# Association between growth factor heregulin -1 α and receptors in growth of ovarian cancer cell line with high potentiality of peritoneal dissemination

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**[Original Article]**

# ASSOCIATION BETWEEN GROWTH FACTOR HEREGULIN -1α AND RECEPTORS IN GROWTH OF OVARIAN CANCER CELL LINE WITH HIGH POTENTIALITY OF PERITONEAL DISSEMINATION

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**Abstract :** Ovarian cancer, one of the poor-prognosis gynecological malignancies, is often associated with extensive peritoneal carcinomatosis when initially treated. The mechanism of the formation of peritoneal carcinomatosis from ovarian cancer is still unknown. It has been reported that overexpression of cancer-related growth factors and/or receptors may worsen the prognosis of diseases. In the previous paper, we had established the human ovarian serous adenocarcinoma cell lines from those with no potentiality of peritoneal dissemination (FOC-3) to high potentiality (MFOC-3), however, the mechanism of its phenotypical change remains unknown.

In this paper, we compared these two cell lines for growth potential and the expression of growth factor heregulin (HRG)-1α and HER-2, HER-3, and HER-4 receptors. In addition, the effect of anti-receptor antibodies on cell growth was investigated. RT-PCR and Western blot analysis found the promotion of the expression of HRG precursor and HRG-1α in MFOC-3. Examination of the number of growing cells over time revealed a statistically significant increase in the number of cells in MFOC-3 compared with FOC-3. In a study using the addition of exogenous HRG-1α, no changes were observed in FOC-3 while statistically significant cell growth was noted in MFOC-3. In a growth inhibition study, statistically significant cell growth inhibition was achieved with the addition of anti-HER2 receptor antibody.

Taken together, the results of this study suggested that HRG may play an important role in the increased growth potential of peritoneal dissemination of ovarian cancer. In particular, HER-2 receptors that can act as a starting point to trigger intracellular signaling pathways are strongly involved in the progression of cancer. Therefore, molecular target drug therapies blocking the HER-2 receptor are promising candidates for ovarian cancer treatment in the future.

**Key words :** Ovarian cancer, Growth Factor, Heregulin, HER2

## INTRODUCTION

In recent years, an increasing number of molecular target drug therapies have been developed, and become available options in addition to surgery and chemotherapy for management of ovarian cancer. However, despite these potent therapies, ovarian cancer is still a poor-prognosis disease that often recurs[1-3]. This is because more than half of ovarian cancer cases are advanced when first treated and often affected by peritoneal carcinomatosis accompanying extensive peritoneal dissemination. Nevertheless, the mechanism of the formation of peritoneal dissemination remains mostly unknown. In order to improve the prognosis for ovarian cancer, we believe that it is necessary to

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elucidate the mechanism of the formation of peritoneal dissemination. Our research group has already succeeded in the establishment of cell lines with the high potentiality of peritoneal dissemination (MFOC-3) using cell line without the potential of peritoneal dissemination derived from human ovarian serous adenocarcinoma (FOC-3) in the peritoneal cavity of mice[4].

Heregulin (HRG) may play an essential role in neurogenesis and cardiogenesis during the fetal stage. HER-2, a receptor of HRG, is overexpressed in approximately one-third of breast cancer and ovarian cancer cases, and the association between overexpression of the HER-2 and the poor prognosis for advanced ovarian cancer has been suggested[14,18,23-26,33]. There have been much research on the expression levels of epidermal growth factor (EGF) and HRG and their receptors, as well as signal transduction, using various breast cancer and ovarian cancer cell lines[14,16-18,25-27,30]. However, no reports have been published regarding substances deeply involved in the change from FOC-3 without the potentiality of peritoneal dissemination into MFOC-3 with the potentiality of peritoneal dissemination, in terms of biological characteristics.

In the present study, we compared these two cell lines with the different potentiality of peritoneal dissemination for the growth potential, as well as the expression of growth factor (HRG)-1α and HRG precursor, and the related HER-2, HER-3, and HER-4 receptors in order to investigate the effect of anti-receptor antibodies on the growth of these cell lines.

## METHOD

### *(1) Cell lines*

FOC-3 cell line : FOC-3 cell line were derived from human ovarian serous adenocarcinoma cell line provided by the Department of Basic Pathology, Fukushima Medical University. FOC-3 cell line fail to form peritoneal dissemination in SCID mice (Formation rate ; 0%(0/8)).

MFOC-3 cell line : FOC-3 cell line were suspended in Matrigel and subcutaneously injected into the inguinal region of SCID mice to produce tumors. MFOC-3 cell line were obtained by subcultures of the produced and grown tumors. MFOC-3 cell line most frequently form peritoneal dissemination in SCID mice (Formation rate ; 81.3%(13/16)).

### *(2) RT-PCR*

When FOC-3 and MFOC-3 cell lines reached 70% to 80% confluence on 100 mm tissue culture dishes, total RNAs were extracted using TRIzol Reagent (Invitrogen) and the phenol/chloroform method. By using the First-strand cDNA synthesis kit (Invitrogen), cDNAs were prepared from FOC-3 and MFOC-3 cell lines. The polymerase chain reaction (PCR) was then performed using these cDNA templates to compare the expression level of HRG. For the PCR reaction, Takara Ex Taq® (Takara), primers : forward 5´-GACCTCTACTTCTCGTGACA-3´ and reverse 5´-TCCAATCTGTTAGCAATGTG-3´[12] were used together with GAPDH as the internal standard. The PCR reaction consisted of 30 cycles, with each one cycle at : 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1.5 minutes. The PCR products were analyzed by 0.8% agarose gel electrophoresis followed by ethidium bromide staining to detect specific bands.

