

Regulation of HRG- β 1-induced proliferation, migration and invasion of MCF-7 cells by upregulation of GPR30 expression

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Abstract. The cooperation and communication between different cell signaling transduction pathways are considered critical in the development of various types of cancer as well as drug resistance. There is evidence of crosstalk between the G protein-coupled receptor 30 (GPR30), the newly discovered estrogen receptor (ER), and the ErbB family. Heregulin (HRG)- β 1, the ligand for ErbB3 and ErbB4, upregulates GPR30 expression in MCF-7, T-47D and BT-474 breast cancer cell lines that express ER α . In the present study, recombinant human HRG- β 1 was used to investigate the upregulation of GPR30 expression by HRGs in MCF-7 breast cancer cells which were ER α -positive. In MCF-7 cells, the ErbB2 inhibitor, AG825, the MAPK inhibitor, PD98059, and the MEK1/2 inhibitor, U0126, blocked the HRG- β 1-induced GPR30 expression. 17- β -estradiol (E2) boosted the HRG- β 1-induced proliferation, migration and invasion of MCF-7 cells. Similar to E2, the specific GPR30 agonist, G-1, promoted HRG- β 1-induced migration and invasion, but inhibited growth. Using the specific GPR30 antagonist, G-15, or the small interfering RNA for GPR30, the functions of GPR30 after treatment with HRG- β 1 were further investigated. The results from our study indicate that the interruption between GPR30 signaling and the ErbB family system may serve as a promising therapeutic strategy for breast cancer.

Introduction

The application of long-term anti-hormonal therapy for estrogen receptor α (ER α)-positive breast cancer benefits the prognosis of patients (1), and has become the standard treatment plan for breast cancer. Nevertheless, the majority of patients relapse with the eventual development of drug resistance, even after having received anti-hormonal therapy. Therefore, endocrine therapy resistance is one of the main challenges in the treatment of ER α -positive breast cancer.

Over the past decade, it has become evident that the communication and cooperation among different signaling networks is critical in the regulation of diverse cellular functions, such as proliferation, differentiation, apoptosis, migration and survival. A number of studies have provided evidence of the existence of crosstalk between ER and growth factor receptor signaling pathways, particularly the ErbB family, which contributes to endocrine therapy resistance in breast cancer (2-4).

The G protein-coupled receptor 30 (GPR30), as a seven-transmembrane domain receptor, belongs to the G protein-coupled receptors (GPCRs) that represent the largest family of membrane receptors. In 2004 and 2005, two studies reported that 17- β -estradiol (E2) binds to and signals through GPR30 with high affinity and *in vitro* potency, respectively (5,6). GPR30 has been speculated to have a potentially important role in cancer progression, including breast, endometrial, prostate, ovarian and thyroid cancers (7-12). A previous study, using a breast cancer patient specimen and cell model, showed that GPR30 signaling is involved in the development of endocrine therapy resistance (13,14). Notably, the ER antagonists found in breast tissue, tamoxifen (TAM) and ICI 182780, act as GPR30 agonists (15,16).

Neuregulins (NRGs; also known as heregulins, neu differentiation factor, acetylcholine receptor-inducing activity and glial growth factor) are a family of ligands for ErbB3 and ErbB4, which consists of NRG1 or heregulin (HRG), NRG2, NRG3 and NRG4. The binding of HRGs to the extracellular domain of the receptor tyrosine kinases, ErbB3 and ErbB4, leads to the formation of ErbB homo- or heterodimers, which in turn activates intracellular signaling pathways and drives cell proliferation, adhesion, differentiation and migration (17). In breast cancer, it has been reported that HRGs contribute to

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Abbreviations: HRG, heregulin; GPR30, G protein-coupled receptor 30; ER, estrogen receptor; siGPR30, small interfering RNA for GPR30; TAM, tamoxifen; GPCRs, G protein-coupled receptors; E2, 17- β -estradiol; MMP, matrix metalloproteinase; FBS, fetal bovine serum; HRP, horseradish peroxidase; CTGF, connective tissue growth factor; FN, fibronectin; CXCR4, chemokine receptor type 4

Key words: G protein-coupled receptor 30, heregulin, proliferation, migration, invasion, breast cancer

the acquisition of anti-hormonal drug resistance characteristic (18,19) and distant metastasis (20-22).

