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School of Life Science and Technology

Department of Life Science and Technology

ROR2 regulates the survival of murine osteosarcoma cells in lung capillary

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11/2020

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Abbreviation List

BL	Bioluminescent
CDC42	Cell division control protein 42
cDNA	Complementary DNA
CRISPR-Cas9	Clustered regularly interspaced short palindromic repeats associated endonuclease 9
CTC	Circulating tumor cells
CYGB	Cytoglobin
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
Fzd	Frizzled
HE	Hematoxylin and eosin
HIF-1α	Hypoxia inducible factor-1α
ID mice	Immune-deficient mice
ITGA6	Integrin α6
ITGB5	Integrin β5
i.v	Intravenous
JNK	c-Jun N-terminal kinase
KO	Knockout
KEGG	Kyoto Encyclopedia of Genes and Genomes
Luc	Firefly luciferase

LRP receptor	Leucine-responsive global transcription regulator receptors
LEF1	Lymphoid enhancer-binding factor 1
mi-RNA	micro-RNA
MMP	Metalloproteinase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B
n.s	not significant
OE	Overexpression
OS	Osteosarcoma
p-AKT	phosphorylated AKT
PCP	Planar cell polarity
PI3K	Phosphatidylinositol 3-kinase
Poly-HEMA	Poly(2-hydroxyethyl methacrylate
qPCR	quantitative polymerase chain reaction
Rho	Ras homolog gene family
RT-PCR	Reverse transcription polymerase chain reaction
rWnt5a	Recombinant Wnt5a
TCF	T-Cell Factor
TGF	Transforming growth factor
TRPM8	Transient receptor potential melastatin member 8
WST-1	Water-soluble tetrazolium salt 1

Chapter 1

General introduction

1.1 Metastatic process

In the early phases of multi-step tumor progression, tumor cells grow at the site where first cells begin to grow unregulated, developing the primary tumor. Only about 10% of death from tumor is contributed by primary tumor, and detection of tumor-caused death from tumors in secondary organs reach 90% [1]. Cells from the primary tumor invade surrounding healthy tissues and make concession their function. Tumors found in tissues in the secondary organs are called metastases. These metastases are conducted by tumor cells leaving the primary tumor and migrating through the body via blood and lymph vessels, looking for new sites throughout the body, escaping and forming colonies. In a various type of tumors, tumor cell dissemination is believed to have already occurred by the time the primary tumor is first detected. However, these disseminated cells are too small to detect at the first diagnosis.

Although expansive studies have been conducted on metastasis, the entire process of metastasis is not yet fully understood. Tumor cells undergo multiple complex metastatic processes: invasion of surrounding tissues, enter into lymphatic or blood circulation, survival in the blood circulation and small capillaries, extravasation into secondary organs and tumor colony formation (Figure 1-1).

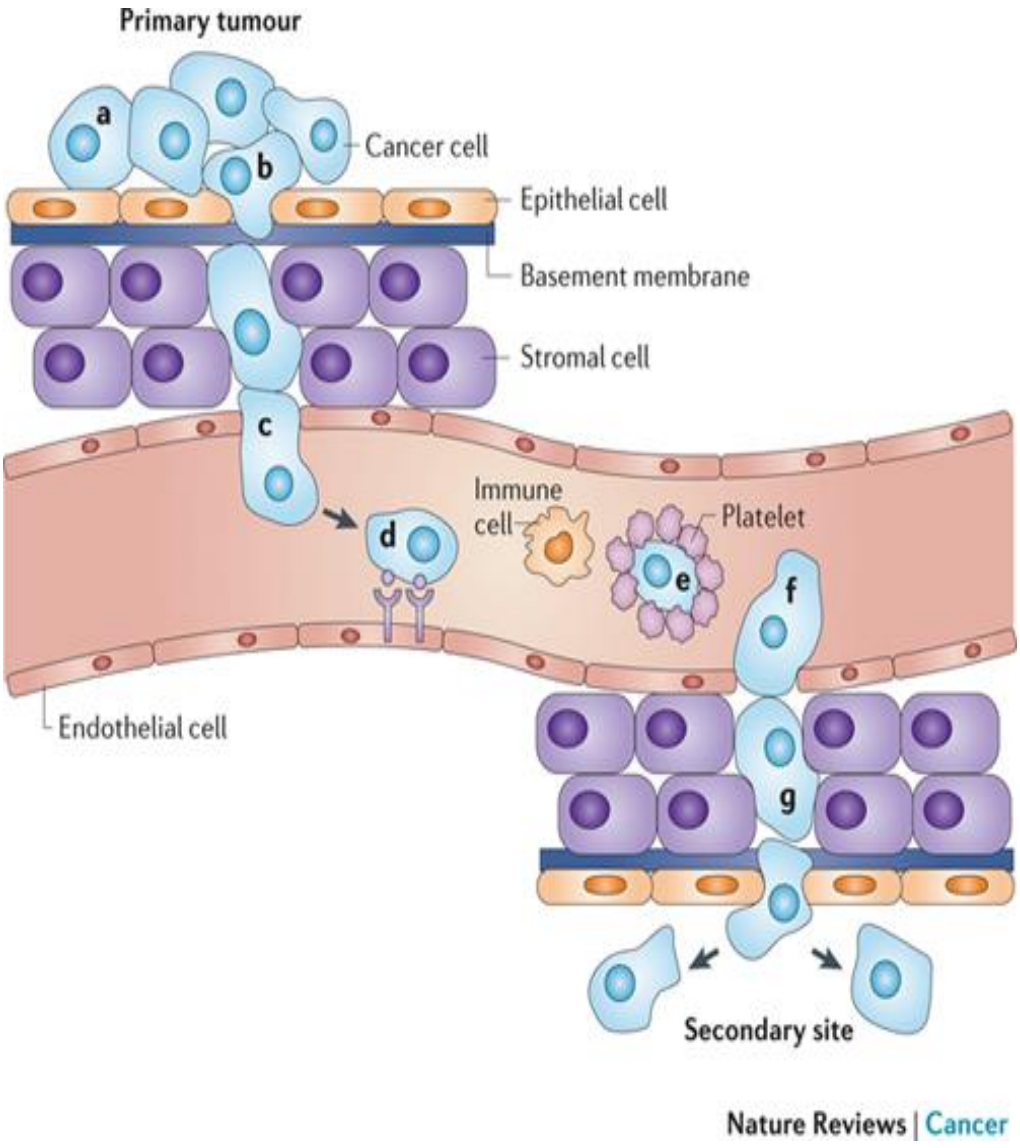

Figure 1-1: Multi-step cascade in cancer metastasis Cited from [2]

Tumor cells a) reduce adhesion to neighboring cells b) degrade extracellular matrix (ECM) for migration into the vasculature-rich stroma c) intravasate into blood circulation d) & e) survival in blood circulation f) extravasate and g) colonize in secondary organs.

1.2 Lung metastasis of osteosarcoma

1.2.1 Osteosarcoma

Osteosarcoma (OS) is mesenchymal-derived tumor cells and characterized by

morphologically abnormal osteoblastic cells producing aberrant osteroid organic portion of the bone matrix. The most common sites are as follows:

- Femur (42%, 75% of which are in the distal femur)
- Tibia (19%, 80% of which are in the proximal tibia)
- Humerus (10%, 90% of which are in the proximal humerus)
- Skull and jaw (8%)
- Pelvis (8%)

A number of types of OS include conventional types (osteoblastic, chondroblastic, and fibroblastic), telangiectatic, multifocal, parosteal, and periosteal. The conventional OS is referred to simply as OS. OS is the most common primary bone malignancy affecting children and young adults.

The exact cause of OS is unidentified. However, several risk factors have been validated. Firstly, rapid bone growth appears to predispose to OS, as evidenced by the increased incidence during the adolescent growth spurt and typical location of OS in the metaphyseal reasion adjacent to the growth plate of long bones. Second factor is genetic mutation. For example, skeletal dysplasia is a genetic disorder that could cause abnormal bone development. Furthermore, high risk of OS could be caused by combination of mutation of Rb gene and radiation treatment. Exposure to radiation is the only known risk factor from environment.

The present understanding of OS outcome and prognosis is driven by specific serum markers, clinical staging, and histologic response to chemotherapeutic agents [3]. Patients with an elevated alkaline phosphatase at diagnosis should probably have lung metastases. p16(INK4a) is determined as a prognostic factor in OS patients in a meta-

analysis of eight studies [4]. Moreover, presence of circulating microRNA (miR)-148a in peripheral blood of OS patients was significantly associated with either tumor size and distant metastasis, or poor overall survival [5]. One study found that high expression of the oncoprotein transient receptor potential melastatin member 8 (TRPM8) predicts poor prognosis in OS patients: TRPM8 is associated with higher clinical stage and distant metastasis as well as with shorter overall survival. TRPM8 may prove to be a useful molecular target for the treatment of patients with OS [6]. Other studies have shown that patients with good histological response to neoadjuvant chemotherapy (>95% necrosis) have a better prognosis than those whose tumors do not respond as well.

OS has a potential to spread to other organs via the blood circulatory system and the lung is the most common site for metastasis [7]. These emphasize a need for developing therapeutic strategies based on understanding molecular mechanism of OS lung metastasis.

1.2.2 Available treatment for OS

Prior to the 1950s, the treatment of OS had been dependent on surgery and the survival 5-year was < 20% in 1972. Current standard treatment of younger OS patients includes preoperative chemotherapy, surgical resection of all tumor and postoperative chemotherapy [8]. Combination of chemotherapeutic reagents, such as adriamycin cisplatin, methotrexate, cyclophosphamide and epirubicin, and surgery significantly improved 10-year survival rate to approximately 70% for patients with localized/regional OS, while for patients with metastatic disease is 24% from 1991 to 2010 [9]. However, there are various side effects caused by chemotherapy. Myelosuppression and gastrointestinal reactions are the most common side effects. Therefore, patients are often

required to stop and modify chemotherapy. Furthermore, current chemotherapy cannot effectively control tumor metastasis. Therefore, suppressing lung metastasis is a major challenge for improving survival in patients with OS.

1.3. Wnt signaling in OS

1.3.1 Cannonical and noncannotinal Wnt signaling

The term “Wnt” is the fusion name of Drosophila segment polarity gene Wingless and the mouse proto-oncogene Int-1 (Wnt-1 in mouse and human cells). The two main groups of Wnt signaling pathways are the canonical or β-catenin-dependent Wnt signaling pathway, and noncanonical or β-catenin-independent Wnt signaling pathway. The Wnt signaling pathway has been shown as an evolutionarily conserved pathway in regards to cell fate, development, self-renewal, and tissue morphogenesis [10]. In addition, Wnt signaling also controls cell proliferation, differentiation, motility, cell polarity, apoptosis, survival and adhesion. These processes require tight regulation, including regulation by extracellular and intracellular antagonistic, inhibitory, and enhancing factors, and by crosstalk with other signaling pathways [11]. Wnt protein comprises a diverse family of secreted lipid-modified signaling glycoprotein palmitoleoylation. Wnt ligands need to bind their carrier protein Wntless, and then can be transported to the plasma membrane for secretion [12]. The action of Wnt ligands on the cell surface elicits canonical or noncanical signals, depending on the cell types, environment and other conditions. Nineteen Wnt ligands have been identified. Typical canonical Wnt ligands are Wnt3, Wnt3a, Wnt7a, Wnt8, and Wnt10, and typical noncanonical Wnt ligands are Wnt4, Wnt5a, Wnt5b, and Wnt11.

Previous studies that overexpression of many components of Wnt signaling such

as Wnt ligands, Frizzled (Fzs) and Leucine-responsive global transcription regulator (LRP) receptors, and silencing of the genes encoding endogenous Wnt pathway inhibitors highlighted the involvement of dysregulated Wnt signaling in the development and progression of OS [13]. Activation of β-catenin-dependent canonical Wnt signaling pathway controls migration and invasion, anoikis–apoptosis resistance and angiogenesis of OS cells.

1. 3.2 β-catenin-dependent canonical Wnt signaling pathway

The canonical Wnt signaling pathway is the Wnt pathway that causes an accumulation of β-catenin in cytoplasma and translocation into nucleus. Its function is a coactivator of T-cell factor / lymphoid enhancer-binding factor (TCF/LEF) transcription factor. Without Wnt ligands, a destruction complex consisting of axin, adenomatosis polyposis coli, protein phosphatase 2A, glycogen synthase kinase 3 and casein kinase 1α degrades β-catenin in cytoplasma. Binding of Wnt ligands to Fzs and LRP5/6 coreceptor triggers a series of continuing events such as translocation of the Wnt regulator Axin and a destruction complex to the plasma membrane. Activation of a cytoplasmic phosphoprotein Dishevelled inhibits glycogen synthase kinase 3 activity and prevents degradation of β-catenin. Stabilized β-catenin is translocated to the nucleus, form a complex with other transcription factors, and function as a transcription factor. The best known factor in the complex are TCF/LEF DNA-binding transcription factor, and their target genes include *c-Myc*, *Ccnd1*, and matrix metallopeptidase 9 (*Mmp9)* [11,14]. The oncogene c-*Myc* encodes a transcription factor that drives cell cycle progression, proliferation, and reduces apoptosis. *Mmp9* is suppressed by Wnt inhibitor and plays an important role in OS metastatic ability [14]. *Ccnd1* encoded cyclin D1 protein controls cell

cycle. It contributes to dysregulation of tumor cell proliferation via canonical Wnt signaling pathway [11]. Therefore, activation of canonical Wnt signaling pathway serves as a genetic driver in many types of tumor, including colorectal, lung, breast, ovarian, prostate, liver, brain, sarcoma, and OS.

1. 3. 2 β-catenin-independent noncanonical Wnt signaling pathway

There are two major signaling pathways in noncanonical Wnt signaling pathway: Wnt/Ca^{2+} and Wnt/ planar cell polarity (PCP) signaling pathway (a DVL-c-Jun N-terminal kinase (JNK) pathway) [15]. They play roles in cytoskeletal remodeling, transcriptional regulation and migration. The PCP pathway acts through JNK protein while Wnt/Ca^{2+} pathway signal transduction is dependent on cytoplasmic Ca^{2+} level. Wnt5a, a Wnt ligand of noncanonical Wnt signaling, binds to Fzs receptor or receptor tyrosine kinase-like orphan receptor 2 (ROR2) that mediates various downstream signaling such as JNK and phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathways.

