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EGFR and HER2 exert distinct roles on colon cancer cell functional properties and expression of matrix macromolecules ☆

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ABSTRACT

Background: ErbB receptors, EGFR and HER2, have been implicated in the development and progression of colon cancer. Several intracellular pathways are mediated upon activation of EGFR and/or HER2 by EGF. However, there are limited data regarding the EGF-mediated signaling affecting functional cell properties and the expression of extracellular matrix macromolecules implicated in cancer progression.
Methods: Functional assays, such as cell proliferation, transwell invasion assay and migration were performed to evaluate the impact of EGFR/HER2 in constitutive and EGF-treated Caco-2 cells. Signaling pathways were evaluated using specific intracellular inhibitors. Western blot was also utilized to examine the phosphorylation levels of ERK1/2. Real time PCR was performed to evaluate gene expression of matrix macromolecules.
Results: EGF increases cell proliferation, invasion and migration and importantly, EGF mediates overexpression of EGFR and downregulation of HER2. The EGF–EGFR axis is the main pathway affecting colon cancer's invasive potential, proliferative and migratory ability. Intracellular pathways (PI3K-Akt, MEK1/2-Erk and JAK-STAT) are all implicated in the migratory profile. Notably, MT1- and MT2-MMP as well as TIMP-2 are downregulated, whereas uPA is upregulated via an EGF–EGFR network. The EGF–EGFR axis is also implicated in the expression of syndecan-4 and TIMP-1. However, glypican-1 upregulation by EGF is mainly mediated via HER2.
Conclusions and general significance: The obtained data highlight the crucial importance of EGF on the expression of both receptors and on the EGF–EGFR/HER2 signaling network, reveal the distinct roles of EGFR and HER2 on expression of matrix macromolecules and open a new area in designing novel agents in targeting colon cancer. This article is part of a Special Issue entitled Matrix-mediated cell behaviour and properties.

1. Introduction

Colorectal cancer is the third most common cancer and the fourth leading cause of death from cancer worldwide [1]. Mitogenic signals transduced by receptor tyrosine kinases (RTKs) play an important role in the pathogenesis of human cancer [2]. The ErbB family of type I receptor tyrosine kinases (Erbb1 or epidermal growth factor receptor-EGFR, HER2/ErbB2, ErbB3 and ErbB4) are widely expressed in epithelial tissues where they play crucial roles in regulation of cell differentiation, proliferation and survival [3,4].

Although all four ErbB receptors have different ligand-binding properties, they are believed to share an overlapping downstream signaling network. When a ligand binds to type I receptors, dimerization occurs. This causes a conformation change in the receptor that activates the kinase domain and results in autophosphorylation and initiation of divergent signal transduction cascades [5]. Four main intracellular pathways activated by ErbB family receptors are the Ras/mitogen-activated protein kinases (MAPKs) [6], the Janus kinase-signal transduction and activators of transcription (JAK-STAT) [7], the phospholipase Cγ [8] and the phosphatidylinositol 3-kinase/Akt pathways [9]. It is important to report that none of the ErbB family ligands identified for ErbB2, which seems to be transactivated following heterodimerization [10,11]. Owing to its lack of kinase activity, the oncogenic function of ErbB3 is predominantly mediated through overexpression and interaction with EGFR and ErbB2 [12].

Abnormal expression levels of ErbB receptors and their prime natural ligands have been reported as one of the causes of cancer progression. Both EGFR and HER2 have been reported to be overexpressed in up to 85% of colon cancers [13,14]; however, increased EGF expression appears

Abbreviations: EGFR, epidermal growth factor receptor; HER2, human epidermal receptor 2; ECM, extracellular matrix; MMPs, matrix metalloproteinases; MT-MMPs, membrane type-matrix metalloproteinases; TIMPs, tissue inhibitor of matrix metaloproteinases; uPA, plasminogen activator of urokinase; SDC-4, syndecan-4; GPC-1, glypican-1

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to be a poor prognostic indicator in some cancer cases [15,16] and locally increased concentration of the ligands in tumor microenvironment seems to be responsible for preserving heterodimers in an activated state even in the absence of receptor overexpression [17–19]. At present, studies have focused on single blockage of EGFR or HER2, but dual inhibition of the receptors may improve the antitumor activity of colon cancer cells. Considering the therapeutic tools targeting EGFR and HER2, there are two well-identified emerging categories of drugs, the one characterized by antibodies against the extracellular domain of the receptor and the other by soluble tyrosine kinase inhibitors (TKIs) [20–23].

The tumor microenvironment plays an important role in the behavior of malignant cells. Several extracellular matrix (ECM) macromolecules have demonstrated their involvement in the multi-functional network of cell–cell and cell matrix interactions that are important for local tumor invasion and subsequent metastasis [24]. Such molecules are the matrix metalloproteinases (MMPs), which play an essential role in tissue remodeling, associated with various physiological and pathological processes, including morphogenesis, angiogenesis and metastasis, and heparan sulfate proteoglycans, such as syndecans and glypicans. In particular, syndecan-4 has been reported to be involved in cell adhesion and proliferation [25] and glypican-1 is possibly implicated in colon cancer progression [26]. On the other hand, the membrane type MMPs, MT-1 and MT2-MMP have been correlated with the invasive potential of colon cancer cells [27,28]. The importance of MT1-MMP for pericellular proteolysis demands a firm control of its catalytic activity at the cell surface. This is partly achieved by the action of endogenous protease inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), which bind to the active site inhibiting catalysis [29]. MT1-MMP seems to activate pro-MMP2 in the presence of TIMP-2, via a triple complex among those molecules [30]. Moreover, in the presence of high levels of TIMP-2, MT1-MMP activity is inhibited. In addition, a direct binding between TIMP-2 and MT1-MMP has been reported, suggesting that TIMP-2 may act as a highly specific inhibitor of MT1-MMP [27]. TIMP-1, a tissue inhibitor of MMP-9, is overexpressed in colon tumor tissues and increased plasma levels serves a strong marker for short survival and recurrence of disease in patients with colorectal cancer [31]. In vitro analysis has indicated an important role of serine proteases group, involved in extracellular matrix degradation, and in particular of urokinase type plasminogen activator (uPA). uPA can be detected in both tumor and the surrounding stromal microenvironment [32] and upon activation is implicated in matrix degradation and tumor invasion [33]. Recent data of our lab suggest that EGFR inhibition, using the fully human IgG2 anti-EGFR monoclonal antibody panitumumab (pmAb) is of importance for colon cancer progression. Notably, it has been reported that pmAb inhibits MT1-MMP, EMMPRIN, uPA and syndecan-4 gene expression, resulting in an inhibitory outcome on colon cancer cell invasion and migration [34].

The purpose of the present study was therefore to evaluate whether multi-targeting of ErbB family receptors is a promising approach in colon cancer. In particular, we aimed to examine the effect of EGFR inhibition alone and in combination with HER2 inhibition, in the presence and absence of natural ligand EGF, in Caco-2 colon cancer cell line. Caco-2 cells were chosen as they originate as wild type concerning the EGFR, kRas and bRaf genes [35]. We then investigated the changes in colon cancer cells' functional properties, such as cell proliferation and migration and cell invasion and the alterations on EGFR, HER2 and matrix macromolecule expression. Moreover, using specific intracellular inhibitors of EGFR-mediated signaling pathways, we hoped to determine the involvement of particular pathways on matrix effectors' expression.

