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Amphiregulin promotes trophoblast invasion and increases MMP9/TIMP1 ratio through ERK1/2 and Akt signal pathways

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ABSTRACT

Aims: The aim of our study is to illustrate the role of amphiregulin in trophoblast invasiveness and underlying signal cascades.

Main methods: An immortalized human early extravillous cell line, HTR-8/SVneo, was used for this investigation. Matrigel-transwell invasion assay was used for testing the effects of amphiregulin on cell invasiveness. MMP9 and MMP2 mRNA expression level and activity were measured using Rt-qPCR and zymographic analysis. Cell signals involved in the invasion process were verified using western blot and specific inhibitors.

Key findings: Our results showed that amphiregulin could promote HTR-8/SVneo cell invasiveness without interfering cell proliferation, and significantly upregulate the expression of MMP9 and TIMP-1 mRNAs as well as the ratio of MMP9/TIMP-1. Using specific inhibitors for MEK and PI3K signaling further indicated that, both ERK1/2 and Akt signal pathways were required for amphiregulin-induced cell invasiveness. The co-ordination between ERK1/2 and Akt signaling pathway was needed for the upregulation of MMP9 mRNA, while ERK1/2 was more essential for the upregulation of TIMP-1 mRNA. Meanwhile, we first put forward that the deficiency of amphiregulin expression in trophoblast might be compensated by the upregulation of epidermal growth factor receptor (EGFR) and heparin-binding EGF (HB-EGF) mRNA.

Significance: ERK1/2 and Akt signaling pathways mediate amphiregulin-induced upregulation of MMP9 mRNA and the MMP9/TIMP-1 ratio, which subsequently contribute to amphiregulin-promotion of HTR-8/SVneo cell invasion.

1. Introduction

After implantation, extravillous trophoblasts (EVTs) penetrate through the epithelium into the decidualized uterus. Normal trophoblast invasion is confined spatially to the inner third of the myometrium and temporally to early pregnancy [1]. Insufficient invasion could lead to higher circulation resistance and cause miscarriages, pre-eclampsia and intrauterine growth retardation(IUGR) [2]. Unlimited invasiveness is involved in the pathogenesis of placenta accrete/increta/percreta [3] and gestational trophoblast disease(GTD) [4]. The invasiveness of EVTs requires the regulation of multiple factors mediated through different signal pathways [5].

The expression and localization of epidermal growth factor (EGF) family members change dynamically in trophoblasts over the course of pregnancy [6–11]. The dysregulation of EGF signaling system is correlated with pre-eclampsia and IUGR [12,13]. Among all the eight EGF-like ligands, amphiregulin and heparin-binding EGF (HB-EGF) are the most abundantly expressed ones in placenta during the first trimester, localized in both trophoblasts and decidua [7,9]. Apart from promoting the production of progesterone [14] and human chorionic gonadotropins [7], promoting the proliferation of trophoblast cells [15,16] and regulating embryo implantation [17], amphiregulin is also a pro-oncogene that could promote the survival, proliferation, epithelial-mesenchymal transition(EMT) as well as invasion in multiple neoplasms

Abbreviations: Areg, amphiregulin; EGFR, epidermal growth factor receptor; HB-EGF, heparin-binding EGF; MMPs, matrix metalloproteinases; TIMPs, tissue inhibitors of matrix metalloproteinases; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mRNA, Messenger RNA; SEM, standard error of the mean; siRNA, small interfering RNA; △△Ct, comparative cycle threshold

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Matrix metalloproteinases (MMPs) is a family of zinc-dependent endopeptidases and can be divided into six subgroups based on the specificity of their substrates, sequence homology, and domain organizations [21]. MMP-2 and MMP-9 belong to the gelatinase group, mainly degrading collagen IV and denatured collagens, the main component of the basement membrane, [22]. MMP2 and MMP9 have been demonstrated as factors that related to the invasive process EVTs [23]. Tissue inhibitors of matrix metalloproteinases (TIMPs) is a group of small secreted glycoprotein that could inhibit the activation of MMPs in a 1:1 ratio by forming stoichiometric complexes [24]. TIMP-1 preferentially inhibits MMP-9, while TIMP-2 has a higher affinity for MMP-2 [25]. The homeostatic balance between MMPs and TIMPs is important for the precise regulation of invasion processes [26,27]. It has been shown that amphiregulin promotes the invasion of different malignant cells through altering the MMPs/TIMPs balance [8,26,28–30]. The proliferation, invasion and EMT of EVTs show great similarities with malignant cells [31,32]. In the present study, we examined the role amphiregulin played on trophoblasts invasiveness and underlying mechanisms.

2. Materials and methods

2.1. Placenta tissue collection

Five normal pregnancies who underwent elective cesarean section at 37–40 weeks were included in this study. The tissue at the center of the placenta on the maternal side were collected (0.5–1 g). Informed consents were obtained from all the patients. The protocol for collecting tissue samples was approved by Human Subject Committees of First Affiliated Hospital of Zhengzhou University.

2.2. Immunofluoresence

After washing thoroughly in cold PBS, tissue was fixed in 4% paraformaldehyde overnight at 4 °C. Paraffin-embedded tissues were sectioned at 3.5 μm thickness, deparaffinized and rehydrated in graded series of ethanol. After blocking with 10% normal goat serum, slides were incubated with primary antibody (mouse anti-amphiregulin, santa cruz, SC-74501; rabbit anti-E-Cadherin, protein tech., 20874-1-AP) at 4 °C overnight. Then sections were washed with PBS for 4 times and incubated with secondary antibodies at room temperature for 2 h. After 4 times washing, slides were stained with DAPI (sigma, D9542). Images were taken using a laser-scanning confocal microscope (Olympus Fluoview FV1000, Japan) and processed with adobe photoshop CC 2017.

2.3. Cell culture

The HTR-8/SVneo cell line was purchased from ATCC (American type culture collection, VA). These cells were derived from the outgrowth of human first trimester extravillous immortalized using the simian virus 40 large T antigens. Cells were cultured in DMEM/nutrient mixture F-12 (Gibco, Grand Island, NY, USA) supplemented with 10% charcoal stripped fetal bovine serum (FBS, Sigma-Aldrich Inc. USA), 100U/ml penicillin and 100 μg/ml streptomycin (Gibco, Grand Island, NY, USA) in an incubator at 37 °C with a humidified 5% CO2. Culture medium was changed every two days. For experiments, cells were seeded in six-well plates and serum free for 24 h after reaching 85% confluent before applying indicated treatments.

2.4. Reagents and antibodies

Recombinant human amphiregulin protein (262-AR-100) was purchased from R&D systems and reconstituted in sterile phosphate-buffered saline (PBS, Gibco). Tyrphostin AG1478 (T4182), U0126 (19–147) and LY 294002 (L9908) were purchased from Sigma-Aldrich (St. Louis, MO) and reconstituted in Dimethyl sulfoxide (DMSO, Sigma-Aldrich Inc.). Phospho-p44/42 MAPK (Thr202/Tyr204, #9106s, mouse monoclonal 1:1000), p44/42 MAPK (Erk1/2, #9102s, rabbit monoclonal, 1:2000), Phospho-Akt (Ser473, #9271, rabbit monoclonal, 1:1000), Akt Antibody (rabbit monoclonal, #9272, 1:2000) were purchased from Cell Signaling Technology Inc. (Danver, CA), EGFR antibody (sc-373746, mouse monoclonal, 1:500) and GAPDH (sc-365062, mouse monoclonal, 1:3000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-conjugated secondary antibodies, goat Anti-Mouse IgG (HRP, ab6789) and goat Anti-Rabbit IgG (HRP, ab6721) were purchased from Abcam (MA, USA).

2.5. Cell proliferation assay

Cell proliferation was assessed using thiazolyl blue tetrazolium blue (MTT, Sigma-Aldrich Inc.). MTT could be converted to water-insoluble MTT formazan by mitochondrial dehydrogenases of living cells. The MTT formazan is soluble in DMSO and produce a purple solution, whose absorbance is proportional to the number of living cells. HTR-8/SVneo cell (1×10^4/well) were seeded in 96-well plates and cultured overnight. The second day, cells were rinsed with PBS twice and then treated with different concentrations of amphiregulin (0, 50, 100, 200 ng/ml) in serum-free medium for 24, 48, and 72 h. At indicated time points, cells were washed with PBS twice and incubated with 200 μl MTT (500 μg/ml) for 4 h at 37 °C. Then, medium was removed and replaced by 200 μl DMSO, shaking for 10 min at room temperature before measuring the optical density (OD) at 570 nm using a SpectraMax M3 (VWR Corporate center, CA). The results of treatment groups were normalized with the untreated control and shown as mean ± SEM. Five replicates were used for each group and tests were repeated for three times.

