STUDY OF MAMMAGLOBIN 1 AS A REGULATOR IN TRASTUZUMAB RESISTANT CELLS' AGGRESSIVENESS

トラスツズマブ耐性細胞の攻撃性における調節因子としてのマンマグロビン 1 に関する研究

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CHAPTER 1
General Introduction

1-1. Cancer

1-1-1. Cancer Cells

Cancer theory suggests that tumors develop from a single cell that starts to divide to form a tumor mass triggered by carcinogenic event. The cells are exposed to further cell division. The daughter cells or subclones undergo DNA replication error that induce genetically different from each other, resulting in tumor cell heterogeneity (Axelrod et al., 2006). Cell proliferation is required for tumors to grow. During proliferation process, cellular metabolism is activated. Cellular metabolism can be influenced by many factors within the tumor microenvironment, resulting in heterogeneous metabolic activity (DeBerardinis et al., 2008).

Human cancer develops as multistep process (Hanahan, 2022). The most fundamental characteristic that distinguishes cancer cells from normal tissue is their ability to sustain chronic proliferation. Normal tissues carefully control cell growth and cell division by of growth-promoting signals that instruct entry into and progression through the cell growth and cell division cycle. These mechanisms ensure a homeostasis of cell number and maintains normal tissue characteristics and functions (Hanahan & Weinberg, 2011). On the other hand, cancer cells deregulate these growth signals, they may produce growth factor ligands themselves, or trigger the surrounding normal cell within tumor-associated stroma to release various growth factor. These sustainable processes result in uncontrollable cell growth. The enabling signals are delivered in large part by growth factors which can bind to cell-surface receptors,

especially containing intracellular tyrosine kinase domains (Hanahan & Weinberg, 2011).

1-1-2. Hallmark of Cancer

Cancer cells have the unique features that distinguish to normal cells. These features are acquired during the development of tumor cells. Hanahan and Weinberg, 2011 proposed 10 hallmarks of cancer to simplify the complexity of cancer biology.

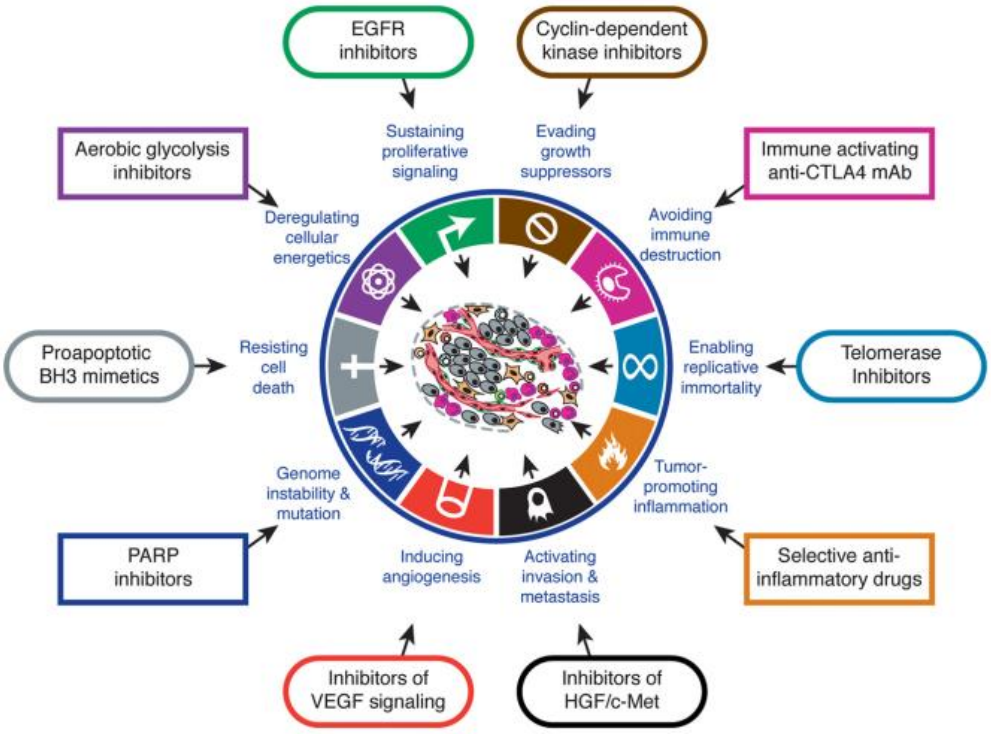


Figure 1-1. The 10 hallmarks of cancer and its targeting therapeutics.

As shown in the Figure 1-1, 10 hallmarks of cancer consist of: **1. Sustaining proliferative signaling**. A sustain chronic proliferation ability becomes a fundamental characteristic of cancer cells. Normally, cells can control the growth factor signal which in turn control the cell proliferation. Cancer cells deregulates growth factor production that leads to uncontrollable cell proliferation through cell cycle and cell growth. **2. Evading growth suppressors.** Along with producing growth factor signal, cancer cells must deregulate tumor suppressor genes, which in normal cells these genes are responsible to limit cell proliferation and cell growth. Cancer cells also abolished cell contact inhibition that leads to evasion from stop producing cells even though cells already confluent **3. Resisting cell death**. Apoptosis (programmed cell death) is one of the mechanisms to limit cell growth which can be triggered by cells by signal imbalance, DNA damage, and other external or internal factors. In cancer cells, "pro-apoptotic signals" are manipulated to maintain cell survival. **4. Enabling replicative immortality.** Cancer cells have unlimited replicative potential through cell growth-and-division cycles to generate macroscopic tumors. **5. Inducing angiogenesis.** Cells need oxygen and other essential nutrient as well as ability to evacuate metabolic wastes and carbon dioxide to survive**.** Angiogenesis is process developing neo vasculature to address these needs. In normal cells, switch on-off of angiogenesis is regulated tightly as tissue needed. In contrast, during tumor progression, an ''angiogenic switch'' always remains on to continually sprout new vessels that help sustain expanding. **6. Activating invasion and metastasis.** Cancer cells typically developed alterations in their shape as well as in their attachment to other cells and to the extracellular matrix (ECM) to invade locally and metastasize to other organs distantly. **7. Genome**

instability and mutation. Simply, certain mutant genotypes confer the selective advantage phenotype on subclones of cells. These new subclones facilitate their immortal growth and eventually dominate local tissue environment. **8. Tumor-promoting inflammation.** Indirectly, tumor-associated inflammatory response enhances tumorigenesis and progression in cancer cells through supplying bioactive molecules to the tumor microenvironment. including growth factors that trigger proliferative signaling, survival factors that resist cell death, angiogenesis inducing factors, extracellular matrix-modifying enzymes that facilitate angiogenesis, invasion, and metastasis, and inductive signals that lead to activation of EMT. **9. Reprogramming energy metabolism.** During the cancer development, cancer cells acquire unique ability to reprogram glucose metabolism to sustain cell growth and division. This process allows the cells largely to glycolysis even in the presence of oxygen, leading to a state that is termed as "aerobic glycolysis". **10. Evading immune destruction**. The last but not least, as another unique feature**,** cancer cells may well evade immune destruction by impairing components of the immune system that being conveyed to eliminate them.

In recent decade, other emerging hallmarks of cancer has been proposed (Hanahan, 2022). Phenotypic plasticity and disrupted differentiation, non-mutational epigenetic reprogramming and polymorphic microbiomes, and senescent cells, of varying origins, has been proposed newly as emerging hallmarks of cancer that functionally affects tumor microenvironment (Hanahan, 2022).

1-2. Key Factor and Molecules

1-2-1. The Human Epidermal Growth Factor Receptor 2 (HER2).

Human epidermal growth factor receptor 2 (HER2), which belongs to the epidermal growth factor receptor (EGFR) superfamily, is a protein involved in one of the most studied signal transduction pathways in cancer (Hynes & Lane, 2005). The amplification or overexpression of HER2 is detected in 15-20% of breast(Hynes & Lane, 2005), mean of 17.9% of gastric and gastroesophageal cancer (Lucas & Cristovam, 2016), in 20-30% of some ovarian cancer (Iqbal & Iqbal, 2014), and is correlated with poor patient survival. The estrogen receptor (ER) and HER2 (*c-erbB2, HER2/neu*) signaling pathways are the dominant drivers of cell proliferation and survival in the majority (85%) of breast cancer cases(Gutierrez & Schiff, 2011).

In the normal physiological condition, HER2 activation is controlled spatial and temporal when the ligand binds with one of the other EGFR family members, leading to heterodimer formation with HER2, which then activates its kinase activity. However, in the abnormal condition, when HER2 is overexpressed, this molecule associates with itself and other EGFR family members and is activated in a ligand-independent manner (Hynes & Lane, 2005; Olayioye et al., 2000; Penuel et al., 2002).

1-2-2. Mammaglobin 1.

Mammaglobin 1 (MGB1), also known as mammaglobin A or *SCGB2A2*, is a member of the secretoglobin family located on a genomic region frequently amplified

in breast cancer, chromosome 11q12.3–13 (Kreutzfeldt et al., 2020; J. Wang & Xu, 2019). Mammaglobin 1 is a promising marker for breast cancer as its specificity has been repeatedly highlighted (O'Brien et al., 2002). Although MGB1 is highly expressed in breast cancer(Galvis-Jiménez et al., 2013; Watson & Fleming, 1996), it has also been detected in gynecological malignancies (Zafrakas et al., 2006).

MGB1 has become a standard marker for detecting disseminated tumor cells in lymph nodes, peripheral blood(Lacroix, 2006), and micrometastases in bone marrow (Talaat et al., 2020). The role of MGB1 in cancer progression has been reported in triple-negative (HER2 negative/ER negative/PR negative) breast cancer cells (Picot et al., 2016). However, the role of MGB1 in cancer progression, especially in HER2 positive/ER negative breast cancer resistant cells, is still not fully understood.

1-2-3. Cyclins

Cyclins are responsible for regulating cell cycle progression (Figure 1-2) by interacting with cyclin-dependent kinases (CDKs), which govern the stage order from the resting stage (G0 phase) to cell division (M phase) (Casimiro et al., 2012). They also play important roles in cancer progression and metastasis through alternate pathway (Casimiro et al., 2012). The cyclin family consists of at least four major types (D, E, A , and B) of the 11 types that have been discovered in mammalian cells (Satyanarayana & Kaldis, 2009).

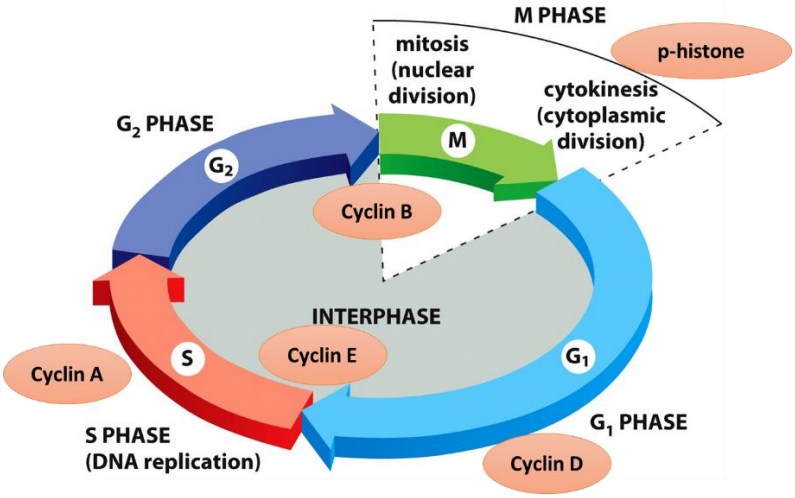


Figure 1-2. Cell cycle process and involvement of cyclins.

D-type cyclins are indirectly associated with a transcriptional role by generating a complex with CDK4 and CDK6. This complex induces phosphorylation of pRb in cyclin D activated the E2F-dependent pathway in cell cycle process (Bendris et al., 2015). Cyclin D1 has another special function in cancer progression. Cyclin D1 that is induced by Rho GTPases signaling activation, is correlating with cellular metastasis. Moreover, it has been reported that membrane-associated cyclin D1 is involved in cell migration, proliferation, and colony formation in cancer cell through RhoA regulation (Chen et al., 2020).

One of the major functions of E-type cyclins, in association with their specific CDKs, is the control of the progression from G1 to S phase, reflected by their accumulation in the end of G1 to most S phase (Bendris et al., 2015). Cyclin E is also involved in glioma cell migration and invasion (Hong et al., 2017). Interestingly,

previously reported that cyclin E involved in trastuzumab resistance (Scaltriti et al., 2011)

Cyclin A2 controls both S phase and G2/M transition in association with Cdk2 and Cdk1 (Arsic et al., 2012). Recently, it has been reported that cyclin A2 is involved in the control of the EMT in cancer. Depletion of Cyclin A2 in epithelial cells leads to loss of cell-to-cell contacts, decreased E-cadherin expression, and a delocalization of p12 catenin to the cytoplasm (Bendris et al., 2015).