2. Materials and methods

2.1. Cell culture and reagents

Caco-2 colon cancer cell line was purchased from the American Type Culture Collection. Caco-2 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate and a cocktail of antimicrobial agents containing 100 IU/mL penicillin, 100 mg/mL streptomycin, 10 mg/mL gentamycin sulfate and 2.5 mg/mL amphoterycin B. All cell culture reagents were obtained from Biochrom KG (Berlin, Germany). Cells were cultured at 37 °C in a humidified atmosphere of 5% (v/v) CO2 and 95% air.

EGF was supplied by Sigma Chemical Co. (St. Louis, MO, USA). Selective inhibitors AG1478 (EGFR) and CP-724,714 (HER2) were supplied by Sigma Chemical Co. (St. Louis, MO, USA) and SelleckBio, respectively. Intracellular pathway inhibitors U0126 monoethanolate (MEK1/2), LY294002 hydrochloride (PI3K) and Tyrphostin AG490 (JAK-2) were also supplied by Sigma Chemical Co. (St. Louis, MO, USA). EGF was diluted in phosphate buffered saline (PBS) from Biochrom KG (Berlin, Germany) and all tested inhibitors were diluted in dimethyl sulfoxide (DMSO), except for AG490 which was diluted in absolute ethanol. The final concentration of both DMSO and ethanol in the culture medium was lower than 0.5% which is not toxic to cells. All other chemicals used were of the best commercially available grade.

2.2. Cell proliferation

To determine whether these agents affect the proliferation of Caco-2 colon cancer cell line, the water-soluble tetrazolium salt (WST-1) assay was used. The assay is based on the reduction of WST-1 by viable cells, producing a soluble formazan salt. Caco-2 cells (6×10^3 per well) were plated in 100 μL cell culture medium with 10% FBS in a 96-well plate. 24 h after plating, cells were washed twice with fresh culture medium serum free. Before addition of EGF (20 ng/mL) cells were incubated with the specific inhibitors of EGFR (AG1478) and HER2 (CP-724,714), alone or in combination for 30 min and also the intracellular inhibitors AG490, LY294002 and U0126 for 45 min. After preincubation of inhibitors, stimuli was added to culture medium for 24 h. For long term incubation, cells (10×10^3 per well) were plated in 500 μL cell culture medium in a 48-well plate. 24 h after plating, cells were washed twice with fresh culture medium serum free. After different periods of incubation, cell proliferation was determined using a microplate reader Infinite 200 (Tecan Austria Gmbtl). The absorbance was measured at a wavelength of 440 nm and a reference wavelength of 650 nm.

2.3. In vitro wound healing and invasion assay

Scratch wound migration assay was conducted on wild-type Caco-2 cells after treatment with inhibitors. Cells were seeded in 24-well plate and cultures until 90% confluence. After starving the cells for 24 h in medium without FBS, the confluent cell monolayer was lightly and quickly scratched with a sterile 10 μL pipette tip to produce a straight line. The debris was removed and the edge of the scratch was smoothed by washing twice with fresh culture medium serum free. EGF stimulation was then performed wherever necessary. The wound healing assay was done in FBS-free medium, further excluding any effect due to a potential proliferation difference. The open gap was then inspected and photographed microscopically at indicated times and is shown in the Figures at a 40× magnification. Scion Image software (Scion Corporation Ltd, Frederick, MD, USA) was used to measure closure of the wound over time.

The invasion of Caco-2 on cancer cells was measured by using the 24-well Transwell chamber (Chemicon, Millipore, CA), according to the manufacturers' protocol. Briefly, the cells were seeded onto the membrane of the upper chamber of the transwell at a concentration of 1.7×10^5 cells/mL. Cells were pretreated with the inhibitors for 30 min and then the stimuli were added wherever necessary. The medium in the upper chamber was serum-free. The medium in the lower chamber contained 10% FBS as a source of chemoattractants. Cells that migrated through the membrane were stained with Cell Stain Solution containing crystal violet supplied in the Transwell Invasion assay.

Table 1
Sequences of forward and reverse primers for real-time PCR and RT-PCR.

Gene	Forward (5′–3′)	Reverse (5′–3′)	Annealing (°C)
Real time PCR primers			
EGFR	ATGCTCTACAACCCCACCAC	GCCCTTCGCACTTCTTACAC	60
HER2	CTGCACCCACTCCTGTGTGCACCTG	CTGCCGTCGCTTGATGAGGATC	60
MT1-MMP	CATGGGCAGCGATGAAGTCT	CCAGTATTTGTTCCCCTTGTAGAAGTA	60
TIMP-2	GGGCACCAGGCCAAGTT	CGCACAGGAGCCGTCAC	60
GAPDH	AGGCTGTTGTCATACTTCTCAT	GGAGTCCACTGGCGTCTT	60
RT-PCR primers			
uPA	ACATTCACTGGTGCAACTGC	CAAGCGTGTCAGCCCTGTAG	*56*
MT2-MMP	ACAACCACCATCTGACCTTTAGCA	AGCTTGAAGTTGTCAACGTCCTTC	*62*
TIMP-1	CTTCCACAGGTCCCACAACC	CAGCCCTGGCTCCCGAGGC	*60*
Syndecan-4	CTCCTAGAAGGCCGATACTTCT	GGACCTCCGTTCTCTCAAAGAT	*60*
Glypican-1	ATCACCGACAAGTTCTGGGGTA	CATCTTCTCACTGCACAGTGTC	*60*
GAPDH	TCAAGATCATCAGCAATGCCTCC	AGTGAGCTTCCCGTTCAGC	

Absorbance was measured at 560 nm by ELISA reader after dissolving of stained cells in 10% acetic acid.

2.4. Preparation of proteins and Western blot analysis

Caco-2 colon cancer cells were incubated with EGF (20 ng/mL) at indicated times. ERK1/2 MAPK activation was evaluated by Western blot analysis using phospho-specific Ab for ERK1/2 and a total-specific antibody for ERK1/2 (Cell Signaling Technology) to demonstrate equal loading. At the end of each experiment, Caco-2 cell monolayers were washed with cold PBS and lysed with RIPA (50 mM Tris-HCl pH7.3, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing protease and phosphate inhibitor cocktails (Sigma Chemical Co). 80 μg of total protein lysed in 2× sample buffer and analyzed by SDS-PAGE electrophoresis in 12% gels and transferred to polyvinylidene difluoride membranes (Macherey Nagel, Germany). Membranes were blocked in 5% (w/v) Bovine Serum Albumin (BSA, Sigma Chemical Co.) in Tris-buffered saline pH 7.4 containing 0.05% Tween 20 (TBS-T) for 1 h at room temperature and were then incubated with a polyclonal anti-phospho-p44/p42 MAPK (Cell Signaling, 1:1000) and the polyclonal p44/p42 MAPK (Cell Signaling, 1:1000) for 16 h at 4 °C. After three washes in TBS-T, membranes were further incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (Chemicon, Millipore CA, dilution 1:5000) for 1.5 h at room temperature. Detection of the immunoreactive proteins was performed by chemiluminescence horseradish peroxidase substrate SuperSignal (Pierce), according to the manufacturers' instructions.

2.5. RNA isolation and cDNA synthesis

Caco-2 colon cancer cells were treated with the tested agents at the indicated concentrations. Cells were harvested as before and total RNA was isolated using NucleoSpin RNA II kit (Macherey-Nagel, Duren, Germany). The amount of isolated RNA was quantified by measuring its absorbance at 260 nm. All total RNA preparations were free of DNA contamination, as assessed by no enzyme controls. cDNA was synthesized from 1 μg of total RNA from each sample using random six mers and 200 U/μL PrimeScript RTase (PrimeScript™ 1st strand cDNA Synthesis kit, TAKARA BIO INC).

