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HER2 mediates epidermal growth factor-induced down-regulation of E-cadherin in human ovarian cancer cells

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ABSTRACT

Overexpression of HER2 is correlated with a poor prognosis in many types of human cancers. Due to the interaction between HER2 and other ErbB receptors, HER2 is implicated in the EGF family of ligands-regulated tumor progression. In ovarian cancer, although the relationships between HER2 amplification and patient prognosis remain controversial, the underlying molecular mechanisms of HER2-mediated tumor progression are not fully understood. Our previous studies demonstrated that EGF induces ovarian cancer cell invasion by down-regulating E-cadherin expression through the up-regulation of its transcriptional repressors, Snail and Slug. It has been shown that overexpression of HER2 down-regulates E-cadherin expression in human mammary epithelial cells. However, whether HER2 mediates EGF-induced down-regulation of E-cadherin remains unknown. In this study, we examined the potential role of HER2 in EGF-induced down-regulation of E-cadherin and increased cell invasion. We show that EGF treatment induces the interaction of EGFR with HER2 and increases the activation of HER2 in human ovarian cancer cells; we also show that these effects are diminished by knockdown of EGFR. Importantly, treatment with HER2-specific tyrosine kinase inhibitor, AG825, and HER2 siRNA diminished the up-regulation of Snail and Slug as well as the down-regulation of E-cadherin by EGF. Finally, we also show that EGF-induced cell invasion was attenuated by treatment with HER2 siRNA. This study demonstrates an important role for HER2 in mediating the effects of EGF on Snail, Slug and E-cadherin expression as well as invasiveness in human ovarian cancer cells.

1. Introduction

The ErbB family comprises four members: epidermal growth factor receptor (EGFR; ErbB1), HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). These receptors belong to the family of receptor tyrosine kinases (RTKs), which have an extracellular ligand-binding domain and a cytoplasmic tyrosine kinase-containing domain. The EGFR family not only regulates normal biological functions such as cell proliferation, migration, invasion and differentiation but also plays important roles in regulating tumorigenesis [1,2]. A number of different EGF family of ligands bind to ErbB receptors, and based on their receptor specificity, they can be divided into three groups [3]. EGF, transforming growth factor α (TGF-α) and amphiregulin belong to the first group, which binds specifically to EGFR [2]. Despite having no direct identified ligand, HER2 can be activated via dimerization with other ligand-bound ErbB family receptors [4,5].

ErbB receptors, in particular EGFR and HER2, have been implicated in the development and progression of many types of human cancer, including ovarian cancer [2,6–8]. In human ovarian cancer, overexpression and activating mutations of EGFR have been detected and correlated with more aggressive clinical behavior and a poor prognosis [7,9–11]. However, a recent study demonstrated that amplification of HER2 is rare in ovarian cancer and is not associated with patient prognosis, contradicting earlier studies [12,13]. Despite this controversy, heterodimerization of HER2 and EGFR potentiates EGFR-mediated signaling [14,15]. Pertuzumab, a monoclonal antibody, binds to HER2 and inhibits the dimerization of HER2 with other ErbB family receptors [16]. In this context, pertuzumab can be used for treatment with tumors expressing HER2 at a lower level. A recent study shows that treatment with pertuzumab modulates the expression of a variety of genes and decreases tumor volume in ovarian cancer xenografts [17]. These results indicate that, regardless of the levels of HER2, dimerization of HER2 with other ErbB receptors also plays important roles in regulating of tumor progression.