2.6. Transwell cell invasion assay

Cell invasiveness was assessed by the number of cells that migrated into Matrigel. After serum free for 24 h, HTR-8/SVneo cells were treated with amphiregulin for another 24 h and then Transwell invasion assay was conducted. Transwell inserts with 8 μm pores of polyethylene terephthalate membranes (Corning Incorporated, CA) were coated with 40 μl of growth factor-reduced Matrigel (1 mg/ml, BD biosciences, CA) and placed into 24-well permeable support companion plates (Corning Incorporated, CA) for 4 h at 37 °C for gel formation. HTR-8/SVneo cells (1×10^5 cells suspended in 250 μl serum-free DMEM/F12) were seeded in the insert with 750 μl 10%FBS DMEM/F12 in each receiver well. After incubated at 37 °C for 24 h, the noninvaded cells were removed with a cotton swab. Invaded cells were fixed using cold methanol for 20min, and then were stained with crystal violet staining solution (Sigma-Aldrich Inc.) at room temperature for 30min. The wells were then rinsed with water thoroughly before air drying. The number of cells invaded was counted in five random fields under LEICA DM IRB microscope (under 10 × 10 magnification) and the average cell number was determined using Image J software. The folds changes of stained cells in treatment groups relative to controls were used for analysis (mean ± SEM). Each experiment was repeated three times.

2.7. Western blot

HTR-8/SVneo cell were rinsed with cold PBS and then lysed using lysis buffer (C3228, Sigma-Aldrich Inc.), supplemented with a protein inhibitor cocktail (P8340, Sigma-Aldrich Inc.). After centrifuged at 14000 × *g* at 4 °C for 15min, protein concentration in the supernatant was determined using pierce BCA protein assay kit (23225, Thermo Fisher Scientific). Equal amounts of protein (30 μg/lane) were separated by SDS-PAGE gel (Thermo Fisher Scientific) and transferred to PVDF membranes. After blocking in Tris-buffered saline (TBS)

containing 5% (wt/vol) nonfat milk at room temperature for 1 h, individual blots were incubated with primary antibodies at 4 °C overnight. The membranes were washed with 0.1% (vol/vol) Tween-20 TBS (TBST) for 3 times, 10 min/time, followed by incubation with appropriate peroxidase-conjugated secondary antibodies for 1 h at room temperature and then washed 3 times with 0.1% TBST. Bands were visualized using pierce ECL western blotting substrate or Supersignal west Femto maximum sensitivity substrate (Thermo Fisher Scientific). When necessary, the membranes were stripped with stripping buffer [62.5 mM Tris, 10 mM dithiothreitol, 2% sodium dodecyl sulfate (pH 6.7)] at 50 °C for 25min and re-probed with antibodies against p44/42 MAPK, Akt or GAPDH.

2.8. Zymographic analysis

After treatment for 24 h, conditioned culture medium was harvested for the detection of enzymatic activity. Culture media from 6-well plates were concentrated using Centrifugal filters (Amicon, MA) with a 10 kDa nominal molecular weight limit. The protein concentration of concentrated culture medium was determined using Pierce BCA protein assay kit (23225, Thermo Fisher Scientific). Tris-glycine SDS sample buffer (Invitrogen) were mixed with 15 μg total protein and incubated at room temperature for 15min before loading on the 10% Novex zymogram plus gel (Thermo fisher scientific). After electrophoresis, gels were washed with renaturing buffer and developing buffer for 30min separately, with gentle agitation. The gels were then incubated at 37 °C overnight. After staining with colloidal blue kit (Cat. LC6025, Thermo Fisher Scientific) for 4 h, the gels were washed with deionized water overnight. The gel was scanned using Universal HOOD II (Bio-Rad laboratories, Italy). Intensity of each band was quantitated using Image J software and normalized to controls. Results are shown as (mean ± SEM) of at least three independent experiments.

2.9. Reverse Transcription-Quantitative Real-time PCR (RT-qPCR)

Total RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer's instruction. Reverse transcription was performed with 1 μg total RNA template using iScript RT Supermix following the manufacturer's instruction (Bio-Rad Laboratories, Hercules, CA). The primers used for SYBR Green (Bio-Rad Laboratories) RT-qPCR were shown in Table 1. The RT-qPCR was performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems) on 96-well optical reaction plates (Applied Biosystems). The thermal cycling protocol is as follows: denaturation at 95 °C for 10s, annealing and extension at 60 °C for 30s, 40 cycles. The specificity of each assay was confirmed by dissociation curve analysis and agarose gel electrophoresis of PCR products. Each sample was assayed in triplicates. A mean value was used for the determination of mRNA levels by the comparative method ($2^{-\triangle\triangle Ct}$) with GAPDH as the reference gene.

2.10. SiRNA transfection

To knockdown the endogenous expression of amphiregulin, HTR-8/SVneo cells were transfected with 20 nM Silencer Select siRNA targeting at amphiregulin (s1547, Invitrogen) using the lipofectamine RNAiMAX transfection reagent (Thermo fisher scientific) according to manufacturer's instruction. The Silencer Select Negative Control siRNA was used as control (Invitrogen). The knockdown efficiency was determined using RT-qPCR analysis.

Table 1
Primers used in this study.

Gene	Forward primer(5′-3′)	Reverse primer(5′-3′)
MMP9	CGCCAGTCCACCCTTGTG	CAGCTGCCTGTCGGTGAGA
MMP2	CGTCTGTCCCAGGATGACATC	ATGTCAGGAGAGGCCCCATA
TIMP-1	CATTGCTGGAAAACTGCAGGA	TCCACAAGCAATGAGTGCCA
TIMP-2	GCTGCGAGTGCAAGATCACG	TGGTGCCCGTTGATGTTCTT
HB-EGF	TTGTGCTCAAGGAATCGGCT	CAACTGGGGACGAAGGAGTC
EGF receptor	GGTGCAGGAGAGGAGAACTGC	GGTGGCACCAAAGCTGTATT
Amphiregulin	GTGTCCCAGAGACCGAGTTG	CCAGCAGCATAATGGCCTGA
GAPDH	GAGTCAACGGATTTGGTCGT	GACAAGCTTCCCGTTCTCAG

2.11. Statistical analysis

The results presented here are mean ± SEM of at least three independent experiments. Independent *t*-test was used to compare differences between two groups, while one-way ANOVA followed by Tukey's multiple comparison test was used for multiple comparisons. Analyses were performed with Prism 6 (GraphPad Software, CA). A P value < 0.05 was considered to be statistically significant.

3. Results

3.1. The localization of amphiregulin in term placenta

It has been well studied that amphiregulin is expressed in the cytoplasm of syncytiotrophoblast in first trimester [7,9]. However, in third trimester and term pregnancy, results are still inconclusive for cell types are indistinct based on immunohistochemistry. So we used the co-staining of E-cadherin to distinguish between cytotrophoblast and syncytiotrophoblast. As shown is Fig. 1, amphiregulin could be detected in the cytoplasm of both cytotrophoblast (including villous and extravillous cytotrophoblast) and syncytiotrophoblast in term placenta.

3.2. Amphiregulin increases HTR-8/SVneo cell invasiveness

As a locally produced factor, the working concentration of amphiregulin is difficult to detect. The detected ~20 ng/ml of amphiregulin in amniotic fluid is the part that has been released [7]. Hence, we checked amphiregulin's function based on two doses (100 ng/ml and 200 ng/ml), referring to published paper [14]. Matrigel invasion assay was performed to examine the effects of amphiregulin on HTR-8/SVneo cell invasiveness. Treatment with amphiregulin for 24 h promoted the invasiveness of HTR-8/SVneo cell in a dose-dependent manner (Fig. 2 A, B). To confirm that the stimulatory effects of amphiregulin on cell invasiveness was not due to differences in cell growth, we used MTT assay to examine the effects of amphiregulin treatment on cell viability and proliferation in HTR-8/SVneo cells. As shown in Fig. 2C different concentrations of amphiregulin (0, 50, 100, 200 ng/ml) had no effect on cell viability and proliferation during 72 h of treatment, suggesting the role of amphiregulin act as a promoter for human trophoblast invasiveness.

3.3. Amphiregulin increases MMP9, TIMP-1 and EGFR expression in HTR-8/SVneo cells

To examine cellular mechanism underlying amphiregulin actions on human trophoblasts, we treated HTR-8/SVneo cells with amphiregulin (100 ng/ml) for different time points and checked the expression level of MMP2/9 and TIMP-1/2, proteins important for cell invasiveness. RT-qPCR results showed that amphiregulin upregulated the expression of MMP9 (Fig. 3A) and TIMP-1 (Fig. 3C) mRNAs starting at 3 h after treatment, reaching the maximum level at 6 h and persisting until 9 h. However, the expression level of MMP2 (Fig. 3B) and TIMP-2 mRNA (Fig. 3D) did not change significantly during 48 h of treatment.