### *(3) Western Blot Analysis*

FOC-3 and MFOC-3 cell lines were incubated on 100 mm tissue culture dishes until they reached 70% to 80% confluence, and washed with PBS, followed by the addition of RIPA buffer (1% Triton X-100, 1% DOC, 0.1% SDS, 150 mM NaCl, 1 mM PMSF, 10 mM Tris·HCl [pH 7.4]) for separation. The supernatant was collected as cell lysates after centrifugation at 10,000 rpm for 10 min at 4°C. The protein concentrations of cell lysates were measured by the Lowry protein assay. 15 $\mu$g of protein were separated by 8-16% SDS-PAGE and proteins were transferred to the nitrocellulose membranes (TEFCO) using semi-dry blotting (150 mA 2 hours). The membranes were soaked in blocking buffer (5% fat-free milk) for 15 minutes. Thereafter, anti-HRG rabbit polyclonal antibody (C-20 Santa Cruz) was used as the primary antibody for both HRG precursor and HRG-1α in 1 to 200 dilution. Furthermore, anti-ErbB2 mouse monoclonal IgG2 antibody (F-11, Santa Cruz), anti-ErbB3 mouse monoclonal IgG2 antibody (G-4, Santa Cruz), and anti-ErbB4 mouse monoclonal IgG2 antibody (C-7, Santa Cruz) were used for HER-2, HER-3, and HER-4, respectively, in 1 to 100 dilution. The reaction was allowed to proceed overnight at room temperature. THP-1 cell (human monocytic leukemia, Cosmo Bio.) was used as the positive control for HRG. Then, after washing with TBST [20 mM Tris (pH 7.5), 138 mM NaCl, 0.1% Tween20], Anti-rabbit IgG, peroxidase-linked species-specific whole

antibody (from donkey) (Habersham Pharmacia Biotech), or Anti-mouseIgG, peroxidase-linked species-specific whole antibody (from sheep), was used as the secondary antibody in 1 to 5,000 dilution. The reaction was allowed to proceed for one hour at room temperature. Chemiluminescence was performed using ECL Western blotting detection reagents and analysis system (Amersham Biosciences), and the light was captured on X-ray film.

*(4) Cell growth of FOC-3 and MFOC-3 cell lines*

A total of $5.0\times10^4$ FOC-3 and MFOC-3 cell lines were seeded on 60 mm tissue culture dishes, and incubated in Dulbecco's Modified Eagle's Medium (SIGMA) containing 10% heat inactivated fetal bovine serum (FBS) using an incubator with temperature, humidity, and $CO_2$ controlled at 37°C, 90%, and 5%, respectively. On day 1 to 4 of incubation, the number of cells was counted using 3 dishes for each cell line. After trypan blue treatment, the number of cells was counted in duplicate for each dish using a hemocytometer (a total of 6 measurements) to prepare the cell growth curve.

*(5) Cell growth assay with the addition of Recombinant Human HRG-1αEGF Domain*

A total of $3.0\times10^4$ FOC-3 and MFOC-3 cell lines were seeded on 60 mm tissue culture dishes, followed by addition of recombinant human HRG-1α EGF Domain (rHRG-1α R&D, Systems Inc.) for cell incubation. A rHRG-1α non-addition group was used as the control group. In a rHRG-1α addition group, FOC-3 and MFOC-3 cell lines were incubated with rHRG-1α addition levels of 1, 10, and 100 ng/ml. On day 5 of incubation, the number of cells was counted in duplicate for 3 dishes from each group (a total of 6 measurements). The number of cells of each rHRG-1α addition group was divided by the number of cells in the control group to examine the growth potential.

*(6) Cell growth inhibition assay with the addition of anti-receptor antibody*

A total of $1.0\times10^4$ FOC-3 and MFOC-3 cell lines were seeded into 24-well plates for incubation. rHRG-1α was added at a concentration of 100 ng/ml. The anti-receptor antibodies shown below were used: 50 μg/ml Anti erbB-2/HER-2 mouse monoclonal IgG1 antibody (Ab-16 LAB VISION)[12], 10 μg/ml Anti erbB-3/HER-3 mouse monoclonal IgG1 antibody (cloneH3.105.5 Upstate) and 10 μg/ml Anti erbB-4/HER-4 mouse monoclonal IgG2a antibody (cloneH4.72.8 Upstate)[13]. The experimental design was as follows ; (a) Control group (rHRG-1α non-addition group), (b) Anti-receptor antibody addition group, (c) rHRG-1α addition group, (d) rHRG-1α+ anti-receptor antibody addition group. In each group,the cells were incubated using 3 wells for each group. After 4 days, the number of cells was counted using a hemocytometer (a total of 3 measurements for each group). The number of cells counted was divided by the number of cells in the control group to examine the growth inhibition.

*(7) Statistical analysis of significant difference*

Student's t-test (two-sided) was used for testing the difference in means between the 2 groups. For the rHRG-1α addition growth assay, Dennett's pairwise multiple comparison t test was used. A $P$ value $< 0.05$ was considered statistically significant.

## RESULT

*(1) RT-PCR*

RT-PCR was performed using primer for HRG for FOC-3 and MFOC-3 cell lines. The results showed that the expression of HRG mRNA was higher in MFOC-3 cell line than in FOC-3 cell line (Fig. 1).

