Biochemical and Biophysical Research Communications 461 (2015) 128–135

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Transforming growth factor-α induces human ovarian cancer cell invasion by down-regulating E-cadherin in a Snail-independent manner

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ARTICLE INFO

Article history:
Received 19 March 2015
Available online 11 April 2015

Keywords:
TGF-α
E-cadherin
Ovarian cancer
Slug
Snail
HER2

ABSTRACT

Transforming growth factor-α (TGF-α), like epidermal growth factor (EGF) and amphiregulin (AREG) binds exclusively to EGF receptor (EGFR). We have previously demonstrated that EGF, AREG and TGF-α down-regulate E-cadherin and induce ovarian cancer cell invasion, though whether these ligands use the same molecular mediators remains unknown. We now show that, like EGF, TGF-α- and AREG-induced E-cadherin down-regulation involves both EGFR and HER2. However, in contrast to EGF and AREG, the transcription factor Snail is not required for TGF-α-induced E-cadherin down-regulation. This study shows that TGF-α uses common and divergent molecular mediators to regulate E-cadherin expression and cell invasion.

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1. Introduction

Ovarian cancer is the fifth most common cause of cancer death in women and the leading cause of death from gynecological cancers in developed countries [1,2]. Epidermal growth factor receptor (EGFR) has been shown to be overexpressed in ovarian cancer and is associated with poor prognosis [3]. EGFR, also known as ERBB1 or HER1, belongs to the ERBB family of receptor tyrosine kinases, which includes three other members, HER2 (ERBB2), HER3 (ERBB3) and HER4 (ERBB4) [4]. Multiple cognate ligands, including transforming growth factor-α (TGF-α), amphiregulin (AREG), epidermal growth factor (EGF), heparin-binding EGF, epiregulin and betacellulin can bind to and activate EGFR. AREG, EGF and TGF-α have been shown to bind exclusively to EGFR while the other ligands can also bind other ERBB family receptors [5–7]. In ovarian cancer, AREG, EGF and TGF-α are expressed and act as autocrine factors to regulate disease progression [8,9].

Although AREG, EGF and TGF-α bind exclusively to EGFR, they have diverse functions and can act either redundantly or differentially. AREG has been shown to have a lower affinity for EGFR compared with EGF or TGF-α [10]. Additionally, binding between AREG and heparin and heparan sulfate proteoglycans can potentiate its bioavailability and activity [11]. A previous study has shown that treatment with AREG, but not TGF-α, induces a spindle-like morphology in MDCK cells [12]. Interestingly, AREG concentrations in peritoneal fluid of ovarian cancer patients are higher than those of TGF-α [13]. Similarly, AREG levels are also higher than EGF or TGF-α levels in ovarian cancer tissues and cell lines [14,15]. These results strongly suggest that these three EGFR ligands (AREG, EGF and TGF-α) may have differing roles in ovarian cancer development and/or progression.

Cadherins constitute a large family of cell membrane glycoproteins that play important roles in mediating cell–cell adhesion. E-cadherin is a prototypical classical cadherin that plays an essential role in maintaining normal epithelial structure [16]. E-cadherin is a well-characterized tumor suppressor, best-known for its important functions in epithelial–mesenchymal transition (EMT). During EMT, down-regulation of E-cadherin expression leads to loss of epithelial characteristics and acquisition of a mesenchymal phenotype, which promotes cell proliferation, motility and invasiveness and contributes to cancer progression [17].

We have shown that EGF induces human ovarian cancer cell invasion by down-regulating E-cadherin expression through

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http://dx.doi.org/10.1016/j.bbrc.2015.03.180
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various signaling pathways [18–20]. Recently, we demonstrated that AREG and TGF-α also suppress E-cadherin and induce cell invasion [21], though whether they use the same molecular mediators remains unknown. Therefore, the aim of this study was to compare the underlying molecular mechanisms mediating the effects of TGF-α, AREG and EGF on E-cadherin expression.

2. Materials and methods

2.1. Cell culture

The SKOV3 human ovarian cancer cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in a 1:1 (v/v) mixture of M199/MCDB105 medium (Sigma–Aldrich, Oakville, ON) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT) at 37 °C in a humidified 5% CO_2 atmosphere.

2.2. Antibodies and reagents

Monoclonal anti-E-cadherin and anti-N-cadherin antibodies were obtained from BD Biosciences (Mississauga, ON). Monoclonal anti-α-Tubulin and polyclonal anti-EGFR antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-Slug and monoclonal anti-HER2 and anti-Snail antibodies were obtained from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Bio-Rad Laboratories (Hercules, CA). Recombinant human EGF and AG1478 were obtained from Sigma–Aldrich. Recombinant human AREG and TGF-α were purchased from R&D Systems (Minneapolis, MN). AG825 was obtained from Tocris Bioscience (Bristol, UK).