1.4. LM8 sublines

LM8 is a murine OS cell line with highly lung metastatic potential from murine Dunn OS established using 8 times of Fidler's procedures [16]. Filder et al claimed that malignant primary tumor contains subpopulations with different metastatic ability. To prove that, he used B16 melanoma syngeneic to C57BL/6 mice. The scheme of experiment is illustrated

in figure 1-2. Isolation of single clones from primary tumors and test metastatic ability of each clone called Fidler's procedure.

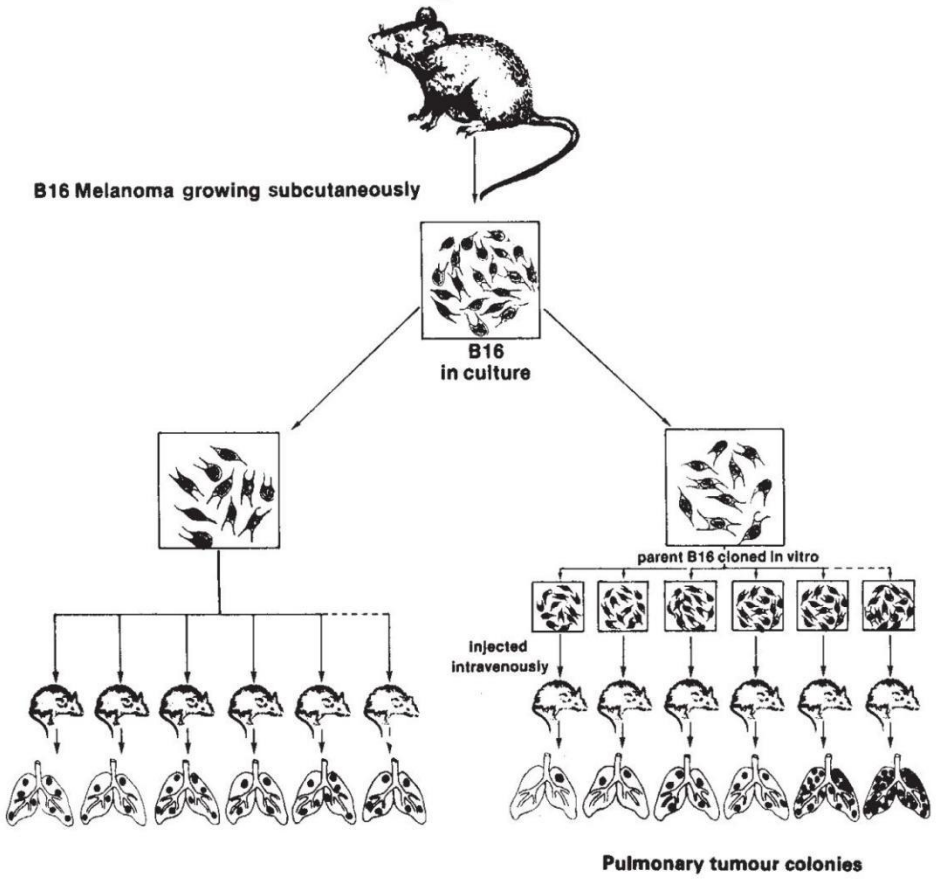

Figure 1-2: The schematic illustration of experiment. Cited from [16].

Cells isolated from the primary subcutaneous tumor were divided into two aliquots and their lung metastatic ability was examined. One aliquot was directly injected into the tail vein (left). The other aliquot was first cloned from a single cell, and then several cloned cells were injected intravenously into the tail vein.

LM8 has many filopodial protrusions, lamellipodial structures surrounding the cell surface, and morphologies with enhanced cell motility. In recent study, lymphoid enhancer-binding factor 1 (LEF1) is proved that cytoglobin (CYGB)-LEF1 axis regulates extravasation ability in lung metastatic process of LM8 sublines [17]. LEF1 has homology with high-mobility group protein 1 and contains a HMG-box domain involved in DNA

binding. LEF1 is a transcription factors that associates with other co-activators or multi-protein enhancer complexes to stimulate expression of target genes. For example, complex of LEF1 and β-catenin plays a critical role in activating the downstream target genes in canonical Wnt signaling. LEF1 has been reported to be involved in the metastatic process of osteosarcoma [18, 19]. Notch1 and LEF1 have a major role in the effects of miR-34c on chemosensitivity and metastasis of human OS cell lines such as 143B, SAOS, U2OS.

Regulatory mechanism of CYGB by LEF1 is not elucidated. CYGB is known to be induced by hypoxia inducible factor 1 (HIF-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and other inflammation-related transcription factors [20]. The interaction of LEF1 with these transcription factors may be important for regulating CYGB expression.

1.5 Research purpose

Current OS treatments are not effective for patients with metastasis, especially lung metastasis. Therefore, I aimed to search for new therapeutic targets related to OS lung metastasis from genes that are differentially expressed between the LM8 sublines with different lung metastatic ability, and elucidate the mechanism by which the targets are involved in the lung metastasis process.

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Chapter 2

ROR2 function is required for lung metastasis of LM8

2.1 Aim of the study

In a previous study, the sublines of murine OS cell line LM8 with high lung metastatic ability (LM8-H) and low lung metastatic ability (LM8-L) were established from the LM8 cell line. Microarray analysis of these sublines showed different gene expression profiles. The purpose of this study was to identify candidate genes that are significantly involved in OS lung metastasis by analyzing genes that are differentially expressed between LM8-H and LM8-L.

2.2 Introduction

2.2.1 Characteristic of LM8 sublines in lung metastasis

LM8 is a murine OS cell line with high metastatic potential to the lungs isolated from murine Dunn OS using eight repeated Fidler's procedures [1]. LM8 consists of cells with various lung metastatic ability. Therefore, to elucidate the mechanism that determine the metastatic ability of LM8, LM8 sublines with different lung metastatic ability were previously isolated by using image-guided *in vivo* screening [2]. LM8 expressing firefly luciferase (LM8/luc) was intracardially injected into immune-deficient (ID) mice. At day 14, LM8 in the hind limb was collected and cultured. Cultured LM8 was reinjected into ID mice. After repeating the procedure four times, LM8-L with high affinity for bones and almost no growth in lung was established. When LM8-L was orthotopically injected into the tibia of ID mice, lung metastasis was detected in a very rare case based on bioluminescent (BL) signal at two weeks after injection. A high lung metastatic LM8 subline was established from the lung metastasis and named as LM8-H.

Previous study revealed that LM8-H has a higher lung metastatic ability than LM8-L. LM8 sublines expressing firefly luciferase were intravenously injected into C3H mice and observed the BL signal at day 0, 4, 8, and 12 by *in vivo* imaging. The BL intensity was recorded and showed in the graph (Figure 2-1). At day 0, BL intensity in lungs of LM8-H and LM8-L-injected groups were similar. However, the BL intensity was different between them on day 3 (early stage). The BL intensity represented the number of viable LM8 in the lungs. It demonstrated that lung metastatic ability of LM8-H was significantly higher than LM8-L.

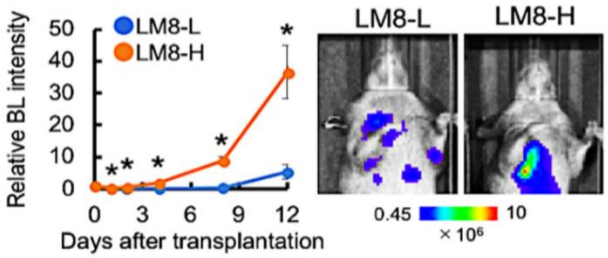

Figure 2-1: Lung metastatic ability of LM8-L and LM8-H. Cited from [2]

Representative *in vivo* BL images on day 12 (right) and quantitative analysis (left) are shown. BL signals from the lungs on indicated days were normalized to those on day 0. n = 5, *p < 0.05.

2.2.2 ROR2

ROR2 is characterized by three main domains: the intracellular tyrosine kinase domain, extracellular Frizzled-like cysteine-rich domain, and membrane-proximal Kringle domain [3-5]. The extracellular domain of ROR2 is assumed to mediate protein-protein interactions, and ROR2 functions as an alternative or coreceptor for Wnt5a, a representative noncanonical Wnt ligand [6,7]. When Wnt5a binds to ROR2, it activates the Wnt-JNK pathway and inhibits the β-catenin-dependent Wnt pathway [7-9]. Moreover,

ROR2 is overexpressed in 73.8% of human samples of OS tumors and correlates with tumor metastasis [10], suggesting its pivotal role in OS progression.

ROR2 expression correlates with migration and invasion activity of various tumors such as OS, ovarian cancer, renal cell carcinoma, and melanoma [11-17]. Many studies have elucidated several molecular mechanisms by which ROR2 signaling is involved in the progression of OS. Wnt5a/ROR2 signaling regulates the formation of lamellipodia and reorientation of microtube organizing center, which induce cell polarized migration, by activating JNK signaling via actin-binding protein filamin A [9]. Moreover, Wnt5a/ROR2 also enhances MMP-3 expression by activating the transcription factor activator protein-1. MMP-3 degrades the ECM and promotes OS cell invasion. However, the role and molecular mechanism of ROR2 in the post-circulatory process of OS lung metastasis has not been completely elucidated.

2.2 Methods

2.2.1 R2 database analysis

The R2 database (Genomics Analysis and Visualization Platform: http://r2.amc.nl) was used to investigate the correlation between ROR2 and integrin genes in OS patients. The data used is a mixed osteosarcoma-Kuijjer-127-vst-ilmnhwg6v2.

2.2.2 Gene-set enrichment analysis and gene mapping

Differentially expressed genes were selected from the microarray data of LM8 sublines [2] and subjected to gene set enrichment analysis and pathway analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG). The analysis was performed with the assistance of John Clyde Co Soriano by the clusterProfiler package using

R/Bioconductor [18]. The reference gene set used was the Wnt signaling pathway (mm0431) set.

2.2.3 RT-PCR

Total RNA was extracted using the RNeasy® Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. One μg of total RNA was reverse-transcribed using Oligo(dT)$_{20}$ primer (Tokyo Co., Osaka, Japan) and ReverTra Ace (Toyobo Co.). qPCR and RT-PCR were carried out using EmeraldAmp® GT PCR Master Mix (Takara, Tokyo, Japan).

Table 2-1. Primers of RT-PCR for screening candidate genes

Name	Sequence	Tm
Ror1_RT_F	TGA GCC GAT GAA TAA CAT CAC AA	58
Ror1_RT_R	CAG GTG CAT CAT TCT TGA ACC A	
Fzd1_RT_F	ACT ATA ACC ATC TTG GCG TTG G	58
Fzd1_RT_R	TGA ACA GAT AAA CGA AGA GAG GC	
Ror2_RT_F	CAC TTC CCA CTC TGA AAG GCT	60
Ror2_RT_R	ACC GGG GCA TCA TTC TTC AG	

2.2.4 Ror2 knock out by CRISPR-cas9 system

The sequence of gRNA (5'-caccgTCGTGGCTCTTGCACAACCG-3') was used for targeting *Ror2*. The *Ror2* gRNA was inserted into a unique BbsI site of the pX330 plasmid (42230; Addgene, Cambridge, MA, USA). The cells whose genomes were correctly edited by CRISPR-Cas9 system was selected by using a fluorescence indicator system using the pCAG/EGxxFP plasmid [19], provided by Dr Ikawa (Osaka University, Osaka, Japan).

GFP-positive cells were picked up and validated ROR2 protein expression level by western blotting.

2.2.5 Western blotting

Cells were lysed in RIPA buffer (50 mM Tris HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease cocktail inhibitor (Nacalai Tesque, Kyoto, Japan) and protein concentration was determined using Pierce BCA protein assay kit (Thermo Fisher Scientific, MA, USA) Protein were separated by electrophoresis on a 12.5% acrylamide gel, transferred to a Hydrophilic polyvinylidene fluoride membrane (Merck, NJ, USA) and blocked with 5% skim milk in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20). The membrane was then probed with relevant primary antibodies [anti-ROR2 (#88639s), anti-GAPDH (#2118s) (Cell Signaling Technology, Danvers, MA, USA)], and secondary antibodies anti-rabbit IgG HRP-linked Antibody (#7074, Cell signaling Technology)]. The resultant membranes were washed with TBST 3 times for 5 minutes and detected by LAS 4000 (Fujifilm, Tokyo, Japan) after processing with Chemi-Lumi One (Nacalai Tesque, Kyoto, Japan).

2.2.6 Proliferation assay

Cell proliferation was evaluated with the water-soluble tetrazolium salt 1 (WST-1) reagent (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Cells (10^3 cells/100 µL culture medium) were seeded in 96-well plates. After culturing for 24, 48, or 72 hours, the medium was removed and 100 µL WST-1 containing medium (10-fold dilution) was added to each well. The cells were further incubated for 3 hours, and then the absorbance of each well was measured at 450 nm with a reference

wavelength of 750 nm after shaking the plates for 1 minute with a microplate reader Model 680XR (Bio-Rad, Hercules, CA, USA).

2.2.7 Mice

Male C3H mice were obtained from Charles River Laboratory, Japan (Yokohama, Japan). All mice used were 6-8 weeks of age and were housed in the animal facilities at Tokyo Institute of Technology. Animal experiments were performed with the approval of the Animal Ethics Committees of Tokyo Institute of Technology (no. D20170004-2) and in accordance with the Ethical Guidelines for Animal Experimentation of Tokyo Institute of Technology.

2.2.8 Histological analysis of OS lung metastasis

Mice, 6-8 weeks of age, were injected intravenously with $1x10^6$ LM8 sublines in 100 μL of PBS. Isolated lungs at 20 days after inoculation were embedded in optimal cutting temperature compound (Sakura Fine Tech, Tokyo, Japan) and store at −80°C overnight. Lungs were performed cryosectioning as 10-μm thick and then fixed in 4% paraformaldehyde. Fixed lung cryosections were then stained with hematoxylin and eosin (HE) and observed under a microscope (BZ-X710; Keyence Corporation, Osaka, Japan). A whole lung image was obtained by stitching together partial lung images using BZ-X analyzer software (Keyence).

