). Cells were incubated at 37°C in a humidified incubator with 5% CO₂.

Reagents

Binding of integrin β1 to ECM was inhibited by the anti-human integrin β1 monoclonal antibody—AIIIB2 (Hall et al., 1990) purchased from the Developmental Studies Hybridoma Bank at the University of Iowa. For immunofluorescence staining of integrin β1, AIIIB2 was used as a primary antibody at a concentration of 350 ng/ml (1:80 dilution), and AlexaFluor-594 goat anti-rat IgG (H+L) (Invitrogen) was used as a secondary antibody at a concentration of 10 μg/ml. AlexaFluor-488 phalloidin (Invitrogen) was used for F-actin staining. Cellmatrix Type I-P (Nitta Gelatin Inc., Osaka, Japan) was used at a concentration of 1.6 mg/ml to make the
collagen gel, and Cellmatrix Type I-C (Nitta Gelatin) was used for collagen-coating on a glass substrate.

**siRNA transfection**

The 21-nucleotide siRNA duplex was synthesized using an *in vitro* Transcription T7 kit (TAKARA, Otsu, Japan). The target sequence to silencing integrin β1 was 5′-TTCAACTGTGATAGATCCAA-3′ (sense sequence). Cells were transfected with the siRNA or a Random RNA duplex by Lipofectamine RNAiMAX Reagent (Invitrogen). Knockdown of integrin β1 mRNA was confirmed by RT-PCR. Cells, transfected with siRNA or random RNA, were fixed after 4 days and roundness index analysis was performed as described later.

**Collagen gel overlay assay and time-lapse observation**

A glass dish of 8.0 mm radius was filled with 400 μl of collagen gel, and 4 × 10^3 cells were seeded onto the collagen gel. After 24 h, 200 μl of collagen sol solution were poured onto the cells, and incubated for 60 min at 37°C to complete gelation. This method allows the cells to be cultured in a 3D environment. Then, the dish was filled with culture medium, and sealed with silicone grease to avoid changes in pH of the medium. A phase-contrast microscope, (TE300,
Nikon Instech., Tokyo, Japan) equipped with a 10× objective and kept at 37°C in an acrylic resin box, was used for time-lapse observations. Image-Pro software (Media Cybernetics Inc., Silver Spring, MD) was used for time-lapse observation by capturing images every 5 min. After 24 h, AIIB2 treatment (300 ng/mL) was performed, and the observation was continued for 24 h. Cell migration rate was calculated by measuring the displacement of the cell center every 30 min.

**Fluorescence microscopy**

A glass dish of 8.0 mm radius was filled with 100 µl of collagen gel, and 4 × 10³ cells were seeded onto the collagen gel. After 24 hours, 50 µl of collagen sol were poured onto the cells and gelled at 37°C. After 24 h, cells were fixed with 4% paraformaldehyde in PBS and blocked with 0.5% bovine serum albumin in PBS (Sigma). For staining of integrin β1 and F-actin, reactions of primary and secondary antibodies were performed at 37°C for 3 h, where dilution of AlexaFluor-488 phalloidin was 1:500. Fluorescence images were obtained by using confocal laser scanning microscopy (C1 confocal imaging system; Nikon Instech.).

**Roundness index analysis**

A glass dish of 17.5 mm radius was filled with 250 µl of collagen gel, and 10⁴ cells were seeded
onto the collagen gel. After 24 h, 125 μl of collagen sol were poured onto the cells and gelled at 37°C. Then, cells were cultured for 24 h in the 3D environment. For inhibition of integrin β1 activity, 30 ng/ml AIIB2 monoclonal antibody was used. After culturing with AIIB2 for 24 h, cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100 in PBS and blocked with 0.5% bovine serum albumin in PBS. F-actin staining was performed for 1 h at room temperature, where dilution of AlexaFluor-488 phalloidin was 1:200. Fluorescence images were obtained by using confocal laser scanning microscopy, and roundness index (RI) was calculated by the Image-Pro software (Media Cybernetics Inc.). Briefly, the roundness index is calculated with the equation $4\pi S/L^2$, where $S$ = surface area and $L$ = perimeter length of the cell. If the RI is larger than 0.5, the cell were categorized as “round”, whereas if the RI is less than 0.5, the cell was considered “spindle”.

**RT-PCR**

Cells were lysed with ISOGEN (Wako, Osaka, Japan) for RNA extraction, and reverse transcription reaction was performed by ReverTra Ace qPCR RT kit (TAKARA). PCR reaction was performed with Taq Polymerase in ThermoPol Buffer (NEB, Ipswich, MA). Primers were as follows; GAPDH, 5′-ACCACAGTCCATGCCATCAC-3′ (upper) and
5′-TCCACCACCCTGTGGCTGTA-3′ (lower); and integrin β1,

5′-AATGAAGGCGTGTTGTAG-3′ (upper) and 5′-CCTGTTGTTCCATTCAGT-3′
(lower).

Statistical analysis

Mean and standard deviation (SD) were calculated, and statistically significant differences were identified using Student’s t-tests.
3-4 Results

*Invasiveness of IR cells was dependent on integrin β1 activity*

The localization of integrin β1 was different in P-3 and IR cells in collagen gels. Immunofluorescence indicated that integrin β1 in P-3 cells was diffused on the cell surface, whereas accumulation of integrin β1 at the head of elongated cell surface was observed in IR cells (Figure 3-1). Therefore, I predicted that integrin β1 activity induced morphological changes and high migration activity in IR cells. Thus, in order to inhibit integrin β1 activity, the cells were treated with the monoclonal antibody AIIB2. Under the collagen gel overlay condition, IR cells treated with AIIB2 showed morphological changes allowing them to assume a round shape, and cell migration was suppressed (Figure 3-2A), whereas P-3 cells treated with AIIB2 did not show any changes in cell shape and movement. Results from the motility assay suggested that treatment with the AIIB2 antibody reduces the migration rate of IR cells significantly (Figure 3-2B).

*Spindle morphology was induced by integrin β1 activity*

The cell roundness index was calculated from F-actin stained images to quantify the proportion of cells exhibiting a particular morphology (Figure 3-3A). The results indicated that more than
70% of P-3 cells showed round morphology, whereas almost 70% of IR cells showed spindle morphology (Figure 3-3B). Furthermore, in IR cells, AIIB2 treatment induced changes in shape from spindle to round, and washout treatment subsequently recovered the spindle morphology.

siRNA transfection also induced round morphology in IR cells (Figure 3-4A). The results of RT-PCR indicated that collagen-coated glass and collagen gel induced the same levels of integrin β1-mRNA expression (Figure 3-4B). The siRNA transfection reduced mRNA expression of integrin β1, and random RNA did not (Figure 3-4C). The proportion of IR cells with a spindle morphology was significantly reduced by siRNA transfection (Figure 3-4D). These results indicate that spindle morphology of IR cells was strongly dependent on integrin β1 expression.
3-5 Discussion

When P-3 and IR cells were buried in a collagen gel, only IR cells indicated spindle morphology and migrated through the collagen matrix, depending on the activity of integrin β1 that clustered in the direction of migration. These results demonstrate that irradiation-tolerant cells can acquire integrin β1 activity and exhibit integrin β1-dependent increased invasiveness in a 3D environment.