*(2) Western Blot Analysis*

SDS-PAGE Western blotting was performed using 15 μg proteins extracted from FOC-3 and MFOC-3 cell lines to investigate the difference in HRG protein expression between the 2 groups. The results showed that the expression of HRG precursor of molecular weight 70 kDa and HRG-1α of molecular weight 44 kDa was promoted in MFOC-3 cell line (Fig. 2). Additionally, other than growth

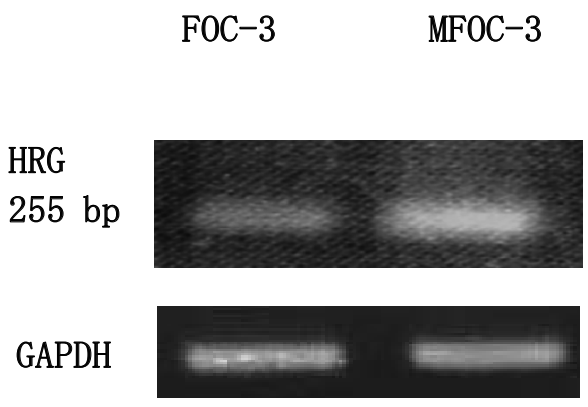


Fig. 1. RT-PC Rof HRG mRNA and GAPDH in FOC-3 and MFOC-3 cell lines

factor, protein expression levels of HRG related receptors, i.e., HER-2 (molecular weight 185 kDa), HER-3 (200 kDa), and HER-4 (180 kDa) were examined. Results found significant protein expressions of all receptors studied in MFOC-3 cell line (Fig. 3).

*(3) Comparison of growth of FOC-3 and MFOC-3 cell lines*

Comparison of FOC-3 cell line and MFOC-3 cell line for cell growth showed that the number of cells significantly increased in MFOC-3 cell line on day 1 to 4 ($p<0.05$, Fig. 4).

*(4) Cell growth assay with the addition of rHRG-1α*

After adding rHRG-1α to FOC-3 cell line and MFOC-3 cell line, the number of cells in the 2 groups was compared (Fig. 5). No changes were observed in the number of cells in FOC-3 cell line after the addition of rHRG-1α. In contrast, in MFOC-3 cell line, the number of cells increased in a concentration-dependent manner, and the increase was statistically significant at the maximum concentration of 100 ng/ml ($p<0.05$).

*(5) Cell growth inhibition assay with the addition of anti-receptor antibody*

The effect of anti-HER-2 antibody and rHRG-1α on cell growth of FOC-3 and MFOC-3 cell lines was shown in Fig. 6. In FOC-3 cell line, no effect of anti-HER-2 antibody on cell growth was observed regardless of the addition of rHRG-1α. On the other hand, in MFOC-3 cell line, significant cell growth inhibition was noted with the addition of anti-HER-2 antibody ($p<0.05$). In particular, the addition of anti-HER-2 antibody remarkably inhibited the cell growth effect produced by the addition of rHRG-1α ($p<0.05$).

The effect of anti-HER-3 antibody and rHRG-1α on cell growth of FOC-3 and MFOC-3 cell lines was shown in Fig. 7. In FOC-3 cell line, no effect of anti-HER-3 antibody on cell growth inhibition was observed regardless of the addition of rHRG-1α. In MFOC-3 cell line, no effect of anti-HER-3 antibody on cell growth inhibition was observed, but significant cell growth inhibition was noted with the addition of anti-HER-2 antibody when rHRG-1α was added ($p<0.05$).

The effect of anti-HER-4 antibody and rHRG-1α on cell growth of FOC-3 and MFOC-3 cell lines was shown in Fig. 8. In both cell lines, no effect of anti-HER-4 antibody on cell growth was observed regardless of the addition of rHRG-1α.

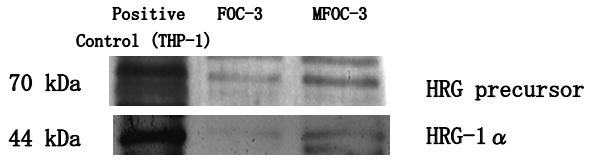


Fig. 2. Western blotting of HRG Precursor and HRG-1α proteins in FOC-3 and MFOC-3 cell lines

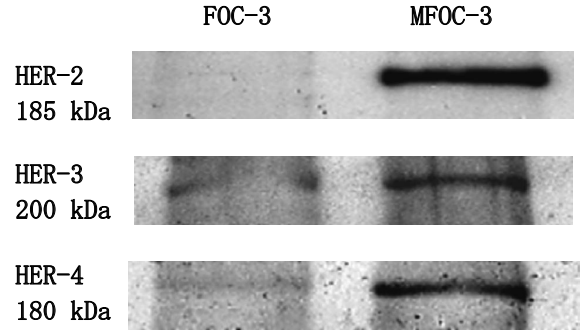


Fig. 3. Western blotting of HER-2, HER-3, and HER-4 proteins in FOC-3 and MFOC-3 cell lines

## DISCUSSION

Conventionally, it has been suggested that adhesion molecules, cell growth factors, angiogenic factors, and matrix metalloproteinase may be involved in the mechanism of the formation of peritoneal dissemination associated with ovarian cancer, but a full elucidation has not yet been achieved. Clarification of the molecular-level mechanism of the formation of peritoneal dissemination, including the intracellular or intermolecular signal transduction mechanism