2.2.9 Statistical analysis

Data are presented as the mean ± standard error of the mean and were statistically analyzed with a two-sided Student's *t*-test. P values of less than 0.05 were considered statistically significant.

2.3 Results

2.3.1 Non-canonical Wnt signaling-related genes were highly expressed in LM8-H

To find candidate genes involved in the noncanonical Wnt signaling during LM8 lung metastasis, gene expression microarray data [2] of LM8-H and LM8-L was used to detect genes that are differentially expressed between them and associated with the noncanonical Wnt pathway. Gene set enrichment analysis [20] identified several Wnt signaling-related genes that showed higher expression in LM8-H than in LM8-L (Figure 2-2A). Mapping of these genes using KEGG [21] indicated that ROR1/2 were receptors for the noncanonical Wnt signaling that is likely involved in the lung metastasis of LM8 (Figure 2-2B). The expression levels of noncanonical Wnt signaling receptor genes *Ror1*, *Ror2*, and *Frizzled class receptor 1* (*Fzd1*) in LM8-H and LM8-L were examined by RT-PCR. *Ror2*, but not *Ror1*, was expressed at a significantly higher level in LM8-H than in LM8-L (Figure 2-3). Furthermore, the mRNA level of *Ror2* was well-correlated with the metastasis-free survival of OS patients (Figure 2-4). Therefore, ROR2 was selected for further analysis.

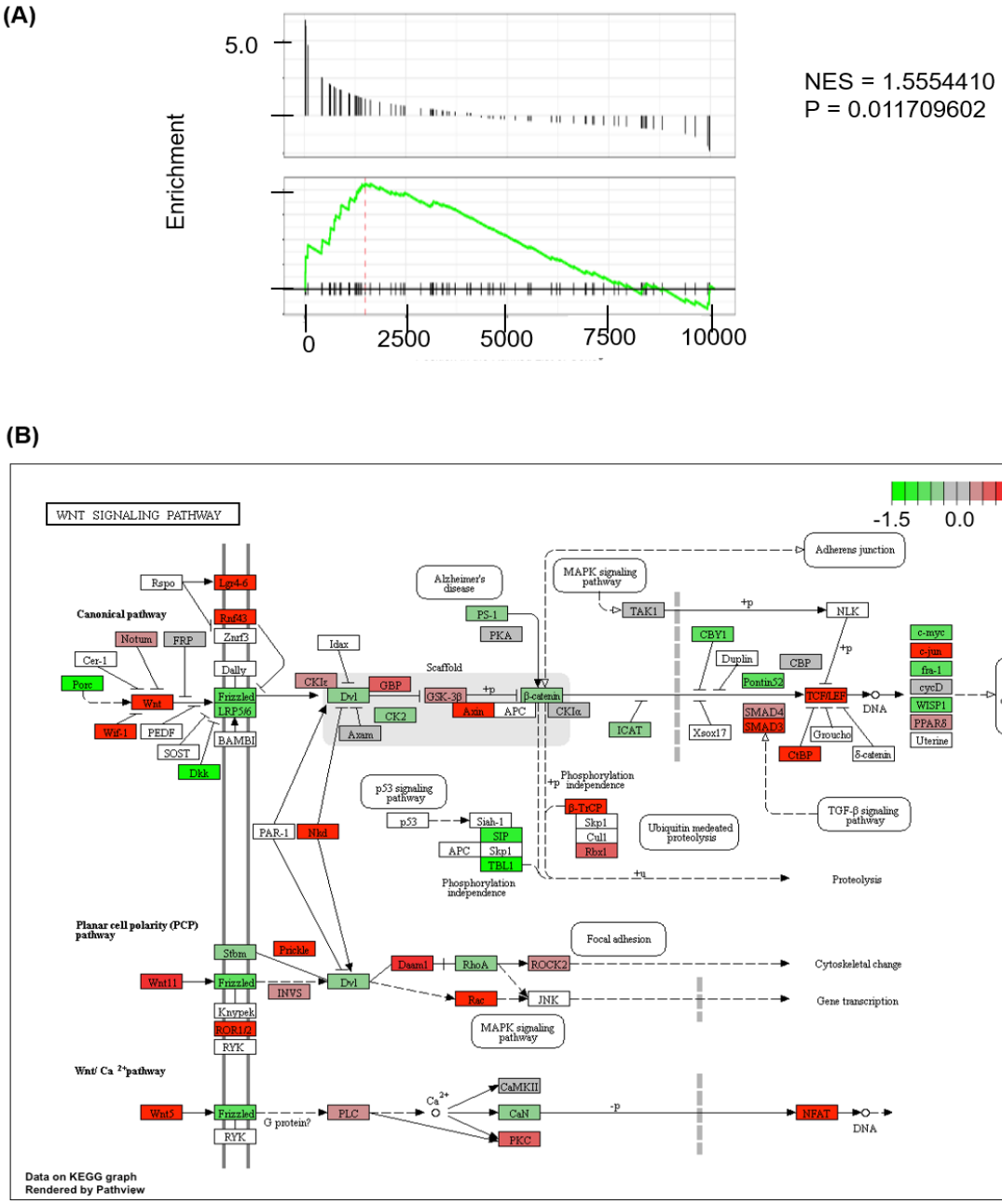

Figure 2-2 Wnt signaling-related gene expression analysis of LM8

(A) Gene set enrichment analysis. Microarray data for LM8-H and LM8-L were analyzed using KEGG software to identify significant gene sets. The enrichment plot shows the distribution of genes in the set that are correlated with the Wnt signaling pathway (mm0431). **(B)** The Wnt signaling pathway in LM8. A putative Wnt signaling pathway of LM8 was constructed based on KEGG mapping. The color of the boxes with gene names corresponds to their mRNA levels from increasing (red) to decreasing (green), as per the color scale shown in the upper right of the figure.

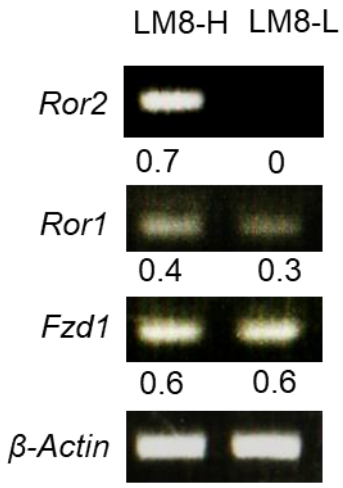

Figure 2-3 mRNA ROR2 expression in LM8 sublines

mRNA expression levels of *Ror2*, *Ror1*, and *Fzd1* in LM8-H and LM8-L were analyzed by RT-PCR. The numbers below the bands indicate the corresponding expression levels relative to β-actin.

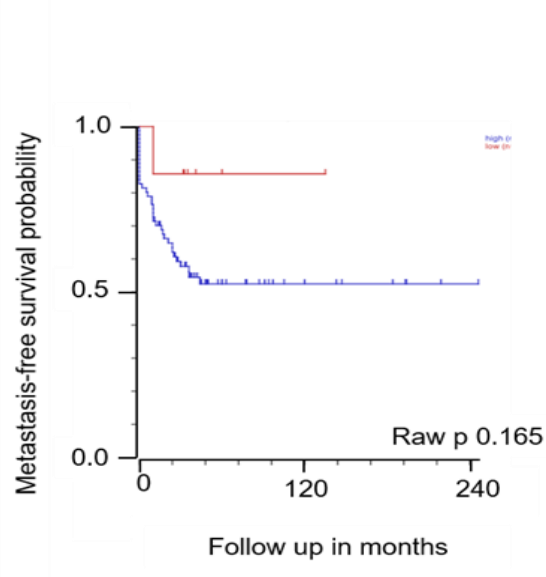

Figure 2-4 Correlation of ROR2 expression with metastasis-free survival of OS pa

Kaplan-Meier metastasis-free survival curves were constructed from data sets of t database (osteosarcoma-Kuijjer-127-vst-ilmnhwg6v2). The red and blue lines sho survival rates of OS patients with low and high ROR2 expression, respectively.

2.3.2 ROR2 signaling regulated the lung metastasis of LM8-H

To determine if ROR2 is involved in lung metastatic ability of LM8. I knocked out *Ror2* gene in LM8-H using CRISPR-Cas9 system. The effect of deletion of *Ror2* (Figure 2-5A) on the proliferation and metastatic potential of LM8-H was first investigated. The proliferation rate of H/*Ror2*-KO was similar to that of LM8-H and LM8-L (Figure 2-5B), indicating that ROR2 function was not involved in proliferation.

Next, I examined the ROR2 role using intravenous injection lung metastasis model. *Ror2*-KO in LM8-H (H/*Ror2*-KO) and parental cell line (LM8-H) was intravenously injected into C3H mice. After 20 days, lungs were collected and performed histological analysis. The size and number of foci in the lungs injected with H/*Ror2*-KO were significantly smaller than those in the lungs injected with LM8-H (Figure 2-6). These data demonstrated the critical role of ROR2 function in LM8 lung metastasis.

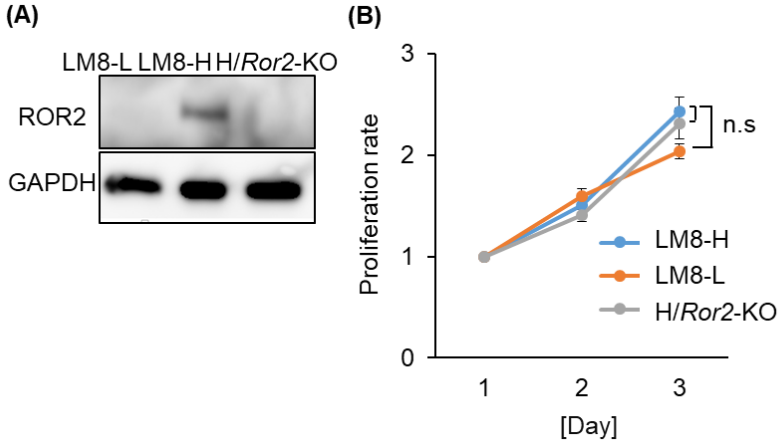

Figure 2-5 Establishment of LM8-H/*Ror2*-KO

(A) ROR2 expression in LM8-L, LM8-H, and H/Ror2-KO were examined by western blotting.

(B) LM8 sublines were cultured under adhesion conditions for 3 days and performed WST-1 assay. n.s: not significant.

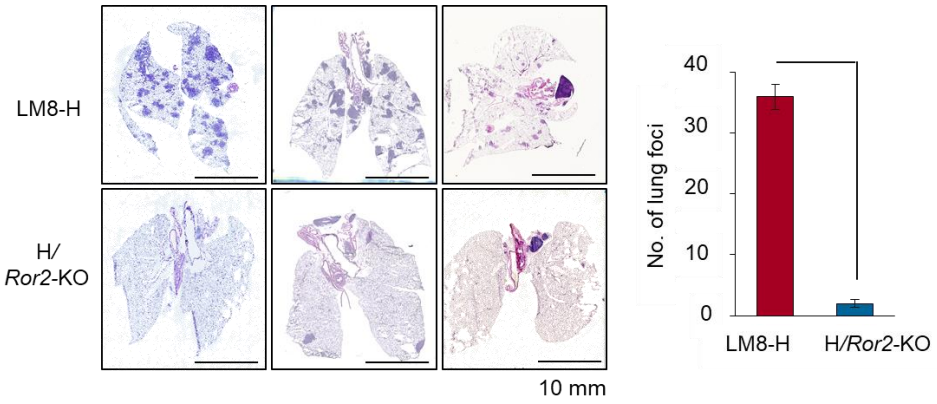

Figure 2-6 Loss of ROR2 expression reduces LM8-H lung metastatic ability

Representative lung images stained with HE (left) and the number of lung foci bigger than 1 mm in diameter (right) at day 20 after intravenous injection of LM8-H and H/*Ror2*-KO. Scale bars: 10 mm, n = 5, **p < 0.01.

2.4 Discussion

Numerous studies have reported a correlation between ROR2 expression and metastatic ability in various cancers [11-16]. Correlation of ROR2 expression and poor survival of OS patient is displayed in Figure 2-4. Moreover, ROR2 is a receptor for noncanonical Wnt signaling that regulates metastatic progression in many types of cancers. In the present study, I found that the number of lung foci in H/*Ror2*-KO-injected mice was smaller compared to that in LM8-H, indicating that ROR2 plays a crucial role in the ability of LM8 to metastasize to the lung. However, the proliferation rates were the same for LM8-H and H/*Ror2*-KO, indicating that ROR2 function is not involved in tumor growth.

In the next chapter, I described the investigation of ROR2 involvement in the transmigration ability of LM8 to understand the function of ROR2 in lung metastasis.

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Chapter 3

ROR2 is an upstream regulator of cytoglobin

3.1 Aim of the study

Previous study has revealed that LEF1- cytoglobin (CYGB) axis plays an important role in regulating LM8 extravasation into lung tissue [1]. In this study, I aimed to elucidate how ROR2 is involved in OS lung metastasis in relation to LEF1-CYGB axis.

3.2 Introduction

3.2.1 Extravasation step in metastatic process

During metastatic process, tumor cells need to cross endothelial barriers of blood/lymph vessels at least twice (intravasation and extravasation, see Figure 1-1). In these steps, the intercellular barrier cell-cell junction of endothelial cells need to be disrupted.

Tumor cell extravasation induces the clustering of endothelial cells and junctions. The direction of blood flow does not affect the movement of tumor cells. Moreover, T cells usually induce extravasation in small capillaries. Cells are easy to be trapped in small capillaries due to the size limitation and create strong adhesion to endothelial cells. In order to perform a cell's extravasation, ligand-receptor complexes are required depending on the type of cell or the type of cancer. There are five groups of receptors on endothelial cells: selectins, integrins, cadherins, CD44 and immunoglobulin superfamily receptors. Many reports have clarified the molecular mechanism of tumor cell extravasation as shown in Figure 3-1.