As reported previously, co-expression of integrin β1 and MT1-MMP in the direction of the migrating site was observed in highly invasive HT1080/MT1 cells (Wolf et al., 2003). Thus, it is possible that not only integrin β1, but also MT1-MMP is activated by irradiation, resulting in increased migration of IR cells. Furthermore, the formation of membrane protrusion sat the leading edge of migrating cells, as a result of actin reorganization, is regulated by one of the small GTPases, Rac1 (Yamazaki et al., 2005), and activated Rac1 induces integrin-dependent migration in mammary epithelial cells (Keely et al., 1997). Therefore, Rac1 activity might be necessary to induce invasiveness in IR cells.

Several studies show that irradiation augments the invasive character of some kinds of cancer cells. Mouse fibrosarcoma QRsP cells exhibit an enhanced invasive activity after survival through 10 Gy irradiation (Nishioka et al., 2007). Moreover, in human non-small lung
cancer H1299 cells, invasiveness is enhanced after 10 Gy irradiation (Tsutumi et al., 2009). Increase in invasive activity after irradiation might be a common phenomenon in cancer cells making it difficult to use radiotherapy as a single modality treatment for the complete cure of many malignant cancers.

It is also reported that a combined therapy of CNTO 95, the anti-human integrin \( \alpha v \) monoclonal antibody and fractionated radiation is more effective than radiation therapy only (Ning et al., 2008). Moreover, integrin \( \beta 1 \) plays an essential role in mediating post-radiation cancer cell survival (Cordes and Park, 2007). Taken together, our results suggest that integrin \( \beta 1 \) can serve as a potentially effective therapeutic target in combination with radiotherapy.
Figures

Figure 3-1. Integrin β1 localization of irradiation-tolerant lung adenocarcinoma cells in a 3D collagen gel.

Fluorescent images of F-actin and integrin β1 in P-3 and IR cells cultured in a collagen gel. P-3: subclonal A549 cells, IR: irradiation-tolerant cells derived from P-3 cells. Cross-sectional views of X–Z and Y–Z directions (axes) are shown together. The white arrow shows the accumulation site of integrin β1. Bar = 20 μm.
Figure 3-2. Invasive activity in IR cells dependent on integrin β1.

(A) Time-lapse phase contrast images of P-3 and IR cells cultured in a collagen gel treated with AIIB2. Numbers in the micrographs represent the observation time. Bar = 50 μm. (B) Statistical analysis of the migrating speed with which cells invade the collagen gel. The mean values of more than 12 cells are shown with SD (shown as error bars) from 3 independent experiments. * P < 0.01.
Figure 3-3. Cell morphology in IR cells treated with AIIB2.

(A) Fluorescent images of F-actin in P-3 and IR cells cultured in a collagen gel. Numbers in the images show the roundness index (R.I.) of the cell morphology. Bar = 20 μm. (B) The proportion of cells categorized as round and spindle. P-3 and IR cells were treated with AIIB2 or washed out after AIIB2 treatment. The mean values of more than 150 cells are shown with SD calculated from at least 3 independent experiments. * P < 0.05, ** P < 0.01.
Figure 3-4. Cell morphology of IR cells transfected with integrin β1 siRNA.

(A) Fluorescent images of F-actin in P-3 and IR cells cultured in a collagen gel transfected with random RNA or integrin β1 siRNA. Bar = 20 μm. (B) mRNA expression of integrin β1 detected by RT-PCR. P-3 and IR cells were cultured under 2 different conditions (glass; on a collagen-coated glass, gel; in a collagen gel) (C) mRNA expression in IR cells cultured in a collagen gel transfected with random RNA or integrin β1 siRNA. (D) The proportion of cell morphology of P-3 and IR cells. The mean values of more than 150 cells are shown with SD calculated from at least 3 independent experiments. * P < 0.05, ** P < 0.01.
Chapter 4

The role of myosin regulatory light chain for invasiveness in irradiation-tolerant lung adenocarcinoma cells
4-1 Abstract

Radiotherapy is one of the major treatment modalities for malignancies by inducing the apoptosis in cancer cells. However, cells surviving irradiation often display high levels of invasiveness. This study shows that irradiation-tolerant lung adenocarcinoma demonstrates high invasive capability depending on dephosphorylation of the myosin regulatory light chain (MRLC). In a collagen gel overlay condition, low-invasive subclones of lung adenocarcinoma (A549P-3) showed a round morphology and diphosphorylation of MRLC. In contrast, irradiation-tolerant A549P-3 cells (A549P-3IR) displayed high invasiveness and a lower level of MRLC diphosphorylation. In addition, inhibition of MRLC phosphatase activity decreased the invasive activity. These findings suggest that A549P-3IR cells acquire high invasiveness through MRLC dephosphorylation.
**4-2 Introduction**

Radiotherapy is often the primary treatment for many types of malignancies. However, it has been reported that growth and metastasis of solid tumors are induced after local tumor irradiation (Von Essen, 1991). Recent studies have revealed that some cancer cells show higher motility and invasiveness after irradiation than prior to irradiation (Tsukamoto et al., 2007; Tsutsumi et al., 2009). It has also been reported that irradiation of cancer cells leads to increased expression of adhesion molecules, integrins (Cordes et al., 2002; Cordes et al., 2003), or matrix metalloproteinases (Nishioka et al., 2007). These findings indicate that irradiation of tumors can evoke malignant properties, although the details of the underlying mechanism is not well understood.

Contractile force is important for many physiological functions such as cell migration, cytokinesis, and morphological change. Contractile force is generated by the contractility mediated by actomyosin, composed of actin filaments and myosin II. In this process, the myosin regulatory light chain (MRLC) is essential for myosin motor activity, and phosphorylation of Ser19 and/or Thr18 of MRLC is crucial for its activation (Mizutani et al., 2006a). Contractile forces also play an important role in cell migration on a 2D substrate. MRLC diphosphorylation induced by RhoA-dependent ROCK activity triggers cellular contractile forces (Ridley, 2001; Yamazaki et al., 2005). On the other hand, in a 3D environment, the relationship between cell
migration and cellular contractile forces is not well studied.

In this study, I investigated the contribution of contractile forces generated by diphosphorylation of MRLC to the invasive behavior of irradiation-tolerant cancer cells in a 3D environment. Chapter 3 revealed that irradiation-tolerant lung adenocarcinoma cells show integrin β1-dependent invasive activity in a 3D collagen matrix (Ishihara et al., 2010). The results in this study showed that high-invasive irradiation-tolerant lung cancer cells demonstrate a lower level of MRLC diphosphorylation than that of low-invasive lung cancer cells in a 3D collagen matrix, and that the invasiveness is dependent on integrin β1 activity. The results also showed that constitutive phosphorylation of MRLC, by use of a phosphatase inhibitor, decreases the invasive activity of irradiation-tolerant cells.
4-3 Materials and methods

Cell culture

The lung adenocarcinoma A549 cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA). A subclonal A549 cell line (P-3) and irradiation-tolerant P-3 cell line (IR) were established, as shown in chapter 2 (Ishihara et al., 2010). Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; JRH Biosciences Inc., Lenexa, KS) and 1% antibiotic/antimycotic solution (Invitrogen, Carlsbad, CA). Cells were incubated at 37°C in a humidified incubator with 5% CO₂.