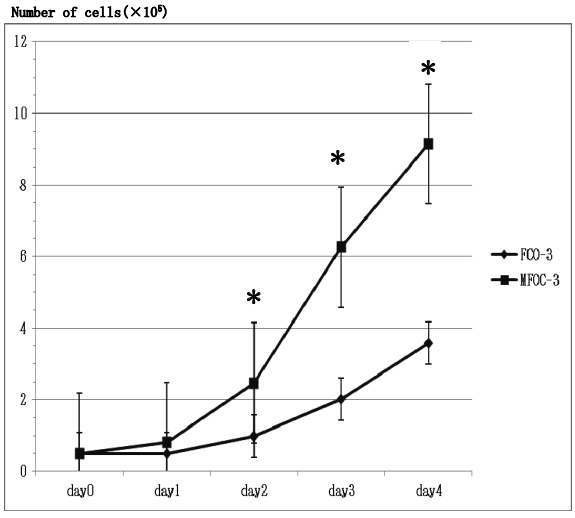


Fig. 4. Curves of cell growth of FOC-3 and MFOC-3 cells. Each value represents the mean±SEM ($n=6$) *$p<0.05$

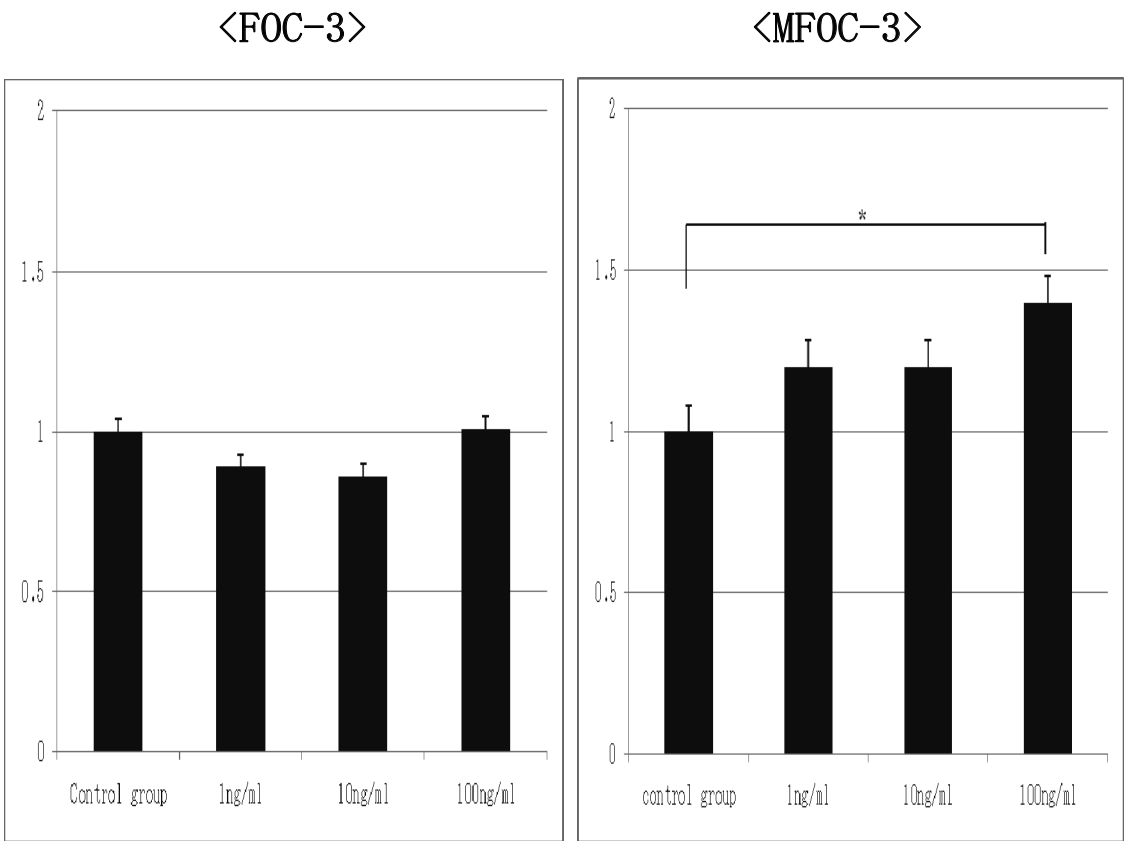


Fig. 5. Concentration-elevation relationships of recombinant human heregulin-1α in cell growth of FOC-3 and MFOC-3 cells.
Each value represents the mean±SEM ($n=6$) $^{*}p<0.05$

is crucially important in the diagnosis, treatment, and management of ovarian cancer[4,5]. In this study, we compared FOC-3 cell line (low potentiality of peritoneal dissemination) and MFOC-3 cell line (high potentiality of peritoneal dissemination) for HRG mRNA expression and protein expression of HRG-1α and related receptors HER-2, HER-3, and HER-4. In addition, we examined the difference in the reactivity for exogenous HRG-1α and conducted research using anti-receptor antibodies for receptors contributing to the promotion of cell growth by HRG-1α.

In 1992, Peles and Wen *et al.* isolated and purified HER-2 (ErbB-2) ligand from culture supernatant of C-Ha-ras transformed rat fibroblast, and named it neu differentiation factor (NDF)[6,7]. Around the same time, Holmes *et al.* isolated and purified a protein from cell culture supernatant of human breast cancer cell lines MDA-MB-231 and named it HRG[8]. Additionally, Falls[9] and Marchionni[11] demonstrated that factors identified as acetylcholine receptor inducing activity (ARIA) or glial growth factor (GGF) and NDF are encoded by the same gene and provided the single unified name neuregulin (NRG) to all these factors[11,12]. In the present study, heregulin (HRG) was used as the unified name.