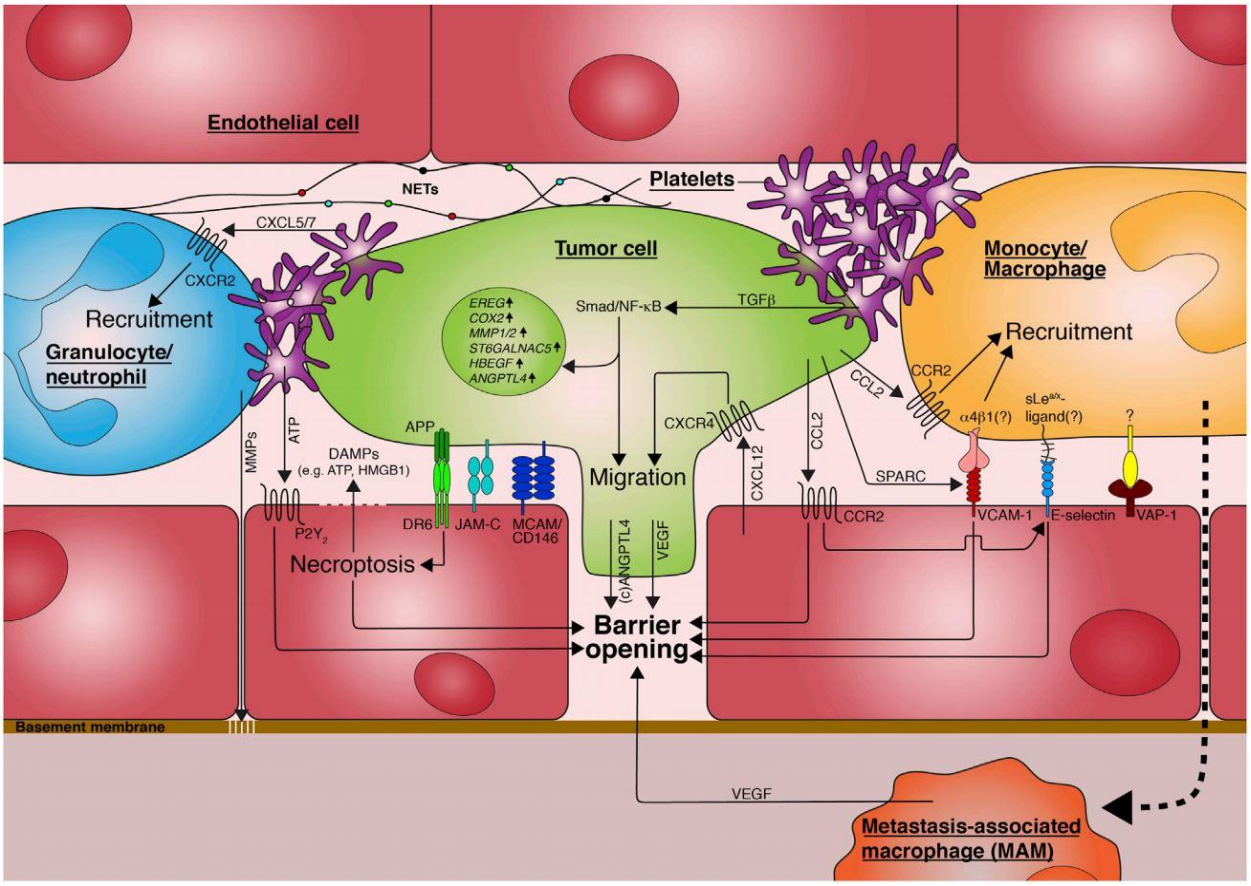

Figure 3-1: Molecular mechanism of tumor cell extravasation from *in vivo* experiments. Cited from [2].

During intravasation and extravasation process, tumor cells induces dynamic change of morphology. This step requires contraction regulation of the cytoskeleton and actomyosin. Ras homology gene (RHO)-family GTPase induces morphological changes of cells [3]. However, it is still unclear how cells transform into round and elongated shapes during extravasation. Elongated tumor cells have been observed to extend protrusions between endothelial cells, disrupting endothelial cell-cell junctions in prostate and mammary gland. RHO-GTPase regulates cell division control protein 42 (CDC42)

which promote the extension of tumor cells when tumor cells adhere to endothelial cells. CDC42 also enhances tumor cell adhesion by regulating integrin-β1 mRNA expression [3].

Furthermore, tumor cells also are protected by interacting with platelets, monocytes, neutrophils, and natural killer cells in the bloodstream to modulate the tumor cell extravasation [4].

Tumor cells can directly promotes extravasation. For example, angiopoietin-like 4 directly interacts with integrin α5β1, VE-cadherin to interrupt vascular endothelial tight junction and promotes metastasis in breast cancer [5,6]. Secretion of osteonectin binds to VCAM-1 and then promotes actin remodeling and opening of endothelial junctions in melanoma cell extravasation [7]. Tumor cells also induce programmed necrosis (necroptosis) in endothelial cell for facilitating extravasation [8].

To study tumor cell extravasation *in vivo*, zebrafish model is used for real-time intravital imaging of injected human tumor cells and mouse model provide the accurate representation of the physiological extravasation such as adhesion to endothelial cells. However, it is difficult to perform real time observation using *in vivo* mouse model. Transwell assays are used for quantifying the number of tumor cells that cross the endothelial cells *in vitro*. Time-lapse microscopy provides information on the time it takes cells to interact and the number of cells interacting in a precise time. The *in vitro* model mimicking extravasation is three-dimensional model. It allows to monitor tumor cells adhesion and invasion in the surrounding ECM.

3.2.2 Function of LEF1 on the lung metastatic ability of LM8

LEF1 is mainly involved in canonical Wnt signaling pathway. LEF1 is a facilitator of epithelial-mesenchymal transition (EMT), which is marker of invasion and migration. Moreover, LEF1 is a biomarker of cancer treatment because presence and dysregulation of LEF1 have been associated with many type of cancer. LEF1 expression is high in LM8-H and low in LM8-L and promotes the lung metastatic ability [1].

Endothelial transmigration assay, which mimics the extravasation step, was used to validate the extravasation ability *in vitro* in previous study [1]. LEF1 facilitates the endothelial transmembrane migration and attachment of LM8-H (Figure 3-2)

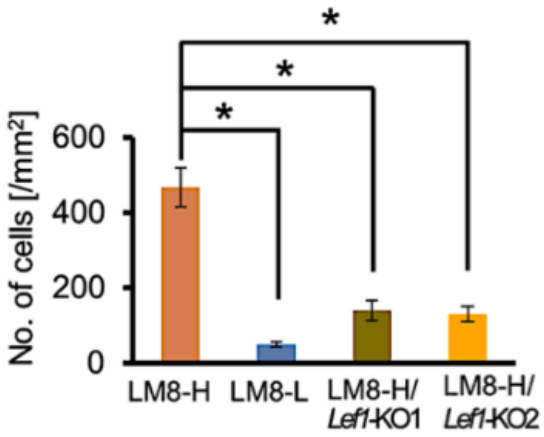

Figure 3-2: Involvement of LEF1 in LM8 transmigration. Cited from [1].

Transmigration ability of LM8 sublines into the vascular endothelial monolayer. The number of fluorescently labeled cell (for each LM8 subline) that migrated through the vascular endothelial monolayer was counted. n = 3, *$p < 0.05$ (vs. LM8-H).

Consistent with the lung metastatic results [1], the extravasation ability to the lungs was significantly impaired in LM8-L and LM8-H/*Lef1*-KO1 cells. These results strongly suggest that there is a difference in the abilities of LM8-L and LM8-H cells to metastasize to the lung during the extravasation stage, and that LEF1 transcription factor is an indispensable factor for extravasation of LM8 into the lungs (Figure 3-3).

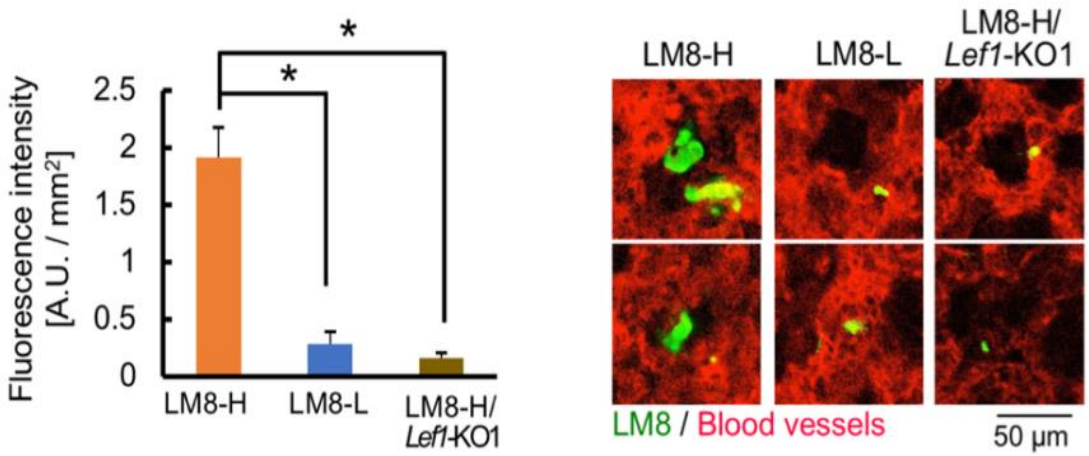

Figure 3-3: Involvement of LEF1 in LM8 extravasation ability. Cited form [1].

Quantitative analysis (left) and representative images (right) of the lungs 48 h after intravenous injection of the green-fluorescently labelled LM8 sublines. n = 9, *p< 0.05 (vs LM8-H). Green: LM8, Red: endothelial cells, Black: Lung parenchyma.

3.2.3 CYGB is a downstream target of LEF1 transcription factor on lung metastasis

CYGB is a member of the globin family, which include hemoglobin and myoglobin [9-11]. CYGB is known to act as a tumor suppressor gene in gliomas, liver cancer [12-14]. High expression of CYGB impairs the metastatic ability of lung cancer under normoxic conditions, while promoting tumorigenicity under stress conditions [15]. Under hypoxic conditions, CYGB is regulated by hypoxia inducible factor-1α and promotes transmigration and anchorage independent growth [16].

To identify the LEF1 downstream target involved in LM8 extravasation ability of lung metastasis, pro-metastasis-related genes were screened by overlapping of RNA microarray data of LM8 sublines and human OS public datasets. The procedure is illustrated in Figure 3-4.

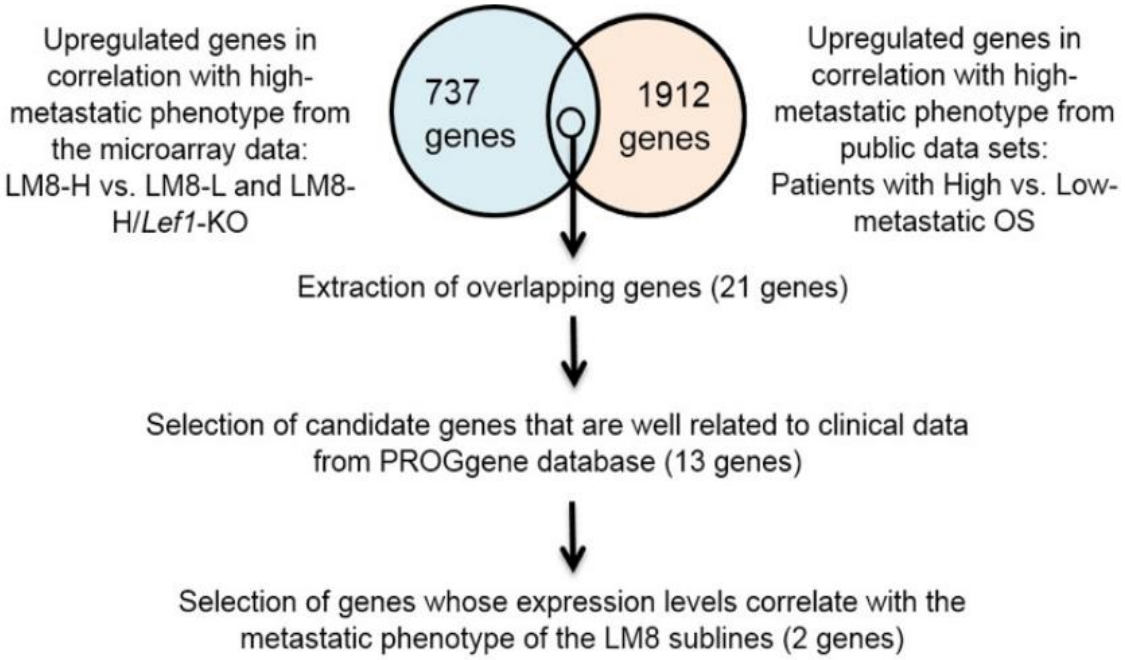

Figure 3-4: Diagram of the process used to narrow down candidate genes by genome-wide meta-analysis. The number of gene indicated for each step is number of selected genes. Cited from [1]

The mRNA and protein expression level of CYGB was sufficiently correlated with the metastatic phenotype of LM8 sublines: CYGB was highly expressed in LM8-H cells but not in LM8-L and LM8-H/*Lef1*-KO cells (Figure 3-5). It suggested that LEF1 regulates *Cygb* expression level. CYGB function promotes the transmigration ability *in vitro* (Figure 3-6). Interestingly, without CYGB function, LM8 failed to transmigrate into the lung tissues and made microcology in the blood vessel [1]. These results suggest that CYGB promotes LM8 to disrupt the endothelial barrier and enter the lung tissues. Function of CYGB in

lung metastasis of LM8 was confirmed by extravasation assay, knock out *Cygb* in LM8-H reduces the fluorescent intensity in lungs at 48 hours after transplantation compared to LM8-L and LM8-H [1].