GPR30, as a member of GPCRs, transactivates epidermal growth factor receptor (EGFR) and its downstream signaling (15,23). In turn, the epidermal growth factor (EGF) and transforming growth factor- α (TGF- α) upregulate GPR30 expression in certain ER α -negative and ER α -positive cancer cells, which enhances the proliferative effects of estrogen (11,12). However, little is known about the correlation between GPR30 and HRGs. In the present study, recombinant human HRG- β 1 was used to investigate the upregulation of GPR30 expression by HRGs in MCF-7 breast cancer cells which were ER α -positive. We show that elevated GPR30 expression is involved in the proliferation, migration and invasion induced by HRG- β 1. Our findings may facilitate future studies on the mechanism of endocrine therapy resistance.

Materials and methods

Cell culture and reagents. MCF-7, T-47D and BT-474 breast cancer cell lines were maintained in MEM, DMEM and RPMI-1640 medium (Gibco, Carlsbad, CA, USA), respectively. All of the growth mediums were supplemented with 10% fetal bovine serum (FBS; Gibco).

The following reagents were solubilized as stock according to the manufacturer's recommendations: Recombinant human HRG- β 1 was obtained from PeproTech (Rocky Hill, NJ, USA), E2 and AG825 were from Sigma-Aldrich (St. Louis, MO, USA), AG1478, LY294002, U0126 and PD98059 were from Cell Signaling Technology (Danvers, MA, USA), G-1 was from Merck (Darmstadt, Germany) and G-15 was from Tocris Bioscience (Bristol, UK). Before use, stock solutions were diluted with phenol red-free growth medium. The final DMSO concentration was <0.1% and had no effect on the parameters analyzed.

Western blot analysis. Cells were lysed in M-PER mammalian protein extraction reagent (Thermo Scientific, Barrington, IL, USA) with 1% protease inhibitors cocktail (Sigma-Aldrich). Protein concentration was determined using the bicinchoninic acid protein assay kit (Thermo Scientific). Equal amounts of whole protein samples from the total cell lysates were separated using a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and were then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The blots were incubated overnight at 4°C with primary antibodies. The membranes were washed three times with Tris-buffered saline with Tween-20 and then incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature. Immunoreactive bands were visualized using the EZ-ECL chemiluminescence detection kit for HRP (Bioind, Kibbutz Beit Haemek, Israel). The following monoclonal (m) and polyclonal (p) antibodies (Ab) were used: anti GPR30 pAb (GeneTex, San Antonio, TX, USA); anti- α -tubulin mAb (Beyotime, Haimen, China).

Real-time quantitative polymerase chain reaction. Total cellular RNA in MCF-7 cells was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase I (Promega, Madison, WI, USA) to eliminate contaminated genomic DNA. Real-time reverse transcription PCR (RT-PCR) was

performed using the PrimeScript RT reagent kit and SYBR Premix ExTaq (Takara, Shiga, Japan) following the manufacturer's instructions. PCR amplification was conducted in a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Relative quantification was defined using the $2^{-\Delta\Delta C_t}$ method. The amount of GPR30 mRNA was normalized to the internal control GAPDH. The primers used were: 5'-GGTGCCAGGAC AATGAAATACTC-3' (GPR30 forward) and 5'-GATCCGCA CATGACAGGTTTATTG-3' (GPR30 reverse); 5'-GAAGGTG AAGGTCGGAGTCAAC-3' (GAPDH forward) and 5'-CCT GGAAGATGGTGATGGGATT-3' (GAPDH reverse).

Small interfering RNA (siRNA) transfection. MCF-7 cells were transiently transfected with the human GPR30 siRNA (siGPR30) or non-specific pre-synthesized control siRNA using Lipofectamine 2000 reagent (Invitrogen), following the manufacturer's instructions. These were used to interfere with GPR30 expression. The sequences of GPR30 siRNA were 5'-GCUGUACAUUGAGCAGAAATT-3' (sense) and 5'-UUU CUGCUCAAUGUACAGCTT-3' (antisense (GenePharma, China). All transfections were conducted in 6-well plates.

Cell proliferation assay. MCF-7 cells were grown in 24-well plates in complete medium for 24 h and then switched to phenol red-free medium with 2% charcoal-stripped FBS and the indicated treatments, which were renewed every two days. After treatment for three days, cells were harvested by trypsinization and the cell number was determined using a hemocytometer.