Reagents

Y27632 (Sigma) was used for inhibition of Rho kinase (ROCK) activity and to reduce MRLC phosphorylation. Calyculin A (CA; Sigma) was used to inhibit phosphatase activity of myosin light chain phosphatase (MLCP; Ishihara et al., 1989). To inhibit integrin β1 activity, monoclonal antibody AIIB2 was used; purchased from the Developmental Studies Hybridoma Bank at the University of Iowa). Rat serum IgG (I8015, Sigma) was used as a control antibody. A 1.6 mg/mL collagen type I gel was prepared with Cellmatrix Type I-P (Nitta Gelatin Inc., Osaka, Japan). For immunofluorescence staining of F-actin, AlexaFluor-488 phalloidin
(Invitrogen) was used at a 1:1000 dilution. Phospho-myosin light chain 2 (Ser19/Thr18) antibody (#3674; Cell Signaling Technology, Beverly, MA) was used as a primary antibody for staining of diphosphorylated MRLC (PP-MRLC) at a 1:150 dilution. AlexaFluor-594 goat anti-rat IgG (H + L; Invitrogen) was used as a secondary antibody for PP-MRLC staining at a 1:500 dilution.

**Immunofluorescence staining**

A glass dish of 8.0 mm radius was filled with 100 μL of collagen gel and $4 \times 10^3$ cells were seeded onto the collagen gel. After 24 h, 50 μL of collagen sol were poured on the dish and incubated at 37°C for 30 min for gelation. This method allowed the cells to be cultured in a 3D substrate. After 24 h, cells were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.5% Triton X-100 in PBS for 10 min, and blocked with 5% skim milk in PBS for 1 h. For PP-MRLC staining, cells were incubated overnight with the primary antibody in 0.5% skim milk in PBS at room temperature. Then, the cells were rinsed 3 times with 0.5% skim milk in PBS and incubated with secondary antibody in 0.5% skim milk in PBS for 1 h at room temperature. After reaction with secondary antibody, cells were rinsed 3 times with 0.5% skim milk in PBS. Fluorescence images were obtained using confocal laser scanning microscopy (C1 confocal imaging system; Nikon Instech., Tokyo, Japan). Fluorescence
intensity was calculated and the intensity ratio of PP-MRLC per F-actin was calculated by Image-Pro software (Media Cybernetics Inc., Silver Spring, MD).

**Roundness index analysis**

A glass dish of 12.5 mm radius was filled with 250 µL of collagen gel, and $1 \times 10^4$ cells were seeded onto the collagen gel. After 24 h, 125 µL of collagen sol was poured on the dish and incubated at 37°C for 30 min for gelation. Then, the cells were incubated with or without Y27632 (20 µM), CA (1 nM), or AIIB2 (30 ng/ml) at 37°C. After 24 h, cells were fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized with 0.5% Triton X-100 in PBS for 10 min. For F-actin staining, the cells were incubated with AlexaFluor-488 phalloidin in PBS for 20 min at 37°C. Then, the cells were rinsed 3 times. Fluorescence images were obtained by confocal laser scanning microscopy and the roundness index was calculated using Image-Pro software, as shown in chapter 3 (Ishihara et al., 2010).

**Time-lapse observation**

A glass dish of 12.5 mm radius was filled with 1000 µL of collagen gel, and $1 \times 10^4$ cells were seeded onto the collagen gel. After 24 h, 500 µL of collagen sol were poured onto the dish and incubated at 37°C for 30 min for gelation. Then, the dish was filled with culture medium and
sealed with silicone grease to avoid exposure to air and a change in the pH of the media. A phase-contrast microscope (TE300, Nikon Instech.), equipped with a 10× objective and a 37°C acrylic resin incubation box, was used for time-lapse observations of cells. Image-Pro software was used for time-lapse observation, which involved capturing images every 5 min. After 12 h, the cells were treated with CA at a concentration of 1.5 nM and the observation was continued. After 12 h, the media was removed and the cells were washed the cells 3 times with fresh DMEM to remove CA from the dish. The observation was continued for 27 h after removing CA.

**Statistical analysis**

Mean and standard deviation (SD) of fluorescence intensities were calculated, and statistically significant differences were identified using Student’s t-tests.
4-4 Results

**Diphosphorylation level of MRLC in P-3 and IR cells**

To investigate differences in the contractile force between low-invasive subclonal A549 lung adenocarcinoma cells (P-3) and high-invasive irradiation-tolerant P-3 cells (IR) cultured in a 3D collagen gel matrix, immunofluorescent staining of diphosphorylated MRLC (PP-MRLC) was performed. PP-MRLC intensity in IR cells was higher than that of P-3 cells (Figure 4-1A). Analysis of the PP-MRLC fluorescence intensities indicated that the PP-MRLC intensity in P-3 cells was significantly higher than that in IR cells (Figure 4-1B). The SD of intensities (shown as error bars in Figure 4-1B) in P-3 cells was larger than that in IR cells. This is because some of P-3 cells had low PP-MRLC fluorescence intensity, whereas that in IR cells consistently demonstrated low PP-MRLC fluorescence intensity. These results suggested that high-invasive IR cells consistently generate lower contractile force compared to low-invasive P-3 cells.

**Spindle morphology induced by dephosphorylation of MRLC in P-3 cells**

The morphological changes in a 3D matrix were observed in P-3 cells treated with Y27632, an inhibitor of MRLC phosphorylation. Treatment with Y27632 significantly reduced PP-MRLC fluorescence intensity in P-3 cells (Figure 4-2). Analysis of roundness index revealed that most of the untreated P-3 cells showed a round morphology in a collagen gel. In contrast, a
significant number of P-3 cells treated with Y27632 displayed spindle morphology (Figure 4-3A, B). These results suggested that dephosphorylation of MRLC induces invasive morphology in P-3 cells cultured in a 3D matrix.

**Invasive behavior induced by diphosphorylation of MRLC in IR cells**

Next, diphosphorylation of MRLC in IR cells was induced by CA, known to be an inhibitor of protein phosphatase (Ishihara et al., 1989). IR cells treated with CA showed a higher intensity of PP-MRLC immunofluorescence than did untreated IR cells in a collagen gel (Figure 4-4). Roundness index indicated that the proportion of IR cells with a round morphology after treatment with CA was significantly greater than that in untreated IR cells in a 3D matrix (Figure 4-5A, B).