2.6. Real time and RT-PCR

Real time PCR was performed in 20 μL mixture consisting of 10 μL KAPA SYBR FAST Master Mix (2×) Universal (KAPABIOSYSTEMS), containing *Taq* DNA polymerase, oligonucleotide primers (0.2 μM each) and 1 μM of template cDNA. The amplification consisted of a two-step procedure denaturation at 95 °C for 3 min and 40 cycles with denaturation at 95 °C for 3 s and then annealing/elongation at 60 °C for 20 s using Rotor Gene Q (Giagen, USA). GAPDH was used as an endogenous control. The sequences of forward and reverse primers used for real time PCR amplification are shown in Table 1.

All reactions were performed in triplicate and a standard curve was always included for each pair of primers for assay validation. In addition, a melting curve analysis was performed for detecting the SYBR Green-based objective aplicon. The relative abundance of mRNA of the gene of interest was deducted from the cycle number at which fluorescence

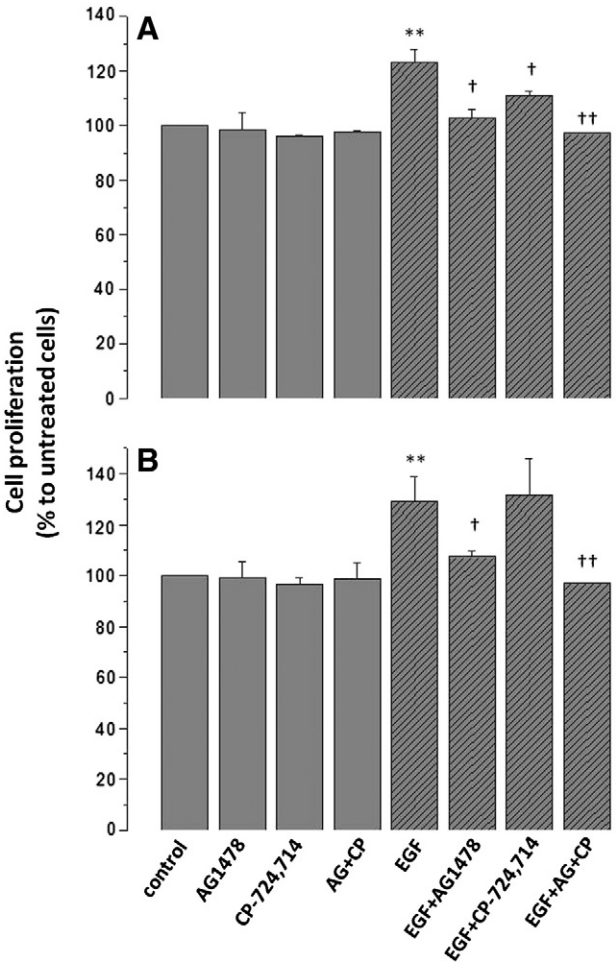

Fig. 1. The effect of EGFR and/or HER2 inhibition on Caco-2 proliferation. Cells were first pre-incubated with AG1478 (1 μM) and CP-724,714 (1 μM) for 30 min, followed by the introduction of EGF (20 ng/mL). Cell proliferation was evaluated for 24 h (A) and 48 h (B). The results are expressed as mean ± SD of three separate experiments in triplicate. Statistically significant differences compared with control and EGF-treated cells are shown by *$p \leq 0.05$, **$p \leq 0.01$ and †$p \leq 0.05$, ††$p \leq 0.01$, respectively.

increased above background level (Ct) in the exponential phase of the PCR reaction, after normalization to the Ct of the calibrator.

Semiquantitative analysis of cDNA sequences was carried out based on simultaneous amplification of a 'housekeeping gene', glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All amplification products were separated by electrophoresis in a 2% agarose gel containing Gel Star stain (BioWhittaker, Rockland, ME, USA). Bands were visualized on a UV lamp and gels were photographed with a charge-coupled device camera. The sequences of primers used for RT-PCR amplification are shown in Table 1. For semiquantitative analysis, gene expression was determined as the relative fluorescence obtained for each molecule compared with the reference gene (GAPDH). Image analysis was performed using the program UNIDOCMV version 99.03 for Windows (UVI Tech, Cambridge, UK).

2.7. Statistical analysis

Differences between groups and controls were tested by unpaired *t* test (one way ANOVA). Each experiment included at least triplicate measurements for each condition tested. All results are expressed as mean ± SD from at least three independent experiments.

3. Results & discussion

3.1. EGF mediates cell proliferation and invasion via the EGF–EGFR axis

A recent study of our lab showed that the blockage of EGFR using a specific monoclonal antibody (panitumumab) leads to the reduction of the EGF-promoting growth in a dose dependent pattern [34]. Here, we first assessed the effect of EGFR inhibitor (AG1478) and HER2 inhibitor (CP-724,714) on cell proliferation of Caco-2 cells, in constitutive and EGF-treated cell cultures. Constitutively, EGFR and/or HER2 inhibition had no significant effect following either 24 or 48 h incubation (Fig. 1A and B). However, EGF treatment significantly increased cell proliferation. The EGF-stimulated cell growth was abolished by both EGFR and combined EGFR/HER2 inhibition reaching levels close to those of control cells. However, inhibition of HER2 following treatment with EGF did not overcome the EGF stimulatory effect (Fig. 1A and B). Considering the fact that EGF selectively binds to EGFR and plays a crucial role in cancer progression [36,37], produced either in an autocrine pattern or in a paracrine one through the microenvironmental cell interactions with cancer cells, it is plausible to suggest that the EGF-mediated activation of Caco-2 cell proliferation is mainly governed via the formation of the homodimer EGFR/EGFR, whereas the formation of the heterodimer EGFR-HER2 has no significant role.

In order to further evaluate the EGF–EGFR effects, we examined whether the EGF-mediated activation via the homodimer EGFR–EGFR is of importance for the invasiveness of colon cancer cells. As shown in Fig. 2, EGF significantly enhanced cell invasion. HER2 inhibition had no significant effect on this EGF stimulatory effect, but the EGFR inhibition abolished the EGF ability to facilitate cell invasion. The obtained data indicated that the EGF-mediated homodimer of EGFR may also be a general mechanism for the invasion potential of these cells.

3.2. Evaluation of intracellular signaling pathways on cell proliferation and migration

To evaluate the intracellular pathways indicated in EGF-mediated cell proliferation, we utilized specific inhibitors either alone or in combination. Particularly, Caco-2 cells were pre-incubated with the inhibitors for JAK2 (AG490, 20 μM), PI3K (LY294002, 20 μM) and MEK1/2 (U0126, 10 μM) and the combination of LY294002/U0126 for 45 min, followed by the addition of EGF for 24 h and 48 h. As shown in Fig. 3A and B, constitutively, all the specific inhibitors appeared to significantly