HRG is highly expressed in the mammary glands, brain, ovaries, testes, and small intestine in normal tissues. The HRG gene is located at 8p12-p22 in the human chromosome. Multiple isoforms are produced by alternative splicing. These isoforms are classified into alpha, beta, or gamma based on the diversity of the EGF domain[14]. To date, many reports regarding HRG and HER-2 receptors using breast cancer and ovarian cancer cell lines have been disclosed, suggesting that HRG may induce cell growth in multiple cancer tissues and cancer cell lines[11]. In the immunohistological study of ovarian cancer, HRG-1α and HRG-1β were expressed in cancer cells, not in interstitial cells, at the frequency of 87% and 77%, respectively[12]. In the same study, the authors reported that HRG mRNA was expressed in 83% of ovarian cancer tissues and in 89% of ovarian cancer cell lines[12]. For the histological type of ovarian cancer, it has been reported that HRG-1α expression was higher in

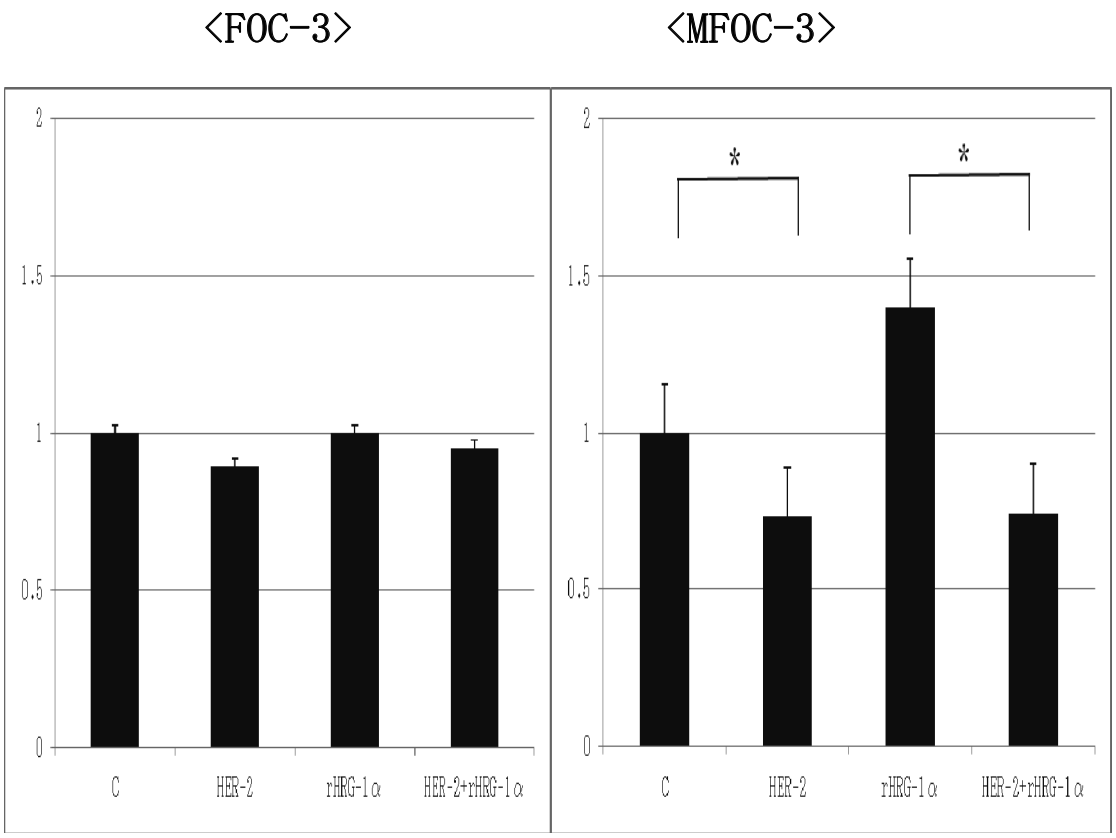


Fig. 6. Assay of cell growth by FOC-3 and MFOC-3 cells with anti-HER-2 antibody or recombinant human heregulin-1α. Each value represents the mean±SEM ($n=6$) $*p<0.05$
C : Control, HER-2 : anti-HER-2 antibody, rHRG-1α : recombinant human heregulin-1α

serous adenocarcinoma than in endometrioid adenocarcinoma, contributing to the promotion of cancer cell growth[14].

In this study, HRG mRNA expression, protein expression of HRG precursor and HRG-1α, and the growth of MFOC-3 cell line were promoted in MFOC-3 cell line suggesting that HRG may play an important role in the increased growth potential of peritoneal dissemination of ovarian cancer.