The loss of E-cadherin is the most important event in epithelial–mesenchymal transition (EMT) and is associated with epithelial tumor cell invasion [18]. We have previously shown that treatment

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with EGF increases invasiveness of human ovarian cancer cells by down-regulating E-cadherin expression [19–21]. The effect of EGF on the down-regulation of E-cadherin is mainly caused by the up-regulation of Snail and Slug, the transcriptional repressors of E-cadherin [19–21]. It has been shown that overexpression of HER2 down-regulates E-cadherin expression in human mammary epithelial cells [22]. However, in ovarian cancer, the involvement of HER2 in EGF-induced down-regulation of E-cadherin and cell invasion remains unknown. In the current study, we tested the hypothesis that HER2 mediates the EGF-induced suppression of E-cadherin expression in human ovarian cancer cells. Our results indicate that EGF treatment increases phosphorylation of HER2 and dimerization of HER2 and EGFR in human ovarian cancer cells. Consistent with our previous study, EGF down-regulates E-cadherin expression and increases cell invasiveness. Moreover, by using HER2 siRNA, our results reveal that HER2 mediates EGF-induced changes in Snail, Slug, E-cadherin and cell invasion.

2. Materials and methods

2.1. Cell culture

The SKOV3 human ovarian cancer cell line was obtained from American Type Culture Collection (Manassas, VA). OVCAR5 ovarian cancer line was kindly provided by Dr. T.C. Hamilton (Fox Chase Cancer Center, Philadelphia, PA). Cells were grown in a 1:1 (v/v) mixture of M199/MCDB105 medium (Sigma–Aldrich, Oakville, ON) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.2. Antibodies and reagents

The monoclonal anti-E-cadherin antibody was obtained from BD Biosciences (Mississauga, ON). The polyclonal anti-phospho-HER2 (1:1000) and monoclonal anti-HER2 (1:3000) antibodies were obtained from Cell Signaling Technology (Danvers, MA). Polyclonal anti-EGFR (1:1000) and anti-actin (1:5000) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Bio-Rad Laboratories (Hercules, CA). Horseradish peroxidase-conjugated donkey anti-goat IgG was obtained from Santa Cruz Biotechnology. Human epidermal growth factor (EGF) was obtained from Sigma.

2.3. Immunoprecipitation

Cells were lysed in lysis buffer (Cell Signaling Technology), and the total cell lysates were used for immunoprecipitation. Immunoprecipitation was conducted using the Catch and Release v2.0 kit (Millipore, Temecula, CA) according to the manufacturer's protocol.

2.4. Small interfering RNA (siRNA) transfection

To knockdown endogenous EGFR and HER2, cells were transfected with 50 nM ON-TARGET*plus* SMART*pool* EGFR or HER2 siRNA (Dharmacon Research, Inc., Lafayette, CO) or siCONTROL NON-TARGETING *pool* siRNA (transfection control, Dharmacon) using Lipofectamine RNAiMAX (Invitrogen, Burlington, ON).

2.5. Western blots

Cells were lysed in lysis buffer (Cell Signaling Technology), and protein concentrations were determined using a DC protein assay kit with BSA as the standard (Bio-Rad Laboratories). Equal amounts of protein were separated by SDS polyacrylamide gel electrophoresis and transferred to PVDF membranes. Following blocking with Tris-buffered saline containing 5% non-fat dry milk for 1 h, membranes were incubated overnight at 4 °C with primary antibodies followed by incubation with HRP-conjugated secondary antibody. Immunoreactive bands were detected with enhanced chemiluminescent substrate (Pierce, Rockford, IL). Membranes were stripped with stripping buffer at 50 °C for 30 min and reprobed with anti-actin as a loading control.

2.6. Reverse transcription quantitative real-time PCR (RT-qPCR)

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed with 3 μg RNA, random primers and M-MLV reverse transcriptase (Promega, Madison, WI). The primers used for SYBR Green RT-qPCR were as follows: E-cadherin, 5′-ACAGCCCCGCCTTATGATT-3′ (sense) and 5′-TCGGAACCGCTTCCTTCA-3′ (antisense); Snail, 5′-CCCCAATCGGAAGCCTAACT-3′ (sense) and 5′-GCTGGAAGGTAAACTCTGGATTAGA-3′ (antisense); Slug, 5′-TTCGGACCCACACATTACCT-3′ (sense) and 5′-GCAGTGAGGGCAAGAAAAAG-3′ (antisense); and GAPDH, 5′-GAGTCAACGGATTTGGTCGT-3′ (sense) and 5′-GACAAGCTTCCCGTT CTCAG-3′ (antisense). RT-qPCR was performed on an Applied Biosystems 7300 Real-Time PCR System (Perkin-Elmer) equipped with a 96-well optical reaction plate. All RT-qPCR results are a mean of at least three independent experiments, each of which was assayed in triplicate. Relative quantification of mRNA levels was performed using the comparative Ct method with GAPDH as the reference gene and with the formula $2^{-\Delta\Delta Ct}$.