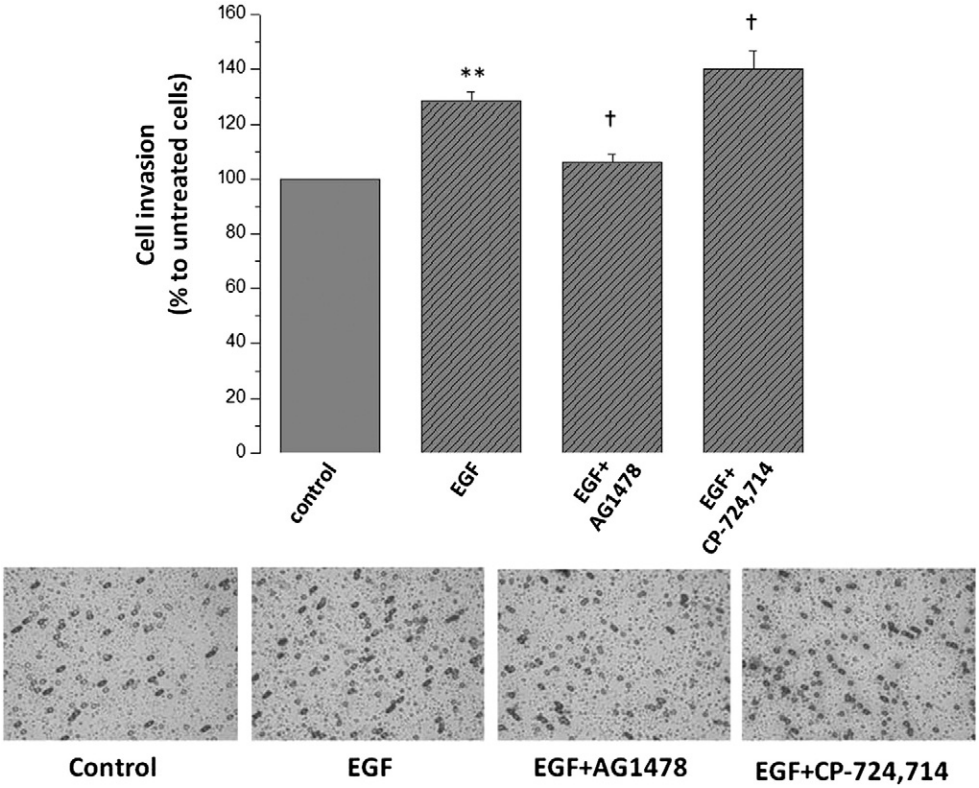

Fig. 2. The effect of EGFR and/or HER2 inhibition on Caco-2 invasive potential. Cells were first pre-incubated with AG1478 (1 μM) and CP-724,714 (1 μM) for 30 min, followed by the introduction of EGF (20 ng/mL). Invasion time was 48 h. Medium supplemented with 10% FBS was used as a chemoattractant. The results are expressed as mean ± SD of three separate experiments in triplicate. Statistically significant differences compared with control and EGF-treated cells are shown by *p ≤ 0.05, **p ≤ 0.01 and †p ≤ 0.05, ††p ≤ 0.01, respectively.

inhibit cell growth for both the 24 and 48 h incubation periods. Moreover, in the EGF-induced cell proliferation, the inhibitions of JAK-2, PI3K and MEK-1/2 abolished the EGF-stimulated cell proliferation. The effect of the combined inhibition of PI3K and MEK1/2 (LY294002 + U0126) was also studied. Notably, this combination significantly attenuated the Caco-2 cell growth at the half of EGF-induced cell proliferation. The above data highlight that the PI3K-Akt, MEK-1/2-Erk and JAK-STAT are all involved in the constitutive and EGF-stimulated cell proliferation.

Cell migration is a highly coordinative process involving precise regulation of cell adhesion and detachment to extracellular matrix proteins [37,38]. In order to assess the role of intracellular pathways downstream of EGFR, in cancer progression, colon cancer cell motility was evaluated with a wound healing assay. As seen in Fig. 4, the PI3K, MEK-1/2 and JAK-2 inhibition, under constitutive conditions, show no significant effect on cell motility, in contrast with the combined inhibition of PI3K and MEK1/2 (LY294002 + U0126), which strongly reduced the migratory ability of Caco-2 cells (≥70%) as compared to that of the untreated cells. EGF, on the other hand, significantly stimulated the cell migration. JAK-2 and PI3K inhibition retarded the cell motility close to the basal levels. Notably the inhibition of MEK1/2 as well the combined inhibition of MEK1/2 with PI3K strongly decreased cell migration as compared to that of EGF-treated cells (−90% and −80%, respectively). The above data indicate that PI3K, MEK1/2-Erk and JAK-2 are all involved in the EGF-mediated Caco-2 cell migration. Moreover, combined blockage of PI3K and MEK1/2 is critical for constitutive and EGF-mediated effects.

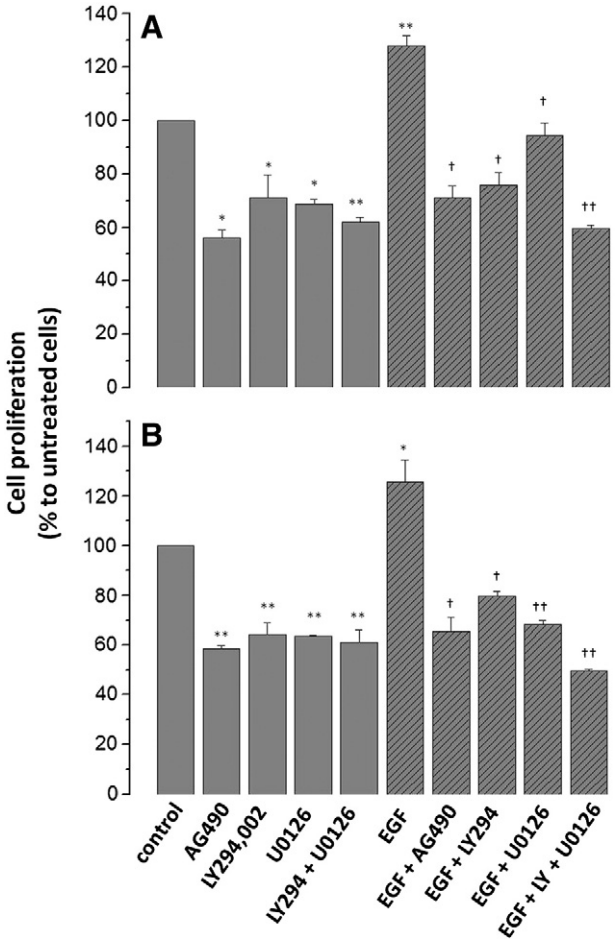

Fig. 3. Evaluation of intracellular pathways on Caco-2 cell proliferation. Cells were first pre-incubated with the inhibitors for JAK-2 (AG490, 20 μM), PI3K (LY294002, 20 μM) and MEK1/2 (U0126 10 μM) for 45 min, followed by the introduction of EGF (20 ng/mL). Cell proliferation was evaluated for 24 h (A) and 48 h (B). The results are expressed as mean ± SD of three separate experiments in triplicate. Statistically significant differences compared with control and EGF-treated cells are shown by $^{*}p \le 0.05$, $^{**}p \le 0.01$ and $\dagger p \le 0.05$, $\dagger\dagger p \le 0.01$, respectively.