The invasive behavior of IR cells in a 3D matrix was also observed. Under untreated condition, IR cells displayed spindle morphology and invaded in a 3D collagen gel (Figure 4-6). However, after CA treatment, IR cells changed to a round morphology, and showed reduced invasiveness. Furthermore, after washout treatment, IR cells recovered to a spindle morphology and demonstrated higher invasiveness. These results indicated that dephosphorylation of MRLC is essential for high invasive activity in IR cells.
Activity of integrin β1 is essential for spindle morphology

As shown in chapter 3, spindle morphology of IR cells in a 3D collagen matrix is dependent on integrin β1 activity (Ishihara et al., 2010). It is investigated that the contribution of integrin β1 activity to the morphological change in P-3 cells to a spindle shape induced by dephosphorylation of MRLC. Roundness index indicated that the majority of P-3 cells treated with both Y23632 and AIIB2, an inhibitory monoclonal antibody, showed a round morphology (Figure 4-7A, B). On the other hand, most of the P-3 cells treated with only Y27632 showed a spindle morphology. Treatment with a control antibody did not induce morphological changes to the round morphology of P-3 cells treated with Y27632 (Figure 4-8). These results indicated that the activity of integrin β1 is necessary for the spindle morphology that is induced by MRLC dephosphorylation in P-3 cells.
4-5 Discussion

This study showed that MRLC dephosphorylation induces spindle morphology and a high-invasive phenotype in A549 lung adenocarcinoma cells. Low-invasive P-3 cells showed a round morphology that was dependent on PP-MRLC in a 3D collagen gel. On the other hand, irradiation-tolerant IR cells displayed high levels of invasive activity and a spindle morphology that was dependent on dephosphorylation of MRLC. The relationship between MRLC phosphorylation and the invasive behavior of irradiation-tolerant cancer cells has not been reported previously.

Because PP-MRLC generates strong contractile forces in actomyosin (Mizutani et al., 2006a), the low invasiveness of P-3 cells in a 3D matrix is probably due to the strong contractile forces of actomyosin. I speculate that the inward contractile forces at the cell periphery maintain a round morphology and prevent protrusions from the cell surface. On the other hand, a previous report suggested that contractile forces are necessary for cancer cells to pull the cell body in the direction of migration (Friedl and Wolf, 2010). Our results indicate that excessive contractile force prevents the invasive activity of P-3 cells.

In addition to MRLC, integrin β1 also contributes to the invasive activity in P-3 and IR cells. This study showed that P-3 cells treated with Y27632 demonstrate an invasive phenotype that is dependent on integrin β1 activity. In addition, as shown in chapter 3,
inhibition of integrin β1 activity prevents an invasive phenotype in IR cells (Ishihara et al., 2010). Another study showed that HT1080 fibrosarcoma cells overexpressing MT1 matrix metalloproteinase show an invasive phenotype in a 3D collagen matrix that is dependent on integrin β1 activity (Wolf et al., 2003). Collectively, integrin β1 activity may be essential for various types of cancer cell to invade in a 3D matrix.

MRLC is a possible therapeutic target in malignant lung adenocarcinoma for preventing invasion. This study showed that CA treatment increases the phosphorylation level of PP-MRLC and inhibits the invasive activity of IR cells in a collagen gel. Thus, CA may be a possible therapeutic agent against malignant lung adenocarcinoma. It is also revealed that Y27632 treatment triggers dephosphorylation of MRLC and induces an invasive phenotype in P-3 cells. This result indicates that Y27632 treatment may facilitate the progression of lung adenocarcinoma. On the other hand, a previous study showed that Y27632 treatment prevented the progression of invasive hepatoma in vivo (Itoh et al., 1999). From these results, it is suggested that both CA and Y27632 have a potential as therapeutic reagents against cancer cells, although the effects of these reagents seem to depend on the type of cancer cells.

This study also suggests a new therapy that targets MRLC in normal tissue cells after radiotherapy. Radiotherapy damages not only tumor tissues but also normal tissues. Wound healing, including migration of fibroblasts, is a key process for repairing normal tissues
damaged by irradiation. However, irradiation disrupts the activity of fibroblasts, including migration, and perturbs the repair process of normal tissues after radiotherapy (Dormand et al., 2005; Gieringer et al., 2011). A previous study showed that myosin II activity is essential for the migration ability of fibroblasts (Petrie et al., 2012). Because myosin II activity is decreased by dephosphorylation of MRLC, this dephosphorylation may decrease the migration ability of fibroblasts, in comparison to that of IR cells. Thus, I speculated that irradiation prevents migration of fibroblasts via dephosphorylation of MRLC, and as a result, inhibits the wound-healing process. A previous study reported that CA treatment of fibroblasts increases mono- or di-phosphorylated MRLC (Nobe et al., 2003). Therefore, CA may help recover migration ability via MRLC phosphorylation in irradiated fibroblasts, and as a result, promote the repair process in normal tissues damaged by irradiation. Thus, CA may be a good therapeutic target, not only to inhibit the invasive ability of IR cancer cells but also to promote the fibroblast-mediated repair process in normal tissues.

In summary, high-invasive IR cells demonstrated invasive activity dependent on the phosphorylation level of MRLC. PP-MRLC inhibited the invasive phenotype in IR cells, while dephosphorylation of MRLC induced an invasive phenotype in low-invasive P-3 cells. Thus, MRLC is a possible therapeutic target for invasive lung adenocarcinoma.
Figures

Figure 4-1. Immunofluorescent images of diphosphorylated myosin regulatory light chain in irradiation-tolerant lung adenocarcinoma cells.

(A) Fluorescent images of F-actin and diphosphorylated MRLC (PP-MRLC) in cells cultured in a 3D collagen gel. P-3: subclonal A549 cells, IR: irradiation-tolerant cells derived from P-3 cells. Cross-sectional views of XZ and YZ directions (axes) are shown together. Bar = 20 μm. (B) Fluorescent intensity of PP-MRLC / F-actin ratio in P-3 and IR cells. The mean values of 20 cells are shown with SD (shown as error bars) calculated from 3 independent experiments. *P < 0.01.
Figure 4-2. Immunofluorescent images of PP-MRLC in P-3 cells treated with Y27632.

(A) Fluorescent images of F-actin and diphosphorylated MRLC (PP-MRLC) in P-3 cells treated with Y27632 and cultured in a 3D collagen gel. Cross-sectional views of XZ and YZ directions (axes) are shown together. Bar = 20 μm. (B) Fluorescent intensity of PP-MRLC / F-actin ratio in P-3 cells and P-3 cells treated with Y27632. The mean values of 20 cells are shown with SD (shown as error bars). *P < 0.01.
Figure 4-3. Cell morphology in P-3 cells treated with Y27632.

(A) Fluorescent images of F-actin in P-3 cells treated with Y27632 and cultured in a 3D collagen gel. Bar = 30 μm. (B) The proportion of cell morphology categorized as round or spindle-shaped. The mean values of more than 150 cells are shown with SD (shown as error bars) calculated from at least 3 independent experiments. *P < 0.05.
Figure 4-4. Immunofluorescent images of PP-MRLC in IR cells treated with Calyculin A.