2.7. Invasion assay

Invasion assays were performed in Boyden chambers with minor modifications [23]. Cell culture inserts (24-well, pore size 8 μm; BD Biosciences, Mississauga, ON) pre-coated with growth factor-reduced Matrigel (40 μL, 1 mg/mL; BD Biosciences) were used for invasion assays. Cell culture inserts were seeded with 1×10^5 cells in 250 μL of medium with 0.1% FBS. Medium with 10% FBS (750 μL) was added to the lower chamber and served as a chemotactic agent. After 24 h incubation, non-invading cells were wiped from the upper side of the membrane, and cells on the lower side were

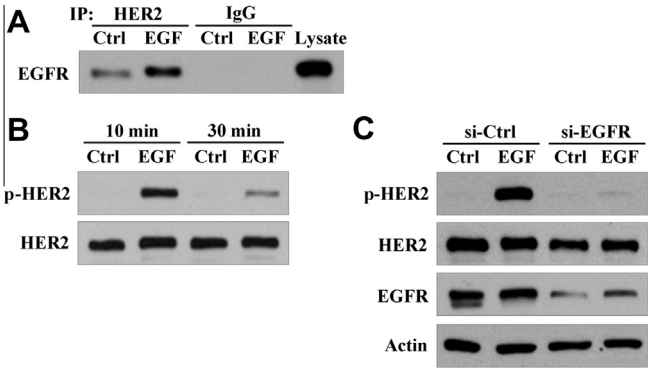

Fig. 1. EGF induces formation of EGFR/HER2 heterodimers and activation of HER2. (A) SKOV3 cells were treated with vehicle control (Ctrl) or 100 ng/mL EGF for 1 h, and the interaction between EGFR and HER2 was analyzed by immunoprecipitation. The total cell lysates were used as a positive control for the Western blot. (B) SKOV3 cells were treated with vehicle control (Ctrl) or 100 ng/mL EGF, and phosphorylation levels of HER2 were analyzed at different time points by Western blot. (C) SKOV3 cells were transfected for 48 h with 50 nM control siRNA (si-Ctrl) or EGFR siRNA (si-EGFR) and then treated for 10 min with vehicle control (Ctrl) or 100 ng/mL EGF. The phosphorylation levels of HER2 were analyzed at different time points by Western blot.

fixed with cold methanol and air dried. Cell nuclei were stained with Hoechst 33258 and counted by epifluorescence microscopy using Northern Eclipse 6.0 software (Empix Imaging, Mississauga, ON). Triplicate inserts were used for each individual experiment, and five microscopic fields were counted per insert.

2.8. MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma) was used to determine cell number. Cells were seeded in a 24-well plate (1×10^4/well) with 500 μL of medium and were treated next day with 100 ng/mL EGF for 24, 48 and 72 h. MTT was added at different time points to a final concentration of 0.5 mg/mL and then incubated for 4 h. The medium was removed, and DMSO was added into each well to dissolve the crystals. The absorbance values were examined by spectrophotometer microplate reader.

2.9. Statistical analysis

The results are presented as the mean ± SEM of at least three independent experiments; they were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Significant differences were defined as $p < 0.05$.