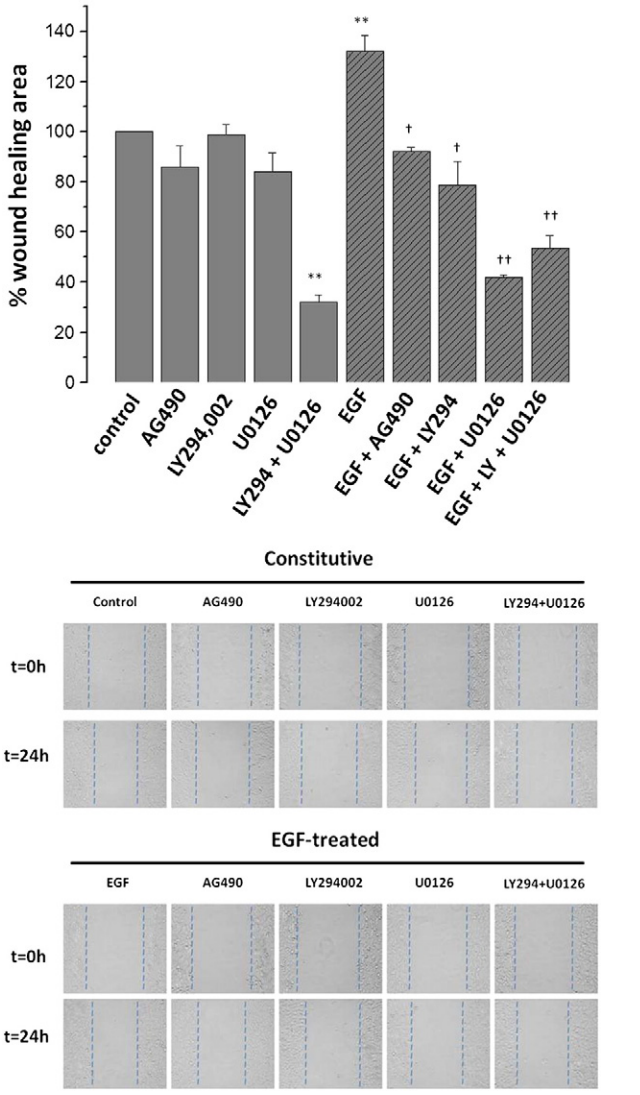

Fig. 4. Effects of EGF and intracellular pathways on migration of Caco-2 colon cancer cells. Confluent monolayers of Caco-2 cells were mechanically wounded with a pipette tip and photos obtained at 0 h and 24 h. Cells were firstly pre-incubated with AG490 (20 μM), LY294002 (20 μM) and U0126 (10 μM) for 45 min, alone and in combination, followed by the introduction of EGF (20 ng/mL). The results are expressed as mean ± SD of three separate experiments in triplicate. Statistically significant differences compared with control and EGF-treated cells are shown by $^{*}p \le 0.05$, $^{**}p \le 0.01$ and $\dagger p \le 0.05$, $\dagger\dagger p \le 0.01$, respectively.

3.3. EGFR-MEK1/2-Erk1/2 signaling axis as regulator of EGF-mediated EGFR expression

Targeting the expression of both ErbB1/EGFR and ErbB2/HER2 may exert additive or even synergistic antitumor activity [39,40]. Therefore, we examined the EGFR mRNA expression levels, by using specific RTKIs against EGFR and HER2 in Caco-2 colon cancer cells. Interestingly, the inhibition of EGFR alone and in combination with the HER2 inhibitor, CP-724,714, constitutively, led to a downregulation of the transcript of the receptor (Fig. 5A). In contrast, the CP-724,714 inhibitor of HER2 alone did not influence the expression levels of EGFR gene. On the other hand, EGF significantly enhanced the transcription of EGFR (Fig. 5A). The EGF stimulatory effect was abolished by AG1478 and the combination of EGFR and HER2 inhibitors, in a significant way. As shown in Fig. 5A, the inhibition of HER2 in the presence of EGF elevated

the EGFR mRNA levels, in comparison with the impact of EGF. These data suggest that the EGFR expression is mediated via an EGF–EGFR activated pathway both in EGF-treated and constitutive conditions.

EGFR activates several downstream pathways including the Ras-MEK-Erk, JAK-STAT and PI3K-Akt. Through these pathways, EGFR regulates the gene expression, proliferation, survival and differentiation of colon cancer cells [41]. To investigate the signaling pathways via which EGFR regulates its gene expression, we used the three selective intracellular inhibitors, as described above, and the mRNA levels of EGFR were assessed by Real-time PCR. As shown in Fig. 5B, the JAK-2 and PI3K inhibition, constitutively, has no significant effect on the gene expression of EGFR. On the contrary, the constitutive inhibition of MEK1/2-Erk pathway alone and in combination with that of PI3K resulted in the upregulation of ErbB1 gene expression, indicating that MEK1/2-Erk axis either alone or in combination with PI3K plays a regulatory role in the constitutive expression of EGFR. However, this is not the case when cells are treated with EGF and, particularly, the inhibition of JAK-STAT and PI3K-Akt axis as well as the combined inhibition of