(A) Fluorescent images of F-actin and diphosphorylated MRLC (PP-MRLC) in IR cells, IR cells treated with DMSO, and IR cells treated with calyculin A (CA), and cultured in a 3D collagen gel. Cross-sectional views of XZ and YZ directions (axes) are shown together. Bar = 20 μm. (B) Fluorescent intensity of PP-MRLC / F-actin ratio in IR cells, IR cells treated with DMSO, and IR cells treated with CA. The mean values of 12 cells are shown with SD (shown as error bars). *P < 0.01.
Figure 4-5. Cell morphology in IR cells treated with CA.

(A) Fluorescent images of F-actin in IR cells treated with calyculin A (CA) and cultured in a 3D collagen gel. Bar = 30 μm. (B) The proportion of cell morphology categorized as round or spindle-shaped. The mean values of more than 150 cells are shown with SD (shown as error bars) calculated from at least 3 independent experiments. *P < 0.05.
Figure 4-6. Invasive activity in IR cells treated with CA.

Time-lapse phase contrast observation of cells cultured in a 3D collagen gel. IR: IR cells, IR + CA: IR cells treated with calyculin A (CA), IR + CA + washout: IR cells after washout of CA.

Numbers on the images indicate the relative time from the start of the observation. The white arrow shows the direction of cell movement. Bar = 50 μm.
Figure 4-7. Cell morphology in P-3 cells treated with Y27632 and AIIB2.

(A) Fluorescent images of F-actin in P-3 cells, P-3 cells treated with only Y27632, or P-3 cells treated with both Y27632 and AIIB2, cultured in a 3D collagen gel. Bar = 30 μm. (B) The proportion of cell morphology categorized as round or spindle-shaped. The mean values of more than 150 cells are shown with SD (shown as error bar) calculated from at least 3 independent experiments. *P < 0.05.
Figure 4-8. Cell morphology in P-3 cells treated with Y27632 and IgG.

(A) Fluorescent images of F-actin in P-3 cells treated with only Y27632 or P-3 cells treated with both Y27632 and IgG control antibody, cultured in a 3D collagen gel. Bar = 30 μm. (B) The proportion of cell morphology categorized as round or spindle-shaped. The mean values of more than 150 cells are shown with SD (indicated by the error bars) calculated from at least 3 independent experiments.
Chapter 5

Invasiveness regulated by activating transcription factor 5 in irradiation-tolerant lung adenocarcinoma cells
5-1 Abstract

Radiotherapy is one of the effective therapies for malignant tumors. However, local irradiation can induce malignant properties such as invasion and metastasis. Here we show that irradiation-tolerant human lung adenocarcinoma cells display high invasive ability dependent on the molecular signaling pathways including activating transcription factor 5 (ATF5). Compared to low-invasive subclones of lung adenocarcinoma cells (P3), irradiation-tolerant P3 cells (IR) displayed high ATF5 expression and activity. In addition, the repression of ATF5 reduced the protein expression of integrin β1 and induced the diphosphorylation of myosin regulatory light chain in IR cells. The repression of ATF5 in IR cells also triggered morphological change to round one that indicates low invasiveness. These findings suggest that irradiation-tolerant lung adenocarcinoma cells acquire high invasiveness, regulated by ATF5.
5-2 Introduction

Radiotherapy is used in many popular ways for treating various types of tumors. However, it has been reported that local tumor irradiation can trigger metastatic phenotypes in tumors (von Essen, 1991). Recent studies revealed that cancer cells after irradiation show much malignant properties such as motility, invasion, and tumorigenesis than non-irradiated cancer cells (Wild-Bode et al., 2001; Tsutsumi et al., 2009; Nishioka et al., 2011). In addition, irradiation to cancer cells can promote the expression of adhesion molecule, integrin β1 (Cordes et al., 2002) and matrix metalloproteinases (Nishioka et al., 2007). These molecules contribute to malignant phenotypes such as invasion and metastasis in cancer cells. These reports suggest that local irradiation to tumors can promote malignant transformation in cancer cells and result in poor prognosis. However, the detail molecular mechanisms on it are not well understood.

Activating transcription factor 5 (ATF5) is a member of the ATF/cAMP response element-binding family of transcription factors (Persengiev and Green, 2003). It is reported that ATF5 regulates differentiation state in neural progenitor cells (Angelastro et al., 2003), oligodendrocytes (Mason et al., 2005), and adipose-derived stem cells (Leong et al., 2012). In addition, ATF5 promotes cell survival in cancer cells with its expression. The expression of ATF5 in transformed cells is greater than that in nontransformed cells in glioblastoma (Angelastro et al., 2006) and breast cancer (Monaco et al., 2007). These studies also showed
that the repression of ATF5 in cancerous cells induces apoptotic cell death whereas ATF5 repression does not kill nontransformed cells. Recently, it has reported that ATF5 increases radioresistance in cancer cells (Nishioka et al., 2009). QRsP mouse sarcoma cells transfected with ATF5 increased survival fractions after 10 Gy irradiation. Therefore, ATF5 is possible therapeutic target for tumors but detail roles of ATF5 for cancer progression have been unclear yet.

Here, I investigated the role of ATF5 for cancer cell invasion, one of the malignant properties of tumors after irradiation. As mentioned in chapter 3 and 4, irradiation-tolerant lung adenocarcinoma cells indicate high invasive ability in a 3D collagen matrix dependent on integrin β1 activity (Ishihara et al., 2010) and MRLC dephosphorylation (Ishihara et al., 2013). It is shown here that ATF5 expression and activity are higher in high-invasive irradiation-tolerant lung cancer cells than in low-invasive non-irradiated lung cancer cells. The repression of ATF5 suppressed invasive phenotype in irradiation-tolerant cells. It is also showed that ATF5 up-regulates integrin β1 expression and promotes MRLC dephosphorylation, which induces invasive phenotype in IR cells.
5-3 Materials and methods

Cell culture

The human lung adenocarcinoma A549 cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA). A subclonal A549 cell line (P-3) and irradiation-tolerant P-3 cell line (IR) were established, as shown in chapter 2 (Ishihara et al., 2010). These cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; BIST TECH) and 1% antibiotic/antimycotic solution (Sigma). Cells were incubated at 37°C in a humidified incubator with 5% CO₂. Phase-contrast images were captured by a phase-contrast microscope (TE300, Nikon Instech.), equipped with a 10 × objective.