1-2-4. Nf-κB

NF-κB family consist of 5 proteins, one of them is RelA/p65 which can generate a heterodimer with NF-κB1 (p50). This complex became the most-studied complex (Joyce et al., 2001). The p65/p50 complex is activated through phosphorylation process then translocate into nucleus to be an important transcription factor of several genes, those are involved in cancer progression, such as proliferation cell (Y. Cao & Karin, 2003). The most explored connection between NF-κB activation and cell cycle progression involves cyclin D1. There is also some evidences that the cyclin A promoter may be transcriptionally activated by NF-κB (Joyce et al., 2001).

In breast cancer, activation of the NFκB-signaling pathway regulates cell proliferation and differentiation, enhanced metastasis, and treatment resistance (Smith et al., 2014; L. Xia et al., 2018). Activation of NFκB through the canonical pathway involves IKKα (Merkhofer et al., 2010). NFκB-signaling pathway also regulates

progression in other cancers through interaction with various protein or signaling factor. Therefore, targeting NF-κB is still to be priority in cancer treatment (L. Xia et al., 2018).

However, in some specific cases, such as liver and skin cancer model, NF-κB seems to be a tumor suppressor. Studies in the chemically induced hepatocellular carcinoma (HCC) model showed that IKK2 targeted deletion in hepatocytes strongly enhanced tumorigenesis. Similarly, depletion of NEMO in hepatocytes promoted liver damage, hepatosteatosis, hepatitis, fibrosis, and finally HCC. Inhibition of NF-κB also shown to be tumor suppressor in both the DMBA/TPA- and Ras-induced keratinocytes which led to increased squamous cell carcinoma (SCC). These results suggest that suppression of NF-κB in keratinocytes might impair cell-cycle arrest upon DNA damage or oncogenic stress (Y. Xia et al., 2014).

1-3. Breast Cancer Therapy and Resistance

Simply, cancer therapy works as a three-component system: (i) a therapy; (ii) a population of cancer cells; (iii) a specific host environment. There are 4 main treatment methods for breast cancer include chemotherapy, radiotherapy, hormone therapy, and surgery (J. Cao et al., 2021). The clinical response or efficacy spectrum of the therapy is dependent on the pharmacological properties of the therapy, together with intrinsic and extrinsic factor. Intrinsic factors consist of acquired physical and molecular parameters of cancer cells, meanwhile the extrinsic factor is its environmental systems (Vasan et al., 2019)

In long-term cancer treatment, one of the major obstacles is overcoming tumor drug resistance that still represents the main reason for therapy failure (Fontana et al., 2021). Generally, drug resistance in cancer have focused on the differences between intrinsic or inherent, which means insensitivity occurs before treatment, and acquired resistance, which means appears following an initial positive response. However, practically, many tumors are or become resistant because of overlapping combinations of these factors (Cosentino et al., 2021; Vasan et al., 2019).

Cancer heterogeneity is predicted to be one of the causes of drug resistance. This phenomenon is driven by genetic, epigenetic, phenotypic modifications, tumor microenvironment and the cell metabolism that subsequently promote alteration on their behavior such as ability to proliferate, migrate, and invade. (Baliu-Pique et al., 2020; Lüönd et al., 2021). These features are critical for malignant tumor progression (Lüönd et al., 2021). Heterogeneity within a tumor increases its capability to readjust into alteration of surrounding pressure, that affect therapy response and clinical outcome (Lüönd et al., 2021). Breast cancer is one of the highly heterogeneous diseases. Therefore it became an obstacle to its therapy (Baliu-Pique et al., 2020).

In the case of estrogen receptor-positive breast cancer Fulvestrant or Tamoxifen is an effective targeted therapy. Expression of estrogen receptor is an important predictor of response to endocrine therapy, therefore lack of estrogen receptor expression by some clones within the tumor results in resistance to therapy (Baliu-Pique et al., 2020). Another example for the kind of breast cancer is HER2 positive breast cancer. Currently, in the case of HER2 positive breast cancer therapy, seven HER2 inhibitors are available.

For example, trastuzumab, pertuzumab, trastuzumab emtansine (T-DM1), trastuzumab deruxtecan (DS-8201a), lapatinib, neratinib, and tucatinib. The inhibitors differ in their mechanisms of actions (Zhang, 2021). Trastuzumab resistance is one of the most resistance observed in HER2-positive breast cancer patient. The resistance appears through the limited expression of HER2 or truncated HER2 that cannot be recognized by Trastuzumab (Scaltriti et al., 2007).

1-4. The Objective

Cancer resistance still becomes a great obstacle to effectiveness of cancer therapy. Resistant cells are well known to have higher aggressiveness that leads to poor prognosis. Therefore, discovering new treatment, as a single treatment or combination treatment, is still challenging to overcome resistance phenomenon. In this study, I aimed to look for new key protein that regulates trastuzumab resistant cells' aggressiveness, especially in HER2-overexpressing breast cancer cell line.

CHAPTER 2
Material and Method

2-1. Cell Culture

SK-BR-3-luc, HER2-overexpressing breast cancer cells without hormone receptors (JCRB Cell Bank, 1627.1, Japan), MCF7 (ATCC, Manassas, VA, USA), and MDA-MB-231 (ATCC) cells were cultured in low-glucose Dulbecco's modified Eagle's medium (Sigma-Aldrich Co. LLC, St Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich Co. LLC, St Louis, MO, USA) and 1% antibiotic/antimycotic solution (Sigma-Aldrich Co. LLC, St Louis, MO, USA). The cells were cultured in a humidified incubator at 37 °C and 5% CO_2. trastuzumab-resistant SK-BR-3-luc cells were obtained as previously described(Zazo et al., 2016). Briefly, SKBR3 wild-type cells A mycoplasma test (Venor™GeM Mycoplasma Detection Kit, PCR-based, Sigma-Aldrich Co. LLC) was conducted every 6 months; the cells were confirmed to be negative for mycoplasma contamination.

2-2. Transfection with Small Interfering RNA (siRNA)

siRNA duplexes and a negative control (non-specific RNA) were synthesized using the in vitro transcription T7 kit (Takara, Otsu, Japan). Cells were transfected with specific siRNA duplexes using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA). Cells (3×10^5) were seeded in a 6 well-culture plate. After 48 h of incubation, the medium was changed to antibiotic-free medium and specific siRNA (1 pmol/100 µL medium/each well in 96-well plate for MTT assay, 5 or 10 pmol/2 mL medium/each well in 6-well plate for

another assay). After 48 h of transfection, siRNA was discarded, and the cells were incubated in complete medium for 24 h.

2-3. Quantitative Polymerase Chain Reaction (qPCR)

RNA extraction was conducted according to the manufacturer's protocol (FG-80250, FastGene™ RNA Basic Kit/Basic Kit, Nippon Genetics, Tokyo, Japan), and reverse transcription reaction was performed using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). qPCR was conducted using SYBR Green (KAPA SYBR Fast qPCR kit, Nippon Genetics, Tokyo, Japan) and an Applied Biosystems StepOnePlus qPCR machine (Thermo Fisher Scientific, Tokyo, Japan). β-actin was used as the control.

2-4 MTT Assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells (3×10^3 cells/well) were cultured in a 96-well plate. After 48 h of incubation, the medium was replaced with the appropriate treatment. After incubation, the medium was replaced with MTT (0.5 mg/mL) (Sigma, Darmstadt, Germany) and incubated for approximately 4 h at 37 °C and 5% CO_2. The reaction was stopped with 10% sodium dodecyl sulfate (SDS) in 0.01 N HCl solution and incubated overnight under dark conditions to dissolve the formazan salt. The cell absorbance was measured using a microplate reader (Bio-Rad Laboratories, Inc., Tokyo, Japan) at 595 nm. The cell absorbance value was converted to the percentage of viable cells.

2-5. Trans-well Invasion and Migration Assay

The invasion assay was performed by seeding 5×10^4 cells on the top of an 8.0 μm pore insert (24 well insert, Corning Inc., Corning, NY, USA), which was coated with type 1 collagen (Cell matrix 1-P Nitta Gelatine, Osaka, Japan), diluted 10× with pH 3 hydrochloric acid or Growth Factor Reduced Matrigel (cat. 354230, Corning, NY, USA) diluted 1:6 with cold phosphate buffer saline (PBS). Non-coated inserts were used to observe migration ability. In the upper chamber, serum-free medium was added, and medium supplemented with 10% FBS was added to the lower wells. The cells were then incubated for 18-20 h. Cells were fixed with 4% paraformaldehyde for 5 min and washed with PBS. Cells on the upper surface of the membrane were removed using a cotton swab. Cells on the lower surface were stained with Hoechst (nuclear dye). Invading and migrating cells were observed using a confocal laser scanning microscope (A1R Confocal Imaging System, Nikon Intech Co. Tokyo, Japan) with a 20× objective lens. The number of cells was counted using the ImageJ software.

2-6. Western Blotting

The samples were prepared as previously described(Nukuda et al., 2016) without ultrasonic fragmentation. The lysates were run on 8, 10, 12, or 13% SDS-polyacrylamide gels and then transferred to polyvinylidene fluoride (PVDF) membranes. The membrane was

incubated with 5% or 10% skim milk in Tris-buffered saline-Tween (TBS-T) for 1 h. The membranes were then incubated with the following specific diluted primary antibodies: anti-GAPDH (1:100,000, AM4300, Thermo Fisher Scientific Baltics UAB, Lithuania), anti-α-tubulin (1:50,000；T5168, Sigma-Aldrich, Japan), anti-cyclin A2 (1:2,000; cat. #4656; Cell Signaling Technology, Danvers, MA, USA), anti-cyclin B1 (1:3,000; cat. #4138; Cell Signaling Technology), anti-cyclin D1 (1:1,000; cat. #2978; Cell Signaling Technology), anti-cyclin E1 (1:10,000; cat. #4129; Cell Signaling Technology), anti-p-histone (1:2,000; cat. #3377; Cell Signaling Technology), anti-mammaglobin A (1:1,000; ab150359, Abcam), and anti-phospho-NF-κB p65 (Ser536) (1:1,000; #3033; Cell Signaling Technology). The secondary antibodies used were HRP anti-rabbit IgG (1:5,000-1:10,000; cat. #7074; Cell Signaling Technology) and horseradish peroxidase (HRP) anti-mouse IgG (1:10,000-1:20,000; cat. #7076; Cell Signaling Technology). GAPDH or α-tubulin were used as internal controls to equalize protein loading. The band signal intensity was quantified using ImageJ software and normalized to that of the control(X. Li et al., 2013).

2-7. Senescence Assay

The senescence assay was performed by seeding 3×10^4 cells in a 6-well culture plate. After 48 h of culture, the cells were transfected with siRNA against *MGB1* or negative control, and then further incubated for 48 h. Then transfection media was discarded and replaced by complete medium for an additional 4 and 5-days period. Senescent cells were detected using a senescence β-galactosidase staining kit (Cell Signaling Technology, Tokyo,

Japan) according to the manufacturer's protocol. To evaluate the senescence phenotype, the cells were observed under a phase-contrast microscope (TS100; Nikon Instech Co., Tokyo, Japan) with a 10X objective lens.

2-8. Cell Death Assay

Dead cells were determined using a trypan blue staining assay, where blue-stained cells are considered non-viable whereas the unstained cells are viable. Wild-type and resistant cells were seeded at a density of 1×10^5 cells/well in a 6 well-plate for 48 h. The cells were then treated with siRNA against *MGB1* or negative control for 48 h. After 48 h, the cells were collected, stained with trypan blue, and counted manually using Neubauer hemocytometer chamber, then cells were observed under a phase-contrast microscope (TS100; Nikon Instech Co., Tokyo, Japan) with a 10X objective lens.

2-9. Generation of MGB1-Overexpressing Wild-type Cells

First, we designed and generated an expression vector encoding MGB1 protein, and its sequence was obtained by the following means. RNA was extracted from SK-BR-3 resistant cells. A complementary DNA (cDNA) pool was obtained by RT-PCR using a ReverTra AceH qPCR RT kit (Toyobo, Osaka, Japan). *MGB1*-encoding DNA was then amplified from this cDNA sequence by PCR using the KOD-Neo kit (Toyobo, Osaka, Japan). The primer set used for the PCR was as follows: 5′-

CTCAAGCTTCGAATTATGAAGTTGCTGATGGTCCTC-3′ (forward) and 5′-GGAGAGGGGCGGATCTTAAAATAAATCACAAAGACTGCTG-3′ (reverse). PCR products were purified using a NucleoSpin® gel and a PCR Clean Up kit (Takara Bio, Osaka, Japan). The pIRES2-ZsGreen1 vector (Clontech Laboratories Inc. Mountain View, CA, USA) was the vector used for *MGB1* overexpression. Subsequently, it was linearized by digestion with EcoR1 and BamHI and purified by electrophoresis. The linearized vector and DNA obtained from the PCR were ligated using the InFusion®HD Cloning kit (Takara Bio). After cloning the *MGB1* sequence into the expression vector, we performed DNA sequencing to check the plasmid sequence. The final construct was introduced into competent *Escherichia coli* cells (HST08 Premium; TaKaRa Bio) and subsequently purified. SK-BR-3 wild-type cells were transfected with the *MGB1*-overexpressing construct (MGB1ox) using Lipofectamine 3000 (Invitrogen). Transfected cells were selected by culturing them in the presence of 1.5 mg/mL G418 (Promega, Madison, WI, USA) for 7 days. Cell populations containing approximately 70% fluorescent protein-positive cells were used for subsequent experiments.