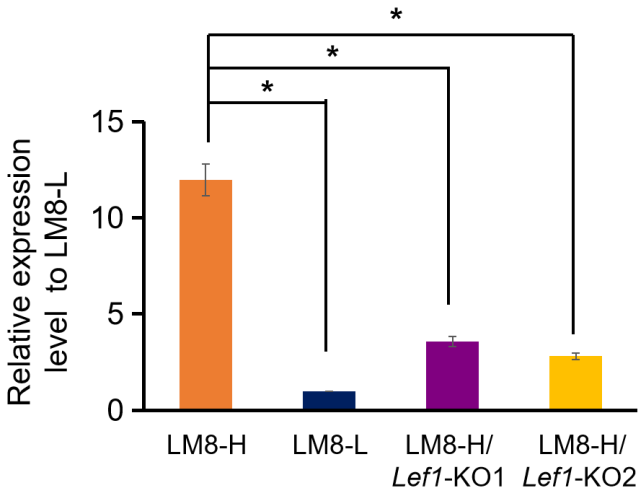

Figure 3-5 LEF1 regulates *Cygb* expression level. Cited form [1].

Relative Cygb mRNA levels in LM8-H, LM8-L, and H/*Lef1*-KO1/2 evaluated by qPCR. n = 3, *p< 0.05

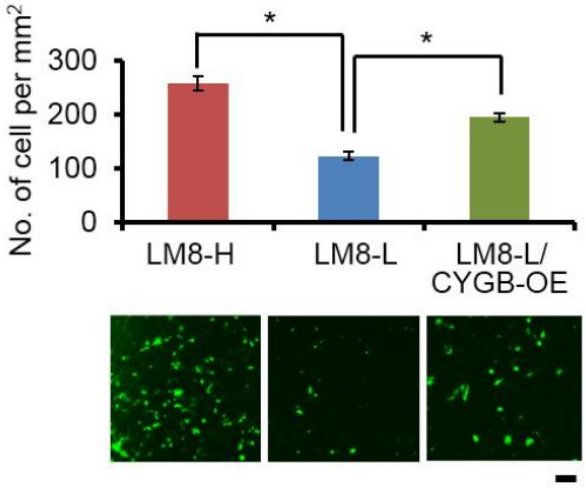

Figure 3-6: The involvement of CYGB in LM8 transmigration ability. Cited from [1]

Cells were quantitatively analyzed (graphs) by determining the number of fluorescently labeled cells transmigrating through a vascular endothelial monolayer. Representative images are shown depicting the fluorescently labeled cells that were counted (photos). n = 3, *p< 0.05. Scale bar: 100 µm.

2.3 Method

2.3.1 Endothelial transmigration assay

bEnd.3 cells (1×10^5) were seeded onto a top filter with an 8 μm pore in a 24-well Transwell® plate (Corning) and grown until the monolayer became confluent. The LM8 sublines were labeled with 25 μM CellTracker® Green for 30 minutes. After washing with PBS, the cells (5×10^4) were seeded onto the bEnd.3 monolayers. After 24 hours of incubation, the untransmigrated cells were wiped off with a cotton swab, and the filter was fixed with 4% paraformaldehyde for 20 minutes. Then, the transmigrated cells on the bottom of the filter were observed under a fluorescence microscope. Partial fluorescent images of each filter were combined, and the number of transmigrated cells were counted using BZ-X analyzer software. For analysis of Wnt5a function in LM8 transmigration, recombinant Wnt5a (R&D System) was dissolved in PBS and used at a final concentration of 5 μg/mL.

2.3.2 qRT-PCR

Total RNA was extracted using the RNeasy® Mini Kit (Qiagen) according to the manufacturer's protocol. One microgram of total RNA was reverse-transcribed using the Oligo$(dT)_{20}$ primer (Toyobo) and ReverTra Ace (Toyobo). qPCR and RT-PCR were carried out using the Thunderbird® SYBR qPCR Mix (Toyobo) and EmeraldAmp® GT PCR Master Mix (Takara Bio, Japan), respectively.

2.3.3 Statistical analysis

Data are presented as the mean ± standard error of the mean and were statistically analyzed with a two-sided Student's *t*-test. P values of less than 0.05 were considered statistically significant.

3.4 Results

3.4.1 ROR2 regulated the *Cygb* transcript level

Although *Ror2* is one of the Wnt signaling-related genes, the ROR2 expression level did not correlate with the LEF1 expression level (Figure 3-7).

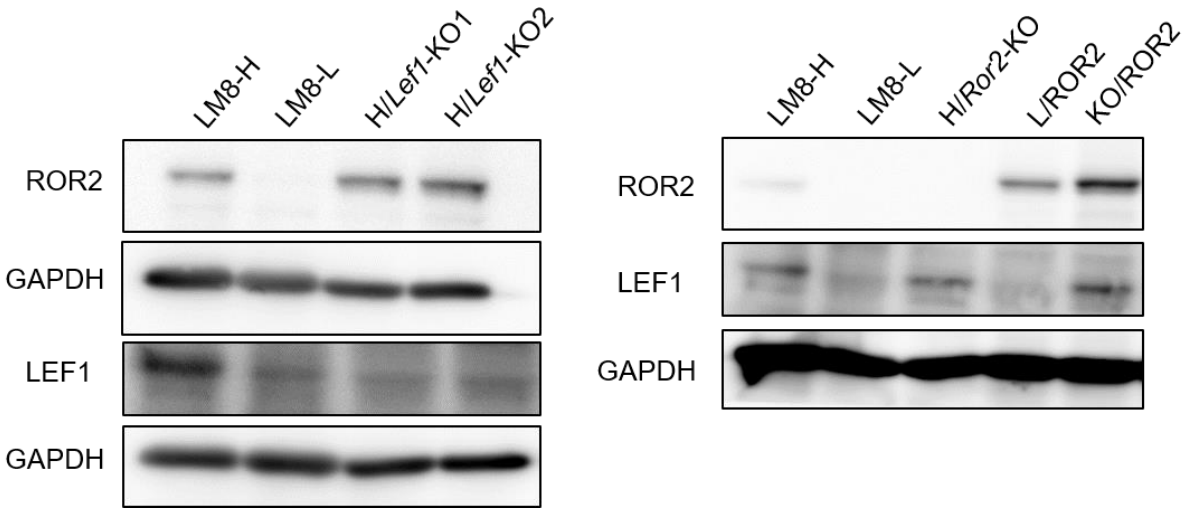

Figure 3-7: Relationship between ROR2 and LEF1 in LM8 sublines

The ROR2, LEF1, and GAPDH protein levels in the indicated LM8 sublines were examined by western blotting. H/*Lef1*-KO1 and H/*Lef1*-KO2 are *Lef1*-knock-out LM8-H sublines.

I investigated the relationship between *Ror2* and *Cygb.* The *Cygb* transcript level was significantly reduced in H/*Ror2*-KO (Figure 3-8), indicating that ROR2 was an upstream component of the signaling pathway related to the LEF1-CYGB axis.

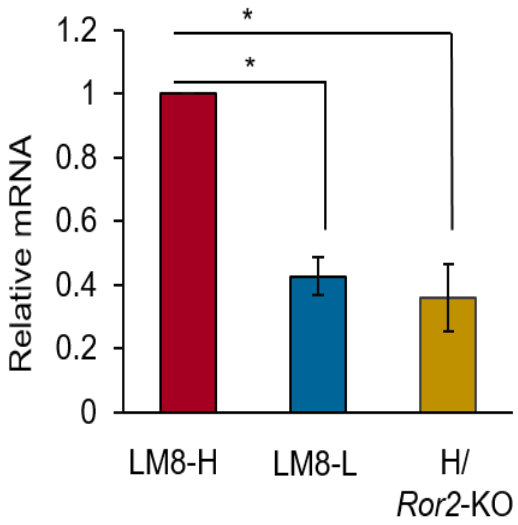

Figure 3-8: Correlation between *Ror2* and *Cygb* mRNA levels.

Cygb mRNA levels in LM8-H, LM8-L, and H/*Ror2*-KO. Data are shown as the mean ± SD of migrated cells. n = 3,*p< 0.05.

3.4.2 ROR2 promoted the endothelial transmigration ability of LM8 sublines

The relationship between *Ror2* and *Cygb* was further confirmed by examining ROR2 function in LM8 transmigration, previously shown to be *Cygb*-dependent. As expected, overexpression and knocking out of *Ror2* in LM8-L and LM8-H resulted in significantly increased and decreased endothelial transmigration abilities, respectively (Figure 3-9). Taken together, ROR2 is an alternative regulator of *Cygb* involved in transmigration ability of LM8.

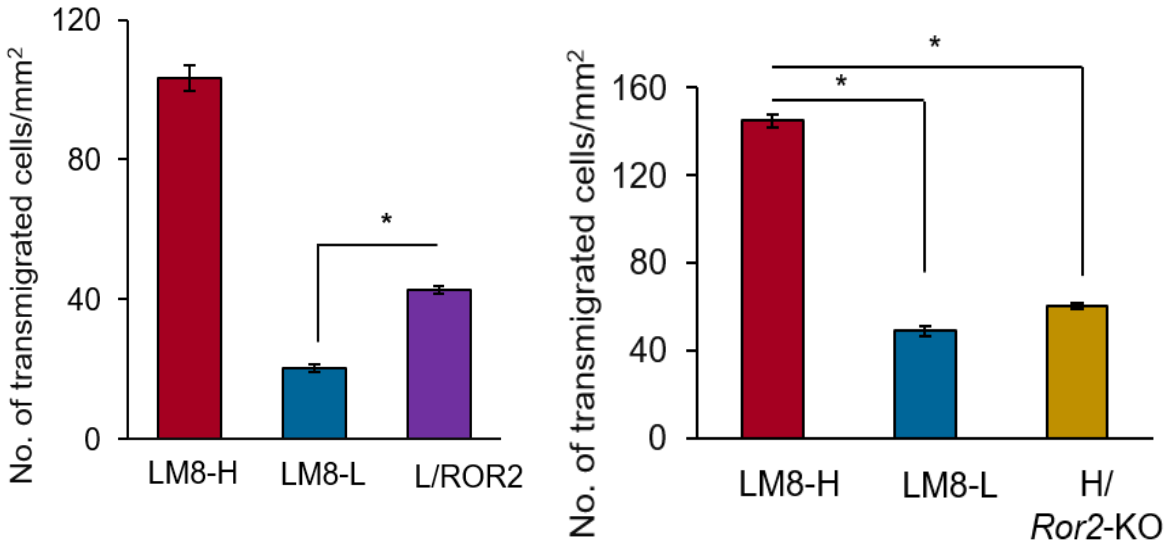

Figure 3-9: *Ror2* regulated the transmigration ability of LM8.

The transmigration activity of LM-H, LM8-L, and H/*Ror2*-KO (left) or of transiently ROR2-expressing LM8-L (L/ROR2) (right) was examined by endothelial transmigration assay. Data are shown as the mean ± SD of migrated cells. n = 3, *p < 0.05.

Although the Wnt5a/ROR2 axis has been reported to induce the migration of human OS cells [17-20], I found that recombinant Wnt5a (rWnt5a) did not promote transmigration of LM8-H (Figure 3-10), suggesting that Wn5a/ROR2 signaling was not involved in the ability of LM8 sublines to metastasize to the lungs.

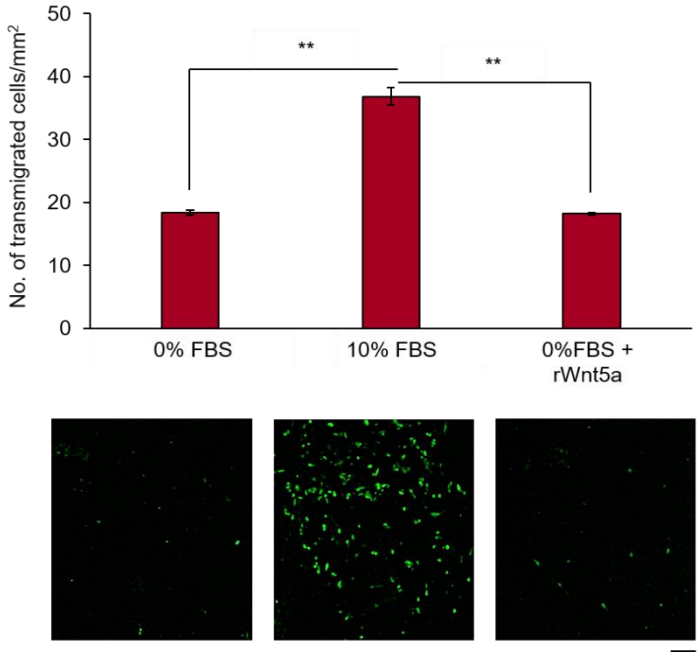

Figure 3-10: ROR2 regulated the transmigration ability of LM8 independent Wnt5a induction

The number of transmigrated LM8-H treated with 5 µg/mL rWnt5a or PBS was examined by an endothelial transmigration assay in serum-free (0% FBS) medium. Data with medium containing 10% FBS is shown as a positive control. The photos below the graph are fluorescent images of the transmigrated LM8-H. n = 3, **p< 0.01. Scale bar: 100 µm.

3.5 Discussion

Previous study using the same lung metastasis model revealed that CYGB function is crucial for LM8 extravasation ability, and *Cygb* was previously identified as a LEF1-regulated gene [8]. Although *Ror2* is one of the Wnt signaling-related genes, the ROR2 expression level did not correlate with the LEF1 expression level (Figure 3-7). In contrast, *Cygb* expression was significantly suppressed in LM8-L and H/*Ror2*-KO, suggesting that ROR2 signaling may be an alternative CYGB regulator independent of LEF-1 signaling in LM8. Together with our previous study of the LEF1-CYGB axis [1], this study provided new insight into the mechanism of Wnt-signaling in OS lung metastasis. Furthermore, finding the mechanism by which ROR2 regulates the *Cygb* mRNA levels is necessary to understand the overall mechanism of CYGB and its extravasation ability.

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Chapter 4

ROR2-AKT axis regulates anoikis resistance of LM8 cells

4.1 Aim of the study

In chapter 2, I show that *Ror2*-KO LM8-H (H/*Ror2*-KO) significantly reduced the number of lung foci compared to LM8-H. The purpose of this study was to investigate the function of ROR2 in the process of lung metastasis. Since I found the differences in the survival of the LM8 sublines with high or low ROR2 expression in lung capillaries, I aimed to elucidate the mechanism in which ROR2 is involved.