Migration and invasion assays. MCF-7 cells were serum-starved for 24 h, and then incubated with the indicated treatments for 24 h in phenol red and serum-free medium. Subsequently, cells were trypsinized, washed and resuspended in phenol red-free MEM with 1% BSA. A total of 1×10^5 cells in 200 μ l MEM/well with indicated reagents were loaded onto the upper compartments of Boyden chambers (Costar, Corning, NY, USA) and allowed to migrate in the presence of 400 μ l of MEM containing 10% FBS which had been added to the lower compartments prior to the experiment. After incubation at 37°C for 48 h, the migratory cells attached to the bottom surface of the membrane were stained with 0.1% crystal for 30 min at room temperature and counted.

The invasion experiments were performed with the chambers coated with 40 μ l Matrigel (BD Biosciences, Bedford, MA, USA), which had been diluted in serum-free medium at the ratio of: matrigel:medium, 1:3. Cells were allowed to invade through the Matrigel membrane for 48 h. The other operations were the same as in the above-mentioned migration experiments.

Statistical analysis. Data were expressed as the means \pm SD. Statistical differences were evaluated by ANOVA followed by Newman-Keuls test. $P < 0.05$ denoted a statistically significant difference.

Results

HRG- β 1 upregulates GPR30 expression in MCF-7 cells. In ER-positive cancer cells, the EGFR ligands, EGF and TGF- α ,