3. Results

3.1. EGF induces formation of EGFR/HER2 heterodimers and activation of HER2

EGF can induce more potent signaling through EGFR/HER2 heterodimers than EGFR homodimers [14,15]. To investigate whether EGF treatment induces the formation of EGFR/HER2 heterodimers, SKOV3 ovarian cancer cells were treated with EGF, and the interaction of EGFR and HER2 was examined by immunoprecipitation. As shown in Fig. 1A, a minimal level of EGFR/HER2 interaction was detected in the control cells, whereas treatment with EGF significantly increased this interaction. Because there is no known ligand for HER2, the activation of HER2 is mainly dependent upon dimerization with other ligand-bound ErbB members [2]. Treat-

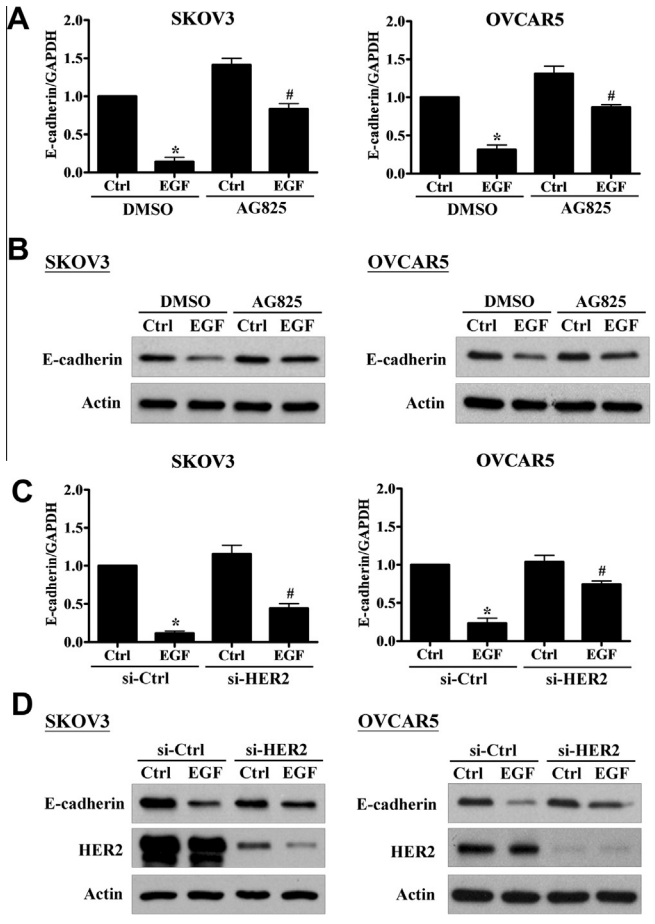

Fig. 2. Inhibition of HER2 attenuates EGF-induced down-regulation of E-cadherin. (A and B), Cells were pretreated with 10 μM AG825 for 30 min and then treated with 100 ng/mL of EGF for 24 h. The mRNA (A) and protein (B) levels of E-cadherin were analyzed by RT-qPCR and Western blot, respectively. (C and D), Cell were transfected for 48 h with 50 nM control siRNA (si-Ctrl) or HER2 siRNA (si-HER2) and then treated for 24 h with vehicle control (Ctrl) or 100 ng/mL EGF. The mRNA (C) and protein (D) levels of E-cadherin were analyzed by RT-qPCR and Western blot, respectively. RT-qPCR results are expressed as the mean ± SEM of at least three independent experiments. $^*p < 0.05$ compared with Ctrl. $^\#p < 0.05$ compared with EGF in DMSO group or EGF in si-Ctrl group.