2-10. Trastuzumab Binding Assay

The trastuzumab-binding assay was performed as previously reported but with slight modification(C. Wang et al., 2021). Briefly, each group of cells was seeded at a cell density of 1×10^5 cells/well in a 6-well plate for 48 h. Cells were then treated with 10 μg/ml trastuzumab for 1 h on ice at 4ºC. After cold PBS washing (3 times), a secondary antibody,

goat anti-human IgG H&L conjugated to FITC (1:100, cat. ab6854; Abcam), was added to the cells, and they were incubated under agitation for 45 min at 4ºC. After cold PBS washing (3 times), fluorescence intensity was measured using Fluoroskan Ascent™ (Thermo Scientific, Thermo Fisher Corporation).

2-11. Statistical Analysis

I used the *p-value* or confidence interval of 95% (CI) to calculate the statistical significance of our experimental data. The normal distribution of the data was analyzed using the Kolmogorov–Smirnov test ($p > 0.05$, indicating that the data met the normal distribution). In the case of normal data, I determined whether the variance of the two datasets was significantly different using the *F*-test ($P < 0.05$, indicating that the variance was significantly different). Based on *F* test results, we used the Welch's *t*-test if the data had statistically different variances or the Student's *t*-test if the data had variances without statistical difference. A 95% CI was also used when the data were compared to the control group. First, the mean of each dataset was calculated. Then the 95% CI was calculated using Microsoft Excel. The two groups were considered to be significantly different if the means +/- 95% CI do not overlap the control value.

CHAPTER 3

Trastuzumab Resistant-cells have Higher Aggressiveness than Wild-type Cells

3-1. Introduction

Trastuzumab (Herceptin®), a recombinant humanized monoclonal antibody against HER2, has been considered as the first-gate therapy for HER2-positive breast cancer patients (Kreutzfeldt et al., 2020). However, in some cases, the effectiveness remains low due to acquired or *de novo* resistance.

Other anti-HER2 therapies that have different actions, such as pertuzumab (inhibitor for heterodimerization of HER2 with HER3), lapatinib (intracellular reversible inhibitor of EGFR and HER tyrosine kinase), and ado-trastuzumab emtansine T-DM1 (an antibody drug-conjugated, anti-HER2 function of trastuzumab and DM1 induced cytotoxicity), are recommended by the U.S. Food and Drug Administration (FDA)(Kreutzfeldt et al., 2020; J. Wang & Xu, 2019). To improve the therapy, in 2012, the FDA approved the combination of trastuzumab and pertuzumab as a first-line therapy for HER2+ metastatic breast cancer. Most recently, the trastuzumab-linked antibody-drug conjugate, DS-8201a, has been approved for the treatment of patients with metastatic HER2+ breast cancer who had previously received two or more anti-HER therapies (Kreutzfeldt et al., 2020).

The general mechanisms of trastuzumab resistance that have been intensively studied include the following: 1. The difficulty associated with trastuzumab binding to HER2 is caused by a structural mutation in HER2, which generates a truncated p95HER2 isoform(Pohlmann et al., 2009; Vu & Claret, 2012); 2. the upregulation of HER2 downstream signaling pathways; 3. signaling through alternate pathways; and 4. failure to stimulate immune-mediated mechanisms to eradicate tumor cells(Pohlmann et al., 2009).

The resistance phenomena remain a major obstacle in cancer treatment owing to the complexity and heterogeneity of the mechanism. Therefore, specific markers of cancer resistance need to be explored to enhance the effectiveness of therapy.

3-2. Result

3-2-1. Resistant Cells Have Higher Proliferation, Invasion, and Migration Abilities than Wild-type Cells

Firstly, I established the resistant cells via treatment with 15 μg/ml trastuzumab for at least 3 months(Zazo et al., 2016). To confirm resistance, I investigated the viability of cells that survived after long-term exposure to trastuzumab (resistant cells), compared to that of untreated cells (wild-type cells), and found that the viability of resistant cells did not change after exposure to 100 μg/mL trastuzumab, whereas that of wild-type cells decreased by more than 30% (Fig. 2-1a). Based on the MTT assay, treatment with a high concentration of trastuzumab had markedly greater effects on the viability of wild-type cells than on resistant cells (Fig. 2-1b). *MKI67* and p-histone have been reported as proliferation markers, and a decrease in *PTEN* is used as a resistance marker (Du et al., 2014; Nagata et al., 2004). Resistant cells showed significantly higher *MKI67* (Fig 2-1c) and p-histone expression (Fig. 2-1d) and lower *PTEN* mRNA levels than those in wild type cells (Fig 2-1c). These results demonstrate that resistant cells gained resistance to trastuzumab.

Based on the *MKI67* mRNA level results, I examined the proliferation ability of

resistant cells by manual counting. Five days after seeding the cells, the number of resistant cells was higher than that of wild-type cells (Fig. 2-1e). Furthermore, they expressed upregulated p-histone suggesting the high proliferation potential.

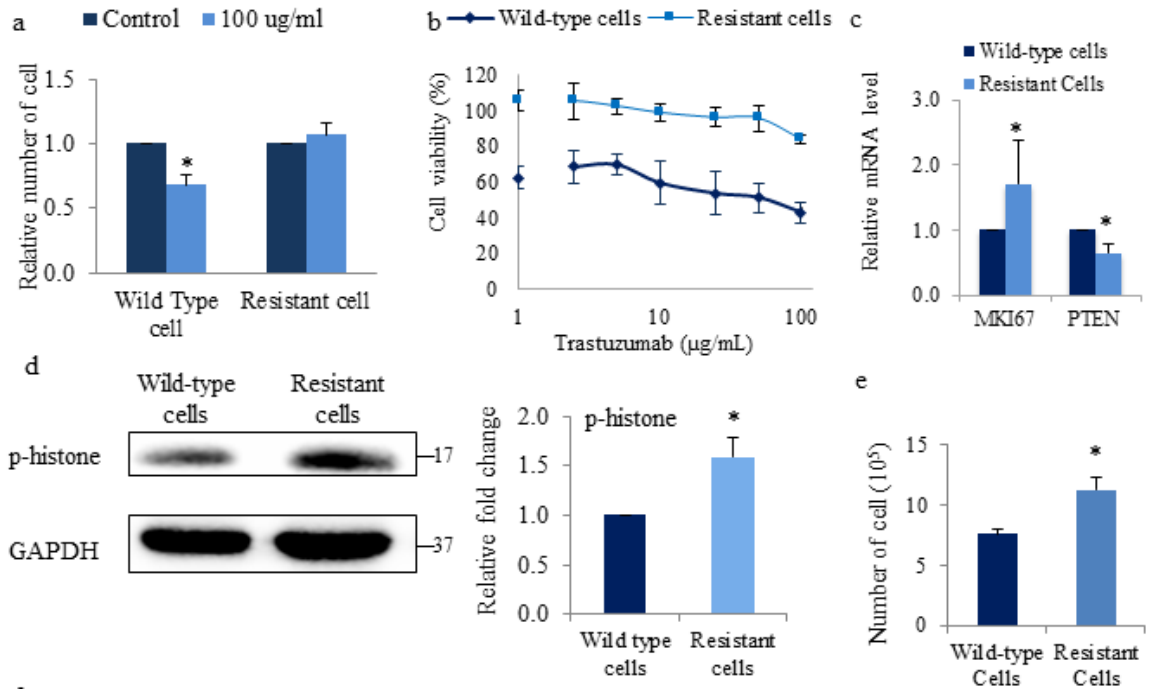


Figure 2.1. Resistant cells are established successfully and have higher proliferation activity. a. Relative number of SK-BR-3 wild-type and resistant cells after treatment with 100 μg/mL trastuzumab for 5 days. The number of cells relative to each control group (0 μg/mL trastuzumab) is shown. **b**. Viability of wild-type and resistant cells after treatment with a series of concentration (0, 1, 2.5, 5, 10, 25, 50, and 100 μg/mL) of trastuzumab for 5 days. The viability of cells treated with 0 μg/mL trastuzumab was used as a basis for the

calculation. **c.** mRNA level of *MKI67*and *PTEN* in resistant cells relatives to wild-type cells. β-actin was used as the control gene. **d.** Representative western blot (left), and quantification (right) of p-histone and GAPDH (loading control) expression in resistant cells relative to wildtype cells. Bar represents standard error mean (SEM), N=3 independent experiments, thrice for each experiment *statistical significance with CI of 95%. **e.** Number of wild-type and resistant cells after culturing for 5 days in complete culture medium without any treatment. The bar represents standard error mean (SEM), N=3 independent experiments, thrice for each experiment, $*p < 0.05$, unpaired Welch's t-test.

Migration and invasion abilities are correlated with cancer progression (Hanahan & Weinberg, 2011). To investigate these, I performed invasion and migration assays on collagen- or Matrigel-coated membranes and on non-coated membrane trans-wells, respectively. After incubating the cells for 18-20 h, I observed the invasion and migration abilities of resistant cells significantly increased compared to those of wild-type cells (Fig. 2-2 a-d). These results suggest that after breast cancer cells gain trastuzumab resistance, their proliferation, invasion, and migration abilities are enhanced.

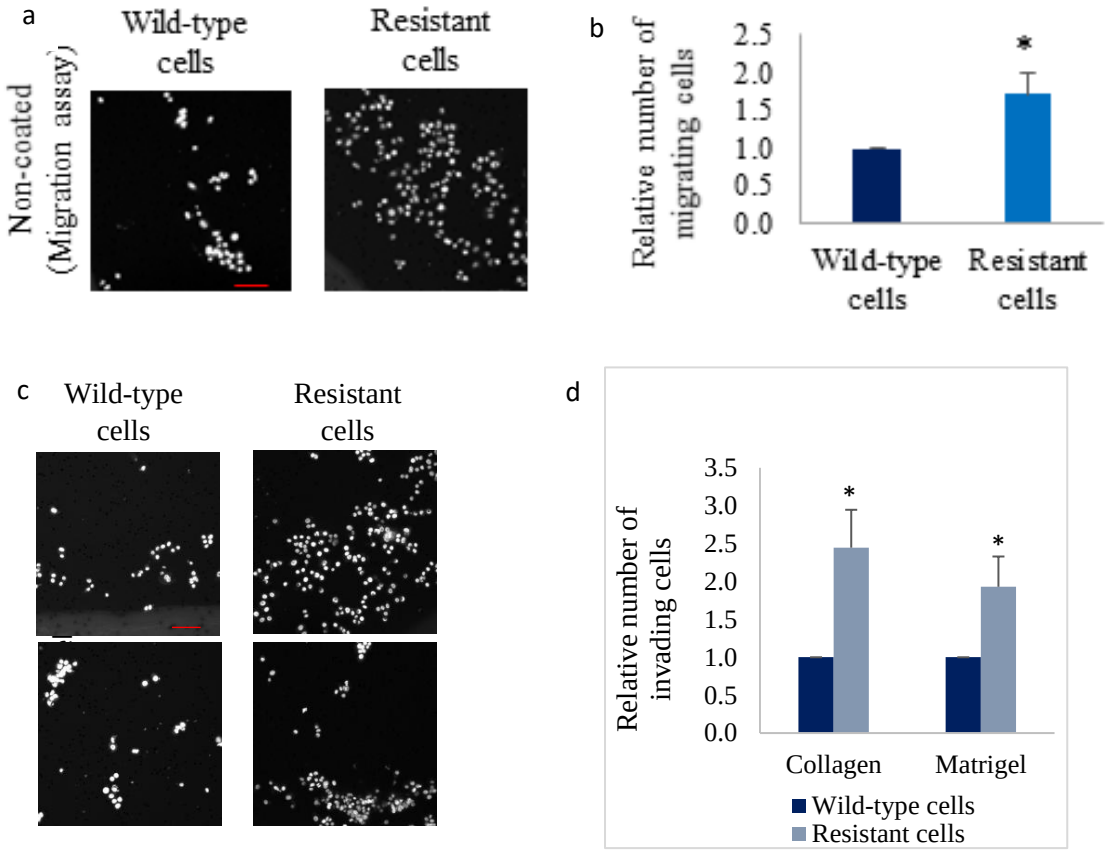


Figure 2.2. Resistant cells have higher migration and invasion ability than those of wild-type cells. a. Representative figure of migrating cells. **b.** Quantification relative number of migrating cells. **c.** Representative figure of invading cells. **d.** Quantification relative number of invading cells.