As to growth factor receptors (HER/ErbB), the presence of epidermal growth factor receptor (EGFR ; ErbB-1), HER-2 (ErbB-2), HER-3 (ErbB-3), and HER-4 (ErbB-4), which possess tyrosine kinase activity, has been identified[14]. Of these, 3 receptors, that is, HER-2, HER-3, and HER-4, are involved as HRG-related receptors. Past literature regarding HER (ErbB) receptors reported that multiple types of HER receptors were more frequently expressed in malignant tumors and advanced cancer than in benign tumors and borderline malignancies, significantly influencing tumor invasion and progression[15]. Notwithstanding the above findings, the specific mechanism responsible for the abnormal growth of cancer cells still remains unclear[16]. Until recently, HER-2 overexpression in various types of cancers has been reported[10]. However, due to lack of identification of HER-2 specific ligands, studies of receptor activation and intracellular signal transduction pathways have been limited[17]. Then, HRG was initially identified as the HER-2 ligand, and thereafter it was demonstrated that HRG binds to HER-3 and HER-4, rather than directly binding to HER-2[11]. Later, it was elucidated that these receptors form homodymers or HER-2/HER-3 and HER-2/HER-4 heterodimers, promoting intracellular downstream signal transduction[16,18,19]. In other words, when HRG or transmembrane HRG precursor with functional activity binds to these receptors, the receptors form dimers, and tyrosine kinase is activated. Specific tyrosine residues undergo phosphorylation, and their receptors activate mitogen activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/Akt, or other elements involved in complex signal transduction pathways (Fig. 9)[38]. As a result, malignant changes in cells, specifically, biological responses, such as cell cycle promotion, cell growth promotion, differentiation induction, apoptosis, and development of resistance to anticancer

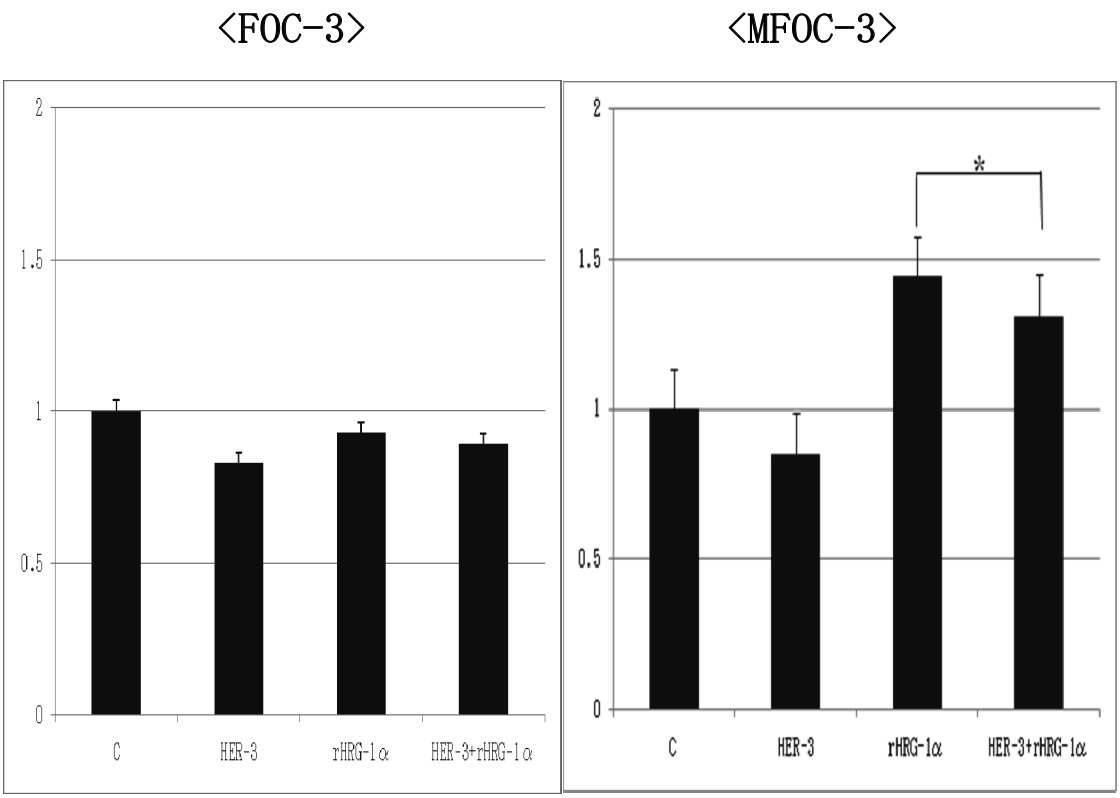


Fig. 7. Assay of cell growth by FOC-3 and MFOC-3 cells with anti-HER-3 antibody or recombinant human heregulin-1α. Each value represents the mean±SEM ($n=6$) $*p<0.05$
C : Control, HER-3 : anti-HER-3 antibody, rHRG-1α : recombinant human heregulin-1α

drugs, are induced[14,17,20,21,37]. The results of a study using multiple cell lines revealed that changes in HER-2 expression or HER-2/HER-3 expression may be involved in the promotion of cellular proliferative potential and the promotion of the colony-forming ability associated with HRG cell cycle progression. It has been reported that HER-2/HER-3-mediated signal transduction may be particularly and strongly involved in cell growth, compared with HER-2/HER-2 or HER-1/HER-2 mediated signal transduction[12,22,37].