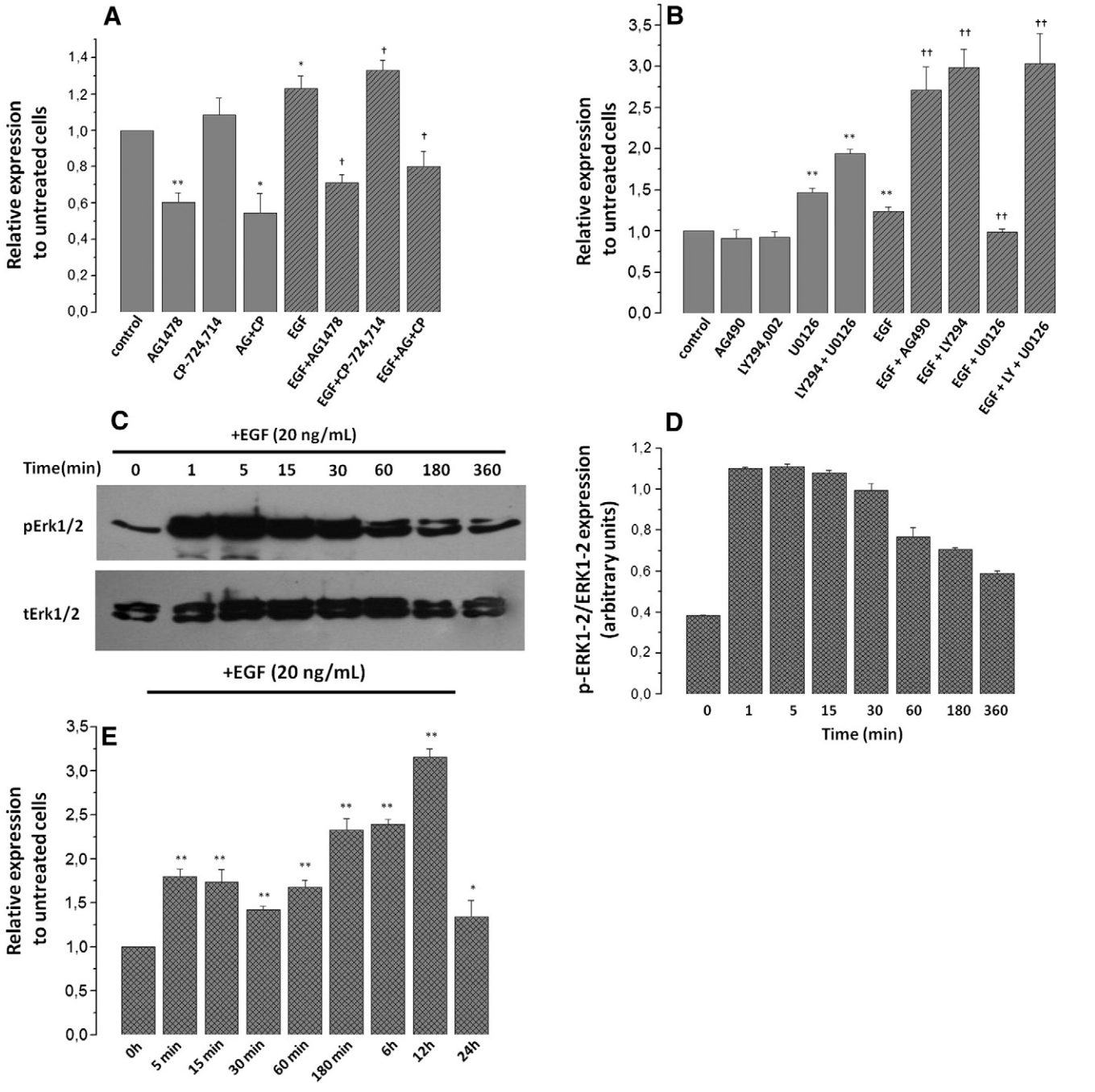

Fig. 5. Evaluation of EGFR gene expression signaling pathway. (A), EGFR and/or HER2 inhibition on EGFR mRNA expression, in Caco-2 colon cancer cells. Cells were treated with AG1478 (1 μM) and CP-724,714 (1 μM), alone and in combination, in the presence and absence of EGF (20 ng/mL). Results are expressed as relative expression and normalized to untreated cells. (B), JAK2, PI3K, MEK1/2 inhibition on EGFR mRNA expression. Cells were treated with AG490 (20 μM), LY294002 (20 μM) and U0126 (10 μM), alone and in combination, in the presence and absence of EGF (20 ng/mL). Results are expressed as relative expression and normalized to untreated cells. (C and D), Caco-2 cells were serum-starved for 24 h and then stimulated with EGF (20 ng/mL) for the indicated time periods. Total protein extracts were analyzed by Western blotting using specific antibodies for total and phosphorylated Erk1/2. (E), Cells were serum-starved (24 h) before stimulation with EGF (20 ng/mL). RNA was isolated at the indicated time points and EGFR mRNA was analyzed by real time PCR. Results are expressed as relative expression and normalized to untreated cells. The results are expressed as mean ± SD of three separate experiments in triplicate. Statistically significant differences compared with control and EGF-treated cells are shown by $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $\dagger p \leq 0.05$, $\dagger\dagger p \leq 0.01$, respectively.

PI3K-MEK1/2, strongly increased the mRNA levels of EGFR (Fig. 5B). Only U0126 (MEK1/2 inhibitor) significantly suppressed the EGF-induced EGFR expression. These results indicate that the intracellular pathways respond differently in the EGF-treated cells as compared to constitutive conditions and that the EGF-mediated internalization of EGFR may be another critical issue in its expression.

To further demonstrate the impact of the MEK1/2-Erk axis on the EGF-induced EGFR expression, we performed a time dependent screening of Erk1/2 protein phosphorylation in the presence of EGF. As shown in Fig. 5C and D, upon treatment of cells at different time points, EGF promoted a strong and rapid, even in 1 min, increase in Erk1/2 phosphorylation that reached a maximum after 5 min and then decreased gradually. In parallel, we examined the expression pattern of EGFR at transcriptional level, following treatment with EGF. In particular, real-time PCR was performed for several time points (including those of Erk1/2 phosphorylation). A gradient increase was noted, with an initial maximum of the EGFR mRNA levels at 5 min and a later at 12 h (Fig. 5E). Taking together the above data, it is suggested that the EGFR-mediated MEK1/2-Erk1/2 signaling axis plays a main and specific role to regulate the expression of EGFR gene in Caco-2 colon cancer cells. Moreover, one could not exclude that these two maxima obtained may be correlated with an initial fast EGFR expression following the activation of EGF–EGFR and a second one utilizing much stronger expression of EGFR due to possible EGF–EGFR internalization.

3.4. HER2 expression is inhibited by EGF

Even if EGFR is of critical importance in colon cancer, HER2/neu also appeared to be significant and has been considered a target molecule in this particular type of cancer [42]. As shown in Fig. 6A, EGF strongly inhibited the mRNA expression of HER2. It is worth noticing that, under constitutive conditions, inhibitions of EGFR, HER2 and of both EGFR and HER2 in combination suppressed the HER2 gene expression.

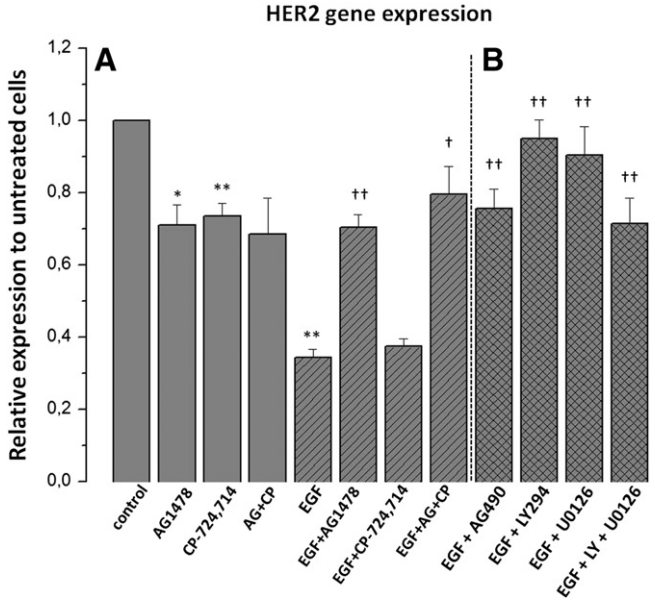

Fig. 6. Evaluation of EGF-mediated expression of HER2. (A), EGFR and/or HER2 inhibition, on HER2 mRNA expression, in Caco-2 colon cancer cells. Cells were treated with AG1478 (1 μM) and CP-724,714 (1 μM), alone and in combination, in the presence and absence of EGF (20 ng/mL). Results are expressed as relative expression and normalized to untreated cells. (B), JAK2, PI3K, MEK1/2 inhibition, on HER2 mRNA expression, in Caco-2 colon cancer cells. Cells were treated with AG490 (20 μM), LY294002 (20 μM) and U0126 (10 μM), alone and in combination, in the presence of EGF (20 ng/mL). Results are expressed as relative expression and normalized to untreated cells. Statistically significant differences compared with control and EGF-treated cells are shown by *$p \leq 0.05$, **$p \leq 0.01$ and †$p \leq 0.05$, ††$p \leq 0.01$, respectively.

In the presence of CP-724,714 however, the levels of HER2 reached those of EGF-treatment cells, suggesting that EGFR exerts its inhibitory effect on HER2 expression.

As HER2 is the most preferred receptor to form heterodimers with EGFR, these receptors may transduce the intracellular signaling using the same downstream signaling pathways. To evaluate the signaling pathway involved in the HER2 gene expression, we used the three specific inhibitors of JAK2, PI3K and MEK1/2, as previously described. EGF treatment led to a significant decrease of HER2 expression, a phenomenon which was abolished in significant profile by all three inhibitors, in the presence of EGF (Fig. 6A and B). These data further indicated that all PI3K-Akt, MEK1/2-Erk and JAK-STAT pathways are involved in the EGF–EGFR mediated inhibitory effect on HER2 expression.

3.5. EGFR and HER2 play distinct roles in the EGF-mediated expression of key matrix macromolecules

Re-organization of the extracellular matrix is an essential process in tumor invasion and metastasis. A major family of ECM degrading enzymes involved MMPs. Among them, MT1-MMP overexpression was noted during multistage tumorigenesis [28] and MT2-MMP inhibition seems to induce inhibitory effect on cell proliferation [43]. We therefore evaluated the expression of MT1- as well as MT2-MMP in colon cancer cells. As shown in Fig. 7A and B, EGF significantly decreased MT1-, MT2-MMP mRNA levels. The blockage of HER2, in the presence of EGF, maintained the expression for both MMPs, whereas the blockage of EGFR resulted in the upregulation of MT1- and MT2-MMP expressions (Fig. 7A, B). Combinatorial inhibition of EGFR and HER2, in the presence of EGF, upregulated the levels of both MMPs. The above data indicate that MT1- and MT2-MMP transcriptions follow a similar profile to that of EGF on HER2 expression and it is plausible to suggest that the EGFR-mediated inhibitory effect on HER2 affects also the expression of MT1-/2-MMPs.