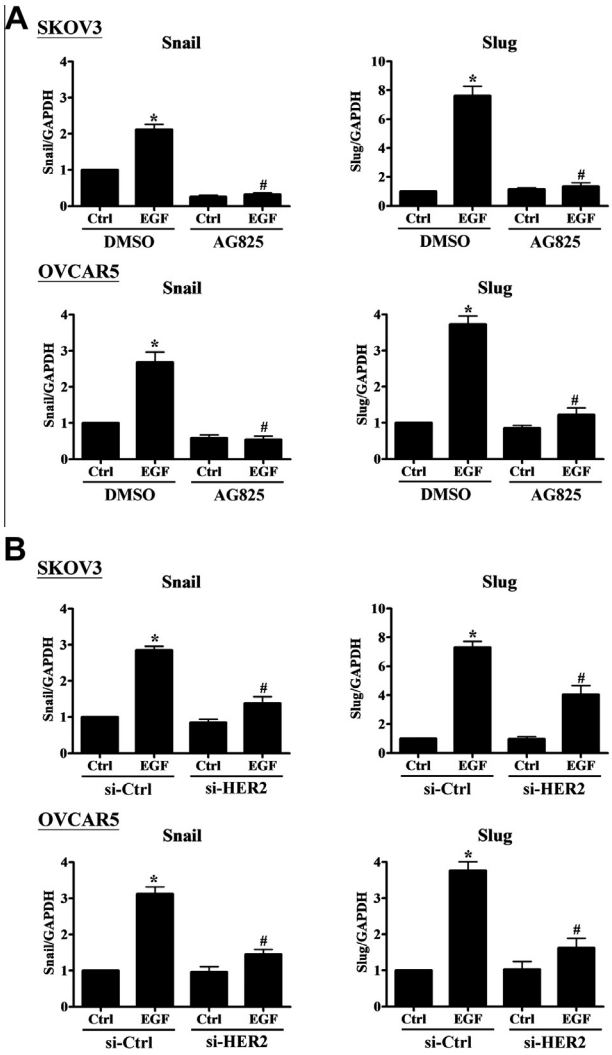

Fig. 3. HER2 mediates EGF-induced Snail and Slug expression. (A) Cells were pretreated with 10 μM AG825 for 30 min and then treated with 100 ng/mL of EGF for 3 h. The mRNA levels of Snail and Slug were analyzed by RT-qPCR. (B) Cell were transfected for 48 h with 50 nM control siRNA (si-Ctrl) or HER2 siRNA (si-HER2) and then treated for 3 h with vehicle control (Ctrl) or 100 ng/mL EGF. The mRNA levels of Snail and Slug were analyzed by RT-qPCR. RT-qPCR results are expressed as the mean ± SEM of at least three independent experiments. $^*p < 0.05$ compared with Ctrl. $^\#p < 0.05$ compared with EGF in DMSO group or EGF in si-Ctrl group.

ment with EGF induced phosphorylation of HER2 in a time-dependent manner (Fig. 1B). To further confirm that this EGF-induced activation of HER2 was mediated by EGFR, specific siRNA for EGFR was used to knockdown the expression of endogenous EGFR. As shown in Fig. 1C, EGFR siRNA significantly down-regulated protein levels of EGFR without affecting HER2 protein levels. In addition, the EGF-induced phosphorylation of HER2 was abolished. These results indicated that EGFR is required for EGF-induced HER2 activation in SKOV3 ovarian cancer cells.

3.2. Inhibition of HER2 attenuates EGF-induced down-regulation of E-cadherin

To investigate the involvement of HER2 in the EGF-induced reduction of E-cadherin, we first used HER2-specific tyrosine kinase inhibitor, AG825, to examine the requirement of HER2 activation in the EGF-induced down-regulation of E-cadherin. Consistent with our previous studies [19–21], EGF treatment decreased E-cadherin mRNA levels in two ovarian cancer cell lines, SKOV3 and OVCAR5. Meanwhile, the EGF-induced down-regulation of E-cadherin mRNA levels was attenuated in the presence of AG825 (Fig. 2A). Western blot analysis showed similar results (Fig. 2B). To avoid the off-target effects of AG825, cells were transfected with siRNA targeting HER2 to knockdown endogenous HER2 expression. As shown in Fig. 2C and D, HER2 siRNA treatment attenuated EGF-induced down-regulation of E-cadherin mRNA and protein levels. Both AG825 and HER2 siRNA did not affect the basal levels of E-cadherin (Fig. 2).