3-2-2. MGB1 was upregulated in resistant cells and important to regulate its aggressiveness

Next, to investigate key molecules involved in the proliferation, migration, and invasion abilities of resistant cells, we performed an RNA microarray. The results demonstrated that *MGB1* expression was upregulated up to 10-fold in resistant cells compared to that in wild-type cells (Fig. 2-3a).

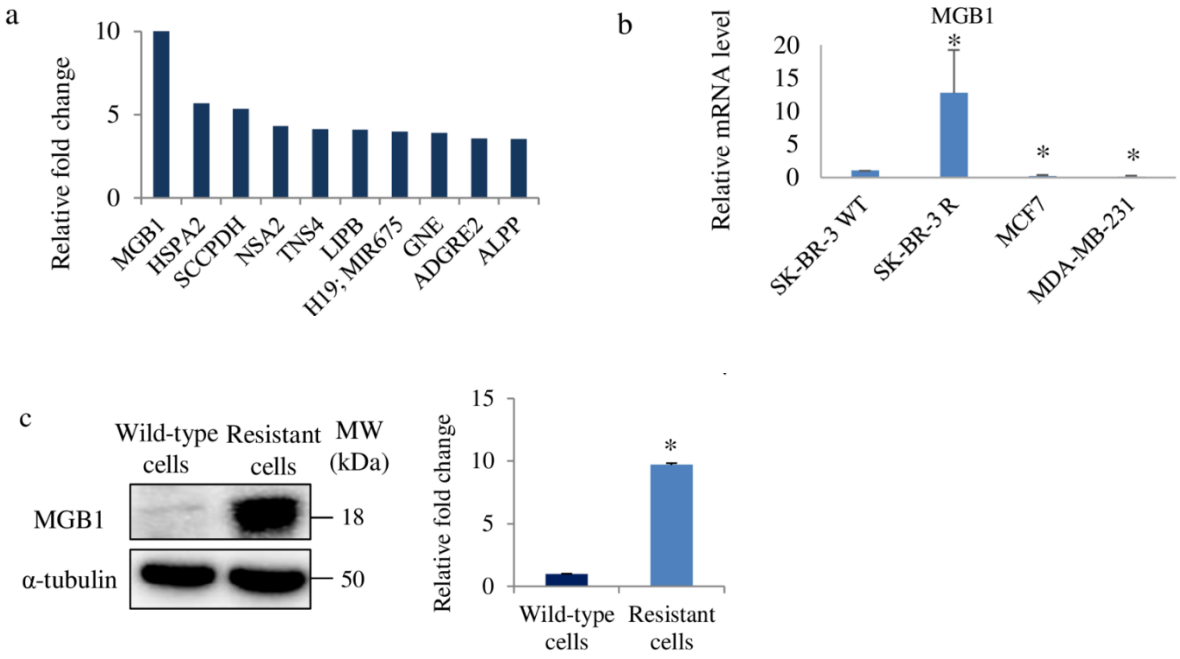


Figure 2-3. Resistant cells upregulated MGB1 expression. **a.** Microarray gene profile in resistant cells relatives to wild-type cells. **b.** Trastuzumab resistant SKBR-3 cells showed highest MGB1 mRNA level compared to wild-type and other breast cancer cell lines. **c.** Representative western blots (left), and quantification (right) of MGB1 and α-tubulin (loading control) expression in resistant cells after relative to the wild-type cells.

To evaluate the cell line specificity for *MGB1*, we detected the mRNA expression of *MGB1* in other breast cancer cells, MCF7 (HER2 negative/ER positive/PR positive) and MDA-MB-231(HER2 negative/ER negative/PR negative). I found that SK-BR-3 cells had higher *MGB1* expression than that in other cells (Fig. 2-3b). Moreover, when the cells gained trastuzumab resistance, the mRNA and protein levels of *MGB1* were significantly elevated (Fig. 2-3b, c).

To examine the role of MGB1 in the resistant cells, we knocked down *MGB1* in resistant cells using siRNA. As the proliferation ability of resistant cells was higher than that of wild-type cells, we investigated the effect of MGB1 on cell proliferation using MTT assay. *MGB1* knockdown was found to significantly decrease the viability of resistant cells, so that the cell viability similar to wild-type cells (Fig. 2-4a). Subsequently, to clarify whether MGB1 regulates the survival of resistant cells after trastuzumab treatment, *MGB1*-depleted resistant cells were exposed to 50 μg/mL trastuzumab for 4 days. There was no difference in the *MGB1*-knocked-down resistant cells' viability with and without trastuzumab (Fig. 2-4b)

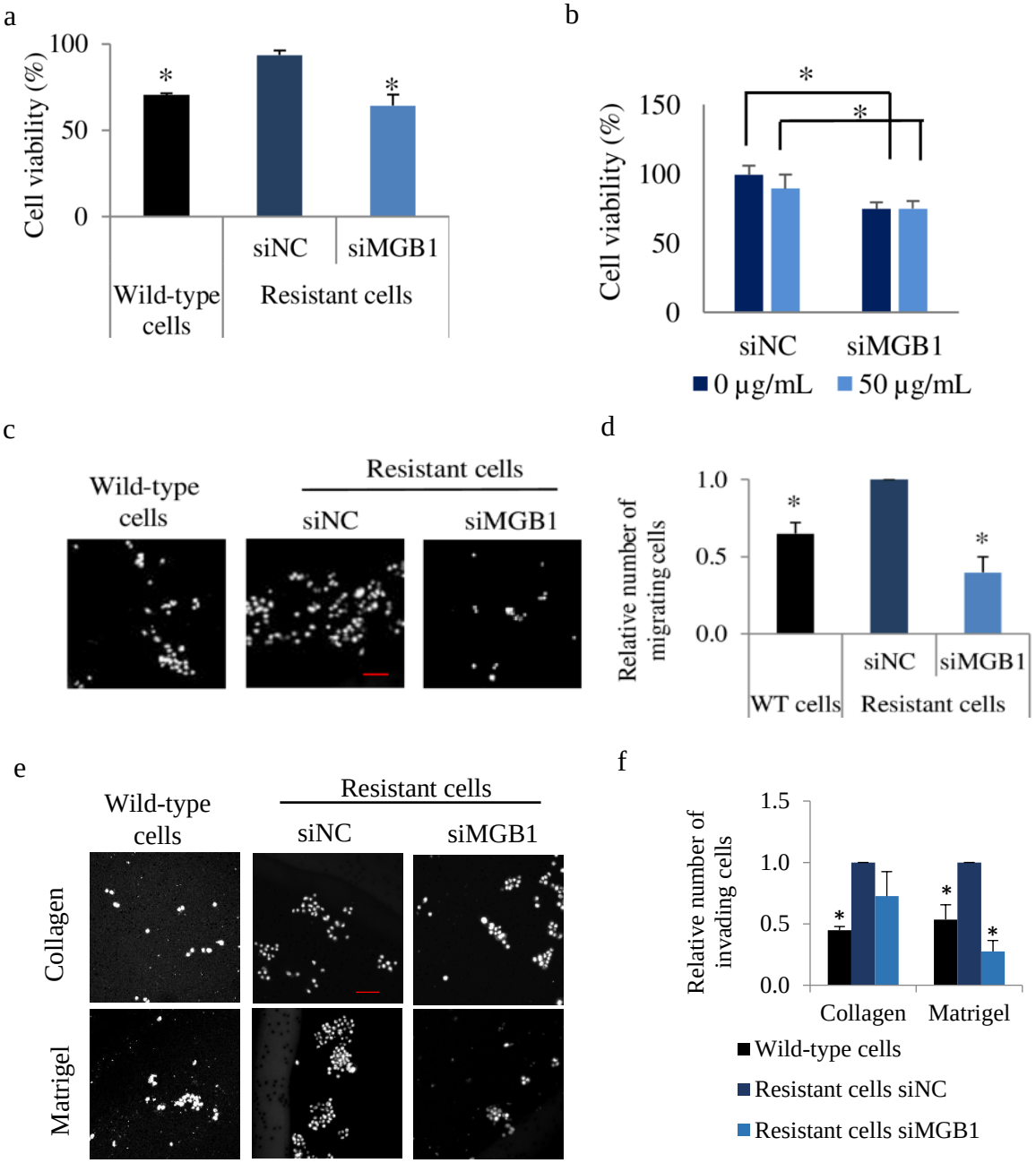

a
Cell viability (%)
100
50
0
*
*
siNC
siMGB1
Wild-type cells
Resistant cells
b
Cell viability (%)
150
100
50
0
*
*
siNC
siMGB1
0 μg/mL
50 μg/mL
c
Wild-type cells
Resistant cells
siNC
siMGB1
d
Relative number of migrating cells
1.0
0.5
0.0
*
*
siNC
siMGB1
WT cells
Resistant cells
e
Wild-type cells
Resistant cells
siNC
siMGB1
Collagen
Matrigel
f
Relative number of invading cells
1.5
1.0
0.5
0.0
*
*
*
Collagen
Matrigel
Wild-type cells
Resistant cells siNC
Resistant cells siMGB1

Figure 2-4. **The effect of MGB1 in resistant cells' proliferation, migration, and invasion ability**. **a.** Cell viability of resistant cells after transfected with siMGB1. The bar represents mean with SEM N=3 independent experiments, thrice for each experiment, *p < 0.05, unpaired Welch's t-test **b.** Viability of resistant cells after transfection with siMGB1 or siNC followed by trastuzumab treatment (50 µg/ml for 4 days). The bar represents mean with SEM. N=2, thrice per experiment with *p value < 0.05 unpaired Student's t-test. **c.** Representative figure of migrating cells. **d.** Quantification relative number of migrating cells. **e.** Representative figure of invading cells. **f.** Quantification relative number of invading cells. The bar represents mean with SEM, N=3 independent experiments, thrice for each experiment, *statistical significance with CI of 95%. Scale bar of 100 µm.

The trans-well assay was performed to compare the migration and invasion abilities of control and MGB1-depleted cells. The migration assay and Matrigel-coated invasion assay revealed that silencing MGB1 expression decreased the migration and invasion abilities of resistant cells, respectively (Fig. 2-4 c-f). These results suggest that MGB1 is a key molecule for the aggressiveness of resistant cells

3-3. Discussion

In this study, I established trastuzumab-resistant cells by chronic treatment with trastuzumab. This approach mimics that used in the clinic, where acquired resistance rises

gradually (Scaltriti et al., 2011) in long-term administration of trastuzumab. After at least 3 months of trastuzumab exposure, cells become less sensitive to trastuzumab relative to wild type cells. Not only less sensitive to trastuzumab, but resistant cells also have higher proliferation ability than wild-type cells. This result was supported by increment of MKI67 mRNA level and p-histone expression that usually used as proliferation marker. Next, I investigate the other features of aggressiveness, migration, and invasion. As expected, migration and invasion ability of cells increased after cells obtain resistance. Drug-resistant cancer cells have been reported to acquire high aggressiveness, which causes poor prognosis in cancer patients (Itamochi et al., 2008).

Along with the aggressiveness, resistant cells have higher expression of Mammaglobin 1 (MGB1) relative to wild-type cells. This result was supported by a previous report, which also showed that MGB1 (*SCGB2A2*) is upregulated in trastuzumab-resistant HER2-overexpressed breast cancer cells (Joshi et al., 2011). However, as there is a lack of evidence regarding the relationship between HER2 and MGB1, it should be further explored. Although it has been reported that MGB1 is highly expressed in various types of breast cancer cell including HER2 positive/ER negative type (Picot et al., 2016), the role of MGB1 in this type has not been reported, moreover when the cells resist to trastuzumab treatment.

The role of MGB1 in resistant cells' aggressiveness was measured through the proliferation, migration, and invasion ability observation. Silencing of MGB1 decreased cell proliferation, migration, and invasion ability in resistant cells significantly. However, a

significant decrease was not observed in the collagen-coated membrane invasion assay (Fig. 2-4f). In some cases, the collagen and Matrigel matrices have opposite roles (Grefte et al., 2012; Sodek et al., 2008). Subsequently, to clarify whether MGB1 regulates the survival of resistant cells after trastuzumab treatment, *MGB1*-depleted resistant cells were exposed to 50 μg/mL trastuzumab for 4 days. There was no difference in the *MGB1*-knocked-down resistant cells' viability with and without trastuzumab (Fig. 2-4b). This result may indicate that MGB1 does not involve in the initial process of resistance.

In summary, I successfully established resistant cells which has higher proliferation, migration, and invasion ability relative to wild-type cells. These features were regulated by MGB1 which also upregulated when the cells getting resistance. However, MGB1 did not promote resistance itself.