The role of MT1-MMP in cancer metastasis has been previously described in several studies. In other studies it has been attempted to associate the role of MT2-MMP expression with the functional properties of cancer cells, too. According to our results, we demonstrate a clear regulatory role of the EGF–EGFR axis on Caco-2 colon cancer cell invasion. Therefore, we first assumed that this may be a synergistic association between MMP expression and metastatic potential of cancer cells through EGFR signaling. However, EGF stimulation did not succeed in upregulating the levels of MT1- and MT2-MMPs. Consequently, we tried to figure the relation between the expression of important ECM molecules and colon cancer cell migration and invasion.

It has been reported that MT1-MMP activates pro-MMP2 in the presence of TIMP-2, whereas in the presence of higher TIMP-2 levels, MT1-MMP activity is inhibited [27]. Although studies on TIMP-1 have been conflicted concerning cancer progression, recent data suggest that elevated TIMP-1 levels in cancer tissue are associated with worse clinical outcomes in many cancer types including prostate and colon cancer [44,45]. We therefore had to investigate the regulatory mechanisms concerning the expression of both TIMPs in response to EGFR and/or HER2 inhibition in Caco-2 colon cancer cells. We found that EGF decreased the mRNA levels of TIMP-2 and that both the EGFR and HER2 inhibitions abolished this reduction by settling the levels close to those of untreated cells (Fig. 7C). These data indicated that the EGF-mediated inhibition of TIMP-2 utilizes both EGFR homodimers and EGFR-HER2 heterodimers. However, a different pattern was observed when the effects on TIMP-1 were evaluated. EGF enhanced TIMP-1 expression, which blocked by EGFR and EGFR + HER2 inhibitions, but not following inhibition of HER2 (Fig. 7D), indicated that it is mainly an EGF–EGFR mediated effect.

The uPA system has been implicated in tumor invasion [46] and upon activation in tumor progression toward the metastatic phenotype [47,48]. Here, we found that EGF stimulated uPA expression via the EGF–EGFR axis (Fig. 7E). The observed EGF-induced invasive potential

of Caco-2 cells may well be correlated with the elevated expression levels of uPA, upon cell activation by EGF. As uPA was previously reported to increase, not only in colon cancer cells, but also in the surrounding microenvironment including stroma [49] and cancer epithelial cells [50,51], this highlights the crucial role of this protease in the extracellular matrix degradation and subsequent invasion. As shown above (Fig. 2), EGFR inhibition abolished EGF-induced cell invasion and here, uPA was also downregulated by AG1478 in the presence of EGF, indicating that colon cancer invasiveness is most probably mediated through the EGF–EGFR signaling axis, resulting in uPA expression.

Moving one step ahead we examined the effects of EGF and EGFR/HER2 in the expression of cell surface heparan sulfate proteoglycans, syndecan-4 and glypican-1, that have been implicated in several functional properties of cancer cells [25,52,53]. Notably, EGF showed an inhibitory effect on syndecan-4 expression and this was found to be EGF–EGFR mediated (Fig. 7F). Syndecan-4 expression levels have been previously reported to be decreased in several colon cancer cell lines, even though with high metastatic potential [25,54,55]. On the other hand, the EGF-mediated expression of glypican-1 was stimulatory. This was found to be mediated mainly by HER2 (Fig. 7G). However,

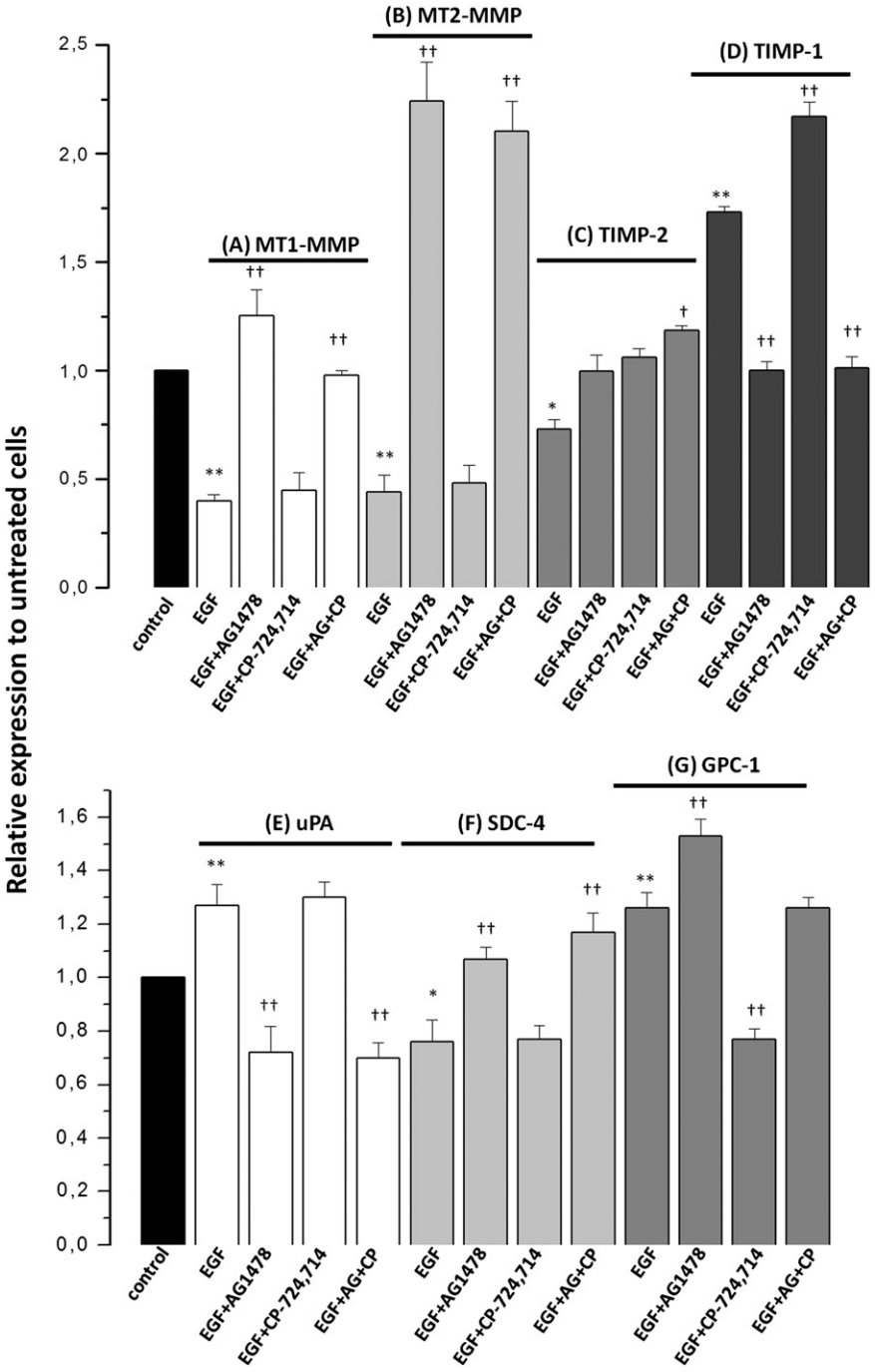

Fig. 7. Evaluation of EGF-mediated expression of matrix macromolecules. EGFR and/or HER2 inhibition, on MT1-MMP (A), MT2-MMP (B), TIMP-2 (C) and TIMP-1 (D), uPA (E), SDC-4 (F) and GPC-1 (G) mRNA expression in Caco-2 colon cancer cells. Cells were treated with AG1478 (1 μM) and CP-724,714 (1 μM), alone and in combination, in the presence of EGF (20 ng/mL). Results are expressed as relative expression and normalized to untreated cells. Statistically significant differences compared with control and EGF-treated cells are shown by $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $\dagger p \leq 0.05$, $\dagger\dagger p \leq 0.01$, respectively.

other receptor-mediated signaling pathways cannot be excluded. Glypican-1 is known to be overexpressed in several cancers and its expression is correlated with the progression of the disease. Taking this into consideration, it may be that the elevated expression of glypican-1 is related to our observation that EGF-mediates a significant increase of the invasiveness of Caco-2 cells.