Studies have reported that the autocrine expression of ligands as well as receptor overexpression could function as mechanisms of increased signaling output from EGFR in the progression of carcinoma [33]. To explore whether amphiregulin could promote invasiveness through above mechanisms in HTR-8/SVneo, the expression levels of amphiregulin and EGFR mRNA were examined. RT-qPCR results

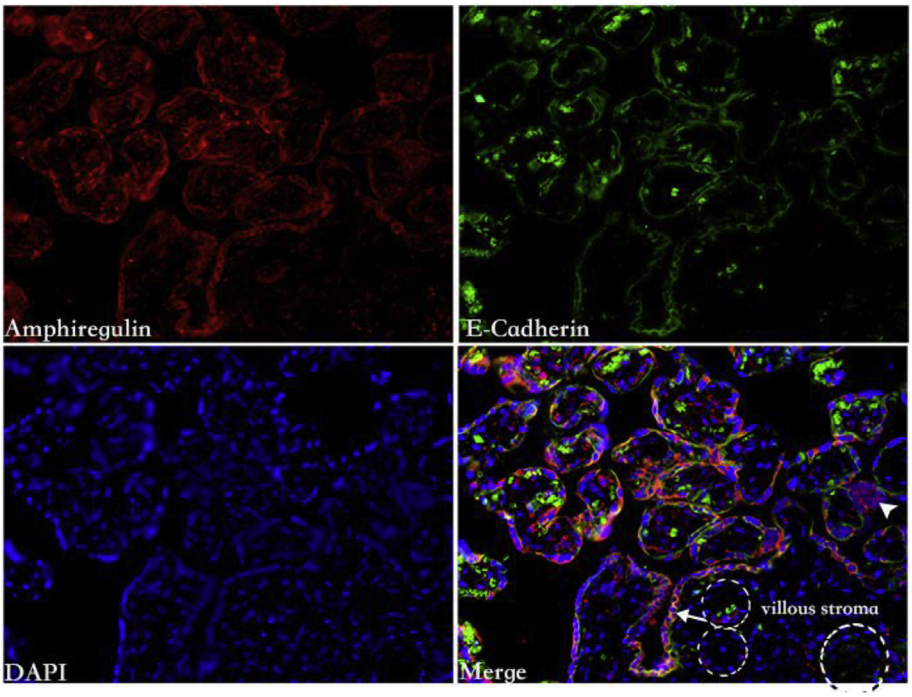

Fig. 1. Amphiregulin is localized in both cytotrophoblast and syncytiotrophoblast in term placenta. Triple staining (merge) colocalizes amphiregulin(red) and E-cadherin(green) in cytotrophoblast (white arrow) and syncytiotrophoblasts (white arrowheads). Dashed white circle: blood vessel. Magnification: × 200. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

showed that, amphiregulin could upregulate the expression of EGFR mRNA (Fig. 3F) in HTR-8/SVneo cells without interfering amphiregulin expression (Fig. 3E).

3.4. The stimulatory effect of amphiregulin on MMP9 mRNA overrides changes in TIMP-1 expression

To further investigate the dose-dependency of amphiregulin in trophoblasts, HTR-8/SVneo cell was treated with different concentrations of amphiregulin (0, 50, 100, 200 ng/ml) for 6 h. RT-qPCR results showed that amphiregulin increased the expression of MMP9 and TIMP-1 mRNA in a dose-dependent manner (Fig. 4 A, C) without affecting MMP2 and TIMP-2 mRNA levels (Fig. 4 B, D). For MMP9 and MMP2 expression, transcript changes were confirmed by zymographic analysis (Fig. 4E). Moreover, the ratio of RT-qPCR results showed that amphiregulin could increase the MMP9/TIMP-1 ratios in a dose dependent manner (Fig. 4C). These results suggested that amphiregulin upregulated MMP9 and TIMP-1 mRNA expression in a similar dose-dependent manner, while the effects of amphiregulin on MMP9 mRNAs could override that on TIMP-1 mRNAs.

3.5. Amphiregulin could activate both ERK1/2 and Akt signal pathway in HTR-8/SVneo cells

We have previously shown that amphiregulin could activate both ERK1/2 and Akt signal pathways through activation of EGFR in human primary granulosa cells [14]. However, the regulatory pathway of amphiregulin in human trophoblasts is still unknown. As shown in Fig. 5A, treatment with amphiregulin also dramatically activated ERK1/2 and Akt signal pathways in HTR-8/SVneo cells. Next, we used

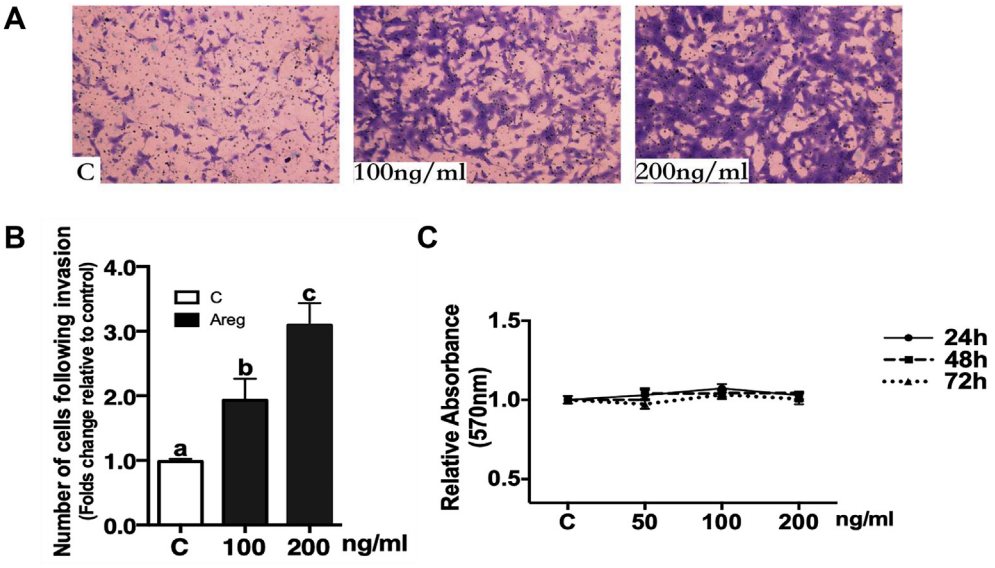

Fig. 2. Amphiregulin promoted the invasiveness of HTR-8/SVneo cells in a dose-dependent manner. A: HTR-8/SVneo cell were treated with different concentrations of amphiregulin (0, 100, 200 ng/ml) for 24 h, cell invasiveness was determined using Matrigel assay. Representative images are shown. B: Summarized quantitative results of cell invasiveness are shown. The folds change of number of stained cells in treatment groups relative to control were used for analysis. C: Cells were seeded in 96-well plates, treated with different concentrations of amphiregulin (0, 50, 100, 200 ng/ml). Cell viability at 24, 48 and 72 h after treatment were examined using MTT. The average absorbance at 570 nm of treatment group was normalized to untreated control group and five replicates were set for each experiment. Results are shown as (mean ± SEM) of three independent experiments. Values with different letters were significantly different ($P < 0.05$). C: PBS as control; Areg: amphiregulin.