CHAPTER 4

MGB1 Regulated Cyclins and p-65 Expression in Resistant Cells and These Regulations are Critical for the Aggressiveness of Resistant Cells

4-1. Introduction

The cyclin family consists of at least four major types (D, E, A , and B) of the 11 types that have been discovered in mammalian cells (Satyanarayana & Kaldis, 2009). Cyclins are responsible for regulating cell cycle progression by interacting with cyclin-dependent kinases (CDKs), which govern the stage order from the resting stage (G0 phase) to cell division (M phase) (Casimiro et al., 2012). They also play important roles in cancer progression and metastasis through alternate pathway (Casimiro et al., 2012).

Additionally, it has been reported that cyclin is connected to NF-κB. The most explored connection between NF-κB activation and cell cycle progression involves cyclin D1. There is also some evidence that NF-κB may activate the cyclin A promoter (Joyce et al., 2001). The NF-κB family consists of five proteins, one of which is RelA/p65. RelA/p65 can generate a heterodimer with NF-κB1 (p50) (Joyce et al., 2001). The p65/p50 complex is activated through phosphorylation and translocated to the nucleus to be a critical transcription factor for several genes involved in cancer progression, such as enabling proliferation (Y. Cao & Karin, 2003). Interestingly, NF-κB is involved in trastuzumab resistance in HER2 positive/ER positive breast cancer cells (Kanzaki et al., 2016).

MGB1 is important for regulating resistant cells' aggressiveness, as described in Chapter III. Because there is lack information about role of MGb1 in cyclins regulation, in this chapter, I looked for the possible involvement of cyclin D1, cyclin E1, cyclin A2 and p65 its regulation pathway in resistant cells 'aggressiveness. Additionally, I also established MGB1-overexpressing wild-type cells, the cells that originally express MGB1 protein

much lower than resistant cells. This investigation is important to ensure the role of MGB1 in resistance induction and cyclins expression.

4-2. Result

4-2-1. MGB1 Regulates Cyclins and p-p65 Expression in Resistant Cells

Cell proliferation is strongly related to cell cycle process. In the chapter III, I showed that MGB1 regulated proliferation of resistant cells. Therefore, to investigate the detailed mechanism, firstly, I detected marker proteins of each phase in the cell cycle process. I found that resistant cells showed higher expression of cyclins (cyclin D1, cyclin E1, and cyclin A2) than wild-type cells (Fig. 4-1a,b).

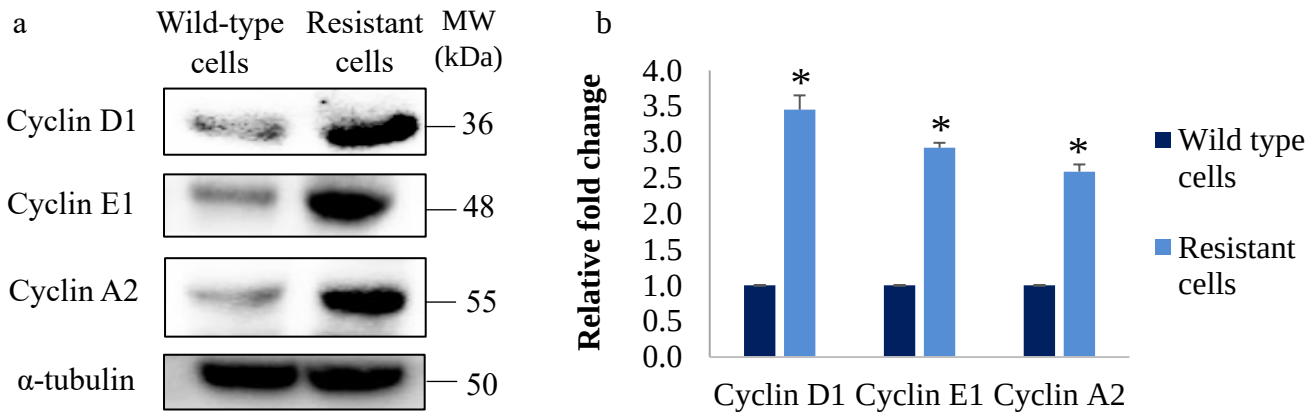


Figure 4-1. Cyclins were upregulated in resistant cells. **a.** Representative western blots and **b.** quantification of cyclin D1, cyclin E1, cyclin A2, and α-tubulin (loading control) in resistant cells relative to wild-type cells.

Then, we investigated the role of MGB1 in cyclin expression in resistant cells. We found that the expression of cyclins and p-histone was significantly downregulated by MGB1 depletion (Fig. 4-2).

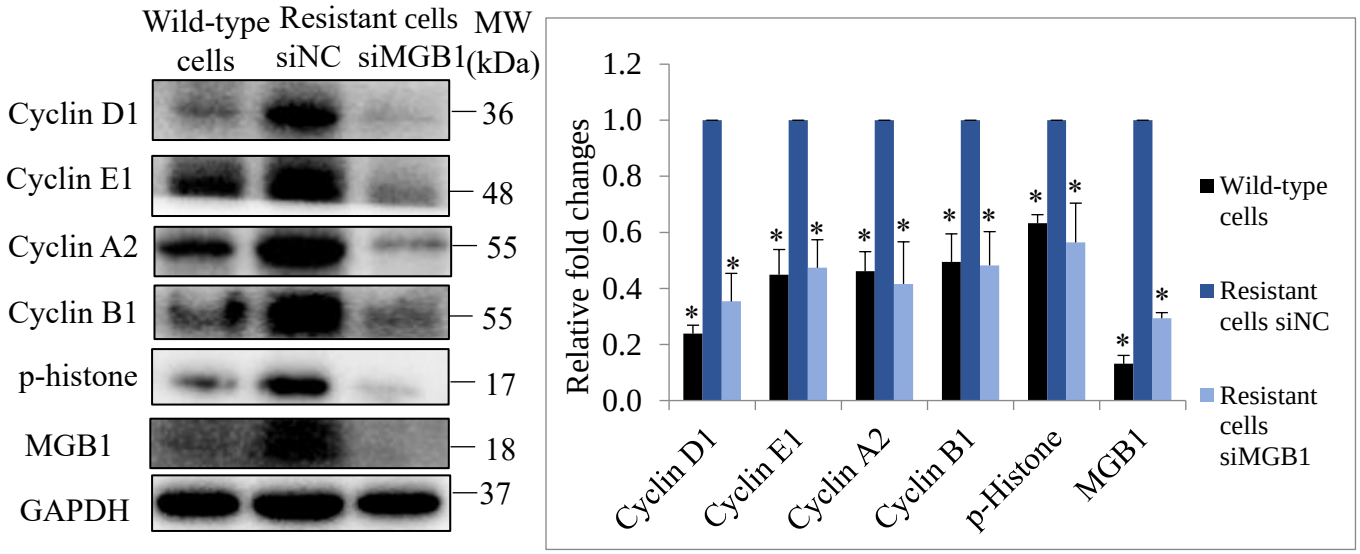


Figure 4-2. Depletion of MGB1 decreased cyclins and p-histone in resistant cells. Representative western blots (left) and quantification (right) of cyclin D1, cyclin E1, cyclin A2, cyclin B1, p-histone, MGB1, and α-tubulin (loading control) in resistant cells after siMGB1 transfection relative to negative control (siNC). The bar represents mean with

SEM, N=3 independent experiments, thrice for each experiment, *statistical significance with CI of 95%.

To determine other mechanisms that reduce cell viability caused by *MGB1* knock-down in resistant cells, I evaluated cleaved-caspase 3 expression for the detection of apoptosis and β-galactosidase activity to analyze senescence. I found that cleaved-caspase 3 expression and β-galactosidase activity decreased in *MGB1*-knocked-down resistant cells (Fig. 4-3a, b). Apoptosis detection using trypan blue assay supports western blotting result (Fig. 4-3c).

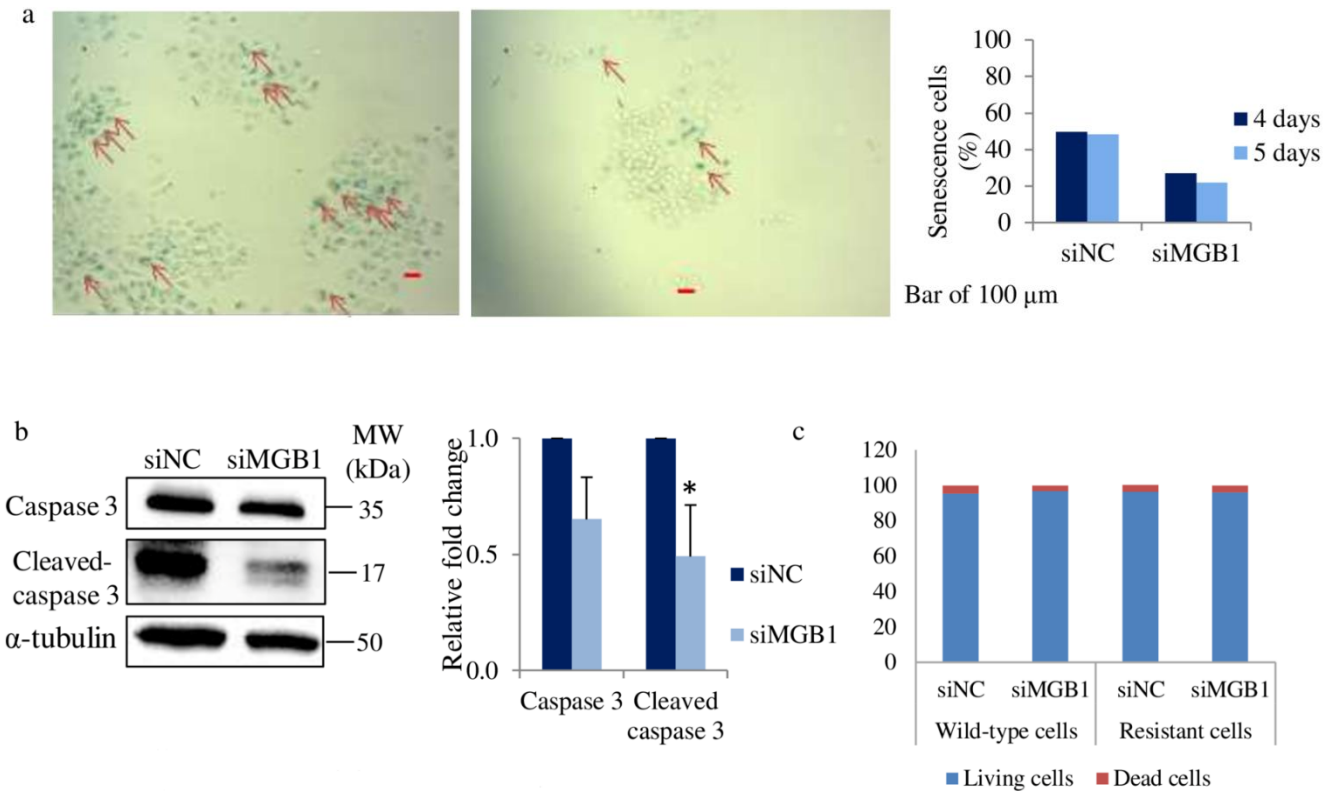
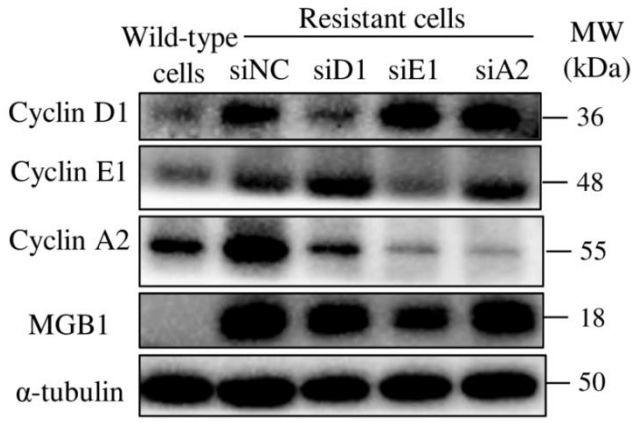


Figure 4-3. MGB1 does not regulate apoptosis and senescence cells in resistant cells. a. Representative images (left) of senescence cells (red arrow), and their quantification

(number of senescence cells per 100 cells) 4- and 5-days post-transfection (right) **b.** Representative western blots (left), and quantification (right) of caspase 3, cleaved caspase 3, and α-tubulin (loading control) expression in resistant cells after siMGB1 transfection, relative to that of the negative control (siNC). **c**. Graph bars showing the percentage of living cells and dead cells in wild-type and the resistant group treated with siMGB1. Bar represents mean with SEM, N=3 independent experiments, thrice for each experiment, *statistical significance with CI of 95%.

4-2-2. Cyclins Regulate MGB1-dependent Aggressiveness in Resistant Cells

Based on the upregulation of cyclin in resistant cells, I investigated the role of cyclin D1, cyclin E1, and cyclin A2 in the regulation of proliferation, migration, and invasion abilities. First, I decreased cyclins expression by siRNA to ensure the decrement of cyclins expression and to figure out the relation among cyclins in resistant cells (Fig. 4-4).