4. Conclusions

Overexpression of EGFR and/or HER2 has been correlated with a poorer clinical outcome in a variety of malignancies [36,40]. Both EGFR and HER2 have been reported to be co-expressed in colorectal cancer cells and that simultaneous targeting of these receptors enhanced apoptosis in preclinical studies [42,56]. There are two different categories of compounds in the current area targeting EGFR network, including monoclonal antibodies and TKIs [57]. Here we examined the impact of EGFR and HER2 on Caco-2 cell proliferation, migration and invasive potential using specific inhibitors. At the functional level, cell proliferation, directional cell migration as well as invasive potential of Caco-2 colon cancer cells was found to be induced by EGF. The EGF–EGFR axis appears to be the crucial mediator in the proliferative ability and invasive capacity of colon cancer cells. uPA system is closely linked with the invasive and metastatic phenotype of cancer cells. Therefore, we suggest a regulatory role of uPA in colon cancer cell invasion via the EGF–EGFR network. In addition, Caco-2 cell migration was suppressed by the blockage of EGFR-mediated signaling pathways including PI3K-Akt, MEK1/2-Erk and JAK/STAT. The migratory ability of colon cancer cells may be promoted by the elevated expression of glypican-1 and TIMP-1. EGF-mediated syndecan-4 expression was found to be abolished, a fact that according to literature is associated with decreased cell adhesion and increased cell migration. On the other hand, HER2 signaling seems to mediate the TIMP-2 and glypican-1 expression through an unknown mechanism. The implication of HER2 in macromolecule expression and the fact that in many studies dual EGFR/HER2 inhibition increases the anticancer impact of EGFR, lead to the need for further investigation of HER2's role in colon cancer. However, EGFR remains the most important and crucial target in colon cancer.

A schematic representation summarizing the obtained data and the most important conclusions, i.e. the crucial importance of EGF on the expression of both receptors and the EGF–EGFR/HER2 signaling network as well as the distinct roles of EGFR and HER2 on expression of matrix macromolecules, is given in Fig. 8. These data improve our knowledge on the EGF-mediated effects on cell functional properties such as proliferation, invasion and metastatic potential as well as on the expression of matrix macromolecules implicated in cancer progression. Moreover, these data open a new area of research to design novel agents for pharmacological targeting of colon cancer.

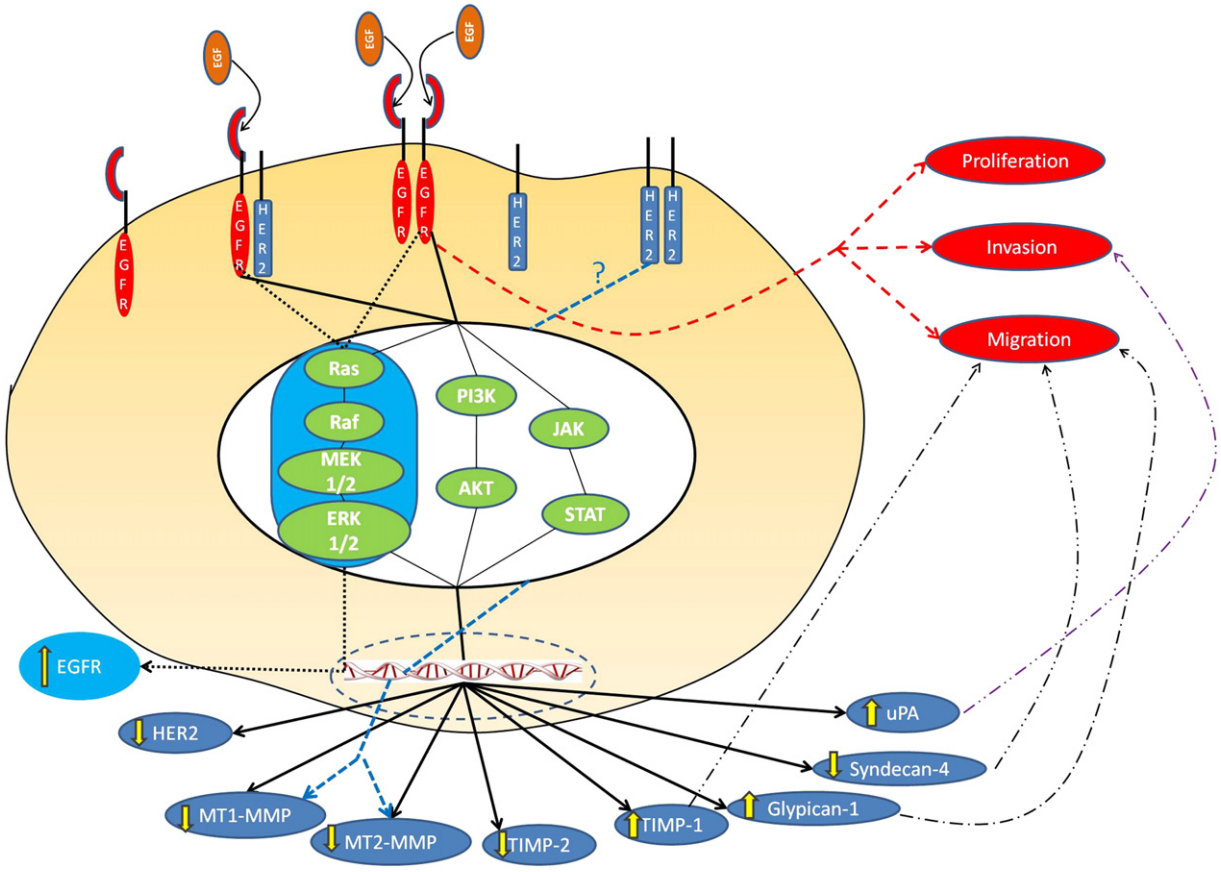

Fig. 8. Schematic representation summarizing the role of EGFR and/or HER2 signaling in this study. In Caco-2 colon cancer cells, EGF binding to EGFR causes either homodimerization (EGFR/EGFR) or heterodimerization (EGFR/HER2) of the receptors. Cell proliferation, invasion and migration are enhanced by EGF, mainly via EGFR/EGFR signaling. EGFR gene expression is regulated through the signaling axis of MEK-Erk, after EGFR phosphorylation. The EGF–EGFR axis is important for the expression of both MT-MMPs as well as TIMP-1. TIMP-2 expression is mediated through both hetero and homodimers of the receptors. uPA, a crucial mediator of tumor cell invasion, is upregulated by EGF, suggesting a significant role of enhanced cell invasion. The EGF-enhanced migratory ability of Caco-2 colon cancer cells is regulated via the EGF–EGFR network and via the elevated expression of glypican-1 and TIMP-1. Syndecan-4 dowregulation may further demonstrate the enhanced migration of cells resulting in reduced adhesion. The direct or indirect role of HER2 in respect to the expression of certain matrix macromolecules needs to be further examined.

Acknowledgments

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