4.2 Introduction

4.2.1 Survival of tumor cells in capillaries

In experiments investigating the dynamics of tumor cells and microbeads in capillaries, 97% of microbeads arrested in the capillaries, only 3% of microbeads were able to escape [1]. The diameter of the beads is 10 μm and larger than the size of capillaries. Therefore they were primarily arrested due to the mechanical trapping. On the other hand, 42% of the flexible tumor cells could escape from the capillary trapping [1]. The tumor cell-endothelial cell interaction also contributed to cell trapping in the lung capillaries [2]. In summary, trapped tumor cells may undergo anoikis due to their lack of interactions between tumor cells themselves and with endothelial cells [2] or may die by mechanical deformation [3]. Some of them may take advantage of their flexibility to leak from blood vessels [1].

4.2.1.1 Resistance to mechanical stress

Tumor cells are exposed to mechanical stresses, such as direct fluid shear forces of blood flow in circulation [4] and mechanical deformation in the capillaries [2]. These mechanical stresses are believed to cause the loss of a significant portion of the tumor

cells that enter the capillaries [5, 6]. Recently, intravital imaging has revealed that tumor cell microparticles are generated in capillaries and taken up by myeloid-derived cells [7]. Tumor cells are protected from mechanical stress by various mechanisms. A common one is the formation of tumor cell-platelet microaggregates, which prevent tumor cells from killing by shear force of blood flow as well as by cellular and humoral immunities [8]. Inhibition of tumor cell-platelet interaction has been shown to reduce metastasis [8-10]. Microaggregates of circulating tumor cells reduces apoptosis by less exposing to mechanical stresses and have a higher metastatic potential [11].

Although many studies have reported the survival mechanism of circulating tumor cells, few reports provide the survival mechanism in capillaries. Cell dead in capillaries is caused by cell deformation or anoikis. Anoikis is programmed cell death which induced by loss of attachment to the extracellular matrix and surrounding cells [12]. To overcome cell deformation, tumor cells need to have an autonomous mechanism which increases cell death resistance. The PANX1 channel releases more ATP from tumor cells when tumor cells enter capillaries. Plasma membrane stretch activates P2y-purinergic receptors whose signaling could inhibit apoptosis in cells and enhance cell survival from deformation in capillaries [13].

4.2.1.2 Resistance to anoikis

When tumor cells enter the capillaries and have no interaction with endothelial cells, they undergo anoikis or migrate to another distant organ [14]. Loss of anchorage from extracellular matrix induces anoikis [15]. In ovarian cancer, silencing of the zinc-finger transcription factor induces anoikis through β1 integrin downregulation [16]. Integrins are also important in the anoikis mechanism as a key mediator of adhesion between cells

and the extracellular matrix protein. They recruit focal adhesion kinase protein and activate pro-survival pathways including phosphatidylinositol 3-kinase (PI3K)/Akt signaling and the Ras/Raf/Mek/Erk pathway. These pro-survival pathways could be activated by insulin-like growth factor 1 receptor, suppressing anoikis due to loss of integrin expression. In addition, tumor cells also tend to microaggregates to reduce anoikis in blood vessel [17].

4.2.2 Overview of the AKT pathway function in lung metastasis of OS

The AKT pathway, one of the most commonly dysregulated pathways in cancer, transduce certain internal and external cell signals that leads to proliferation and resistance to apoptosis. Many receptor tyrosine kinases such as epidermal growth factor receptor, vascular endothelial growth factor receptor 2, platelet-derived growth factor receptor, and G protein-coupled receptors activate the AKT pathway in cells.

Aberrant expression of phosphorylated AKT (p-AKT) is a common feature in both early and advanced cancers. AKT is a protein conserved among animal species and has three isoforms encoded by three different genes: *Akt1, Akt2,* and *Akt3*. Knockout of the three genes has been achieved by targeted homologous recombination in mice and has provided some insight into their functions. Knocking out one of *Akt* genes lead to abnormal development of mice.

The more evidence there is for the PI3K / AKT pathway in OS lung metastasis, the more important that pathway is justified. Enrichment of AKT activity has been found from 24 OS patients with lung metastasis [18].

A recent study has demonstrated that TGFβ–induced (also known as TGFBI or βig-h3) is a TGFβ1-inducible protein and known to be secreted by many types of cells. It can enhance the metastatic potential of Saos-2 cells via PI3K/AKT signal pathway mediated by integrin α2β1 [19]. TGFα is a member of the epidermal growth factor family of cytokines and functions as an activator of the PI3K/AKT signaling. It regulates human OS metastasis by activating PI3K/AKT/NF-κB pathway and downregulating intercellular adhesion molecule-1 (ICAM-1) expression [20]. Nude mice transplanted with LM8 developed lung metastasis and show higher p-AKT levels. Inhibition of the PI3K/ AKT signaling using LY294002 (a PI3K inhibitor) or a dominant negative form of AKT markedly reduces lung metastasis [21]. It has been demonstrated that inhibition of AKT decreased MMP-2 secretion and retarded the development of lung metastasis in nude mice transplanted with LM8 on their backs [22]. Deactivation of the Ras/PI3K/Akt signaling reduced the expression and activities of MMP-1, MMP-2 and MMP-9, resulting in reduced LM8 metastatic activity [23]. It is consistent with AKT signaling-related gene expression profile in LM8 sublines: LM8-H shows high expression of the AKT signaling-related genes based on the gene set enrichment analysis (Table 6-1) [24].

Table 6-1 List of candidate genes that enriched in LM8-H

Gene name	**Full name**
Itga1	Integrin Subunit Alpha 1
Itga6	Integrin Subunit Alpha 6
Itgb3	Integrin beta-3
Itgb5	Integrin beta-5
Pten	Phosphatase and tensin homolog
Cerk	Ceramide Kinase
Nfkb2	Nuclear Factor Kappa B Subunit 2
Tmbim6	Transmembrane BAX Inhibitor Motif Containing 6
Tmbim1	Transmembrane BAX Inhibitor Motif Containing 1
Bcl11a	B-cell lymphoma/leukemia 11A
Bcl3	B-cell lymphoma 3
Bcl6	B-cell lymphoma 6
Nfkb1	Nuclear Factor Kappa B Subunit 1

ROR2 expression is associated with anchorage independent growth ability of renal cell carcinoma [25]. In human OS cells, activation of AKT signaling via Wnt5a/ROR2 axis promotes migration and invasion. However, relationship between ROR2-induced AKT activation and anoikis resistance have not been elucidated yet. In recent years, MK2206, one of allosteric AKT inhibitors, has been developed and currently has been applied in Phase II clinical trials. It is shown that MK2206 significantly suppressed breast cancer growth with [26] and also reduced the proliferation of U2OS and HOS cells [27].

4.2.3 Integrin

4.2.3.1 Integrinβ5

Integrin β5 (*Itgb5*) encodes integrin β chain that can interact with various integrin α chains. *Itgb5* mediates transforming growth factor (TGF) β-induced epithelial mesenchymal transition and contributes to the tumorigenic potential of breast tumor cells [28, 29]. Interaction of Itgb5 and β-catenin promotes tumorigenesis in hepatocellular carcinoma [30]. In addition, ITGB5 expression was enriched in liver metastatic pancreatic cancer exosomes [31]. These indicate the potential role of ITGB5 in intercellular communication during tumor progression and metastasis. In glioblastoma, *Itgb5* was shown to regulate infiltration of macrophages in the local microenvironment via modulation of osteopontin [32]. However, the role of *Itgb5* in the OS progression is not understood.

4.2.3.2 Integrin α6

Integrin α6 (ITGA6) encodes integrin α chain. It associates with integrin β1 chain to form the α6β1, and with integrin β4 chain to form the α6β4 complex. Both complexes are important laminin receptor and are involved in cell-matrix and cell-cell interactions [33]. These activate intracellular signaling pathways involved in the regulation of various cellular processes, including cytoskeletal arrangement, growth factor signaling and gene transcription [34]. ITGA6 is abnormally expressed in breast cancer [35]. The majority of these studies have shown that ITGA6 is involved in tumor progression because high expression of ITGA6 is associated with tumor cell metastasis and invasion. Moreover, ITGA6 regulates anoikis resistance in Madin-Darby Canine Kidney cells [36] and human

thyroid carcinoma [37]. In OS, inhibition of ITGA6 reduces cell growth and invasion ability [38].

4.3 Method

4.3.1 Analysis of tumor cells in lungs

Cells were labeled with 100 μM CellTracker® Green and intravenously injected into C3H mice (1×10^6 cells/100 μL PBS). DyLight® 594-labeled isolectin B4 (6 mg/kg; Vector Laboratories, Burlingame, CA, USA) was injected intravenously to stain endothelial cells 5 minutes before dissecting the mice. The lungs were collected and observed under confocal microscopy (Carl Zeiss). The number of fluorescently labeled cells in the three microscope fields of each lung was quantified using ImageJ software [39]. Results are shown as the average number of cells per field. Each group was analyzed in triplicates.

For measuring the fluorescence intensity of the lungs, lung lysate was prepared with RIPA buffer (50 mM Tris HCl, pH 8.0; 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) and well-homogenized. The supernatant was then collected, and the fluorescence intensity was measured using Infinite F500 (Tecan, Mannedorf, Switzerland) with a filter set for CellTracker® Green (Ex/Em = 480 nm/517 nm).

4.3.2 Hypotonic assay

LM8-H and H/*Ror2*-KO (1×10^4) were seeded in 24-well plates and cultured overnight. The medium was then replaced with either hypotonic solution (12.5% PBS) or isotonic solution (100% PBS) and incubated for 30 minutes. Live and dead cells were identified by trypan blue staining and then counted.

4.3.3 Anoikis assay

Poly(2-hydroxyethyl methacrylate) (poly-HEMA; Thermo Fisher Scientific) was completely dissolved in 95% ethanol (20 mg/mL). Plates were coated with poly-HEMA solution and dried on a clean bench overnight. Cells (5×10^4) in serum-free medium were seeded in a poly-HEMA-coated 24-well plate. After 24 hours, calcein AM (Thermo Fisher Scientific) and ethidium homodimer-1 (Thermo Fisher Scientific) were added at final concentrations of 2 µM and 4 µM, respectively, and the cells were further incubated for 30 minutes. The fluorescent signal was observed under fluorescence microscopy and quantified with the BZ-X Analyzer (Keyence). To investigate the effect of AKT on LM8 anoikis, the AKT inhibitor MK2206 (Nacalai Tesque) was prepared with dimethyl sulfoxide (DMSO), and the cells were cultured for 24 hours in poly-HEMA-treated plates containing 50 nM MK2206 in 5% FBS DMEM.

4.3.4 Western blotting

Cells were lysed in RIPA buffer containing a protease cocktail inhibitor (Nacalai Tesque), and protein concentration was determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific). Proteins were separated by electrophoresis on a 12.5% acrylamide gel, transferred to a hydrophilic polyvinylidene fluoride membrane (Merck) and blocked with 5% skim milk in TBST (20 mM Tris, pH 7.5; 150 mM NaCl, 0.1% Tween 20). The membrane was then probed with relevant primary antibodies [anti-AKT (#2920S), anti-phosphor-AKT (Ser473; #4060s), anti-ROR2 (#88639s), and anti-GAPDH (#2118s; Cell Signaling Technology)], and secondary antibodies [anti-mouse IgG HRP-linked Antibody (#7076) and anti-rabbit IgG HRP-linked Antibody (#7074; Cell Signaling Technology)]. The resultant membranes were washed with TBST 3 times for 5 minutes

and detected by ImageQuant LAS 4000 (GE Healthcare Life Science) after processing with Chemi-Lumi One (Nacalai Tesque). To investigate the effect of AKT inhibition, cells were treated with 50 nM MK2206 in 5% FBS DMEM for 24 hours.

4.3.5 Statistical analysis

Data are presented as the mean ± standard error of the mean and were statistically analyzed with a two-sided Student's *t*-test. P values of less than 0.05 were considered statistically significant.

4.4 Results

4.3.1 ROR2 promoted cell survival in lung capillaries

To determine how ROR2 was involved in the extravasation process *in vivo*, the LM8 sublines in the lungs were examined at 30 minutes and 48 hours post-injection, at which points the tumor cells had reached the lungs and extravasated to lung tissues, respectively. The LM8 sublines were labeled with a green fluorescent dye before *i.v* injection, and removed lungs were observed under an inverted confocal fluorescence microscope. Similar numbers of fluorescently labeled cells were observed in the lungs injected with LM8-H and H/*Ror2*-KO 30 minutes after injection (Figure 4-1), indicating that a lack of ROR2 function did not influence the survival of LM8 in circulation. However, 48 hours after injection, the number of fluorescently labeled cells in lungs injected with H/*Ror2*-KO was significantly reduced compared to that in lungs injected with LM8-H (Figure 4-1), suggesting that ROR2 was involved in LM8 cell survival in the lung capillaries.