3.3. HER2 mediates EGF-induced Snail and Slug expression

We have previously shown that EGF induces Snail and Slug expression to repress the expression of E-cadherin in ovarian cancer cells [19–21]. Therefore, AG825 and HER2 siRNA were used to investigate whether HER2 mediates the effects of EGF on Snail and Slug. RT-qPCR results demonstrated that treatment with EGF significantly increased Snail and Slug mRNA levels, while these effects were diminished in the presence of the AG825 or HER2 siRNA (Fig. 3).

3.4. HER2 participates in EGF-induced cell invasion

We have previously shown that down-regulation of E-cadherin by EGF enhances ovarian cancer cell invasion [19–21]. Therefore, we used HER2 siRNA to investigate whether HER2 is also involved in EGF-induced cell invasion. Consistent with our previous studies, treatment with EGF for 24 h increased SKOV3 and OVCAR5 cell invasion. Treatment with HER2 siRNA attenuated the level of EGF-induced cell invasion but did not affect basal invasion (Fig. 4A). To confirm that the stimulatory effect of EGF on cell

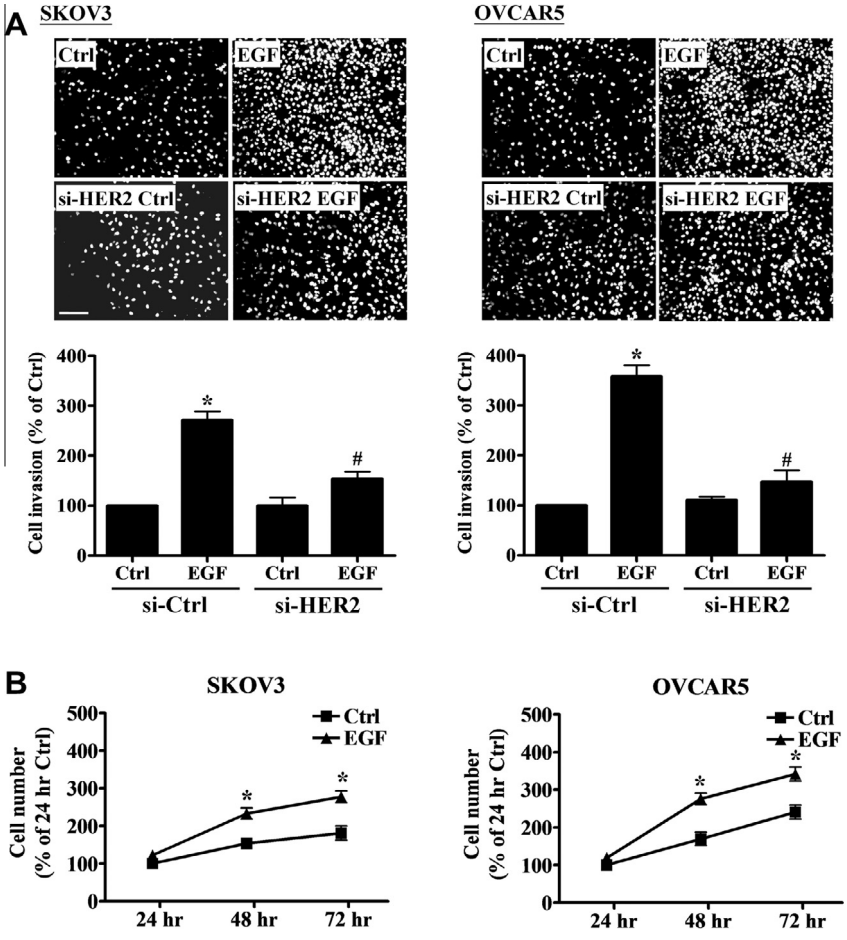

Fig. 4. HER2 participates in EGF-induced cell invasion. (A) Cell were transfected with 50 nM control siRNA (si-Ctrl) or HER2 siRNA (si-HER2) for 48 h. Following transfection, the cells were treated with vehicle control (Ctrl) or 100 ng/mL EGF, seeded into Matrigel-coated transwell inserts and cultured for an additional 24 h. Non-invading cells were wiped from the upper side of the filter, and the nuclei of invading cells were stained with Hoechst 33258. The top panel shows representative fluorescence images of the invasion assay. Scale bar represents 200 μM. The bottom panel shows summarized quantitative results, which are expressed as the mean ± SEM of at least three independent experiments. (B) Cells treated with 100 ng/mL EGF every 24 h and cultured up to 72 h. The MTT assay was used for measuring cell number. $^{*}p < 0.05$ compared with Ctrl or Ctrl in si-Ctrl group. $^{\#}p < 0.05$ compared with EGF in si-Ctrl group.

invasion was not due to the effects of EGF-induced cell growth, cell proliferation after EGF treatment was examined using a MTT assay. In both SKOV3 and OVCAR5 cells, treatment with EGF for 24 h did not significant increase cell proliferation, whereas EGF significantly increased cell proliferation after 2 days of culture (Fig. 4B). Taken together, these results indicate that HER2, at least in part, mediates EGF-induced cell invasion.

4. Discussion