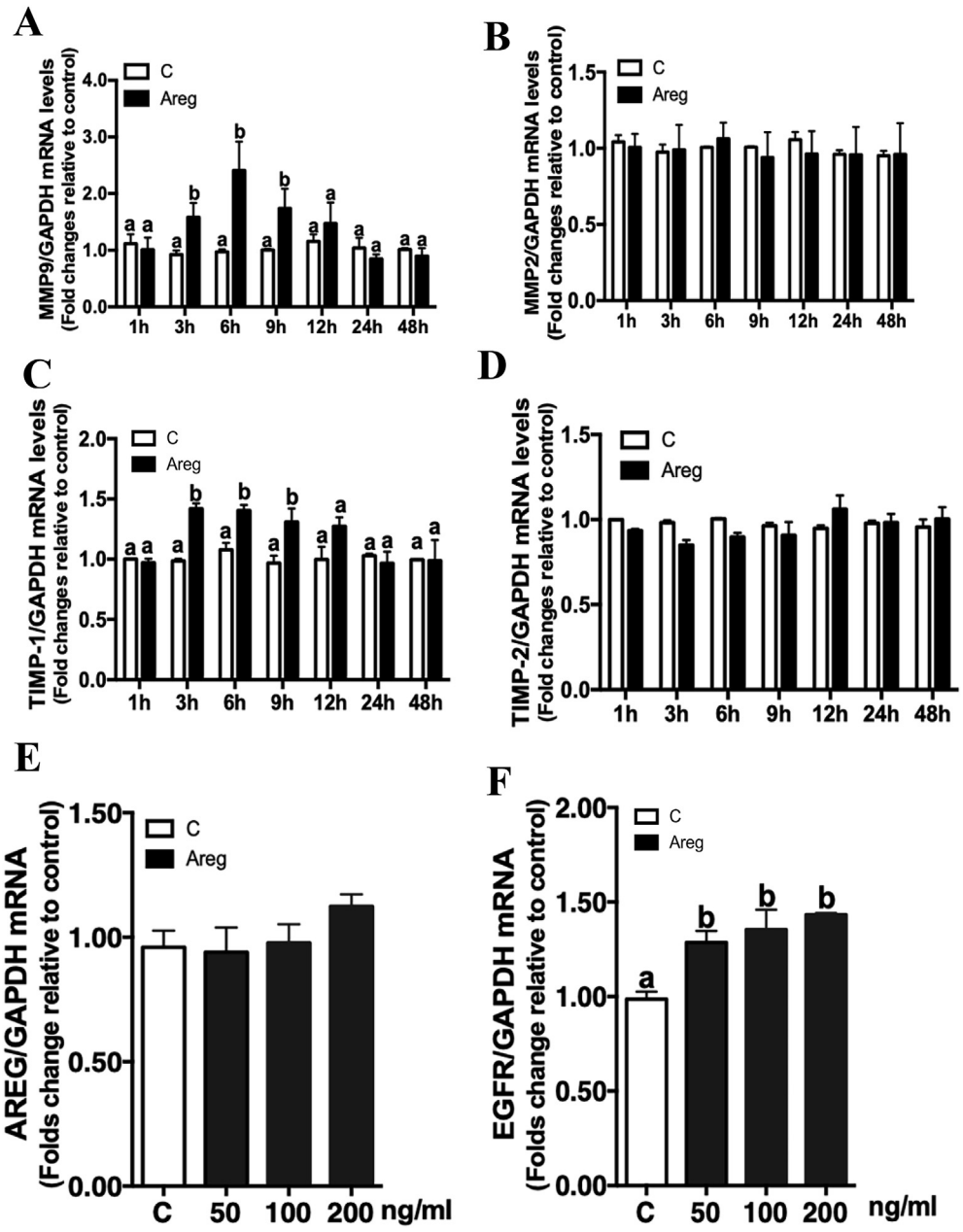

Fig. 3. Amphiregulin upregulates MMP9 and TIMP-1 expression in HTR-8/SVneo cell in a similar temporal pattern. A, B: HTR-8/SVneo cells were treated with amphiregulin (100 ng/ml), the expression level of MMP9 and MMP2 mRNA was checked at different time points using RT-qPCR. C, D: HTR-8/SVneo cells were treated with amphiregulin (100 ng/ml), the expression levels of TIMP-1 and TIMP-2 were checked at different time points using RT-qPCR. E, F: HTR-8/SVneo cells were treated with different concentrations of amphiregulin (0, 50, 100, 200 ng/ml) for 6 h, the expression levels of amphiregulin and EGFR mRNA were checked using RT-qPCR. Results are shown as (mean ± SEM) of at least three independent experiments. Values with different letters were significantly different ($P < 0.05$). C: PBS as control; Areg: amphiregulin.

the specific pharmacological inhibitors of EGFR (AG1478) to block the function of EGFR. Pretreatment with 10 μM AG1478 could abolish the phosphorylation of ERK1/2 and Akt signal pathway induced by amphiregulin. Meanwhile, the amphiregulin-induced phosphorylation of ERK1/2 and Akt could be specifically abolished by U0126 and LY294002 respectively (Fig. 5B).

3.6. EGFR, ERK1/2 and Akt signal pathways are required for amphiregulin-promoted HTR-8/SVneo cell invasiveness

Pretreatment with 10 μM AG1478, could abolish amphiregulin-induced upregulation of MMP9, TIMP-1 mRNA and MMP9/TIMP-1 ratios (Fig. 6A, C, D). Zymographic analysis further confirmed changes of MMP9 activity after AG1478 pre-treatment (Fig. 6B). Meanwhile, the invasiveness of HTR-8/SVneo cell was also largely inhibited by AG1478(Fig. 6E), indicating the activation of EGFR is required for amphiregulin-promoted invasiveness.

To investigate the individual roles of ERK1/2 and Akt signal pathways in amphiregulin-promoted HTR-8/SVneo cell invasiveness, 10 μM U0126 and 10 μM LY294002 were used to specifically block MEK and PI3K, respectively. Western blotting results in Fig. 5B showed that U0126 and LY294002 did not have off-target effects on PI3K and MEK signal pathways, respectively. Amphiregulin-upregulated MMP9 expression and MMP9/TIMP-1 ratio could be abolished by both U0126 and LY294002 (Fig. 6 A, D). Zymographic analysis further confirmed the change of MMP9 activity (Fig. 6B). In contrast, amphiregulin-upregulated TIMP-1 mRNA could be abolished by U0126, but not LY294002 (Fig. 6C). These results indicate that the ERK1/2, but not Akt, signal pathway mediates amphiregulin-upregulated TIMP-1 mRNA. Also, treatment with LY294002 or U0126 completely abolished the effects of amphiregulin on HTR-8/SVneo cell invasiveness (Fig. 6E).

3.7. Upregulation of HB-EGF and EGFR mRNA may compensate for the knockdown of amphiregulin

Amphiregulin siRNA was used to knockdown endogenous amphiregulin expression. As shown in Fig. 7A, transfection with amphiregulin siRNA significantly down-regulated amphiregulin mRNA expression.

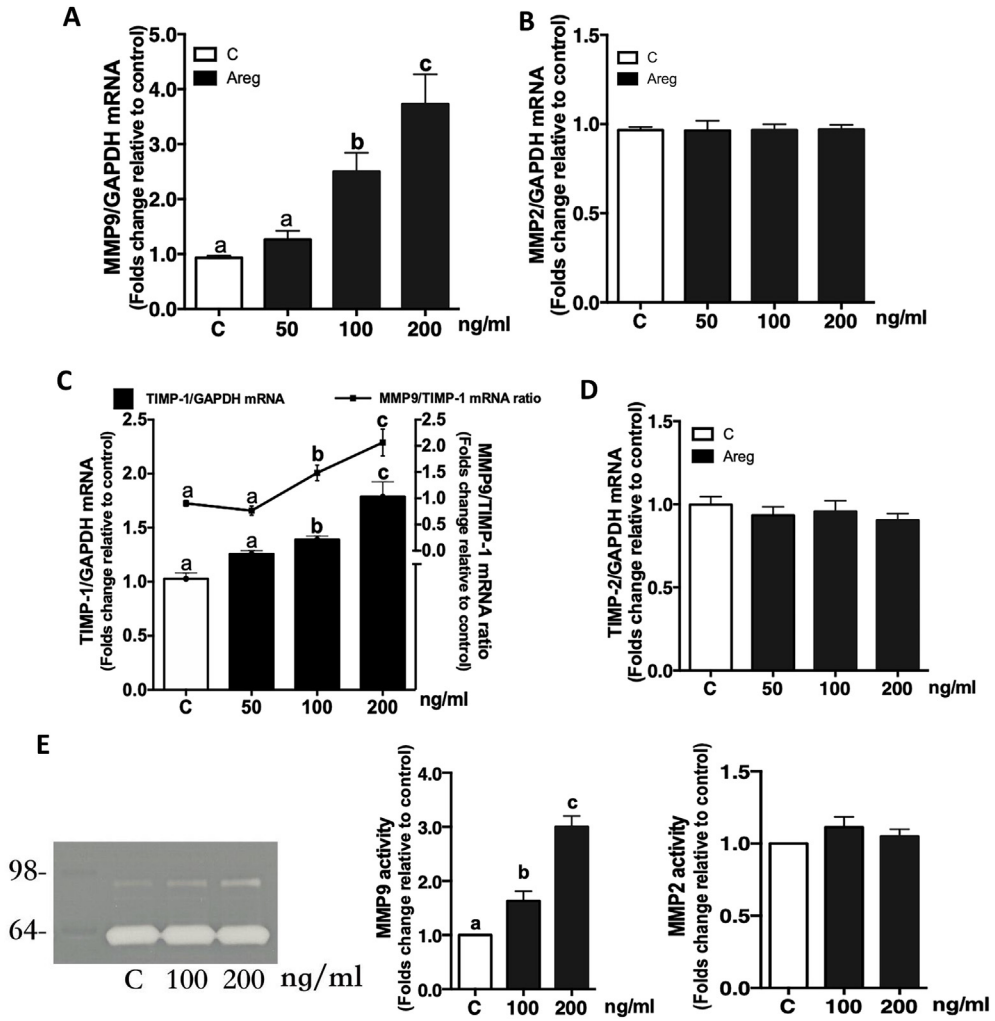

Fig. 4. Amphiregulin-induced upregulation of MMP9 mRNA overrides that of TIMP-1 mRNA. A,B: HTR-8/SVneo cells were treated with different concentrations of amphiregulin (0, 50, 100, 200 ng/ml) for 6 h, the expression levels of MMP9 and MMP2 were checked using RT-qPCR. C,D: HTR-8/SVneo cells were treated with different concentrations of amphiregulin (0, 50, 100, 200 ng/ml) for 6 h, the expression levels of TIMP-1 and TIMP-2 were checked using RT-qPCR. The ratio of MMP9/TIMP-1 was calculated in each dose-group. E: HTR-8/SVneo cells were treated with different concentrations of amphiregulin (0, 100, 200 ng/ml) for 24 h and then zymographic analysis was conducted. Left panel, representative result of zymographic analysis is shown. Middle and right panels, summarized quantitative results of MMP9 and MMP2 band intensity. Results are shown as (mean ± SEM) of at least three independent experiments. Values with different letters were significantly different ($P < 0.05$). C: PBS as control; Areg: amphiregulin.