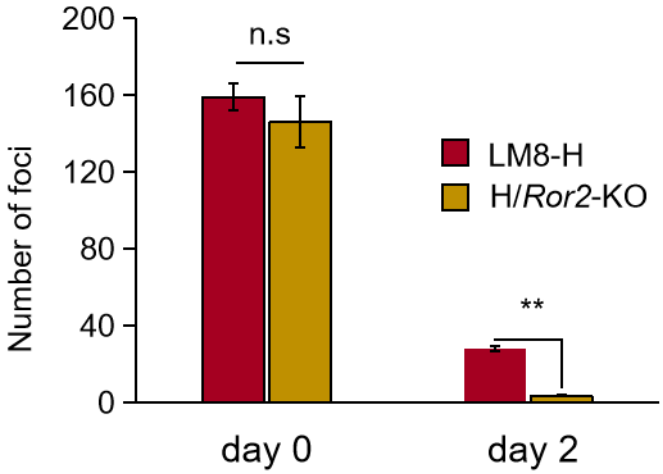

Figure 4-1: Fluorescently labeled LM8 in the lungs were quantified using a confocal fluorescence microscope. Data are shown as the mean ± SD of three fields of fluorescently labeled cells in each lung on the indicated days. n = 3, **p < 0.01

To confirm ROR2's function, LM8-L, LM8-H, H/*Ror2*-KO, and *Ror2*-overexpressing LM8-L and H/*Ror2*-KO (L/ROR2 and KO/ROR2, respectively) were examined in terms of their survival in the lung capillaries. Significantly higher fluorescence intensity was detected in lung lysates from mice injected with the LM8 sublines expressing high ROR2 (LM8-H, L/ROR2, and KO/ROR2) compared to that in the lung lysates from mice injected with the LM8 sublines expressing low ROR2 (LM8-L and H/*Ror2*-KO) (Figure 4-2). These

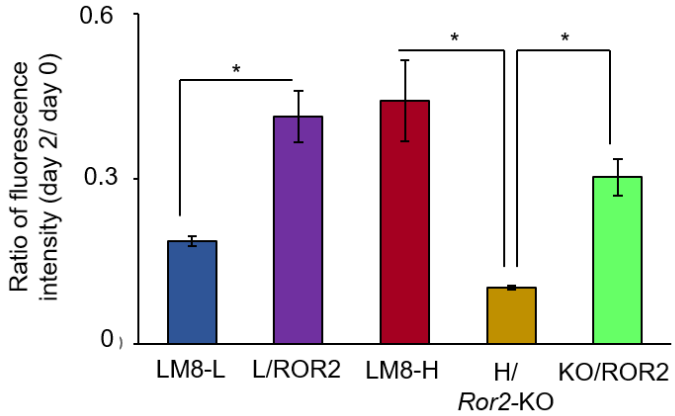

Figure 4-2: Fluorescence intensity in lung lysates prepared from mice injected with the indicated LM8 sublines was quantified on day 0 and day 2. Data are shown as the ratio of day 2 fluorescence intensity normalized to day 0. n = 3, *p < 0.05.

results confirmed a correlation between ROR2 expression and LM8 survival in the lung capillaries.

4.3.2 ROR2 prevented anoikis under low adhesion conditions

Tumor cells that have reached the lung capillaries are stressed mechanically because the blood vessels are smaller in diameter and are less compliant. This mechanical stress causes cell death in up to more than 90% of tumor cells entering the capillaries. The LM8 sublines were examined for sensitivity to mechanical stress *in vitro* using a hypotonic buffer that causes hypotonic cell swelling, an established perturbation method that induces plasma membrane stretching in a well-controlled manner [13]. Incubation of LM8-H and H/*Ror2*-KO for 30 minutes in hypotonic buffer reduced viability by less than half compared to incubation in isotonic buffer (Figure 4-3), indicating that ROR2 was not involved in the resistance to mechanical stress in the lung capillaries. The differences in anoikis resistance among the LM8 sublines with various ROR2 expression levels (Figure 4-4) were examined in terms of viability after culturing for 24 hours on poly-HEMA-coated dishes (under low adhesion conditions) using fluorescent dyes that differentially label live and dead cells. The LM8 sublines with low ROR2 expression (LM8-L and H/*Ror2*-KO) were associated with significantly increased anoikis, and those with high ROR2

expression (L/ROR2 and KO/ROR2) was associated with significantly decreased anoikis (Figure 4-5).

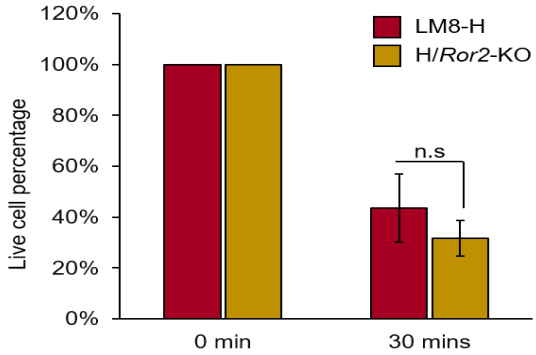

Figure 4-3: Hypotonic assay

LM8-H and H/*Ror2*-KO were incubated for 30 minutes in an extremely hypotonic (12.5% PBS) solution, and then live and dead cells were identified by trypan blue staining. The viability in hypotonic buffer was normalized by the viability in isotonic buffer (100% PBS). The live cell percentage is shown as the percentage of the viability of cells treated with isotonic buffer. n = 3, ***p < 0.001.

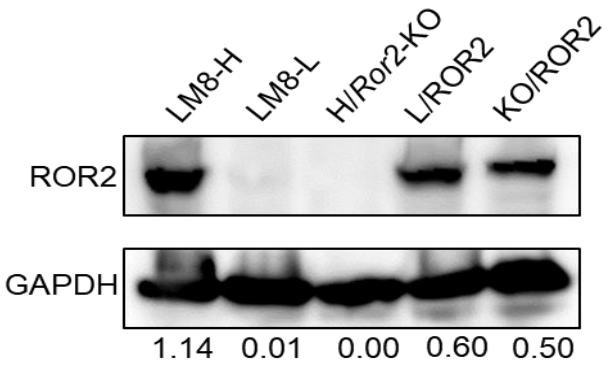

Figure 4-4: ROR2 protein level

ROR2 protein was examined in the indicated LM8 sublines. The numbers below the bands indicate the corresponding ROR2 expression levels relative to GAPDH.

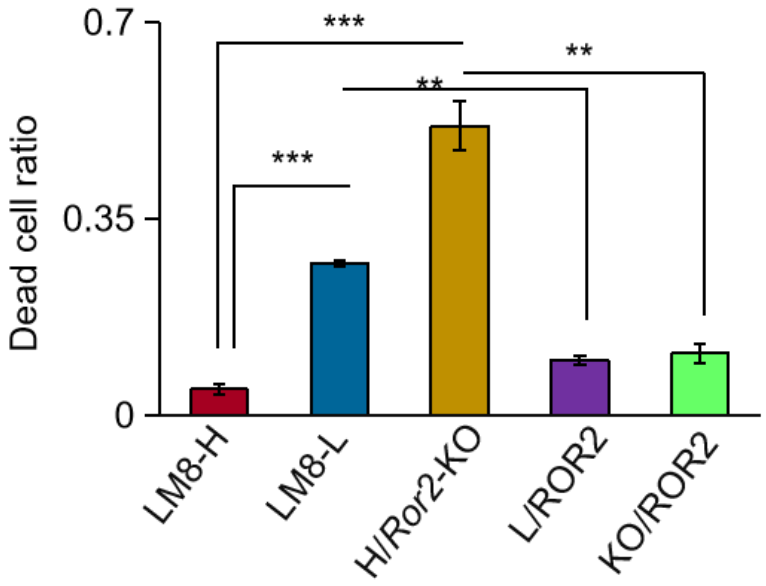

Figure 4-5: Involvement of ROR2 in LM8 anoikis resistance ability

Fluorescence intensities of live and dead cells labeled with different dyes were measured using appropriate filters after 24 hours of culture under low adhesion conditions as described in the Methods. The dead cell ratio was calculated by dividing the fluorescence intensity of the dead cells by the total fluorescence intensity. n d= 3, *p < 0.05, **p < 0.01, ***p < 0.001.

4.3.3 ROR2 activated AKT signaling pathway in LM8 sublines under low adhesion conditions

The interaction between ECM molecules and integrin activates focal adhesion kinase (FAK) [12], which then activates anoikis-suppressing pro-survival pathways such as the PI3K/AKT signaling pathways [40] Therefore, we investigated the involvement of AKT signaling in LM8 ROR2-induced anoikis resistance. First, AKT activation (Ser473 phosphor-AKT levels) was examined in LM8 sublines with different ROR2 expression levels. AKT activation did not occur in LM8 sublines without any stimulation and cultured on ordinal culture dishes (under adhesion conditions). Conversely, AKT activation did occur in the LM8 sublines that express ROR2 and that were cultured under low adhesion conditions (Figure 4-6). Second, the correlation between AKT activation and anoikis was examined using MK2206, an AKT inhibitor. AKT activation in LM8-H under low adhesion conditions was significantly suppressed by MK2206 (Figure 4-7), resulting in a significant increase in anoikis in these cells (Figure 4-8).

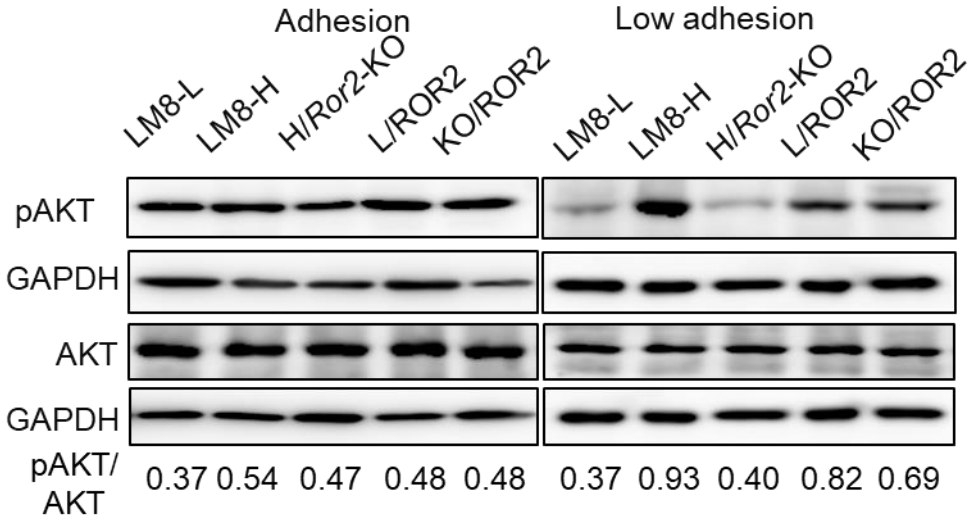

Figure 4-6: pAKT and AKT levels in LM8 sublines cultured under adhesion (left) and low adhesion (right) conditions were analyzed by western blotting. The numbers shown below each western blot indicate the ratios (pAKT/AKT) of the GAPDH (internal control)-normalized densities of the corresponding pAKT and AKT bands.

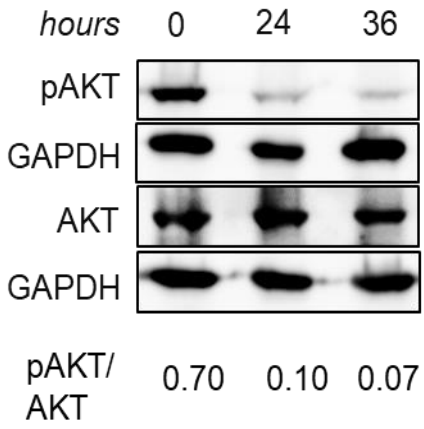

Figure 4-7*:* pAKT and AKT levels in LM8-H under low adhesion conditions were examined at the indicated times after MK2206 treatment. The western blotting experiments were repeated three times, and representative data are shown. The numbers shown below each western blot indicate the ratios (pAKT/AKT) of the GAPDH (internal control)-normalized densities of the corresponding pAKT and AKT bands.

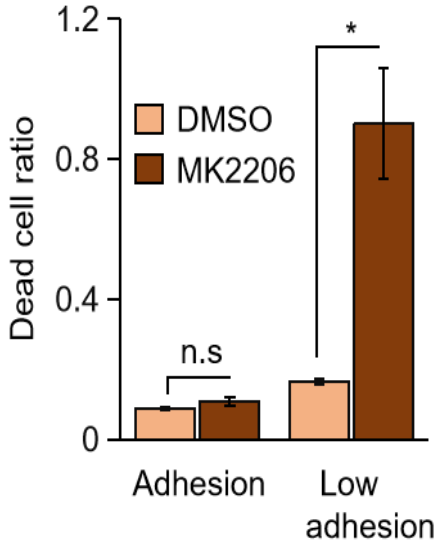

Figure 4-8: LM8-H cells were cultured under adhesion and low adhesion conditions with or without MK2206 for 24 hours. Graph shows dead cell ratios. n = 3, *p < 0.05.

4.3.4 ROR2 differentially regulated *Itgb5* and *Itga6* expression through AKT activation under low adhesion conditions

Since integrin molecules are known to be involved in anoikis resistance [41], I examined four integrin subunit genes (*Itgb1, Itgb3, Itgb5*, and *Itga6*), which are

differentially expressed in LM8-H and LM8-L according to microarray data [42]. Expression of *Itgb5* and *Itga6* was significantly higher in L/ROR2 and KO/ROR2 compared to that in LM8-L and H/*Ror2*-KO under low adhesion conditions (Figure 4-9). *ITGB5* and *ITGA6* expression also showed correlation with *ROR2* expression in clinical data (Figure 4-10). These results suggested a potential contribution of *Itgb5* and *Itga6* to the anoikis resistance function of ROR2 in LM8.

The involvement of AKT activation in ROR2-induced *Itgb5* and *Itga6* expression was examined under both adhesion and low adhesion conditions. *Itgb5* expression decreased 2 fold, but *Itga6* increased 2.5 fold in LM8-H with MK2206 treatment under low adhesion conditions (Figure 4-11). However, under adhesion conditions, both gene expressions were increased when AKT activation was inhibited with MK2206 (Figure 4-11). Interestingly, AKT activation was not regulated by ROR2 in LM8 sublines under adhesion conditions (Figure 4-6). These results demonstrated that ROR2 function in LM8 changes in response to extracellular matrix attachment.