From a clinical perspective, an association between HER-2 overexpression, observed in 25% to 30% of human breast cancer, ovarian cancer, and lung cancer, and the prognosis for the diseases has been pointed out[14,18,23,26]. In particular, it is known that the prognosis for HER2-positive breast cancer tends to be poor and that any therapeutic effect may be enhanced with a regimen consisting of chemotherapy plus an anti-HER2 antibody, an anticancer agent such as Trastuzumab[25,27,28]. In vivo studies using ovarian cancer cell line SKOV-3 has shown that Trastuzumab inhibited growth[12]. Regarding ovarian cancer, it has been reported that HER-2 overexpression was noted in 71% of the patients with stage 3 advanced cancer[29]. And in those with recurrent ovarian cancer after chemotherapy[30], but the extent of the association between HER-2 overexpression and the prognosis for the disease still remains mostly unclear[15,25,31,32]. Unfortunately, in a report of the result of a clinical study by Michael A *et al.* in 2003, the response rate of treatment with Trastuzumab alone was low at 7.3%[36]. Nevertheless, in patients with advanced or recurrent ovarian cancer, suppression of tumor progression may be achieved with a combination of anti-HER2 antibody, anticancer agents with other anticancer drugs, or co-administration with molecular target drugs targeting both EGFR/HER-2 receptors, such as gefitinib and erlotinib which may provide a cooperative inhibitory effect.

In fact, in vitro and in vivo studies have shown that erlotinib has activity against human colorectal, head and neck, NSCLC (Non-small cell lung cancer) and pancreatic tumor cells. Further preclinical studies suggest that erlotinib may also have activity against tumors that are dependent on HER2 activation for growth and survival[34]. Akita has reported that combining erlotinib with cisplatin, doxorubicin, gemcitabine or low-dose paclitaxel had an additive

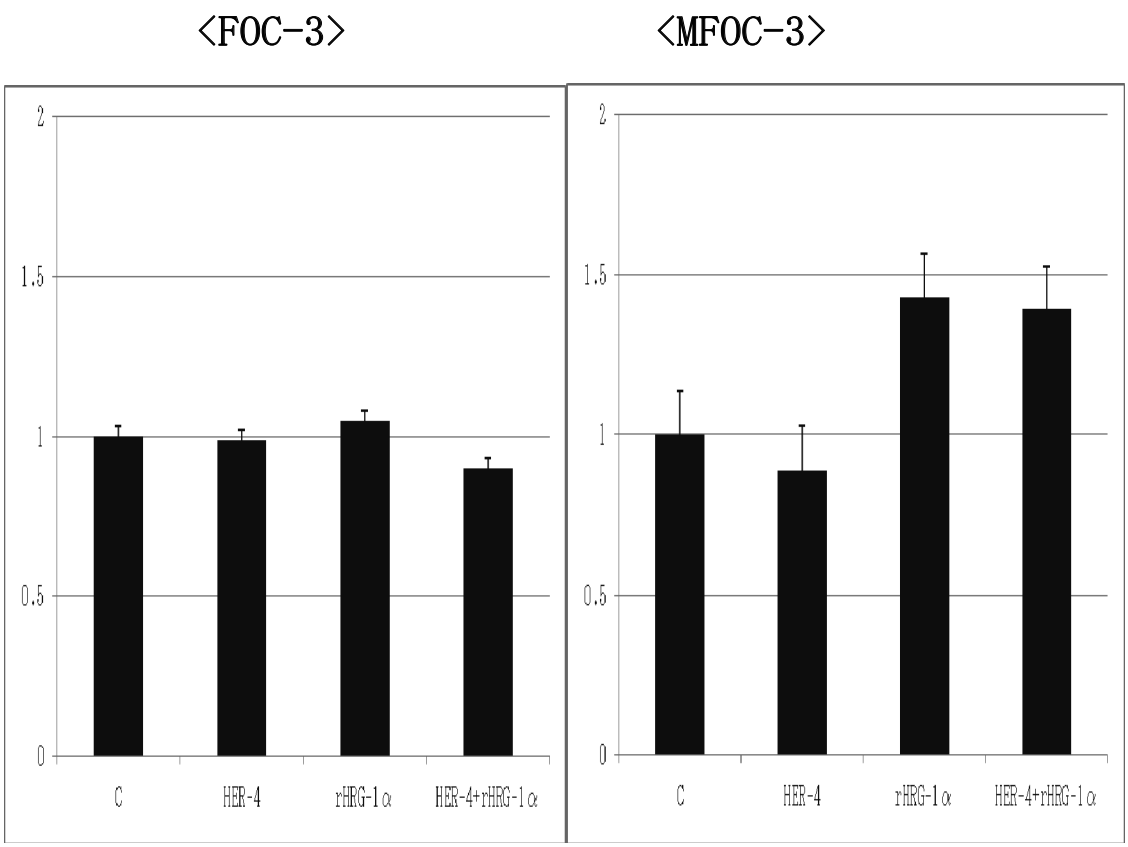


Fig. 8. Assay of cell growth by FOC-3 and MFOC-3 cells with anti-HER-4 antibody or recombinant human heregulin-1α. Each value represents the mean±SEM ($n=6$) $*p<0.05$
C : Control, HER-4 : anti-HER-4 antibody, rHRG-1α : recombinant human heregulin-1α

effect on antitumor acitivity[35].

An in vitro research study reported that heregulin responses were inhibited by inhibited binding of HER-2 to high-affinity receptors such as HER-3 or HER-4 through the use of anti-HER2 antibody in breast cancer cell lines T47D or MCF7[11]. In the colon cancer cell line GEO anti-HER2 antibody may block intracellular heregulin (HRG) induced signal transduction to MAPK and Akt[17]. In view of these findings, HER-2 may play a role as a primary coordinator of the signal network, rather than a ligand binding receptor[14,16,37]. Furthermore, it has been reported that HER-2/HER-3 heterodimers play a crucial role in HRG induced signal transduction[16,37].