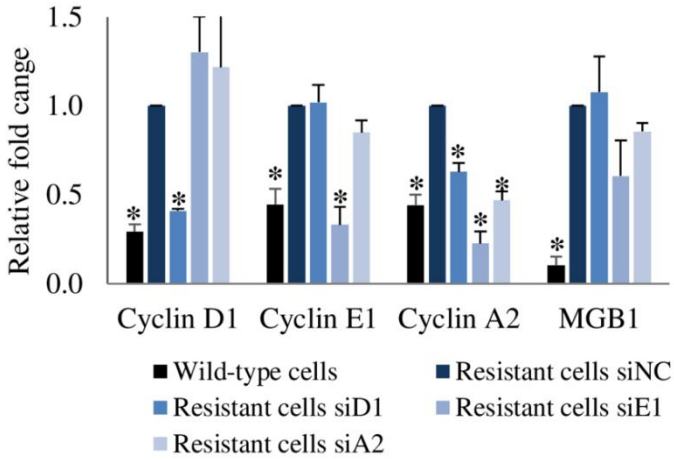


Figure 4-4. Regulation among cyclins expression. Representative western blots (left) and quantification (right) of cyclin D1, cyclin E1, cyclin A2, MGB1, and α-tubulin (loading control) in resistant cells after siCyclin D1 (siD1), siCyclin E1 (siE1), and siCyclin A2 (siA2) transfection relative to the negative control (siNC). Bar represents mean with SEM, N=3 independent experiments, thric for each experiment, *statistical significance with CI of 95%.

After confirming that cyclins expression was down-regulated successfully, I checked the role of each cyclin in cell proliferation, migration, and invasion ability. Cell viability was decreased by silencing each cyclin in the resistant cells (Fig. 4-5a), as well as migration and invasion abilities (Fig. 4-5c, d, e). These findings suggested that the downregulation of cyclin D1, cyclin E1, and cyclin A2 decreased the viability, migration, and invasion ability of resistant cells.

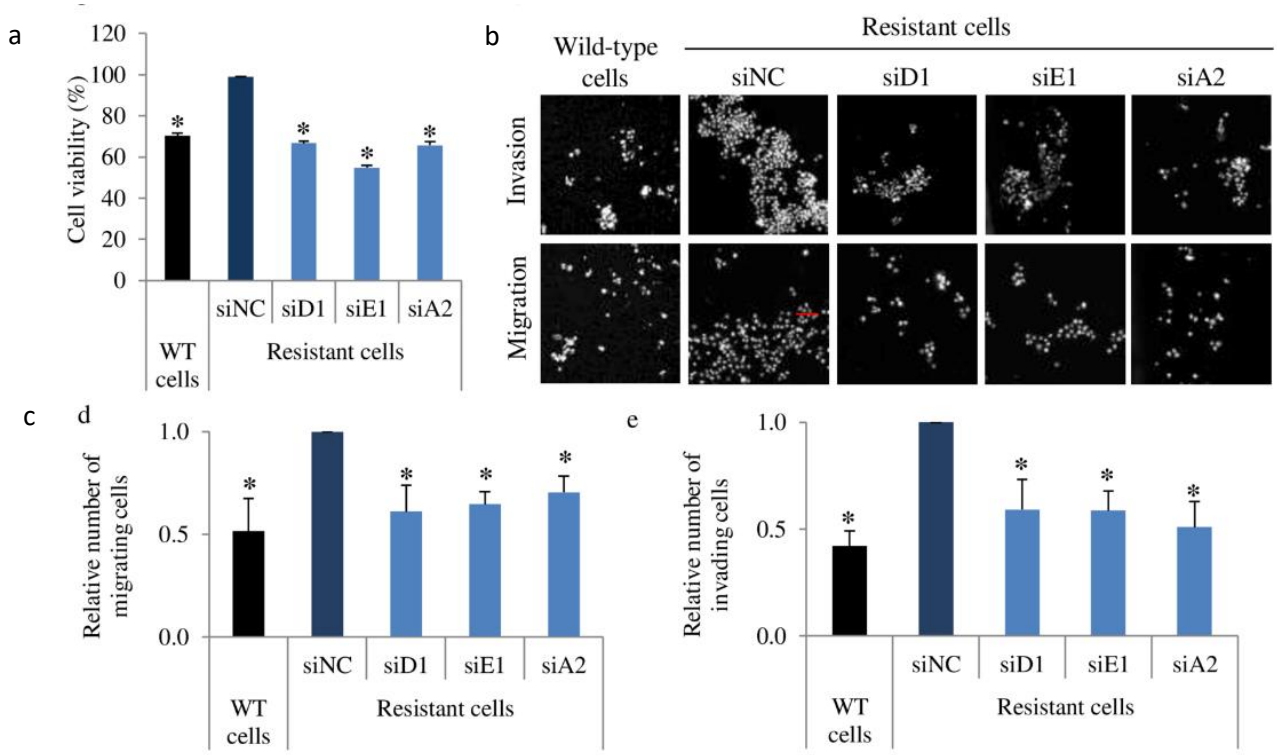


Figure 4-5. Cyclin D1, E1, and A2 are involved in MGB1-regulated aggressiveness in resistant cells. a. Cell viability. Bar represents mean with SEM N=3 independent experiments, thrice for each experiment, **p* value < 0.05 unpaired Welch's t-test., **b**. Representative figure of migration and invasion assay with 20x magnification. Scale bar of 100 µm **c**. Quantification of migration, and **d**. invasion of resistant cells after transfection with siD1, siE1, and siA2 relative to the negative control (siNC). Bar represents mean with SEM, N=3 independent experiments, thrice for each experiment, *statistical significance with CI of 95%.

4-2-3. p-p65 Triggers MGB1-regulated Aggressiveness in Resistant Cells through the Induction of Cyclins

One of the crucial factors reported to be involved in breast cancer cell malignancies is NF-κB (Smith et al., 2014; L. Xia et al., 2018). Based on this finding, I investigated the expression and role of the most-studied NF-κB family member, p65(Y. Cao & Karin, 2003; Joyce et al., 2001) especially phosphorylated-p65 (p-p65), the active state of p65 protein, in resistant cells. The expression of p-p65 in resistant cells was significantly higher than that in wild-type cells (Fig. 4-6a, b). Then, I examined whether MGB1 expression was involved in p-p65 expression. The results showed that a decrease in MGB1 downregulated p-p65 expression (Fig. 4-6 c,d). These findings suggest that MGB1 contributes to the regulation of cyclins and p-p65 expression in resistant cells.

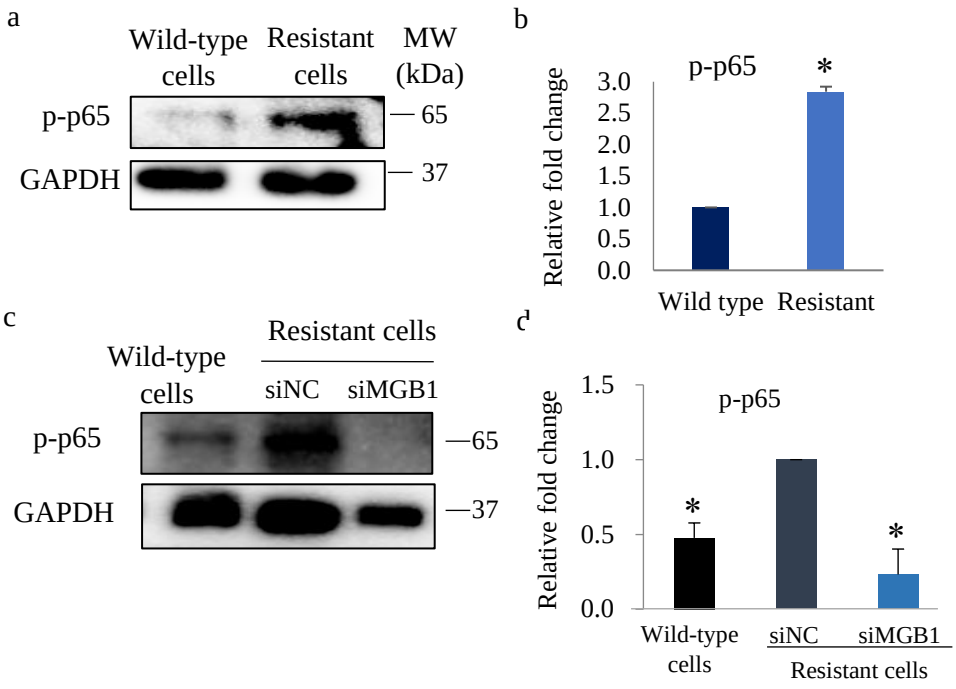


Figure 4-6. MGB1 regulated p-p65 expression. a. Representative western blots and **b.** quantification of p-p65 and GAPDH (loading control) expression in resistant cells relative to that of wild-type cells. **c.** p-p65 and GAPDH (loading control) representative expression and **d.** its quantification in resistant cells after siMGB1 transfection, relative to that of the negative control (siNC). The bar represents mean with SEM, N=3 independent experiments, thrice for each experiment, *statistical significance with CI of 95%.

Since p-p65 was upregulated in resistant cells, I investigated the viability, migration, and invasion abilities of p65-depleted resistant cells. I discovered that cell viability decreased by more than 60% (Fig. 4-7a). Moreover, migration and invasion abilities of resistant cells decreased significantly by silencing the p65 gene (Fig 4-7b, c, d).

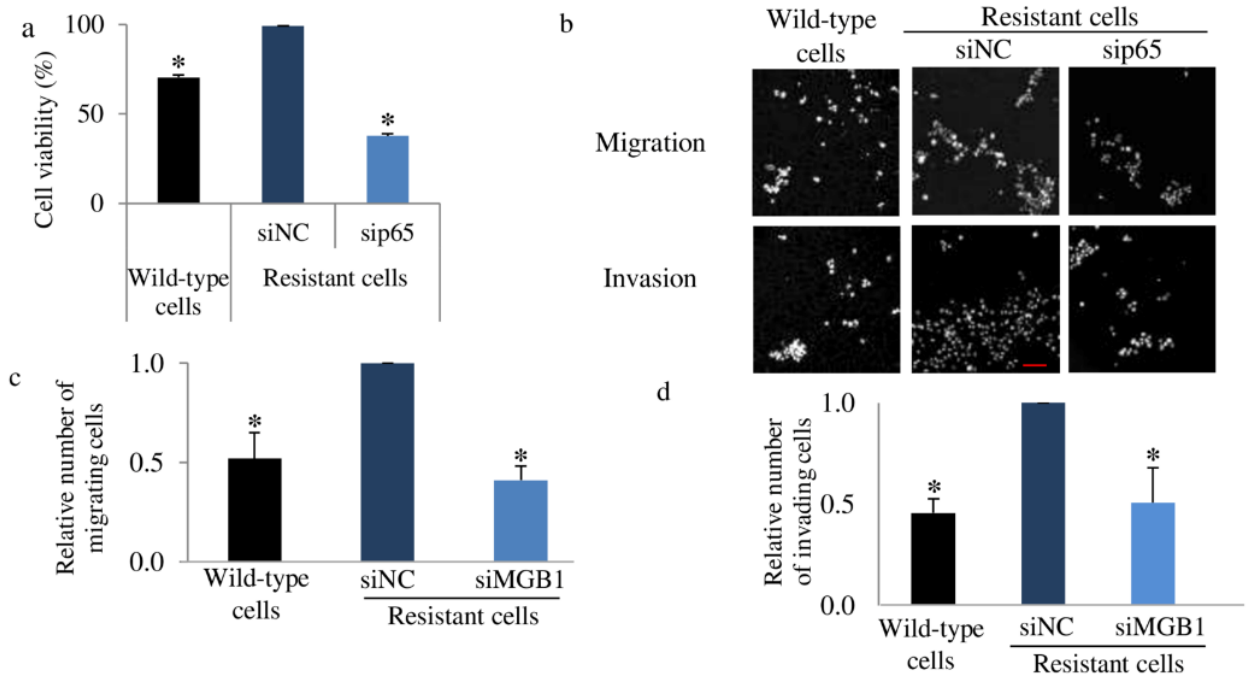


Figure 4-7. p-p65 is involved in MGB1-regulated aggressiveness in resistant cells. a. Cell viability, Bar represents mean with SEM, N=3 independent experiments, thrice for each experiment, *p value < 0.05 unpaired Welch's t-test. **b.** Representative figure of migration and invasion assay with 20x magnification. Scale bar of 100 µm **c**. Quantification of migration, and **d.** invasion of resistant cells after transfection with sip65 relative to the negative control (siNC). Bar represents mean with SEM, N=3 independent experiments, thrice for each experiment, *statistical significance with CI of 95%.