ErbB receptors and their ligands have been detected in both human ovarian cancer cell lines and tissue specimens and are involved in the development of progression of ovarian cancer [7,8]. Overexpression of EGFR and the elevated activated form of HER3 are detected in the mammary tumors of transgenic mice that overexpress HER2 [24,25]. In addition, TGF-α and HER2 exhibit a synergistic effect in the transformation of the mouse mammary epithelium [26]. Moreover, treatment with an EGFR tyrosine kinase inhibitor inhibits the development of mammary tumors in HER2 transgenic mice [27]. In humans, co-expression of EGFR, HER2 and HER3 in breast cancer is associated with poor prognosis and survival [28,29]. Similarly, in lung cancer, co-expression of EGFR and HER2 results in a worse prognosis compared with patients expressing a single receptor [30]. These results clearly indicate that the interaction of HER2 with other ErbB receptor family members may enhance other ErbB receptor-regulated tumorigenesis.

In human ovarian cancer, tumor grade and poor patient outcome are correlated with the high expression levels of EGFR [31]. However, several clinical trials that used EGFR monoclonal antibodies or small molecule tyrosine kinase inhibitors only induced minimal response [6]. Due to the interaction of EGFR with other ErbB receptors and the similar signaling pathways that are induced by all ErbB receptors, treatments that only target EGFR will likely be insufficient. Therefore, several recent trials that target EGFR in combination with other ErbB receptors are under investigation [6]. To date, although some studies have demonstrated the association of HER2 expression and worse prognosis in ovarian cancer, the underlying molecular mechanism of HER2-mediated tumor progression remains largely unknown [7]. Our previous studies have shown that EGF-induced Snail and Slug expression contributes to the EGF-induced down-regulation of E-cadherin and cell invasion in ovarian cancer cells [19–21]. In the present study, we showed that upon EGF stimulation, HER2 dimerized with EGFR and mediated the EGF-induced down-regulation of E-cadherin and cell invasion by increasing Snail and Slug expression. These results provide evidence that HER2 is involved in EGF-induced ovarian cancer invasion and support the current idea that targeting more than one ErbB receptor may be a more effective strategy to treat ovarian cancer.

In summary, this study simply examined the involvement of HER2 in the EGF-induced down-regulation of E-cadherin and cell invasion in ovarian cancer cells. Our results demonstrate that HER2 is activated by EGF treatment, which in turn mediates EGF-induced down-regulation of E-cadherin and cell invasion. Moreover, this study suggests that nuclear EGFR and HER2 contribute to the EGF-induced up-regulation of Snail and Slug and down-regulation of E-cadherin. Consequently, targeting both EGFR and HER2 could be an useful therapeutic strategy for the prevention of ovarian cancer metastasis.

Acknowledgments

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