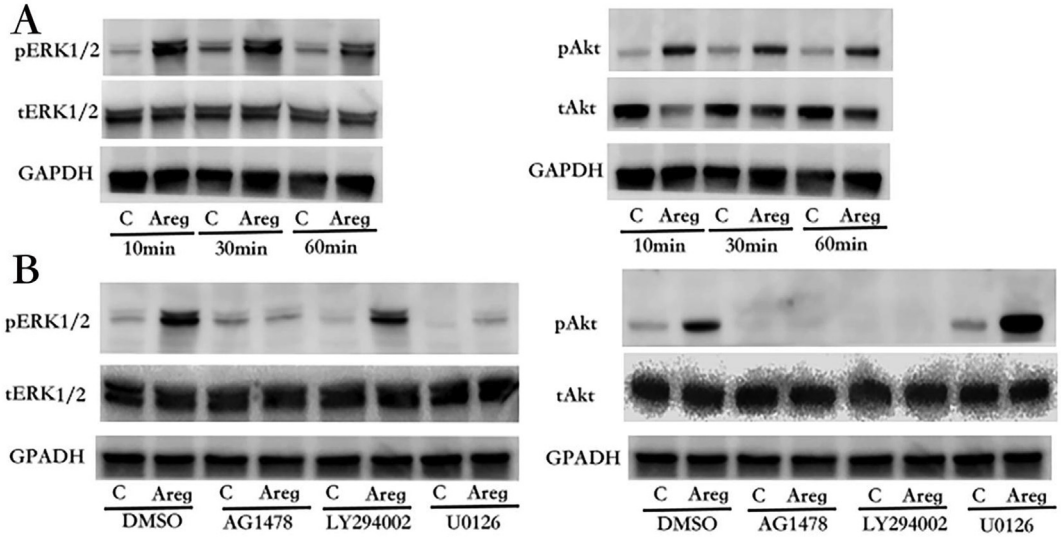

Fig. 5. Amphiregulin activates ERK1/2 and Akt signal pathway through binding EGFR. A: HTR-8/SVneo cells were treated with 100 ng/ml amphiregulin for 10, 30 and 60 min. Levels of phosphorylated ERK1/2 (pERK1/2) and phosphorylated Akt (pAkt) were examined using western blot. Membranes were stripped and re-probed with antibodies for total ERK1/2 (tERK1/2) and total Akt (tAkt1/2). B: HTR-8/SVneo cells were pretreated with vehicle control (DMSO) or 10 μM AG1478, 10 μM LY294002 or 10 μM U0126 for 1 h before treated with 100 ng/ml amphiregulin for 30min. Levels of phosphorylated and total ERK1/2, Akt were examined using western blot. Representative images are shown. C: control; Areg: amphiregulin.

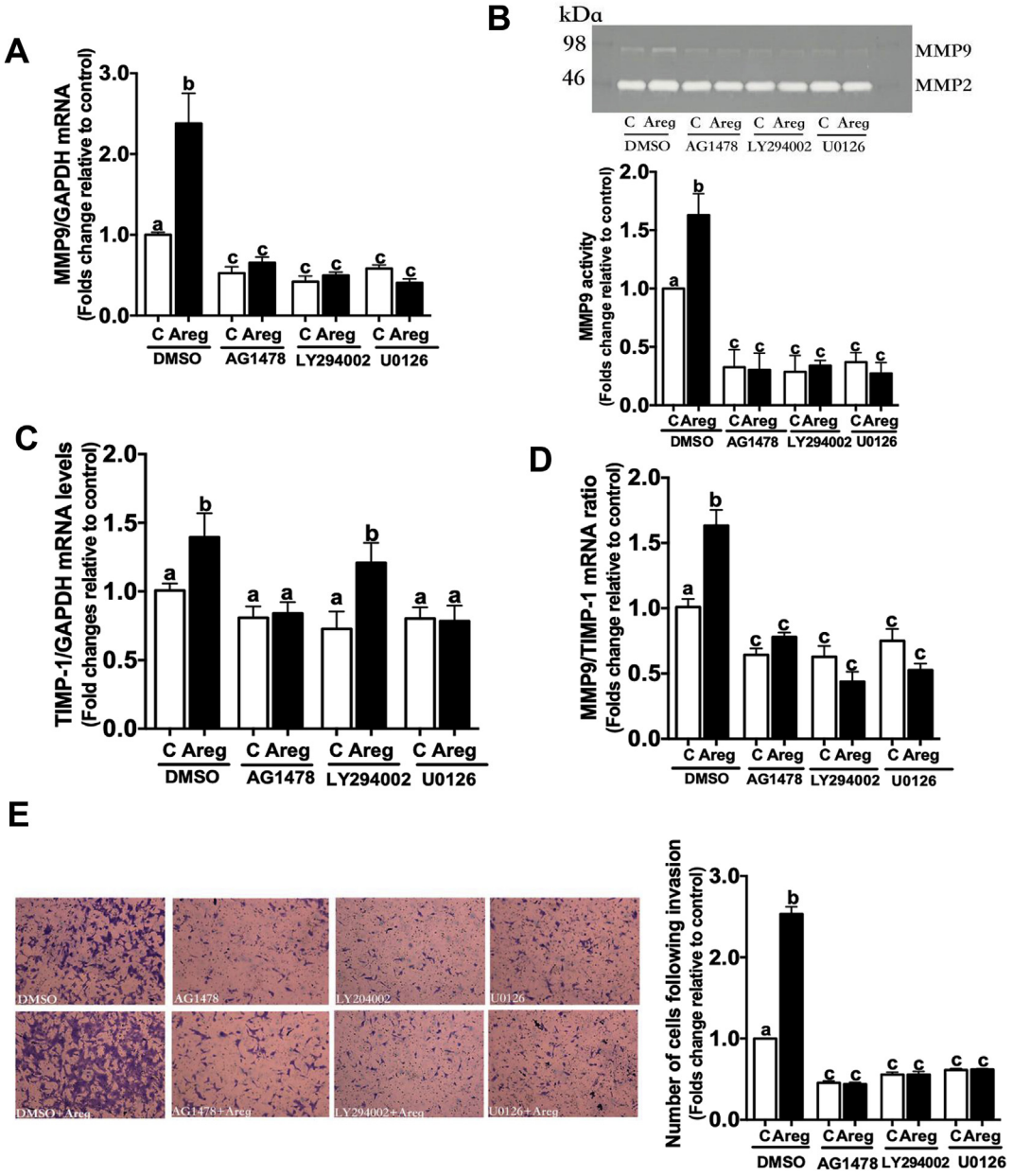

Fig. 6. Amphiregulin-promoted cell invasiveness could be abolished by specific inhibitors of EGFR, PI3K and MEK. A, C: HTR-8/SVneo cells were pretreated with a vehicle control (DMSO), 10 μM AG1478, 10 μM LY294002 or 10 μM U0126 for 1 h before treated with 100 ng/ml amphiregulin for 6 h. MMP9, TIMP-1mRNA levels were examined using RT-qPCR. B: HTR-8/SVneo cells were pretreated with a vehicle control (DMSO), 10 μM AG1478, 10 μM LY294002 or 10 μM U0126 for 1 h before treated with 100 ng/ml amphiregulin for 24 h. MMP9 and MMP2 activities were examined using zymographic analysis. Right panel, summarized quantitative results of MMP9 band intensity. D: The ratio of MMP9/TIMP-1 was calculated based on Rt-qPCR results in each group. E: HTR-8/SVneo cells were pretreated with a vehicle control (DMSO), 10 μM AG1478, 10 μM LY294002 or 10 μM U0126 for 1 h before treated with 100 ng/ml amphiregulin for 24 h. Matrigel transwell assay was conducted for the invasiveness of HTR-8/SVneo cells. Representative images are shown. Right panel, summarized quantitative results of invasion. Results are shown as (mean ± SEM) of at least three independent experiments. Values with different letters were significantly different ($P < 0.05$). C: control; Areg: amphiregulin.

However, the invasiveness of HTR-8/SVneo (Fig. 7B) as well as the expression of MMP9 and TIMP-1 mRNA were not impaired (Fig. 7C). Zymographic analysis further confirmed the change of MMP9 activity after amphiregulin siRNA knockdown (Fig. 7D). Earlier data showed that increase of amphiregulin expression could override HB-EGF deficiency in embryo implantation [34]. Hence, we put forward the idea that HB-EGF might have complementary effects for amphiregulin deficiency during trophoblast invasion. As shown in Fig. 7E, the expression of HB-EGF and EGFR mRNA were upregulated significantly after the knockdown of the endogenous amphiregulin, suggesting deficiency of amphiregulin production might be compensated by the upregulation of HB-EGF and EGFR mRNA.

4. Discussion

During trophoblast differentiation, some cytotrophoblasts fused to form syncytiotrophoblast, involved in the process of hormone secretion and nutriments exchange; some underwent the EMT to differentiate into EVTs and acquired the invasive properties, implicated in the process of chorionic villi anchor [35]. Amphiregulin has been shown to be an implantation-specific and progesterone-regulated gene in mouse uterus [17,34] and could promote the secretion of human chorionic gonadotropin (hCG) in human syncytiotrophoblast [7]. We extended amphiregulin's possible physiological role during pregnancies by demonstrating that amphiregulin could affect EVTs (HTR-8/SVneo cells)