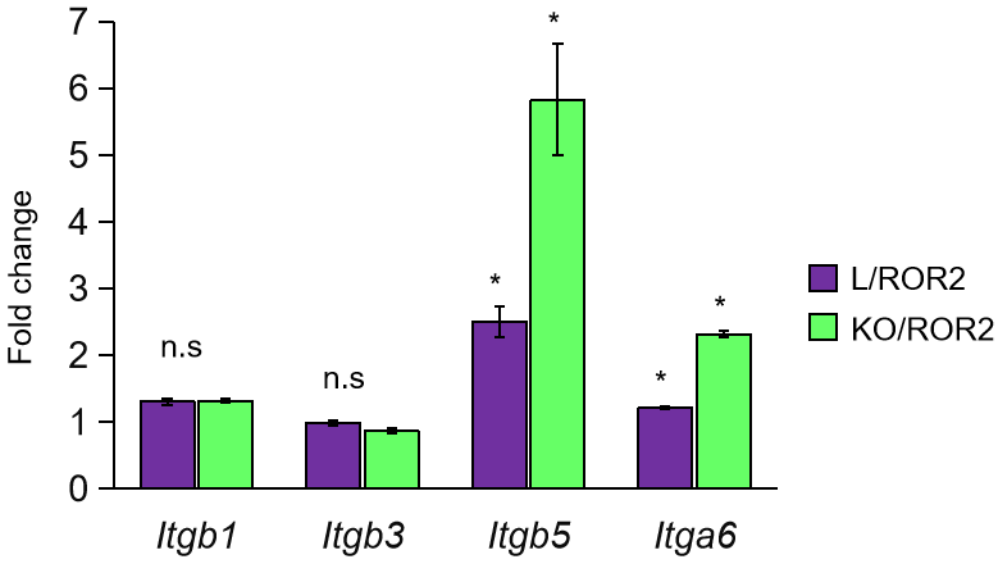

Figure 4-9: Correlation of Ror2 expression and integrin expression

Expression of the indicated genes of LM8 sublines cultured for 24 hours under low adhesion conditions was analyzed by qRT-PCR. The results are shown as the fold change after normalizing Ror2-overexpressing LM8-L (L/ROR2) and H/Ror2-KO (KO/ROR2) values with LM8-L and H/Ror2-KO values, respectively. n = 3,*p < 0.05.

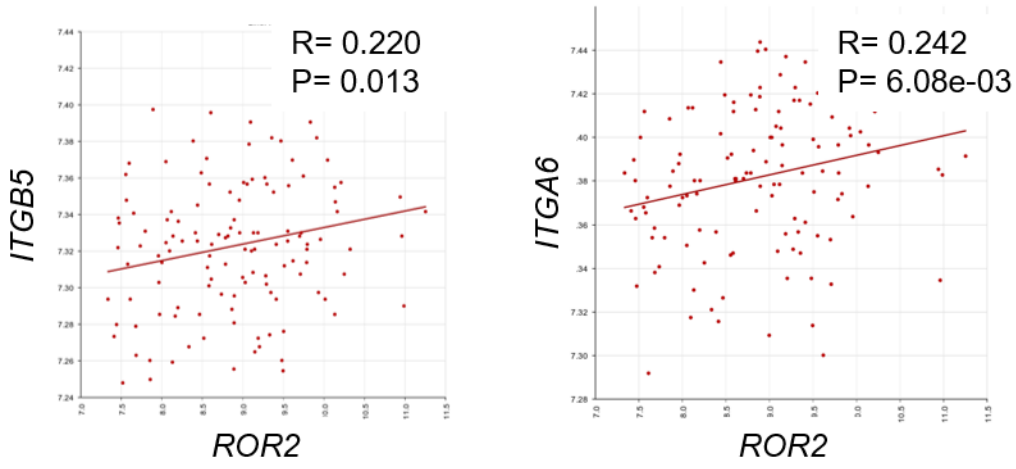

Figure 4-10: Correlation among ITGB5, ITGA6, and ROR2 mRNA levels was examined in OS patients using the R2 database (osteosarcoma-Kuijjer-127-vst-ilmnhwg6v2). The Pearson correlation coefficient (R) and p value are shown along with a linear regression line displaying the correlation.

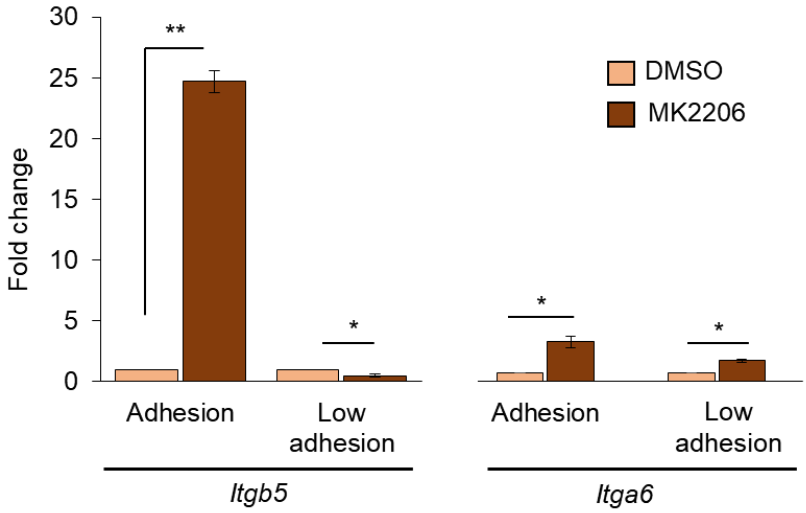

Figure 4-11: The amounts of *Itga6* and *Itgb5* mRNA in LM8-H cultured in MK2206 (50 nM) for 24 hours under adhesion and low adhesion conditions were normalized with those in LM8-H similarly treated with DMSO and are shown as the fold changes. n = 3, *p < 0.01.

4.5 Discussion

The number of cells with fluorescence in the lungs 30 minutes after *i.v* injection of LM8-H and H/*Ror2-KO* was similar, indicating that viability of these cells in circulation is about same. However, relative fluorescence intensity of lungs at 48 hours compared to 30 minutes showed significant correlations with ROR2 expression levels among the LM8 sublines. These results were consistent with the results of lung foci in Chapter 2. The results of anoikis assay indicated presence of ROR2 in LM8 could enhance LM8 viability under low adhesion conditions. I found the importance of ROR2 function in LM8 survival in lung capillaries. Here, I suggest that one of causes of LM8 death in the lung capillaries is an improper adherent environment. The requirement of ROR2 for LM8 cell survival under low adhesion conditions supports my hypothesis that ROR2 has another function in addition to promoting extravasation during lung metastases.

I demonstrated that the anoikis resistance regulated by ROR2 was dependent on AKT activation and that AKT inhibition reduced ROR2-induced anoikis resistance. However, Ror2 did not regulate the proliferation of LM8 under adhesion conditions (Chapter 2). Therefore, Ror2-induced anoikis resistance in LM8 might not relate to the proliferation of LM8. The AKT inhibitor used here, MK2206, is currently in phase II clinical trials for recurrent and advanced endometrial cancer. In addition to inhibiting tumor growth in neuroblastoma and colorectal cancer, MK2206 also impairs the proliferation of human OS cells such as U-2 and HOS. MK2206 was found to reduce LM8-H viability only under low adhesion conditions, but low levels of cell death were observed as a result of the AKT inhibitor under adhesion conditions. In other words, these results indicate that MK2206 might be used to specifically kill OS cells that have lost anchorage dependency, such as cells that have acquired the ability to invade and metastasize.

Since integrin-stimulated intercellular aggregation prevents anoikis, I focused on the involvement of integrins (transmembrane adhesion receptors consisting of an α and β chain complex) as one of the mechanisms by which blocking AKT signal increases anoikis. Integrin αVβ5 contributes to the adhesion and movement of some cell types on vitronectin. Moreover, integrin αVβ5 is correlated with survival factors such as Ki67 and HIFα [43] and with the adhesion of circulating tumor cells to the cerebral microvasculature, a crucial step in extravasation. In addition, integrin β5 promotes the growth of breast cancer through the Src-FAK signaling pathway, and Src activity contributes to the PI3-AKT-mediated anoikis resistance of human OS SAOS-2 cells. Therefore, the existing literature suggests that ROR2 may promote anoikis resistance in the lung capillaries of LM8 by enhancing intracellular aggregation through integrin β5. This ROR2-mediated anoikis

resistance mechanism was supported by our finding that under low adhesion conditions, *Itgb5* mRNA levels were increased by AKT activity, which was regulated by ROR2.

MK2206 treatment significantly increased both *Itgb5* and *Itga6* mRNA levels under adhesion conditions, suggesting that the AKT signal suppressed integrin expression in the presence of appropriate ECM attachments. Interestingly, *Itga6* expression was suppressed by the AKT signal both under adhesion and low adhesion conditions, indicating that regulation of Itga6 expression is independent of ECM attachment. Further studies are necessary to clarify the adhesion-dependent mechanism by which the expression of Itgb5 is regulated through the AKT activity.

Even though *Itgb5* is known to be a downstream gene in the signaling pathway of Wnt-1 (a canonical Wnt signaling-related gene product) and to prolong the survival of G1-arrested cells, the mechanism by which the Wnt-1 signal regulates *Itgb5* is still unclear. The Wnt-1 signal also mediates cell survival by activating the β-catenin/LEF1 transcription factor, which blocks cytochrome c release and caspase 9 activation. Therefore, in order to survive under low adhesion conditions, AKT activation might somehow activate the Wnt-1 signaling pathway in LM8-H cells, leading to the upregulation of *Itgb5* mRNA levels. Further analysis of integrin protein levels in LM8 sublines and of LM8 with engineered integrin genes is required to explore anoikis regulatory mechanisms under low adhesion conditions in relation to integrins and signaling by AKT and Wnt.

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Chapter 5

Discussion and prospect

Chapter 1 describes the research background and purpose of this study. Lung metastasis is a major cause of mortality in patients with osteosarcoma (OS). Surgical and chemotherapeutic treatments have significantly improved survival in patients with non-metastatic osteosarcoma. However, metastatic disease interferes with long-term cure and can decrease 5-year survival rate to 20%. A better understanding of the molecular mechanism of OS lung metastasis may facilitate development of new therapeutic strategies to prevent the metastasis. Wnt signaling consists of two main groups: the canonical or β-catenin-dependent Wnt signaling pathway, and noncanonical or β-catenin-independent Wnt signaling pathway. Although the Wnt signaling pathway is involved in the progression of OS, the involvement of noncanonical Wnt signaling in OS progression has not been studied as much as canonical Wnt signaling. Therefore, my research focused on noncanonical (β-catenin-independent) Wnt signaling pathway. I aimed to identify pro-metastatic regulators associated with noncanonical Wnt signaling pathway in the sublines of murine OS cell line LM8. I believed that the data presented would provide new therapeutic targets useful for developing treatments of patients with metastatic OS.

Chapter 2 describes the identification and analysis of receptor tyrosine kinase-like orphan receptor 2 (ROR2) as a key factor associated with lung metastasis in LM8. Based on analysis of gene expression profiles between LM8-H andLM8-L, I have identified ROR2, a receptor of the non-canonical Wnt signaling pathway, as a gene that plays an important role in lung metastasis of OS. LM8-H knocked out of Ror2 (H/Ror2-KO) significantly reduced lung metastasis.

Chapter 3 describes ROR2 as an alternative regulator of cytoglobin (CYGB) in LM8. Previous study has shown that lymphoid enhancer-binding factor 1 (LEF1), a compensate transcription factor of canonical Wnt signaling, promotes the extravasation ability of LM8 during lung metastasis via regulating CYGB expression. In vitro studies revealed that CYGB plays a crucial role in the transmigration ability of LM8. In chapter 3, I have found that loss of ROR2 expression in LM8-H reduced Cygb mRNA level while ROR2 expression did not correlate with the expression of LEF1. Moreover, knockout or overexpression of Ror2 significantly reduced or enhanced transmigration ability of LM8-H or LM8-L, respectively. These data suggested that ROR2 is an upstream regulator of Cygb.

Chapter 4 describes the function of ROR2 in cell survival in lung capillaries and its underlying molecular mechanism. In an experiment in which fluorescently labeled LM8 sublines were intravenously administered, the ratio of fluorescence intensity day 2 to day 0 in the lung was correlated with the expression level of ROR2: the fluorescence intensity on day 2 was significantly reduced in the lungs administered with cells having low ROR2 expression level. In vitro studies revealed that ROR2 function is involved in the resistance of LM8 to anoikis, a type of cell death induced by loss of proper extracellular matrix attachment. Furthermore, I showed that ROR2 contributes to LM8 anoikis resistance via AKT activation that the anoikis resistance is associated with Itga6 and Itgb5 expression, which was altered by AKT inhibition by MK2206 under low adhesion conditions: Under low adhesion conditions, MK2206 significantly reduced Itgb5 expression but increase Itga6 expression. These results suggest that the ROR2-AKT-ITGB5 axis may play an important role in lung metastasis of LM8 through anoikis resistance in lung capillaries.

ROR2 has been reported in human OS cell as a regulator of migration and invasion, key steps in the initiation of metastasis. I successfully elucidated the novel function of ROR2 involved in cell survival in lung capillaries through AKT activation and transmigration ability through CYGB function which are the post-circulatory lung metastasis process of murine OS cells. To my knowledge, this is the first report describing the involvement of ROR2-AKT signaling in OS anoikis resistance. To explore anoikis regulatory mechanisms under low adhesion conditions in relation to ROR2-AKT-ITGB5 axis, further analysis of integrin protein levels in the sublines of LM8 and of LM8 cells with engineered integrin genes is required.

These results of ROR2 function in anoikis resistance, invasion and transmigration, and MK2206 function for the sublines of LM8 cell survival under low adhesion conditions suggest novel therapeutic strategies: the combination of MK2206 and blocking ROR2 signaling may contribute to therapeutic treatments for OS patients with lung metastasis.

Achievement

Poster presentation

- Tran Diem*, Pongsuchart M, Kuchimaru T, Kondoh S. Wnt5a induces a signal to suppress lung metastasis in the extravasation of murine OS cell line LM8. The 41th annual meeting of the Molecular Biology Society of Japan, November 28, 2018, Yokohama.
- Tran Diem*, Yonezawa S, Pongsuchart M, Kuchimaru T, Kondoh S. Analysis of regulatory mechanisms OSlung metastasis via Wnt singaling. The 76th Annual Meeting of Japanese Cancer Association, September 28, 2017, Yokohama.
- Tran Diem*, Yonezawa S, Pongsuchart M, Kuchimaru T, Kondoh S. Analysis of Wnt signaling involvement in lung metastasis by using murine OScell lines. The 75th Annual Meeting of Japanese Cancer Association, October 7, 2016, Yokohama.

Publication

Pongsuchart M, Kuchimaru T, Yonezawa S, Tran DTP, Kha NT, Hoang NTH, et al. Novel lymphoid enhancer-binding factor 1-cytoglobin axis promotes extravasation of OScells into the lungs. Cancer Sci. 2018;109:2746–2756.

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