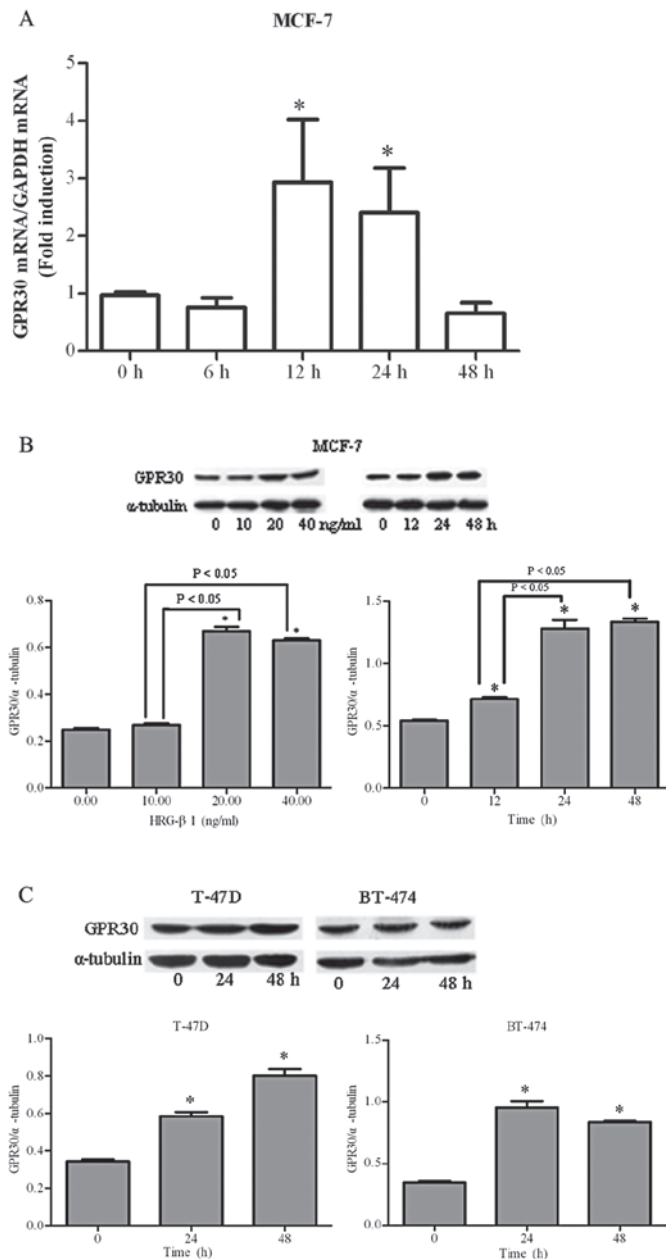


Figure 1. Heregulin (HRG)- β 1 upregulates G protein-coupled receptor 30 (GPR30) expression in MCF-7 cells. (A) After treatment with 20 ng/ml HRG- β 1 for various periods of time, GPR30 mRNA levels in MCF-7 cells were detected by real-time PCR. The error bars are expressed as the means \pm SD of three independent experiments. GPR30 expression was normalized to that of GAPDH. * $P < 0.05$, for cells that received treatment for different times vs. 0 h. (B) After treatment with various concentrations of HRG- β 1 for 24 h or with 20 ng/ml HRG- β 1 for different periods of time, GPR30 protein expression in MCF-7 cells was evaluated by western blot analysis. α -tubulin served as the loading control. Data shown are representative of three independent experiments. The error bars are expressed as the means \pm SD of three independent experiments. * $P < 0.05$, for cells that received treatment vs. the control group. (C) After treatment with 20 ng/ml HRG- β 1 for 0, 24 and 48 h, GPR30 protein expression in T-47D and BT-474 cells was examined by western blot analysis. α -tubulin served as the loading control. Data shown are representative of three independent experiments. The error bars are expressed as the means \pm SD of three independent experiments. * $P < 0.05$, for cells that received treatment for different times vs. 0 h.

have been shown to upregulate GPR30 expression in Ishikawa endometrial cancer cells and TAM-resistant MCF-7 breast cancer cells (11,12). HRGs, as ligands for ErbB3 and ErbB4,

possibly have the similar ability to regulate GPR30 expression in breast cancer. In our study, a time course investigation in MCF-7 cells following HRG- β 1 treatment was performed by real-time quantitative RT-PCR at first. As shown in Fig. 1A, the GPR30 mRNA expression increased after treatment with 20 ng/ml HRG- β 1 for 12 h and persisted for more than 24 h. Then, with HRG- β 1 being depleted, GPR30 mRNA decreased, even lower than the untreated cells. Next, GPR30 protein levels were examined after treatment with various concentrations of HRG- β 1 for different periods of time. GPR30 protein levels were elevated in a time- and concentration-dependent manner. After ~24 h of treatment, GPR30 protein levels significantly increased; this increase in protein levels occurred at a later stage compared to the increase in mRNA levels (Fig. 1B). We observed that GPR30 protein also increased in both T-47D and BT-474 breast cancer cell lines which expressed ER α and basic levels of GPR30 (Fig. 1C). Therefore, HRG- β 1 has the ability to upregulate GPR30 expression in ER α -positive breast cancer cells.

Upregulation of GPR30 expression by HRG- β 1 is mainly associated with the activation of ErbB2-ERK signaling transduction pathway in MCF-7 cells. It has been established that ErbB3 and ErbB4 prefer to heterodimerize with ErbB2 when binding to HRG- β 1, which leads to receptor tyrosine phosphorylation and activates downstream signaling transduction primarily, including the MAPK/ERK and PI3K/Akt pathways (24). Considering the upregulation of GPR30 expression by EGF and TGF- α through the EGFR-ERK transduction pathway (11,12), we hypothesized that HRG- β 1 may increase GPR30 expression through the ErbB2-ERK signaling system. Therefore, specific pharmaceutical inhibitors were used to elucidate the signaling pathway connected with the regulation of GPR30 expression. As expected, the ErbB2 inhibitor, AG825, the MAPK inhibitor, PD98059, and the MEK1/2 inhibitor, U0126, inhibited the HRG- β 1-induced GPR30 expression, whereas the EGFR inhibitor, AG1478, and the PI3K inhibitor, LY294002, did not exhibit any inhibitory effect (Fig. 2B). These results indicate that HRG- β 1 mediates GPR30 expression primarily through the ErbB2-ERK signal transduction pathway.

GPR30 plays an inhibitory role in the proliferative effect induced by HRG- β 1 in MCF-7 cells. E2 is not only the ligand for ER, but is also the agonist for GPR30 (16). In our study, the proliferative effects in MCF-7 cells induced by E2, HRG- β 1 alone or in combination, were further promoted by the specific GPR30 antagonist, G-15 (Fig. 3A). Likewise, G-15 boosted the growth of MCF-7 cells when they were treated with the specific GPR30 agonist, G-1, HRG- β 1 alone or the combination of both, although G-1 itself inhibited MCF-7 cell proliferation (Fig. 3C). These results were further confirmed after GPR30 knockout using siGPR30 (Fig. 3B and D). Previous studies have shown that the upregulation of GPR30 by EGF engages E2 to boost the proliferation of ER α -negative breast cancer cells and ER α -positive Ishikawa endometrial cancer cells (11,12). However, GPR30 has been shown to have a growth inhibitory effect on certain cell types (25-29). Our findings were consistent with those from other reports demonstrating that the activation of GPR30 signaling inhibits MCF-7 cell growth (26,29).