Reagents

Y27632 (Sigma) is used at a concentration of 20 μM to decrease Rho kinase (ROCK) activity and to inhibit MRLC phosphorylation. To repress integrin β1 activity, monoclonal antibody AIIB2 was used at a concentration of 300 ng/mL (Hall et al., 1990; purchased from the Developmental Studies Hybridoma Bank at the University of Iowa). A 1.6 mg/mL type-I collagen gel was prepared with Cellmatrix Type I-P (Nitta Gelatin Inc., Osaka, Japan). For western blotting, anti-ATF5 (N-17, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, sc-46935),
anti-integrin β1 (BD Biosciences, Franklin Lakes, NJ, 610467), Phospho-myosin light chain 2 (Ser19/Thr18) antibody (anti-PP-MRLC, Cell Signaling Technology, Beverly, MA, #3674), and anti-GAPDH (Ambion, Foster City, CA, 1103016) were used as a primary antibody. HRP anti-goat IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, 305-035-003), HRP anti-mouse IgG (Bio-Rad Hercules, CA, 70-6516), and HRP anti rabbit IgG (Cell Signaling Tech, #7074) were used as a secondary antibody. For immunofluorescence staining, anti-PP-MRLC and Myosin light chain 2 antibody (anti-total-MRLC, #3672, Cell Signaling Tech) were used as a primary antibody and AlexaFluor-594 donkey anti-rabbit IgG (H + L; Invitrogen, A-11012) was used as a secondary antibody. For fluorescence staining of F-actin, MFP 488-phalloidin (Mo Bi Tee, Göttingen, Germany) was used.

**Western blotting**

A plastic cell culture dish of 17.5 mm radius was filled with 500 μL of collagen gel. 5 × 10⁴ cells were seeded onto the collagen gel. After 24 h, 250 μL of collagen sol was poured on the dish and incubated at 37°C for 30 min for gelation. This method allowed the cells to be cultured in a 3D substrate. Then, the sample was filled with culture medium with or without reagents. After 24 h, the sample was fixed by 10% trichloroacetic acid in PBS and washed 3 times with PBS on ice. Then, the sample was incubated with 375 μL of 0.1% collagenase L (Nitta Gelatin
Inc.) in PBS at 37°C for 1 h to digest the collagen gel. The sample was collected into micro tubes and centrifuged at 14,000 rpm for 3 min at 4°C. After removing the supernatant, the sample was lysed with SDS sample buffer (0.25 M Tris-HCl, 5% dithiothreitol, 2.3% sodium dodecyl sulfate, 10% glycerol, 0.01% bromophenol blue, pH 8.8). The sample was treated with supersonic wave and boiled for 5 min. Polyacrylamide gels (12.5%) were prepared for SDS-PAGE (20 mA per gel, 1 h). After SDS-PAGE, blotting to PVDF membranes was performed (92 mA per gel, 60 min). After blotting, the membranes were incubated with 5% skim milk in TBS-Tween solution for 2 h (ATF5), with 5% skim milk in TBS-Tween solution for 1 h (integrin β1 and GAPDH), and with 0.5% skim milk in TBS-Tween solution for 1 h (PP-MRLC) at room temperature. Membranes were incubated with primary antibodies (ATF5 1:7500, integrin β1 1:1000, PP-MRLC 1:300, and GAPDH 1:100000) overnight at 4°C. After 3 washes with TBS-Tween, membranes were incubated with secondary antibodies (HRP anti goat IgG 1:20000 for ATF5, HRP anti mouse IgG 1:10000 for integrin β1, HRP anti rabbit IgG 1:10000 for PP-MRLC, and HRP anti mouse IgG 1:200000 for GAPDH) for 1 h at room temperature. After 3 washes with TBS-Tween, signals were detected with Immobilon Western Chemiluminescent HRP substrate (Millipore).

*Luciferase reporter assay*
Luciferase plasmids (300 ng/mL pCRE-Luc, 30 ng/mL pRL-TK, 1200 ng/mL pcDNA3) were transfected with Lipofectamine 2000 to semi-confluent P-3 or IR cells on a 24 well plastic plate covered with 100 μL of collagen gel. Cells were cultured with non-antibiotic media supplemented with 10% fetal bovine serum. After 24 h, luciferase activities were detected using Dual Luciferase Reporter Assay System (Promega). The activities of pCRE per RL-TK were calculated.

**siRNA transfection**

The 21-nucleotide siRNA duplex was synthesized using an *in vitro* Transcription T7 kit (TAKARA, Otsu, Japan). The target sequence to silencing ATF5 was 5′-CAAAAATAAAACGAAACATT-3′ (sense sequence). Cells were transfected with the ATF5 siRNA or a negative control RNA duplex by Lipofectamine RNAiMAX Reagent (Invitrogen). Cells transfected with RNA were seeded onto collagen gels 24 h after transfection.

**Immunofluorescence staining**

A glass dish of 8.0 mm radius was filled with 100 μL of collagen gel and 4 × 10³ cells were seeded onto the collagen gel. After 24 h, 50 μL of collagen sol were poured on the dish and incubated at 37°C for 30 min for gelation. Then, the sample was filled with culture medium.
After 24 h, cells were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.5% Triton X-100 in PBS for 10 min, and blocked with 0.5% skim milk in PBS for 1 h. For PP-MRLC or total-MRLC staining, cells were incubated overnight with the primary antibody (1:150 dilution) in 0.5% skim milk in PBS at room temperature (for PP-MRLC) or 4°C (for total-MRLC). Then, the cells were rinsed 3 times with 0.5% skim milk in PBS and incubated with secondary antibody (1:500 dilution) and MFP 488-phalloidin (1:1000 dilution) in 0.5% skim milk in PBS for 1 h at room temperature. After reaction with secondary antibody, cells were rinsed 3 times with 0.5% skim milk in PBS. Fluorescence images were obtained by confocal laser scanning microscopy (C1 confocal imaging system; Nikon Instech., Tokyo, Japan). The intensity ratio of PP-MRLC or total-MRLC per F-actin was calculated by Image-Pro software (Media Cybernetics Inc., Silver Spring, MD).

**Roundness index analysis**

A glass dish of 12.5 mm radius was filled with 250 μL of collagen gel, and 1 × 10^4 cells were seeded onto the collagen gel. After 24 h, 125 μL of collagen sol was poured on the dish and incubated at 37°C for 30 min for gelation. Then, the sample was filled with culture medium with or without reagents. After 24 h, cells were fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized with 0.5% Triton X-100 in PBS for 10 min. For F-actin staining, the cells
were incubated with MFP-488-phalloidin in PBS for 20 min at 37°C. Then, the cells were rinsed 3 times. Fluorescence images were obtained by confocal laser scanning microscopy and the roundness index was calculated using Image-Pro software, as shown in chapter 3 (Ishihara et al., 2010).

**Statistical analysis**

Mean and standard deviation (SD) were calculated, and statistically significant differences were identified using Student’s *t*-tests.
5-4 Results

ATF5 expression and activity are higher in IR cells than in P-3 cells

I detected ATF5 expression in low-invasive subclonal A549 lung adenocarcinoma cells (P-3) and high-invasive irradiation-tolerant P-3 cells (IR) cultured in a 3D collagen gel matrix by western blotting. The expression of ATF5 in IR cells was higher than that in P-3 cells (Figure 5-1A). Furthermore, to investigate the transcriptional activity of ATF5, luciferase reporter assay was performed to analyze the transcriptional activity of cAMP response element (CRE), which is negatively regulated by ATF5 (Angelastro et al., 2003; Liu et al., 2012). The CRE transactivity in IR cells was lower than in P-3 cells (Figure 5-1B). Furthermore, ATF5-knockdowed IR cells by ATF5 siRNA transfection (KD cells) showed higher CRE activity than IR cells transfected with negative control RNA (NC cells; Figure 5-1C). These results indicated that IR cells express more ATF5 proteins and show higher ATF5 activity than P-3 cells.