Additionally, it has been known that NF-κB regulates cyclin activation (Joyce et al., 2001). Therefore, I examined the relationship between p-p65 and cyclin expression. The downregulation of p65 decreased cyclin D1 and cyclin A2 expression but did not decrease cyclin E1 and MGB1 expression (Fig. 4-8 a, b). Meanwhile, the downregulation of each cyclin had no significant effect on p-p65 expression (Fig. 4-8 c, d). These findings suggest that p-p65 regulates cyclin D1 and A2, whereas cyclins are not critical for p-p65 expression.

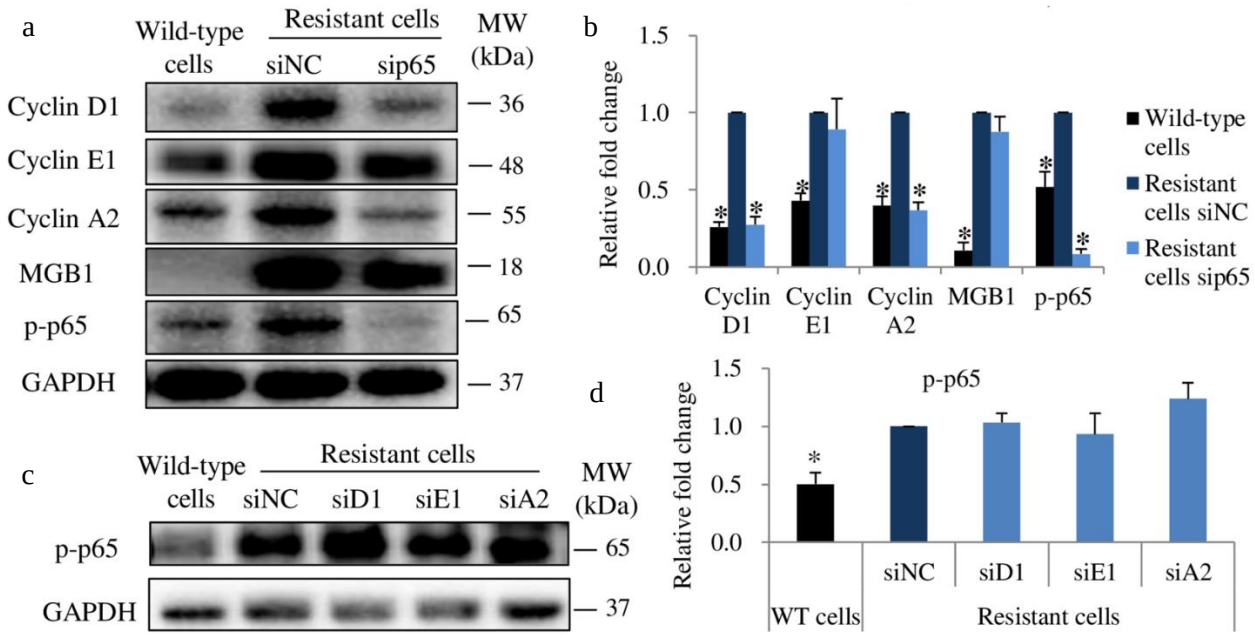


Figure 4-8. p-p65 is involved in cyclins regulation of resistant cells. a. Representative western blots and **b.** quantification of cyclin D1, cyclin E1, cyclin A2, MGB1, p-p65, and

GAPDH (loading control) in wild-type cells, siNC and sip65 resistant cells, relative to siNC resistant cells. **c.** Representative western blots and **d.** quantification of p-p65 and GAPDH (loading control) in wild-type cells, and resistant cells after siD1, siE1, and siA2 transfection relative to the resistant cells negative control. Bar represents mean with SEM, N=3 independent experiments, thrice for each experiment, *statistical significance with CI of 95%.

4-2-3. Induced MGB1 in Wild-type Cells Results Different Regulation in Resistance and Cyclins Expression.

To investigate the role of MGB1 in the resistance process, we induced *MGB1* overexpression in wild-type cells, which expressed MGB1 at lower levels than that in resistant cells. MGB1 wild-type cells overexpressing MGB1 approximately three-fold (MGB1ox wild-type cells) were successfully generated, as shown in Fig. 4-9a. MGB1ox wild-type cells were exposed to trastuzumab at concentrations up to 100 µg/mL, and we found that MGB1 overexpression did not induce trastuzumab resistance (Fig. 4-9b). These findings may suggest that an increase in MGB1 expression may occur in breast cancer cells after developing trastuzumab resistance in a trastuzumab-independent manner. Additionally, even though MGB1 overexpression in resistant cells can trigger cyclins expression, an increase in cyclin expression was not observed in MGB1ox wild-type cells (Fig. 4-10). This result may indicate that MGB1-dependent increment of cyclins only happened after cell getting resistance.

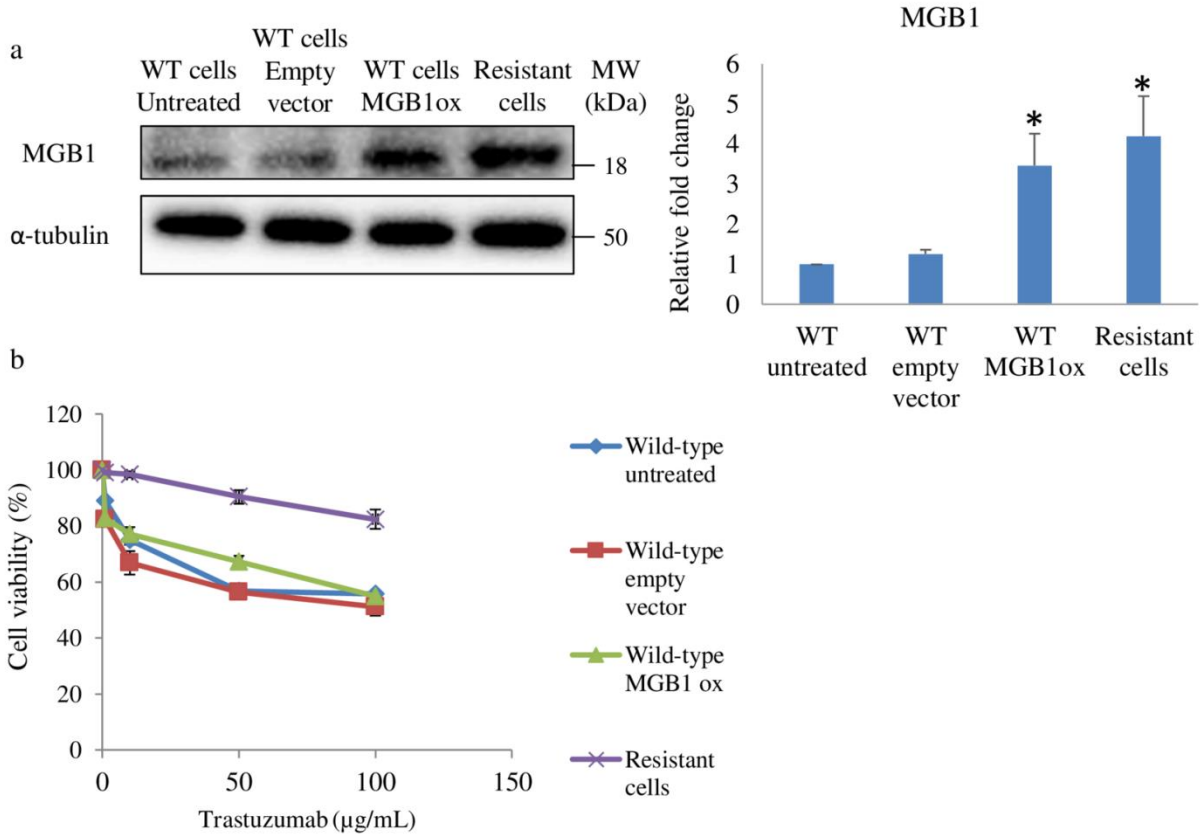


Figure 4-9. MGB1 overexpression in wild-type cells does not promote trastuzumab resistance. a. Representative western blots (left), and quantification (right) of MGB1 and α-tubulin (loading control) expression in wild-type cells (untreated, empty-vector, and *MGB1*-overexpressing (MGB1ox) and resistant cells relative to wild-type cells untreated. The bar represents the mean with SEM; N = 3 independent experiments. **b**. Viability of wild-type cells (untreated, empty-vector, and MGB1ox) and resistant cells after the treatment with different concentrations (1, 10, 50, and 100 µg/mL) of trastuzumab for 5 days. The viability of treated cells was calculated relative to that of untreated cells. * Statistical significance with confidence interval at 95%.

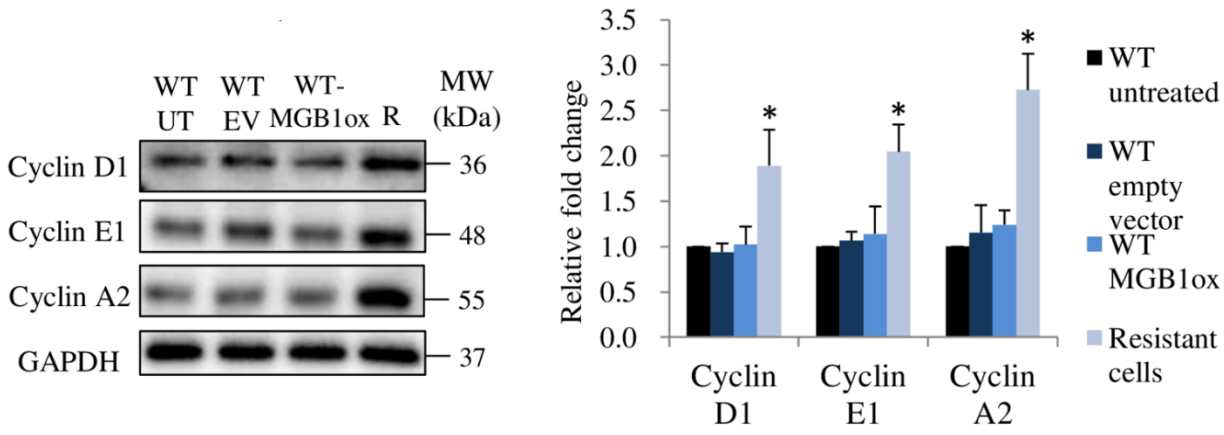


Figure 4-10. Induced MGB1-overexpression in wild type cells does not trigger cyclins expression. Representative images of western blots (left) and quantification (right) of cyclins and GAPDH (loading control) in untreated wild-type cells (WT UT), wild-type cells with empty vector (WT-EV), wild-type cells with MGB1 overexpression (WT-MGB1ox), and resistant cells (R). The bar represents mean with SEM, N=3 independent experiments, thrice for each experiment, *statistical significance with CI of 95%.

4-3. Discussion

Firstly, in the Chapter 3, it showed that *MKI67* and p-histone expression were upregulated in resistant cells. Upregulation of these molecules was strongly related to the elevation of cell proliferation (Kim et al., 2017). This result was supported by upregulation of Cyclin D1, Cyclin E1, and Cyclin A2 expression in resistant cells. It suggested that cell proliferation in resistant cells was regulated through cell cycle. Based on the senescence assay and cell death assay, I suggested that the decrease in cell viability by *MGB1* knockdown in resistant cells was due to the prevention of cell proliferation rather than apoptosis or senescence induction. In senescence assay, I prolonged the culture period of cells deliberately to make sure that senescence will not occur in MGB1 depletion resistant cells. It has been reported that trastuzumab does not induce senescence in SK-BR-3 wild type cells (McDermott et al., 2019). This phenomenon occurs even if SK-BR-3 cells confer trastuzumab resistance.

Overcoming cell cycle arrest is one of the mechanisms of trastuzumab resistance (Vu & Claret, 2012). Numerous studies have reported that in addition to cell cycle progression, cyclin D1(Chen et al., 2020; Z. Li et al., 2006; Zhong et al., 2010), cyclin E1(Luo et al., 2013), and cyclin A2 (Arsic et al., 2012; Tu et al., 2019) are also involved in the regulation of cell migration and invasion. Based on these findings, I examined whether each cyclin regulated the expression of another cyclin. Cyclin D1 and cyclin E1 expression did not change when other cyclins were downregulated, whereas cyclin A2 expression level was decreased by silencing cyclin D1 or E1. I also found that MGB1 expression was not affected by a decrease in cyclin expression. These results indicate that MGB1 is an

upstream regulator of cyclins in trastuzumab-resistant HER2 positive/ER negative breast cancer cells.

Increment of all major cyclin (D1, E1, and A2) in resistant cells may therefore render cells independent of cell cycle arrest mediated by trastuzumab. A previous study reported that the downregulation of MGB1 decreased the viability of triple-negative breast cancer cells(Picot et al., 2016). This finding was consistent with our results, which used different types of breast cancer cells. Cyclins play an essential role in cell cycle regulation. However, there is no evidence of a relationship between MGB1 and cyclins.