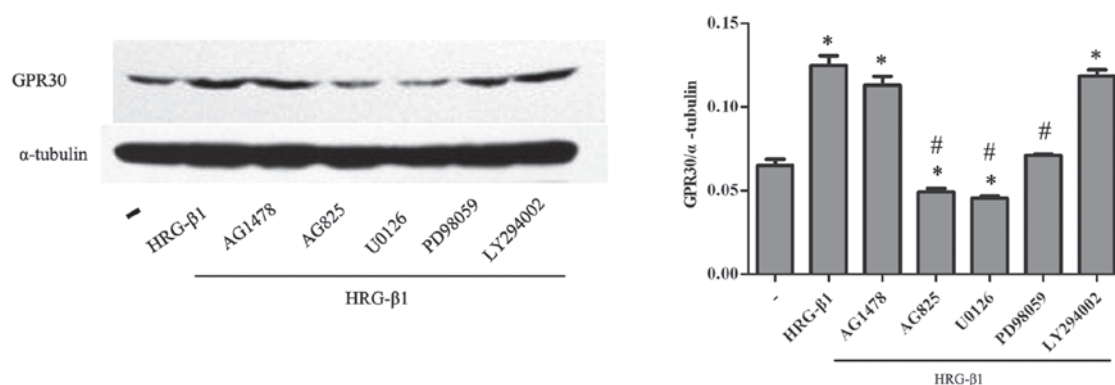


Figure 2. Heregulin (HRG)- β 1 induces G protein-coupled receptor 30 (GPR30) expression in MCF-7 cells primarily through the ErbB2-ERK signaling transduction pathway. Cells were treated with 20 ng/ml of HRG- β 1 alone or in combination with the EGFR inhibitor, tyrphostin AG1478 (10 μ M), the ErbB2 inhibitor, tyrphostin AG825 (10 μ M), the MAPK inhibitor, PD98059 (10 μ M), the MEK1/2 inhibitor, U0126 (10 μ M), and the PI3K inhibitor, LY294002 (10 μ M) for 24 h. All inhibitors were pre-treated for 1 h. GPR30 expression was examined using immunoblot analysis. α -tubulin served as the loading control. Data represent three independent experiments. The error bars are expressed as the means \pm SD of three independent experiments. * P <0.05, for cells that received treatment vs. vehicle (-).