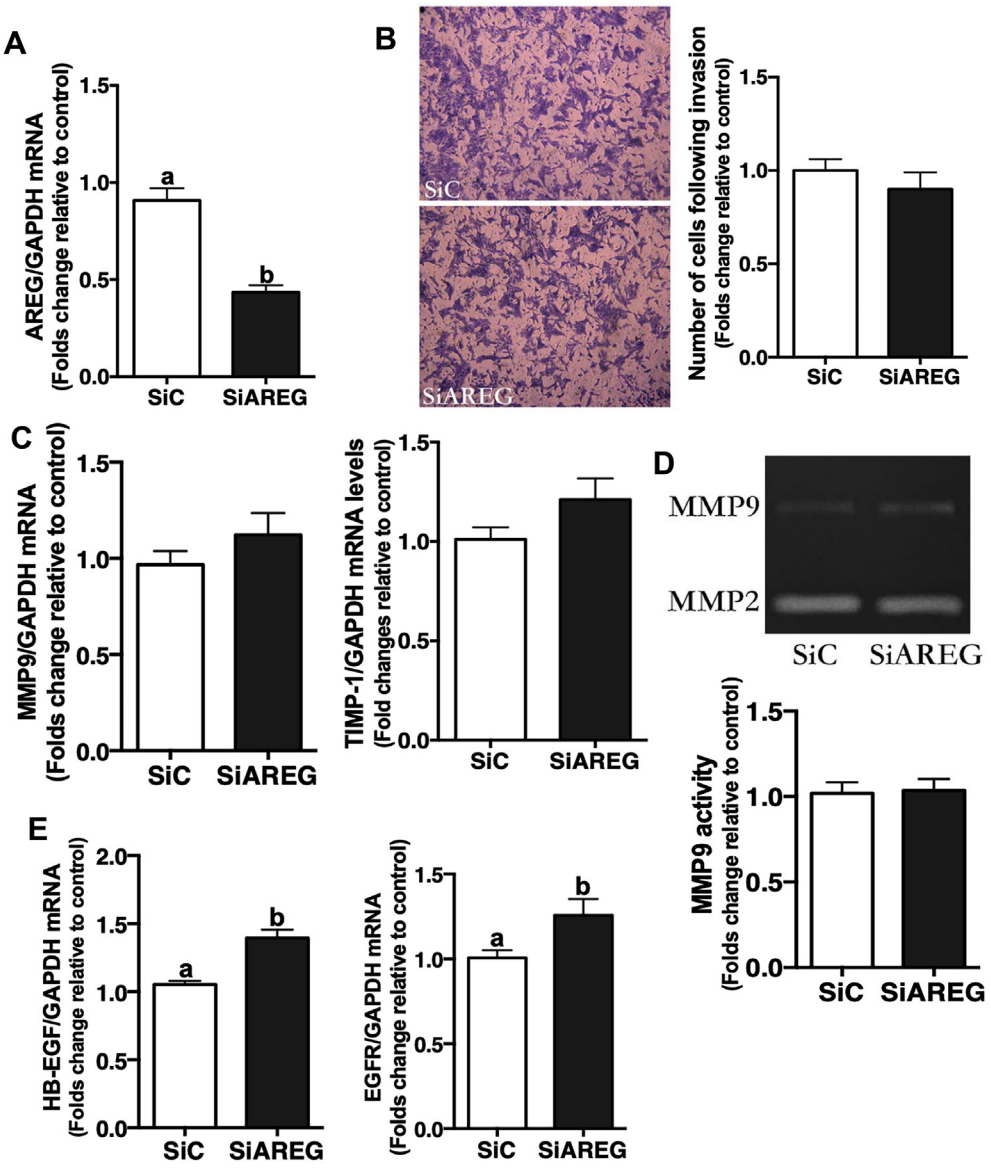

Fig. 7. HB-EGF and EGFR mRNA are upregulated after knocking down endogenous amphiregulin expression. A) HTR-8/SVneo cell were transfected with 20 nM control siRNA (SiC) or amphiregulin siRNA for 24 h and then transfect efficiency was determined using RT-qPCR. B) HTR-8/SVneo cell were transfected with 20 nM SiC or amphiregulin siRNA for 24 h and then cell invasiveness was examined using Matrigel transwell assay. Left panel, representative images are shown; right panel, summarized quantitative results of invasion. C) The expression level of MMP9 and TIMP-1 mRNA were examined using RT-qPCR 24 h after amphiregulin siRNA transfection. D) RT-qPCR results of MMP9 were further confirmed by zymographic analysis. Upper panel, representative images are shown; Lower panel, summarized quantitative results of MMP9 band intensity. E) The expression level of HB-EGF (left panel) and EGFR (right panel) mRNA after transfected with amphiregulin siRNA for 24 h were examined using RT-qPCR. Results are shown as (mean ± SEM) of at least three independent experiments. Values with different letters were significantly different ($P < 0.05$).

invasion through upregulating MMP9 and TIMP-1 mRNAs, and increasing MMP9/TIMP-1 ratios. In addition, our inhibitor studies indicated that, the co-ordination between ERK1/2 and Akt signal pathways are needed for amphiregulin-induced upregulation of MMP9 as well as MMP9/TIMP-1 ratios; whereas, ERK1/2, but not Akt signal pathway, is essential for the amphiregulin-induced upregulation of TIMP-1 mRNA. Due to the compensatory effects of EGFR and HB-EGF, the knockdown of endogenous amphiregulin do not significantly impair the invasiveness of HTR-8/SVneo cells.

The precise regulation of transiently invasive behavior of trophoblasts is crucial for normal pregnancies. This process includes complex molecular and genetic factors, intricate discourses between stimulatory and inhibitory factors and different signal pathways [36,37]. EGF signaling system has formed an intricate and complementary network for the regulation of trophoblast proliferation, apoptosis, differentiation as well as invasion [38]. The expression of several members of the EGF family members (HB-EGF, TGF-α and EGF) were decreased in patients with pre-eclampsia when comparing with non-preeclamptic pregnancies [12]. To date, EGF has been proved to promote HTR-8/SVneo cell invasiveness through the upregulation of MMP9/TIMP-1 ratios by activating both PI3K/Akt and MAPK/ERK signal pathway [39]. As a locally produced factor, amphiregulin is much more abundantly expressed in trophoblast than EGF [7], whether it plays a role in regulating trophoblast invasiveness remain unknown. In present study, our results demonstrated that amphiregulin had similar invasiveness-promotion effects on HTR-8/SVneo cell as EGF, but in different temporal patterns. The effects of amphiregulin reached maximum at 6 h of treatment and vanished at 24 h, the time when the effects of EGF were obvious [39].

It has been shown that human stromal cells secreted more amphiregulin after embryo implantation [40]. Immunostaining of amphiregulin was reported to be restricted to the cytoplasm of syncytiotrophoblast, but not in cytotrophoblast, during early gestation [7,9]. The co-localization of amphiregulin and E-cadherin using immunostaining in term placenta indicates that, amphiregulin is abundantly expressed in both cytotrophoblast and syncytiotrophoblast. Accordingly, in trophoblasts, amphiregulin might work in autocrine, paracrine and juxtacrine ways by binding and activation of EGFR. The phosphorylation of EGFR is often accompanied by its degradation [41,42]. The upregulation of EGFR mRNA after amphiregulin treatment demonstrated that, amphiregulin might intensify signaling output from EGF family ligands by receptor overexpression, similar to that found during progression of carcinoma [33].

In HTR-8/SVneo cells, amphiregulin could activate both ERK1/2

and Akt signal pathways, previously found to be important signaling cascades mediating trophoblast proliferation, differentiation and invasion [43]. In our study, we found that amphiregulin had no effect on the proliferation of HTR-8/SVneo cells. However, amphiregulin had been reported to promote the proliferation of trophoblast cells in porcine embryo during preimplantation development [15]. Meanwhile, a recently published study reported that amphiregulin could promote the proliferation of BeWo cell, a syncytiotrophoblast cell line, through ERK1/2 and Akt signaling pathway [16]. Whereas, EGF, which could also activate the ERK1/2 signaling pathway in BeWo cell [44], has no effect on BeWo cell proliferation [45]. The potential reason for the contradictories among above studies might lie in the fact that the cell components in each model are different (HTR-8/SVneo cells are a mixture of both extravillous cytotrophoblast and stromal cell [46], while BeWo is a combination of choriocarcinoma and decidual tissue [47] and the trophoblasts on the outer layer of blastocysts have the potential to differentiate into syncytio- and cyto-trophoblast during culture). What is more, even as choriocarcinoma cell lines, JEG3 and BeWo cells respond differently to inducers too [45].

MEK and PI3K signal pathways mediate cell motility [48]. Using of specific inhibitors of EGFR, MEK and PI3K (AG1478, U0126 and LY294002 respectively) could dramatically inhibit the invasiveness of HTR-8/SVneo cells as well as amphiregulin-induced upregulation of MMP9 mRNA and MMP9/TIMP-1 ratios. However, amphiregulin-induced upregulation of TIMP-1 mRNA can be blocked by AG1478 and U0126, but not by LY294002. Above results indicated that the transcription of MMP9 requires EGFR-mediated activation of both MAPK/ERK and PI3K/Akt signal pathway, whereas amphiregulin-induced upregulation of TIMP-1 mRNA is regulated mainly through EGFR-mediated activation of MAPK/ERK signal pathway. It can be explained by the fact that, the promoters of MMP9 and TIMP-1 share the same AP-1 binding site [49,50], and both MAPK/ERK and PI3K/Akt could activate AP-1, whereas MAPK/ERK mediates the PI3K/Akt-induced AP-1 activation [51]. Meanwhile, the transcription of MMP9 gene also need the recruitment of nuclear factor-κB (NF-κB) to the promoter region, which is mediated by PI3K/Akt signal pathway [52].

The effects of amphiregulin on MMP9 and TIMP-1 mRNA expression are specific since the MMP2 and TIMP-2 levels are not changed. Of particular interest is TIMP-1, due to its complex functions [53]. In addition to its inhibition of MMP-9 [54], TIMP-1 is also recognized as a cancer promoting factor [55], due to its anti-apoptotic effects [55], mitogenic activity [56] as well as angiogenesis enhancement function [57]. TIMP-1 are abundantly expressed in trophoblasts, and participate in the precise regulation of trophoblast invasion depth [58]. We demonstrated that amphiregulin could increase the MMP9/TIMP-1 ratio in a dose-dependent manner. It can be interpreted as, amphiregulin increases HTR-8/SVneo invasiveness by increasing net MMP9 production; when invasion depth is excessive, amphiregulin restrict invasiveness by increasing TIMP-1. However, above interpretation and corresponding mechanism need further investigation.