In this study, we added exogenous HRG-1α to MFOC-3 cell line and found that cell growth was promoted. Moreover, in an anti-receptor antibody addition study intended to suppress intracellular signal transduction, it was demonstrated that anti-HER2 receptor antibody, which failed to produce a cell growth inhibitory effect in FOC-3 cell line, exerted a favorable inhibitory effect on MFOC-3 cell line. In addition, anti-HER3 receptor antibody also showed a growth inhibitory effect only on MFOC-3 cell line under the conditions where excessive rHRG-1α was added. Compared with anti-HER2 receptor antibody, the effect of anti-HER3 receptor antibody was less potent. The possible reason behind this may include the following : anti-HER3 receptor antibody is a monoclonal antibody, the antibody titer differs, and the effect of HER-3-non-independent growth factor is present in the media. Finally, the signal transduction pathway mediated by growth factor HRG-1α and HER-2 is found to be associated with the promotion of cell growth in MFOC-3 cell line.

In conclusion, it was suggested that HRG may play an important role in the increased growth potential of peritoneal dissemination of ovarian cancer. In particular, HER-2, which can act as a starting point to trigger intracellular signal transduction pathways, is strongly involved in the progression of cancer. Therefore, molecular target drug therapies blocking the HER-2 receptor are promising candidates for ovarian cancer treatment in the future.

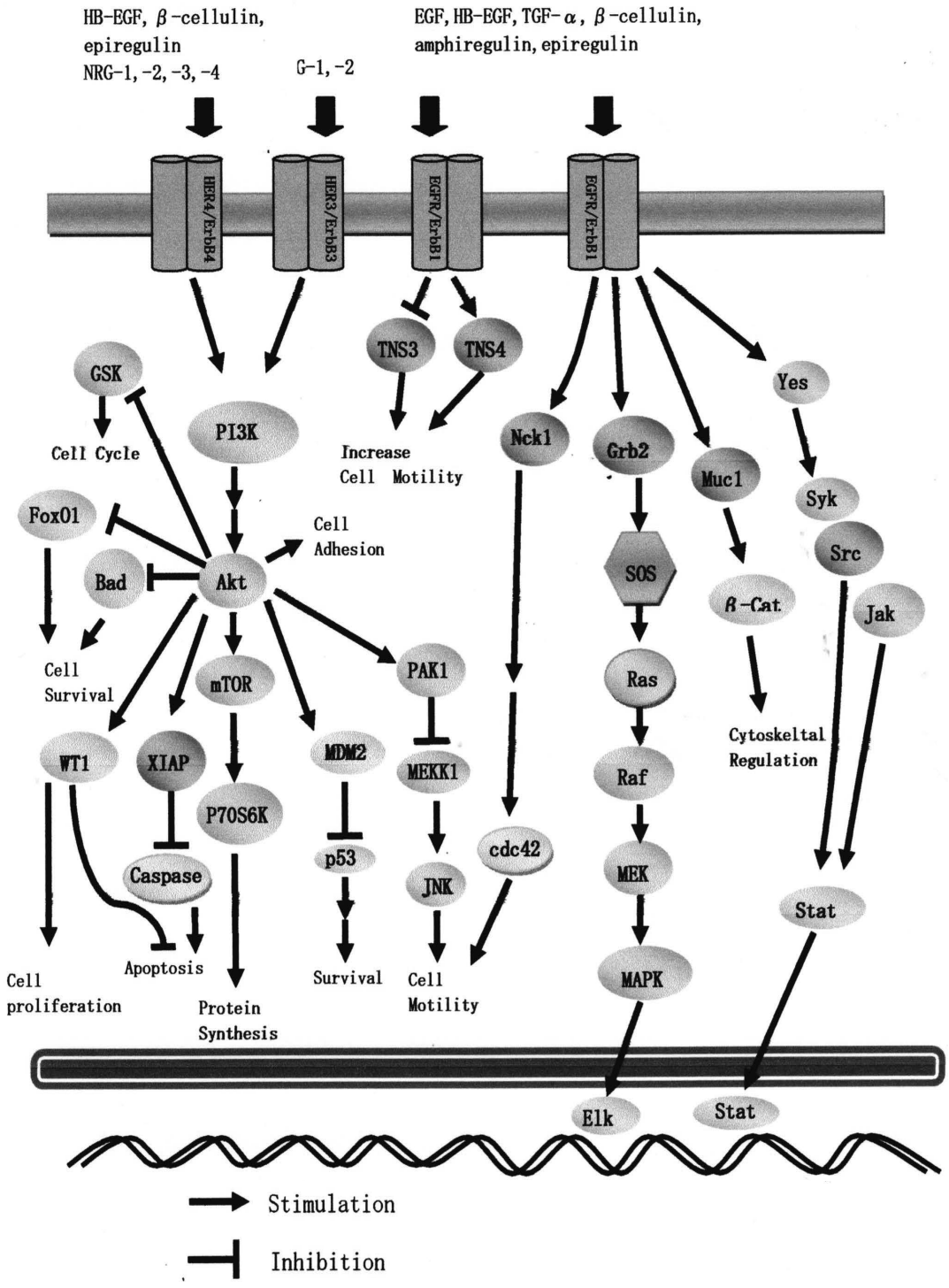


Fig. 9. ErbB/HER signaling pathway
Cell Signaling Technology, Inc. <http://www.cstj.co.jp/pathways/tyrosine.php modification>

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