Knockdown of ATF5 induces round morphology in IR cells

Because IR cells indicated invasive ability with spindle cell shape in a 3D collagen gel as shown in chapter 2 (Ishihara et al., 2010), ATF5 knockdown by siRNA transfection in IR cells was performed. KD cells displayed round morphology whereas NC cells indicated spindle
morphology (Figure 5-2A). These results indicated that ATF5 plays a crucial role for spindle morphology in IR cells.

**Knockdown of ATF5 reduces integrin β1 expression in IR cells**

Next, to investigate whether ATF5 regulates the protein expression that contributes to invasiveness in IR cells, western blotting analysis was performed in KD and NC cells. Western blotting analysis showed that KD cells displayed lower expression of ATF5 and integrin β1 than NC cells (Figure 5-2B). As mentioned above, integrin β1 plays an important role for cancer cell invasion by regulating the adhesions between cells and collagen fibers, which is major component of connective tissues (Wolf *et al.*, 2003). In addition, the data in chapter 3 revealed that IR cells showed high invasive activity dependent on integrin β1 (Ishihara *et al.*, 2010). Taken together, it is suggested that ATF5 induces integrin β1 expression, and as a result, promotes invasive ability in IR cells.

**ATF5 repression induces MRLC diphosphorylation in IR cells**

In is shown in chapter 4 that IR cells presented high invasive activity dependent on dephosphorylated MRLC (Ishihara *et al.*, 2013). It is also reported that IR cells treated with calyculin A, which induce diphosphorilation of MRLC (PP-MRLC), indicated low invasive
phenomena in a 3D collagen gel. Thus, the role of ATF5 for MRLC phosphorylation in IR cells was investigated. Fluorescent staining of PP-MRLC showed that KD cells displayed higher fluorescent signals of PP-MRLC at cell periphery than NC cells in a 3D collagen gel (Figure 5-3A). Analysis of the relative PP-MRLC fluorescent intensities indicated that the PP-MRLC intensity in KD cells significantly greater than in NC cells (Figure 5-3B). Next, fluorescent staining of total-MRLC in KD and NC cells was performed. Both of KD and NC cells displayed the localized signals of total-MRLC at the cell periphery in 3D collagen gels (Figure 5-3C). The relative fluorescent intensities of total-MRLC were not significantly different between KD and NC cells (Figure 5-3D). These results suggested that ATF5 does not decrease the amounts of total MRLC but down-regulate diphosphorylation status of MRLC.

**MRLC dephosphorylation does not affect ATF5 and integrin β1 expression in P-3 cells**

Next, the protein expressions of PP-MRLC, ATF5, and integrin β1 were detected by western blotting in P-3 cells treated with Y27632, an inhibitor of MRLC diphosphorylation. Y27632 treatment induced spindle morphology and decreased the signal of PP-MRLC, as shown in chapter 4 (Figure 5-4A, B). However, Y27632 treatment did not affect the expressions of ATF5 and integrin β1 (Figure 5-4B). These results suggested that phosphorylation status of MRLC does not change the protein expressions of ATF5 and integrin β1.
**Inhibition of integrin β1 activity does not affect ATF5 expression and MRLC diphosphorylation in IR cells**

Next, western blotting analysis of ATF5 and PP-MRLC was performed in IR cells treated with AIIB2, inhibitory monoclonal antibody of integrin β1, in a 3D collagen gel. AIIB2 treatment induced round morphology in IR cells, as indicated in chapter 3 (Figure 5-5A). On the other hand, the signals of ATF5 and PP-MRLC were not affected by AIIB2 treatment in IR cells (Figure 5-4B). These results suggested that the activity of integrin β1 does not regulate the ATF5 expression and MRLC diphosphorylation.

**MRLC dephosphorylation induces spindle morphology in ATF5-knockdowned IR cells.**

Finally, the cell morphology in KD cells treated with Y27632, which induces MRLC dephosphorylation, was observed and analyzed. Analysis of roundness index indicated that the proportion of spindle cells in KD cells was significantly lower than that in NC cells (Figure 5-6A, B), as mentioned above. Furthermore, Y27632 treatment to KD cells recovered the proportion of spindle cells (Figure 5-6A, B). These data suggested that ATF5 expression induces spindle morphology via repression of MRLC diphosphorylation in IR cells.
5-5 Discussion

This study revealed that irradiation-tolerant A549 lung adenocarcinoma cells presented the greater ATF5 expression and activity than non-irradiated A549 cells. Previous study reported that ATF5-transfected subclone of QRsP mouse sarcoma cells gained higher radioresistance than control ones (Nishioka et al., 2009). This data indicates that ATF5 expression enhances the radioresistance in QRsP mouse sarcoma cells. In the same way, A549 lung adenocarcinoma cells that highly express ATF5 may indicate high irradiation tolerance, and as a result, preferentially survive after irradiation and form a subset of irradiation-tolerant cells.

It is also shown that the repression of ATF5 expression in irradiation-tolerant IR cells induced a morphological change from a spindle cell shape to a round morphology in a 3D collagen matrix. As shown in chapter 2, non-irradiated P-3 cells displayed low invasiveness with round cell morphology whereas IR cells showed high invasiveness with spindle morphology in a 3D collagen gel (Ishihara et al., 2010). Taken together, it is suggested that IR cells indicate high invasiveness with a spindle cell shape dependent on ATF5 expressions.

ATF5 regulates invasion ability in IR cells via biochemical signaling pathways involving integrin β1 and MRLC. This study showed that the repression of ATF5 suppressed the expression of integrin β1 protein and induced diphosphorylation of MRLC. In contrast, the inhibition of integrin β1 activity by AIIB2 in IR cells did not affect the expression levels of ATF5 and dephosphorylated MRLC. The induction of MRLC dephosphorylation by Y27632 in
P-3 cells also did not change the expression of ATF5 and integrin β1. These results suggest that ATF5 independently induces integrin β1 expression and MRLC dephosphorylation whereas integrin β1 and MRLC do not regulate ATF5 expression. Chapter 3 and 4 showed that the activity of integrin β1 and dephosphorylation of MRLC are essential for the invasive ability in IR cells (Ishihara et al., 2010; Ishihara et al., 2013). In addition, Y27632 treatment to inhibit MRLC dephosphorylation increased the proportion of spindle morphology in ATF5-knockdowned IR cells. Therefore, it is suggested that ATF5 induces the expression of integrin β1 and dephosphorylation of MRLC, and as a result, promotes invasive ability in IR cells, although the detail biochemical pathways are unclear.