It is intriguing to see that the HTR-8/SVneo invasiveness as well as MMP9 and TIMP-1 mRNA expression was not compromised after knocking down the endogenous amphiregulin expression using siRNA. Interestingly, the expression of EGFR mRNA and HB-EGF mRNA, which also utilizes EGFR, were upregulated after amphiregulin knockdown, consistent with the complementary effects of amphiregulin on HB-EGF ablation in pregnant mice reported in a previous study [34]. It further indicates that, the cooperation and complementary effects among specific EGFR ligands ensure the normal progression of pregnancies. HB-EGF has been reported to function through the activation of EGFR and the phosphorylation of ERK1/2 and AKT signal pathway [59–61]. The unimpaired phosphorylation of ERK1/2 and AKT signal pathway may not reduce cell mobilities, which may account for the uncompromised invasiveness of HTR-8/SVneo cells. All in all, it is remarkable to find that the compensatory effects HB-EGF could override amphiregulin insufficiency during invasion. Low molecular weight heparin (LMWH) has been widely used for the prevention of placenta-mediated pregnancy complications, like preeclampsia, IUGR and recurrent miscarriages. HB-EGF has been proved to be a necessary downstream target for LMWH [59]. However, whether amphiregulin work alone, or coordinately with HB-EGF to mediate the effect of LMWH during treatment worth further investigation. The compensatory effects between HB-EGF and amphiregulin provides new insights into the management of placenta-mediated pregnancy complications.

Recently, the usage of HTR-8/SVneo cell line as a model for the EVT invasion study has been doubted, for it is a mixture of both extravillous cytotrophoblasts and stromal cells [46]. However, HTR-8/SVneo cell is compatible with the investigation of amphiregulin, who works by autocrine, paracrine and juxtacrine manners during pregnancies. Nevertheless, lack of primary trophoblasts experiment is still one of the most important drawbacks of our study.

5. Conclusion

Our study indicated that the increase of MMP9/TIMP-1 ratio contributed to amphiregulin-induced HTR-8/SVneo invasiveness. Although the amphiregulin-induced upregulation of TIMP-1 mRNA is mediated through ERK1/2 signaling, amphiregulin-induced upregulation of MMP9 mRNA as well as increases of MMP9/TIMP-1 ratios need both ERK1/2 and Akt signaling. This study provides molecular evidence that amphiregulin is an important regulator during trophoblast invasion.

Author contributions

Y.P.S, L.L.F and Y.P.Y designed the experiments and wrote the manuscript. Y.P.Y, S.J.W, Y.R.L and Y.J.G performed the experiment and data analysis, review and edited the manuscript.

Declarations of interest

None.

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References

[1] R. Pijnenborg, W. Robertson, I. Brosens, G. Dixon, Review article: trophoblast invasion and the establishment of hemochorial placentation, Placenta 2 (1981) 71–92 https://www.ncbi.nlm.nih.gov/pubmed/7010344.
[2] R. Pijnenborg, J. Bland, Wa Robertson, I. Brosens, Uteroplacental arterial changes related to interstitial trophoblast migration in early human pregnancy, Placenta 4 (4) (1983) 397–413 https://www.ncbi.nlm.nih.gov/pubmed/6634666.
[3] T. Hannon, B. Innes, G. Lash, J. Bulmer, S. Robson, Effects of local decidua on trophoblast invasion and spiral artery remodeling in focal placenta creta–An immunohistochemical study, Placenta 33 (12) (2012) 998–1004.
[4] S. Lele, S. Crowder, M. Grafe, Asymptomatic intraplacental choriocarcinoma diagnosed on routine placental examination, J. Perinatol.: Off. J. California. Perinatal.