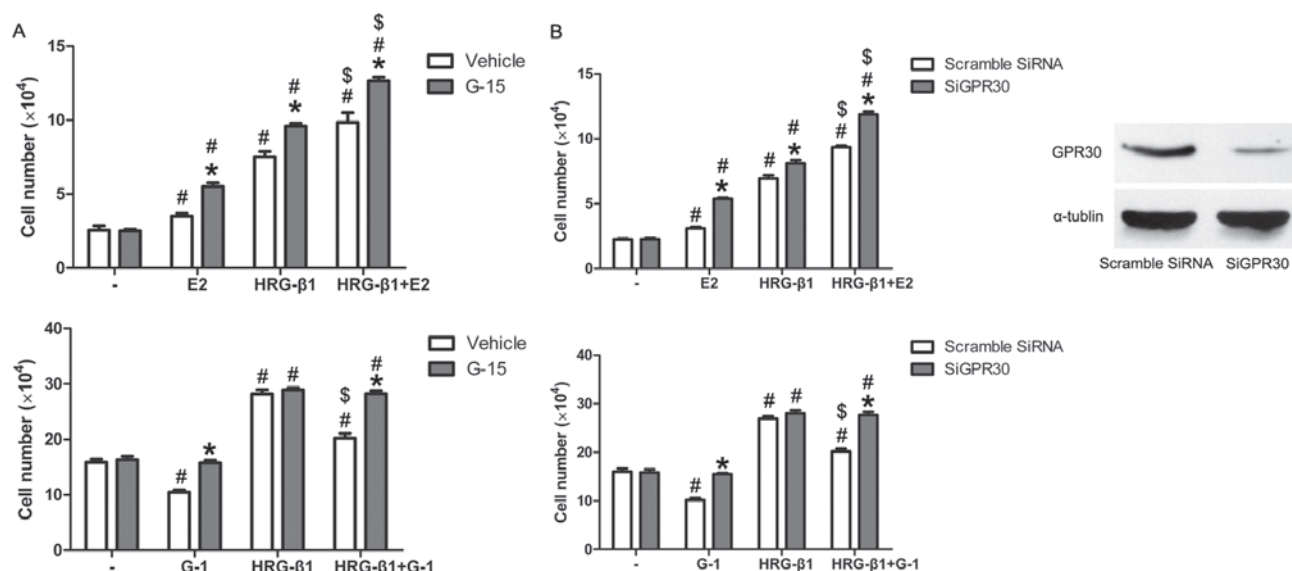


Figure 3. G protein-coupled receptor 30 (GPR30) upregulation inhibits the proliferative effects induced by heregulin (HRG)- β 1 in MCF-7 cells. On the first day, 1×10^4 [17- β -estradiol (E2) group] or 3×10^4 (G-1 group) cells per well were loaded into 24-well plates. Then, cells were incubated with medium containing 2% charcoal-stripped FBS and the indicated reagents. Each data point is the mean \pm SD of three independent experiments performed in triplicate. (A and B) Growth effects influenced by 10 nM E2 alone or in combination with 20 ng/ml HRG- β 1 were boosted by 1 μ M G-15 or GPR30 silencing siRNA (siGPR30). (C and D) Growth effects influenced by 1 μ M G-1 alone or in combination with 20 ng/ml HRG- β 1 were boosted by 1 μ M G-15 or GPR30 silencing siRNA (siGPR30). * P <0.05, for cells that received vehicle vs. G-15, or for cells that were transfected with scrambled siRNA vs. siGPR30. # P <0.05, for cells that received E2 (or G-1) and HRG- β 1 alone or a combination of E2 (or G-1) and HRG- β 1 vs. vehicle (-). \$ P <0.05, for cells that received HRG- β 1 alone or in combination with E2 (or G-1).

Elevation of GPR30 promotes the migration and invasion induced by HRG- β 1 in MCF-7 cells. Since GPR30 and HRG- β 1 signaling has been reported to play a role in tumor metastasis (8,22,30-32), we investigated whether GPR30 plays a role in the migration and invasion induced by HRG- β 1. The migration of MCF-7 cells was analyzed with a Boyden chamber migration assay. In this experiment, the number of cells that had migrated or invaded through the polycarbonate filter during the 48-h treatment period were counted. Indeed, E2, G-1 and HRG- β 1 alone had the ability to stimulate the migration and invasion of MCF-7 cells. With the combination treatment of E2 (or G-1) and HRG- β 1, the migration ability (Fig. 4A and C) and invasion (Fig. 4E and G) were enhanced, which were inhibited after the addition of G-15. Finally, we further confirmed this result

using siGPR30. The knockdown of GPR30 also reduced the migration and invasion induced by HRG- β 1 in MCF-7 cells (Fig. 4B and D). These results support our hypothesis that GPR30 boosts the HRG- β 1-induced migration and invasion of MCF-7 cells.

Discussion

Increasing evidence has demonstrated that the crosstalk between the ErbB family and GPCRs generates a complex signaling network, which plays an important role in cancer progression (33-39). A number of studies have addressed the transactivation of ErbB family members, especially EGFR, due to the activation of GPCRs signaling (37,40,41). Positive