My findings revealed that MGB1 silencing downregulated cyclin expression. The decrease in MGB1 and cyclin D1, E1, and A2 decreased the viability of resistant cells. Additionally, MGB1 and cyclins contributed to the regulation of the migration and invasion abilities of resistant cells. A previous study reported that the upregulation of cyclin A2 enhanced the migration of hepatocellular carcinoma (HCC), while its downregulation decreased the migration of DLD-1 and SW620 colorectal cancer cell lines (CRC) (Fu et al., 2020; Tu et al., 2019). Cyclin D1 enhances cell migration and invasion activity in fibroblasts by regulating RhoA (Chen et al., 2020) as well as tumor cells (Fusté et al., 2016; Z. Li et al., 2006; Zhong et al., 2010). These findings are consistent with our results. However, cyclin A2 depletion enhanced the migration of fibroblasts and the invasiveness of CRCs and triple-negative breast cancer cells by downregulating RhoA activity (Arsic et al., 2012). These contradictory results are possibly due to the difference in the molecular profile of each cell, or the type of matrix used for the experiments. Three studies used Matrigel, while the latest study used collagen as the matrix. In some cases, the collagen and

Matrigel matrices have opposite roles (Grefte et al., 2012; Sodek et al., 2008). Contradicting results were also found for cyclin D1 function, even though the same breast cancer cell line was used (Lehn et al., 2010; Zhong et al., 2010). The expression levels of cyclin A and E were correlated with each other, but not with cyclin D1(Boström et al., 2009). Cyclins have diverse functions, however, their regulatory function involves various signaling pathways and different conditions (Arsic et al., 2012; Hong et al., 2017; Joyce et al., 2001; Tu et al., 2019).

Activation of NF-κB through HER2 signaling is essential for HER2-mediated cancer resistance (Ahmed et al., 2006). NF-κB regulates cyclin D1 and cyclin E1 expression to regulate glioma cell growth and invasion (Hong et al., 2017). A previous study reported that a lack of MGB1 decreased NF-κB transactivation by increasing IκB-α (inhibitor of NF-κB alpha) in triple negative breast cancer cells (Picot et al., 2016). These previous results support our finding that the regulation of NF-κB and cyclin through MGB1 plays a crucial role in the aggressiveness of resistant cells.

Based on a mouse model, cyclin redundancy has been reported to occur in cell cycle regulation (Satyanarayana & Kaldis, 2009). In the absence of D-type cyclin, the cell cycle process can still be run by the presence of cyclin E or cyclin A. Cyclin D and cyclin A can substitute for the absence of cyclin E to maintain cell survival (Satyanarayana & Kaldis, 2009). In contrast, cyclin A2 is one of the most non-redundant cyclins (Satyanarayana & Kaldis, 2009). These findings support our data, which showed that a decrease in cyclin D1 through NF-κB, or cyclin E1 decreased cyclin A2 expression. Through this regulation, at least two types of cyclins (D1 and A2 or E1 and A2) decreased the viability, migration, and

invasion abilities of resistant cells. Meanwhile, the depletion of cyclin A2 did not influence other cyclins, NF-κB, and MGB1 expression, however, single cyclin A2 depletion decreased cell viability, migration, and invasion abilities. Further research should be conducted to determine whether the depletion of a single cyclin can have a similar function. Moreover, the correlation of each cyclin in resistant cells should be further explored.

CHAPTER 5
Antibody-dependent cellular cytotoxicity AADC

5-1. Introduction

In addition to antigen recognition, antibody-dependent cellular cytotoxicity (ADCC) is an immune response by which Fc portion of membrane-attached specific antibody binds to Fc receptor (FcR) of immune cells that triggers cell death. Natural killer (NK) cell is the most-well known immune cell that involved in ADCC process. FcR of NK cell binds to Fc portion of antibody which then release different cytotoxic molecules, such as granzyme and perforin, that provoke the death of the target cell (Rivero et al., 2021).

Besides ADCC, the induction of antibody-dependent cellular phagocytosis (ADCP) that is mediated by macrophage, showed clinical efficacy and to be a major mechanism for the anticancer effects of many therapeutic antibodies. The binding of the Fc portion of antibodies to Fcγ receptors (FcγRs) on macrophages induces ADCP. This process involves phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) of FcγRs, which activates downstream signaling through Rac–GEFs (guanine nucleotide exchange factors) to induce phagocytosis (X. Cao et al., 2022).

Trastuzumab mediates innate and adaptive immune response (Nuti et al., 2011). Failure to stimulate immune-mediated mechanisms to eliminate tumor cells is one of the characteristics of trastuzumab-resistant cancer cells (Pohlmann et al., 2009). ADCC and ADCP are the dominant immune-based antitumor effects of trastuzumab. An important process in ADCC and ADCP is the binding of trastuzumab to the HER2 receptor. Therefore, ADCC and ADCP can still occur if tumor cells express HER2 target antibody-binding epitopes (Upton et al., 2021).

5-2. Result

Based on the findings that ADCC and ADCP are still occurred in the presence of HER2 target antibody-binding epitopes. Here, I performed a trastuzumab binding assay to observe the ability of trastuzumab to bind to the HER2 receptor in resistant cells and MGB1-depleted resistant cells. The results demonstrated that resistance to trastuzumab did not affect its binding to the resistant cells. Likewise, MGB1 depletion did not affect trastuzumab binding efficiency (Fig. 5-1), indicating that ADCC and ADCP may still occur in resistant cells even when MGB1 is silenced.

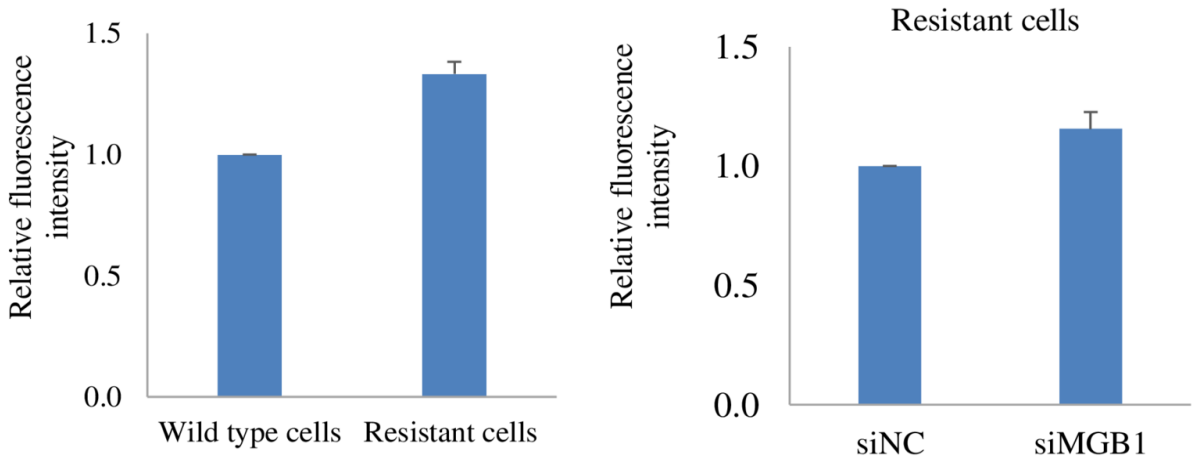


Figure 5-1. Detection of HER2 receptor in resistant cells and siMGB1-treated resistant cells. The fluorescence intensity values indicate the binding of trastuzumab to the HER2 receptor. Detection of HER2 receptor ectodomain by trastuzumab binding assay in resistant cells relative to wild-type cells (left), and in siMGB1-resistant cells relative to the

negative control (right). Graph bars represents mean ± SEM; N = 3. No significant differences were observed in any of the comparisons

5-3. Discussion

It is well-known that trastuzumab has significant anticancer effects on HER2 receptor function. Additionally, immune system contributes to critical role in trastuzumab anticancer effects through involvement of macrophages and NK cells. Both cells use cell surface high affinity activating FcRs to bound to the Fc domains of antiHER2 antibodies for their subsequent effector functions. Following these crucial interactions, NK cells release killer vesicles such as perforin and granzymes A and B, meanwhile macrophages perform cell phagocytosis and intracellular death (phagoptosis).

Interaction between FcRs of immune cells and Fc of HER2 receptor is a key mechanism on ADCC and ADCP. Therefore, based on this reason, I conducted trastuzumab binding assay that can detect the presence of HER2 in resistant cells and MGB1 depleted-resistant cells. Principally, trastuzumab will bind to Fc portion of HER2 receptor, then FITC-conjugated anti-human IgG binds to trastuzumab. The fluorescence released by this binding is measured. The result showed that fluorescence intensity of resistant cells relative to wild-type cells has no difference significantly (Fig.5-1). It indicates that HER2 receptor expression did not elevate in resistant cells, even though resistant cells more aggressive than wild-type cells. It also indicates that ADCP and ADCC may not be affected by

resistance. Similar to our result (Fig. 5-1), it has been reported that even after relapse upon treatment with trastuzumab, resistant cells still overexpress HER2.

Since MGB1 is important to promote aggressiveness in resistant cells, I checked if MGB1 involved in ADCC and ADCP mechanism. As shown (Fig. 5-1), the results revealed that ADCC and ADCP might still occur even if MGB1 is depleted. However, further experiments should be conducted to confirm this hypothesis. In any case, I can conclude that MGB1 could be a promising therapeutic target for HER2-positive breast cancer patients with trastuzumab resistance.

CHAPTER 6
Summary and Remaining Questions

Summary

In this study, I established trastuzumab-resistant cells by culturing SKBR3 cells in long-term exposure of trastuzumab. Resistant cells showed higher proliferation, migration, and invasion ability than wild-type cells. I also found that HER2 positive/ER negative breast cancer cells enhanced MGB1 expression up to 10-fold after trastuzumab resistance. However, as there is a lack of evidence regarding the relationship between HER2 and MGB1, it should be further explored.

I demonstrated that the upregulation of MGB1 was induced by trastuzumab resistance in HER2-positive/ER negative breast cancer cells, and MGB1 increased cell viability, migration, and invasion abilities by upregulating cyclin and NF-κB expression. I observed that MGB1 overexpression in wild-type cells did not trigger cyclin upregulation, indicating no correlation between cyclin and MGB1 expression. This may indicate that regulation of cyclins expression by MGB1 only occurs after the cells acquire resistance to trastuzumab. Further study should be conducted to clarify this phenomenon. Briefly, this study showed that resistant cells MGB1 regulated proliferation, migration, and invasion abilities through cyclin and p-65 signaling (Fig. 6)

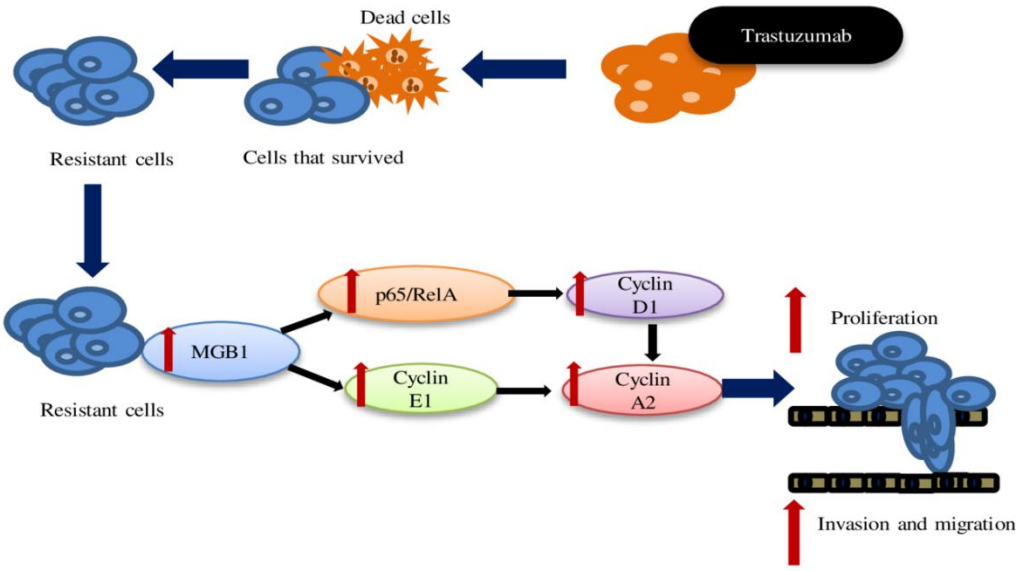


Figure 6. Schematic depicting how MGB1 regulates the progression of resistant cells through cyclin and NF-κB regulation

One of the recent strategies of trastuzumab combination therapy is immune-based biomarker. Further, MGB1 has previously been developed as a vaccine for breast cancer. Therefore, these findings indicate that MGB1 could be a prospective marker for detecting resistance in breast cancer patients receiving long-term trastuzumab treatment.

Failure to stimulate immune-mediated mechanisms to eliminate tumor cells is one of the characteristics of trastuzumab-resistant cancer cells. ADCC and ADCP are the immune-mediated anticancer action by trastuzumab. In this study, the result showed that ADCP and ADCC process are independent to resistance status and MGB1 expression.

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