ATF5 is a possible therapeutic target for cancer therapy. It is shown in this study that repression of ATF5 expressions inhibited the invasiveness in irradiation-tolerant lung adenocarcinoma cells. Previous study reported that ATF5 plays an important role for irradiation resistance in QRsP mouse sarcoma cells (Nishioka et al., 2009). In addition, combined therapy of fractionated radiation and molecular inhibitor such as the anti-human integrin αv monoclonal antibody is more effective than radiation therapy only (Ning et al., 2008). Thus, the combined therapy of irradiation and ATF5 inhibitors may be an effective therapy to treat malignant tumors.
Figure 5-1. Expression and activity of activating transcription factor 5 (ATF5) in P-3 and IR cells.

(A) The protein expression of ATF5 and GAPDH in P-3 and IR cells cultured in a 3D collagen gel detected by western blotting. P-3: subclonal A549 cells, IR: irradiation-tolerant cells from P-3 cells. (B) The pCRE transactivity of P-3 and IR cells on a 2D collagen gel detected by luciferase reporter assay. Relative activities of pCRE / RL-TK are shown. pCRE transactivity is
negatively regulated by ATF5. *: P < 0.01. Error bar = SD. n = 9 (P-3) or 8 (IR) samples in three independent experiments. (C) The pCRE transactivity of non-treated IR cells (NT), negative control RNA transfected IR cells (NC), and ATF5 siRNA transfected IR cells (KD) on a 2D collagen gel detected by luciferase reporter assay. Relative activities of pCRE / RL-TK are shown. pCRE transactivity is negatively regulated by ATF5. *: P < 0.01. Error bar = SD. n = 3 (NT, NC) or 2 (KD) samples.
Figure 5-2. Cell morphology and integrin β1 expression in IR cells transfected with ATF5 siRNA.

(A) Phase-contrast images of IR cells transfected with negative control RNA (NC) or ATF5 siRNA (KD) in a 3D collagen gel. Bar = 100 μm. (B) Protein expressions of ATF5, integrin β1, and GAPDH in the cells cultured in a 3D collagen gel detected by western blotting.
Figure 5-3. Immunofluorescence images of diphosphorylated- and total- MRLC in IR cells transfected with ATF5 siRNA.

(A) Fluorescent images of F-actin and diphosphorylated MRLC (PP-MRLC) in IR cells transfected with negative control RNA (NC) or ATF5 siRNA (KD) in a 3D collagen gel. Cross-sectional views of XZ and YZ (axes) directions are shown together. A white arrow indicates the localization of PP-MRLC. Bar = 20 μm. (B) Fluorescent intensities of PP-MRLC / 

106
F-actin ratio in the cells. The relative mean values of intensities are shown with SD as error bars.

*: P < 0.01. n = 20 cells. (C) Fluorescent images of F-actin and total-MRLC of the cells in a 3D collagen gel. Cross-sectional views of XZ and YZ (axes) directions are shown together. Bar = 20 μm. (D) Fluorescent intensities of total-MRLC / F-actin ratio in the cells. The relative mean values of intensities are shown with SD as error bars. n = 10 cells.
Figure 5-4. Expression of ATF5 and integrin β1 in P-3 cells treated with Y27632.

(A) Phase-contrast images of non-treated (NT) or Y27632 treated (Y27632) P-3 cells in a 3D collagen gel. Bar = 100 μm. (B) Protein expressions of ATF5, integrin β1, PP-MRLC, and GAPDH in the cells detected by western blotting. The cells were cultured in a 3D collagen gel.
Figure 5-5. Expression of ATF5 and PP-MRLC in IR cells treated with AIIB2.

(A) Phase-contrast images of non-treated (NT) or AIIB2 treated (AIIB2) IR cells in a 3D collagen gel. Bar = 100 μm. (B) Protein expressions of ATF5, PP-MRLC, and GAPDH in the cells detected by western blotting. The cells were cultured in a 3D collagen gel.
Figure 5-6. Cell morphology in IR cells transfected with ATF5 siRNA and treated with Y27632.

(A) Fluorescent images of F-actin in IR cells transfected with negative control RNA (NC), IR cells transfected with ATF5 siRNA (KD), or IR cells transfected with ATF5 siRNA and treated with Y27632 (KD+Y27632) in a 3D collagen gel. Bar = 30 μm. (B) The proportion of cell morphology categorized as round or spindle shape. The mean values of the proportion calculated from 50 cells are shown with SD as error bars. *: P < 0.01. n = 4 (NC and KD) or 3 (KD+Y27632) in four or three independent experiments.
Chapter 6

Summary and remaining questions
I revealed that subclonal lung adenocarcinoma cells (P-3) show low invasiveness with round morphology whereas irradiation-tolerant P-3 cells (IR) display high invasiveness with spindle cell shape. The invasiveness in IR cells was dependent on the activity of integrin β1, dephosphorylation of myosin regulatory light chain (MRLC), and the expression of activating transcription factor 5 (ATF5; Figure 6-1). From the results of this study, I established a model of biochemical signaling, which regulates the invasiveness of IR cells (Figure 6-2). IR cells show higher expression and activity of ATF5 than P-3 cells. ATF5 enhances the expression of integrin β1, which promotes invasive ability by regulating cell-ECM adhesion in IR cells. ATF5 also induces dephosphorylation of MRLC, and as a result, triggers invasive phenotype in IR cells by reducing actomyosin contractions. However, there have still been remaining questions to be solved.

First, the mechanisms in P-3 cells to survive after irradiation are unclear. Previous study showed that ATF5 expression enhances radioresistance in QRsP mouse sarcoma cells (Nishioka et al., 2009). As mentioned above, IR cells showed higher expression level of ATF5 than P-3 cells. Thus, ATF5 may strengthen radioresistance not only QRsP cells but also P-3 cells. P-3 cells that express high levels of ATF5 gene may survive after irradiation and behave as IR cells. Further studies are required to reveal detail molecular mechanisms on it.

Second, the mechanisms of ATF5-dependent regulation in the expression of integrin
β1 are unclear. There have been no reports to be shown that ATF5 regulates integrin β1 expression. Because ATF5 proteins can bind to pCRE regions on DNA and suppress the transactivity of it, this association may regulate the expression of integrin β1. The biochemical molecules intervening between ATF5 and integrin β1 may exist.

Third, the mechanisms on ATF5-dependent MRLC dephosphorylation are still unclear. MRLC phosphorylation is enhanced by kinases such as Rho kinase (ROCK), myosin light chain kinase (MLCK), and integrin linked kinase (ILK) (Somlyo and Somlyo, 2003). In contrast, MRLC phosphorylation is weakened by myosin light chain phosphatase (MLCP). ATF5 may inhibit kinase activities and/or promote phosphatase activities to decrease phosphorylation levels of MRLC.

In this study, I revealed the biochemical signal pathways regulating the high invasiveness in IR cells. The key molecules; integrin β1, MRLC, and ATF5; are possible therapeutic targets for malignant tumors, which re-arise after irradiation. The treatment with drugs that target these molecules may be effective therapy in combination with radiotherapy.
Figure 6-1. A model of mechanisms in the invasion of IR cells.
Figure 6-2. A model of biochemical signaling regulating the invasion of IR cells.
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