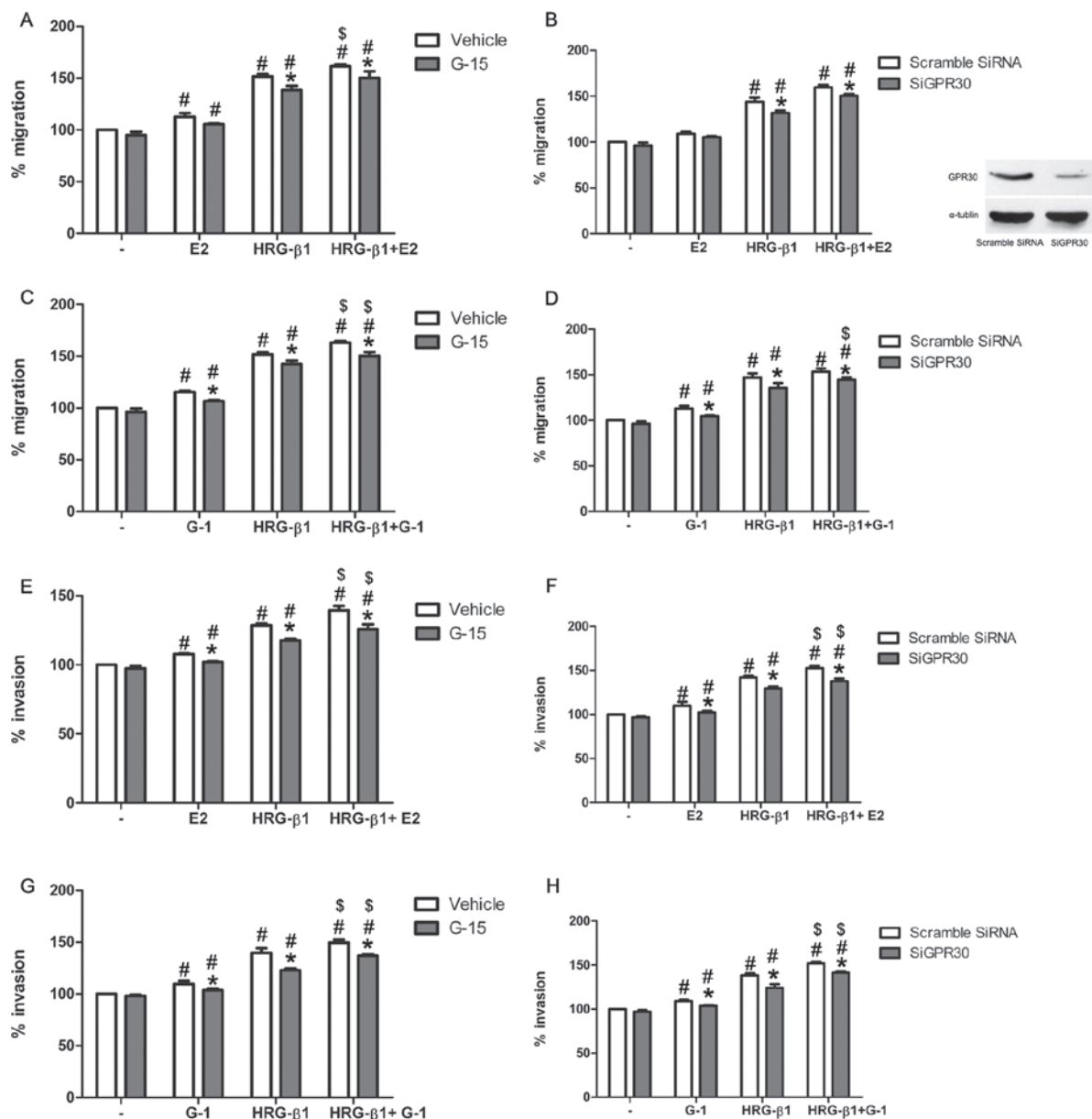


Figure 4. G protein-coupled receptor 30 (GPR30) is involved in the migration and invasion promoted by heregulin (HRG)- β 1 in MCF-7 cells. After being incubated with the indicated reagents for 48 h, cells were counted under eight high-power fields per filter. Each data point is the mean \pm SD of three independent experiments performed in triplicate. (A, B, E and F) Migrations and invasion induced by 10 nM 17- β -estradiol (E2) alone or in combination with 20 ng/ml HRG- β 1 were abolished by 1 μ M G-15 or GPR30 silencing (siGPR30). * P <0.05, for cells that received vehicle vs. G-15, or for cells that were transfected with scrambled siRNA vs. siGPR30. # P <0.05, for cells that received E2 and HRG- β 1 alone or a combination of E2 and HRG- β 1 vs. vehicle (-). \$ P <0.05, for cells that received HRG- β 1 alone vs. a combination of E2 and HRG- β 1. (C, D, G and H) Migrations and invasion induced by 1 μ M G-1 alone or in combination with 20 ng/ml HRG- β 1 were abolished by 1 μ M G-15 or GPR30 silencing (siGPR30). * P <0.05, for cells that received vehicle vs. G-15, or for cells that were transfected with scrambled siRNA vs. siGPR30. # P <0.05, for cells that received G-1 and HRG- β 1 alone, or a combination of G-1 and HRG- β 1 vs. vehicle (-). \$ P <0.05, for cells that received HRG- β 1 alone vs. a combination of G-1 and HRG- β 1.

feedback loops are ubiquitous in cancer; therefore, whether the ErbB family regulates GPCRs is a pressing question. However, only a few reports on the upregulated expression of GPCRs by ErbB family members are available (42-45). The present study focused on the possible regulation of GPR30 by HRGs, another important group of ligands for ErbB receptors. As expected, HRG- β 1 upregulated GPR30 expression in the MCF-7-, T-47D- and BT-474-cultured breast cancer cell lines.