Assoc. 19 (3) (1999) 244–247, https://doi.org/10.1038/sj.jp.7200140.
[5] J. Pollheimer, M. Knöfler, Signalling pathways regulating the invasive differentiation of human trophoblasts: a review, Placenta 26 (2005) S21–S30, https://doi.org/10.1016/j.placenta.2004.11.013.
[6] K.E. Bass, et al., Human cytotrophoblast invasion is up-regulated by epidermal growth factor: evidence that paracrine factors modify this process, Dev. Biol. 164 (2) (1994) 550–561.
[7] T. Fukami, et al., Amphiregulin regulates the production of human chorionic gonadotropin in trophoblasts, Life Sci. 84 (23–24) (2009) 796–804, https://doi.org/10.1016/j.lfs.2009.03.009.
[8] S.B. Kondapaka, R. Fridman, K.B. Reddy, Epidermal growth factor and amphiregulin up-regulate matrix metalloproteinase-9 (MMP-9) in human breast cancer cells, Int. J. Cancer 70 (6) (1997) 722–726.
[9] J. Lysiak, G. Johnson, P. Lala, Localization of amphiregulin in the human placenta and decidua throughout gestation: role in trophoblast growth, Placenta 16 (4) (1995) 359–366, https://doi.org/10.1016/0143-4004(95)90093-4.
[10] J.J. Lysiak, I.H. Connelly, N.K. Khoo, W. Stetler-Stevenson, P.K. Lala, Role of transforming growth factor-α (TGFα) and epidermal growth factor (EGF) on proliferation and invasion by first trimester human trophoblast, Placenta 15 (1994) 455–467, https://doi.org/10.1016/S0143-4004(05)80366-2.
[11] J. Lysiak, V. Han, P. Lala, Localization of transforming growth factor in the human placenta and decidua: role in trophoblast growth, Biol. Reprod. 49 (5) (1993) 885–894, https://doi.org/10.1095/biolreprod49.5.885.
[12] D.R. Armant, et al., Reduced expression of the epidermal growth factor signaling system in preeclampsia, Placenta 36 (3) (2015) 270–278, https://doi.org/10.1016/j.placenta.2014.12.006.
[13] R.E. Leach, et al., Pre-eclampsia and expression of heparin-binding EGF-like growth factor, The Lancet 360 (9341) (2002) 1215–1219, https://doi.org/10.1016/S0140-6736(02)11283-9.
[14] L. Fang, Y. Yu, R. Zhang, J. He, Y.-P. Sun, Amphiregulin mediates hCG-induced StAR expression and progesterone production in human granulosa cells, Sci. Rep. 6 (2016) 24917, https://doi.org/10.1038/srep24917.
[15] J.H. Lee, et al., Amphiregulin promotes the proliferation of trophoblast cells during preimplantation development of porcine embryos, Theriogenology 72 (8) (2009) 1023–1031, https://doi.org/10.1016/j.theriogenology.2009.05.021.
[16] L.V. Pires, et al., Lapatinib inhibits amphiregulin-induced BeWo choriocarcinoma cell proliferation by reducing ERK1/2 and AKT signaling pathways, Anticancer Res. 39 (5) (2019) 2377–2383.
[17] S. Das, et al., Amphiregulin is an implantation-specific and progesterone-regulated gene in the mouse uterus, Mol. Endocrinol. 9 (6) (1995) 691–705, https://doi.org/10.1210/mend.9.6.8592515.
[18] C. Berasain, M.A. Avila, Amphiregulin. *Seminars in Cell & Developmental Biology*, Elsevier, 2014, pp. 31–41.
[19] E. Elinav, et al., Inflammation-induced cancer: crosstalk between tumours, immune cells and microorganisms, Nat. Rev. Cancer 13 (2013) 759, https://doi.org/10.1038/nrc3611.
[20] N. Bles, L. Di Pietrantonio, J.-M. Boeynaems, D. Communi, ATP confers tumorigenic properties to dendritic cells by inducing amphiregulin secretion, Blood 116 (17) (2010) 3219–3226.
[21] R. Visse, H. Nagase, Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry, Circ. Res. 92 (8) (2003) 827–839, https://doi.org/10.1161/01.RES.0000070112.80711.3D.
[22] M. Cohen, A. Meisser, P. Bischof, Metalloproteinases and human placental invasiveness, Placenta 27 (8) (2006) 783–793, https://doi.org/10.1016/j.placenta.2005.08.006.
[23] S. Campbell, J. Rowe, C. Jackson, E. Gallery, In vitro migration of cytotrophoblasts through a decidual endothelial cell monolayer: the role of matrix metalloproteinases, Placenta 24 (4) (2003) 306–315, https://doi.org/10.1053/plac.2002.0911.
[24] D.E. Woolley, D.R. Roberts, J.M. Evanson, Inhibition of human collagenase activity by a small molecular weight serum protein, Biochem. Biophys. Res. Commun. 66 (2) (1975) 747–754, https://doi.org/10.1016/0006-291X(75)90573-2.
[25] J.-Y. Zhu, Z.-J. Pang, Y-h Yu, Regulation of trophoblast invasion: the role of matrix metalloproteinases, Reviews in obstetrics and gynecology 5 (3–4) (2012) e137, https://doi.org/10.3390/ijms18071448.
[26] C.S. Moore, S.J. Crocker, An alternate perspective on the roles of TIMPs and MMPs in pathology, Am. J. Pathol. 180 (1) (2012) 12–16, https://doi.org/10.1016/j.ajpath.2011.09.008.
[27] T. Collette, R. Maheux, J. Mailloux, A. Akoum, Increased expression of matrix metalloproteinase-9 in the eutopic endometrial tissue of women with endometriosis, Hum. Reprod. 21 (12) (2006) 3059–3067, https://doi.org/10.1093/humrep/del297.
[28] Z. Liu, J. Klominek, Regulation of matrix metalloprotease activity in malignant mesothelioma cell lines by growth factors, Thorax 58 (3) (2003) 198–203, https://doi.org/10.1136/thorax.58.3.198.
[29] S. Menashi, et al., Regulation of extracellular matrix metalloproteinase inducer and matrix metalloproteinase expression by amphiregulin in transformed human breast epithelial cells, Cancer Res. 63 (22) (2003) 7575–7580.
[30] O. Pornchai, H. Modjtahedi, P. Rhys-Evans, G.M. Box, S.A. Eccles, Epidermal growth factor-like ligands differentially up-regulate matrix metalloproteinase 9 in head and neck squamous carcinoma cells, Cancer Res. 60 (4) (2000) 1121–1128.
[31] J.E. Davies, et al., Epithelial-mesenchymal transition during extravillous trophoblast differentiation, Cell Adhes. Migrat. 10 (3) (2016) 310–321, https://doi.org/10.1080/19336918.2016.1170258.
[32] C. Ferretti, L. Bruni, V. Dangles-Marie, A. Pecking, D. Bellet, Molecular circuits shared by placental and cancer cells, and their implications in the proliferative, invasive and migratory capacities of trophoblasts, Hum. Reprod. Update 13 (2) (2007) 121–141, https://doi.org/10.1093/humupd/dml048.
[33] T. MUKOHARA, et al., Activated Akt expression has significant correlation with EGFR and TGF-α expressions in stage I NSCLC, Anticancer Res. 24 (1) (2004) 11–18, https://doi.org/10.1073/pnas.0707909104.
[34] H. Xie, et al., Maternal heparin-binding-EGF deficiency limits pregnancy success in mice, Proc. Natl. Acad. Sci. 104 (46) (2007) 18315–18320, https://doi.org/10.1073/pnas.0707909104.
[35] V.D. Winn, et al., Gene expression profiling of the human maternal-fetal interface reveals dramatic changes between midgestation and term, Endocrinology 148 (3) (2007) 1059–1079.
[36] D. Evain-Brion, Growth factors and trophoblast differentiation: a review, Placenta 13 (1992) 1–18, https://doi.org/10.1016/S0143-4004(05)80305-4.
[37] P. Bischoff, A. Meisser, A. Campana, Paracrine and autocrine regulators of trophoblast invasion—a review, Placenta 21 (2000) S55–S60, https://doi.org/10.1053/plac.2000.0521.
[38] M.J. Large, et al., The epidermal growth factor receptor critically regulates endometrial function during early pregnancy, PLoS Genet. 10 (6) (2014) e1004451, , https://doi.org/10.1371/journal.pgen.1004451.
[39] Q. Qiu, M. Yang, B. Tsang, A. Gruslin, EGF-induced trophoblast secretion of MMP-9 and TIMP-1 involves activation of both PI3K and MAPK signalling pathways, Reproduction 128 (3) (2004) 355–363, https://doi.org/10.1530/rep.1.00234.
[40] B. Gellersen, A. Wolf, M. Kruse, M. Schwenke, A.-M. Bamberger, Human endometrial stromal cell-trophoblast interactions: mutual stimulation of chemotactic migration and promigratory roles of cell surface molecules CD82 and CEACAM1, Biol. Reprod. 88 (3) (2013), https://doi.org/10.1146/annurev.bi.56.070187.004313 80, 81-13.
[41] G. Carpenter, Receptors for epidermal growth factor and other polypeptide mitogens, Annu. Rev. Biochem. 56 (1) (1987) 881–914.
[42] S. Cohen, G. Carpenter, L. King, Epidermal growth factor-receptor-protein kinase interactions. Co-purification of receptor and epidermal growth factor-enhanced phosphorylation activity, J. Biol. Chem. 255 (10) (1980) 4834–4842.
[43] P. Jessmon, B.A. Kilburn, R. Romero, R.E. Leach, D.R. Armant, Function-specific intracellular signaling pathways downstream of heparin-binding EGF-like growth factor utilized by human trophoblasts, Biol. Reprod. 82 (5) (2010) 921–929, https://doi.org/10.1095/biolreprod.109.082305.
[44] H.E.M. zu Schwabedissen, et al., Epidermal growth factor-mediated activation of the map kinase cascade results in altered expression and function of ABCG2 (BCRP), Drug Metab. Dispos. 34 (4) (2006) 524–533.
[45] S. Al-Nasiry, B. Spitz, M. Hanssens, C. Luyten, R. Pijnenborg, Differential effects of inducers of syncytialization and apoptosis on BeWo and JEG-3 choriocarcinoma cells, Hum. Reprod. 21 (1) (2005) 193–201, https://doi.org/10.1093/humrep/dei272.
[46] W. Abou-Kheir, J. Barrak, O. Hadadeh, G. Daoud, HTR-8/SVneo cell line contains a mixed population of cells, Placenta 50 (2017) 1–7.
[47] R.A. Pattillo, G.O. Gey, The establishment of a cell line of human hormone-synthesizing trophoblastic cells in vitro, Cancer Res. 28 (7) (1968) 1231–1236.
[48] Y. Gan, et al., Differential roles of ERK and Akt pathways in regulation of EGFR-mediated signaling and motility in prostate cancer cells, Oncogene 29 (35) (2010) 4947–4958, https://doi.org/10.1038/onc.2010.240.
[49] P. Borden, R.A. Heller, Transcriptional control of matrix metalloproteinases and the tissue inhibitors of matrix metalloproteinases, Crit. Rev. Eukaryot. Gene Expr. 7 (1–2) (1997), https://doi.org/10.1615/CritRevEukarGeneExpr.v7.i1-2.90.
[50] P. Huhtala, et al., Complete structure of the human gene for 92-kDa type IV collagenase. Divergent regulation of expression for the 92-and 72-kilodalton enzyme genes in HT-1080 cells, J. Biol. Chem. 266 (25) (1991) 16485–16490.
[51] J. Li, et al., PI-3K and Akt are mediators of AP-1 induction by 5-MCDE in mouse epidermal Cl41 cells, J. Cell Biol. 165 (1) (2004) 77–86, https://doi.org/10.1083/jcb.200401004.
[52] C.Y. Cheng, C.T. Kuo, C.C. Lin, H.L. Hsieh, C.M. Yang, IL-1β induces expression of matrix metalloproteinase-9 and cell migration via ac-Src-dependent, growth factor receptor transactivation in A549 cells, Br. J. Pharmacol. 160 (7) (2010) 1595–1610, https://doi.org/10.1111/j.1476-5381.2010.00858.x.
[53] E. Lambert, E. Dasse, B. Haye, E. Petitfrere, TIMPs as multifacial proteins, Crit. Rev. Oncol.-Hematol. 49 (3) (2004) 187–198, https://doi.org/10.1016/j.critrevonc.2003.09.008.
[54] L. Guedez, et al., In vitro suppression of programmed cell death of B cells by tissue inhibitor of metalloproteinases-1, J. Clin. Investig. 102 (11) (1998) 2002–2010, https://doi.org/10.1172/JCI2881.
[55] Y. Jiang, I.D. Goldberg, Y.E. Shi, Complex roles of tissue inhibitors of metalloproteinases in cancer, Oncogene 21 (14) (2002) 2245, https://doi.org/10.1038/sj.onc.1205291.
[56] T. Hayakawa, K. Yamashita, K. Tanzawa, E. Uchijima, K. Iwata, Growth-promoting activity of tissue inhibitor of metalloproteinases-1 (TIMP-1) for a wide range of cells A possible new growth factor in serum, FEBS Lett. 298 (1) (1992) 29–32, https://doi.org/10.1016/0014-5793(92)80015-9.
[57] H. Yoshiji, et al., Mammary carcinoma cells over-expressing tissue inhibitor of metalloproteinases-1show vascular endothelial growth factor expression, Int. J. Cancer 75 (1) (1998) 81–87, https://doi.org/10.1002/(SICI)1097-0215(19980105)75:1<81::AID-IJC13>3.0.CO;2-G.
[58] B. Dimo, et al., Comparative study of the immunohistochemical expression of tissue inhibitors of metalloproteinases 1 and 2 between clearly invasive carcinomas and “in situ” trophoblast invasion, Med. Oncol. 29 (3) (2012) 2270–2275, https://doi.org/10.1007/s12032-011-0032-8.
[59] A.D. Bolnick, et al., Enhancement of trophoblast differentiation and survival by low molecular weight heparin requires heparin-binding EGF-like growth factor, Hum. Reprod. 32 (6) (2017) 1218–1229, https://doi.org/10.1093/humrep/dex069.

[60] V.B. Wali, et al., Convergent and divergent cellular responses by ErbB4 isoforms in mammary epithelial cells, Mol. Cancer Res. 12 (8) (2014) 1140–1155, https://doi.org/10.1158/1541-7786.MCR-13-0637.
[61] L. Fang, G. Li, G. Liu, S.W. Lee, S.A. Aaronson, p53 induction of heparin-binding EGF-like growth factor counteracts p53 growth suppression through activation of MAPK and PI3K/Akt signaling cascades, EMBO J. 20 (8) (2001) 1931–1939, https://doi.org/10.1093/emboj/20.8.1931.