The binding of ligands results in the homo- and/or heterodimerization of ErbB receptors and activation of their intrinsic tyrosine kinase activity. Although ErbB2 is an orphan receptor

with no known ligand, it is the preferred heterodimerization partner of other ErbB family members. On the other hand, ErbB3 requires dimerization with other ErbB members to activate its signaling due to an impaired intracellular tyrosine kinase domain (46). Moreover, ErbB3 constitutes a high-affinity HRG binding receptor only when co-expressed with ErbB2 (47); therefore, HRG binding to ErbB3 is stable and maintains the activation of the downstream signaling pathway (48). ErbB2-ErbB3 has been suggested to be the most potent heterodimer in cancer development (49,50). In previous reports, the activation of the ErbB2-ERK signaling pathway has been

shown to be involved in the regulation of GPCRs expression (39,43,45,51,52). In our study, the ErbB2/neu tyrosine kinase inhibitor, AG825, as well as the MAPK inhibitor, PD98059, and the MEK1/2 inhibitor, U0126, decreased GPR30 expression in MCF-7 cells, which implies that ErbB2/3-MAPK/ERK is the main signaling pathway related to the regulation of GPR30 by HRG- β 1.

Almost half of breast cancer patients with TAM treatment have clinical benefits. Nevertheless, a significant proportion of TAM-responsive breast cancer patients relapse with an anti-estrogen-resistant phenotype, wherein GPR30 overexpression may be involved (53-55). Previous reports have demonstrated that cell models of acquired endocrine resistance have a high migratory capability (56-58), and those patients with acquired endocrine resistance also present with local and/or regional recurrences, frequently with distant metastases (59,60). GPR30 expression is not only associated with cancer growth, but also with metastases development (61). In primary breast tumors (10), epithelial ovarian cancer (62) and endometrial cancer (7), tumors with higher GPR30 expression are more metastatic compared to GPR30-negative tumors. Likewise, aggressive cancer cell lines express high GPR30 levels, whereas their associated normal cell lines show little or no GPR30 expression (63,64). On the other hand, aberrant expression and activation of the ErbB system (e.g., phosphorylated EGFR, ErbB2 and/or ErbB3) and overexpressed ErbB ligands (e.g., TGF- α and amphiregulin) are linked to endocrine therapy resistance in both breast cancer cells and breast tumors (65-67). Previous studies have suggested that HRGs are sufficient for breast cancer initiation and progression by promoting metastasis and angiogenesis; even in the absence of estrogen stimulation and the independence of ErbB2 overexpression (21,22,32,67-69). The blockage of HRG expression suppresses the aggressive phenotype by inhibiting tumor growth and metastasis (32). It has been reported that common target genes relative to metastasis between GPR30 and HRG- β 1 exist, such as connective tissue growth factor (CTGF) (70), fibronectin (FN) (71), chemokine receptor type 4 (CXCR4) (72) and matrix metalloproteinase (MMP)-9 (73). Our results showed that E2 or G-1 boosted the migration and invasion induced by HRG- β 1 in MCF-7 cells. As mentioned above, GPR30 was regulated by HRG- β 1; therefore, all these results imply that GPR30 may be involved in the endocrine therapy resistance and tumor metastasis as a downstream molecule of HRG- β 1.

The recent clinical use of specific signal transduction inhibitors is one of the most promising therapeutic approaches in breast cancers. The MEK inhibitor, PD98059, and the PI3K inhibitor, LY294002, have both been shown to enhance the cytostatic effect of OH-TAM or fulvestrant in MVLN sensitive cells, and the inhibition of MAPK or PI3K/Akt pathways associated with endocrine therapy has been shown to be sufficient to reverse OH-TAM or fulvestrant resistance (74). In our study, the specific GPR30 antagonist, G-15, inhibited the migration and invasion induced by E2, HRG- β 1 alone or the combination of both. Our results imply that the therapy targeting GPR30 signaling may be a promising way to treat breast cancer.

In conclusion, HRG- β 1 upregulates GPR30 expression primarily through the ErbB2-ERK pathway. Considering that the stimulation of GPR30 transactivates EGFR signaling, HRG- β 1 induces a positive loop formation between the ErbB

family and GPR30, EGF and TGF- α (11,12). Moreover, GPR30 boosts HRG- β 1-induced migration and invasion in MCF-7 cells after combination treatment with E2 or G-1, but decreases the proliferation. Therefore, our results suggest that the cooperation between GPR30 and HRG- β 1 may play an important role in endocrine therapy resistance and tumor recurrence. The present findings may facilitate the development of new strategies in controlling human breast cancer.

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