2.3. Small interfering RNA (siRNA) transfection and E-cadherin overexpression

To knockdown endogenous EGFR, HER2, Snail or Slug, cells were transfected with 50 nM ON-TARGET*plus* SMARTpool siRNA (Dharmacon Research, Inc., Lafayette, CO) using Lipofectamine RNAiMAX (Invitrogen, Burlington, ON). siCONTROL Non-targeting siRNA (Dharmacon) was used as a transfection control. For E-cadherin overexpression, cells were transfected with empty pcDNA3.1-EGFP (Invitrogen) or pcDNA3.1-human E-cadherin-EGFP (plasmid 28009; Addgene, Cambridge, MA) vectors using Lipofectamine LTX (Invitrogen).

2.4. Western blots

Equal amounts of protein were separated by SDS polyacrylamide gel electrophoresis and transferred to PVDF membranes. After being blocked for 1 h with 5% non-fat dry milk in Tris-buffered saline (TBS), the membranes were incubated overnight at 4 °C with primary antibodies diluted in 5% non-fat milk-TBS. Following primary antibody incubation, the membranes were incubated with the appropriate HRP-conjugated secondary antibody and immunoreactive bands were detected using an enhanced chemiluminescent substrate and X-ray film.

2.5. Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed with 3 μg RNA, random primers and M-MLV reverse transcriptase (Promega, Madison, WI). The primers used for SYBR Green RT-qPCR were as follows: E-cadherin, 5′-ACAGCCCCGCCTTATGATT-3′ (sense) and 5′-TCGGAACCGCTTCCTTCA-3′(antisense); EGFR, 5′-GGTGCAGGAGAGGAGAACTGC-3′ (sense) and 5′-GGTGGCACCAAAGCTGTATT-3′ (antisense); Snail, 5′-CCCCAATCGGAAGCCTAACT-3′ (sense) and 5′-GCTGGAAGGTAAACTCTGGATTAGA-3′ (antisense); Slug, 5′-TTCGGACCCACACATTACCT-3′ (sense) and 5′-GCAGTGAGGGCAAGAAAAAG-3′ (antisense); HER2, 5′-AACTGCACCCACTCCTGTGT-3′ (sense) and 5′-TGATGAGGATCCCAAAGACC-3′ (antisense); and GAPDH, 5′-GAGTCAACGGATTTGGTCGT-3′ (sense) and 5′-GACAAGCTTCCCGTTCTCAG-3′ (antisense). RT-qPCR was performed using an Applied Biosystems 7300 Real-Time PCR System equipped with 96-well optical reaction plates. All RT-qPCR results represent the mean from at least three independent experiments conducted in triplicate. Relative quantification of mRNA levels was performed by the comparative Cq method using GAPDH as the reference gene and the formula $2^{-\Delta\Delta Cq}$.

2.6. Invasion assay

Transwell invasion assays were performed as previously described with minor modifications [22]. Cell culture inserts (24-well, pore size 8 μm; BD Biosciences, Mississauga, ON) pre-coated with growth factor reduced Matrigel (40 μl, 1 mg/ml; BD Biosciences) were seeded with 1×10^5 cells in 250 μl of medium supplemented with 0.1% FBS. Medium with 10% FBS (750 μl) was added to the lower chamber and served as a chemotactic agent. After incubation for 48 h, non-invading cells were removed from the upper side of the membrane and the cells on the lower side of the membrane were fixed with cold methanol and air dried. The cell nuclei were stained with Hoechst 33,258 and counted by epifluorescence microscopy using Northern Eclipse 6.0 software (Empix Imaging, Mississauga, ON).

2.7. Statistical analysis

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

3. Results

3.1. TGF-α increases cell invasion by down-regulating E-cadherin

Previous studies on MDCK kidney cells demonstrated differential effects of AREG and TGF-α on cell morphology [12], however to date, comparative effects of EGFR ligands on ovarian cancer cell morphology have not been examined. Similar to EGF, treatment of SKOV3 cells with TGF-α or AREG induced EMT-like morphological changes from a cobblestone-like shape to a fibroblast-like, spindle-shaped appearance (Fig. 1A). Next, we compared the time-dependent effects of EGFR ligands on E-cadherin mRNA levels in SKOV3 cells. As shown in Fig. 1B, treatment with TGF-α, AREG or EGF induced equivalent reductions in E-cadherin mRNA levels after 6 h, with maximal reductions observed after 24 h. Western blot analysis showed that treatment with these EGFR ligands also produced comparable decreases in E-cadherin protein levels (Fig. 1C) though, like AREG [21], neither EGF nor TGF-α affected N-cadherin protein levels (Fig. 1D). To confirm that E-cadherin loss contributes to TGF-α-induced cell invasion, we overexpressed E-cadherin in SKOV3 cells and examined cell invasiveness in response to TGF-α treatment. As shown in Fig. 1C, compared to cells transfected with empty vector, cells transfected with E-cadherin expression vector displayed increased E-cadherin protein levels that were insensitive to treatment with TGF-α, AREG or EGF. Invasion assay results

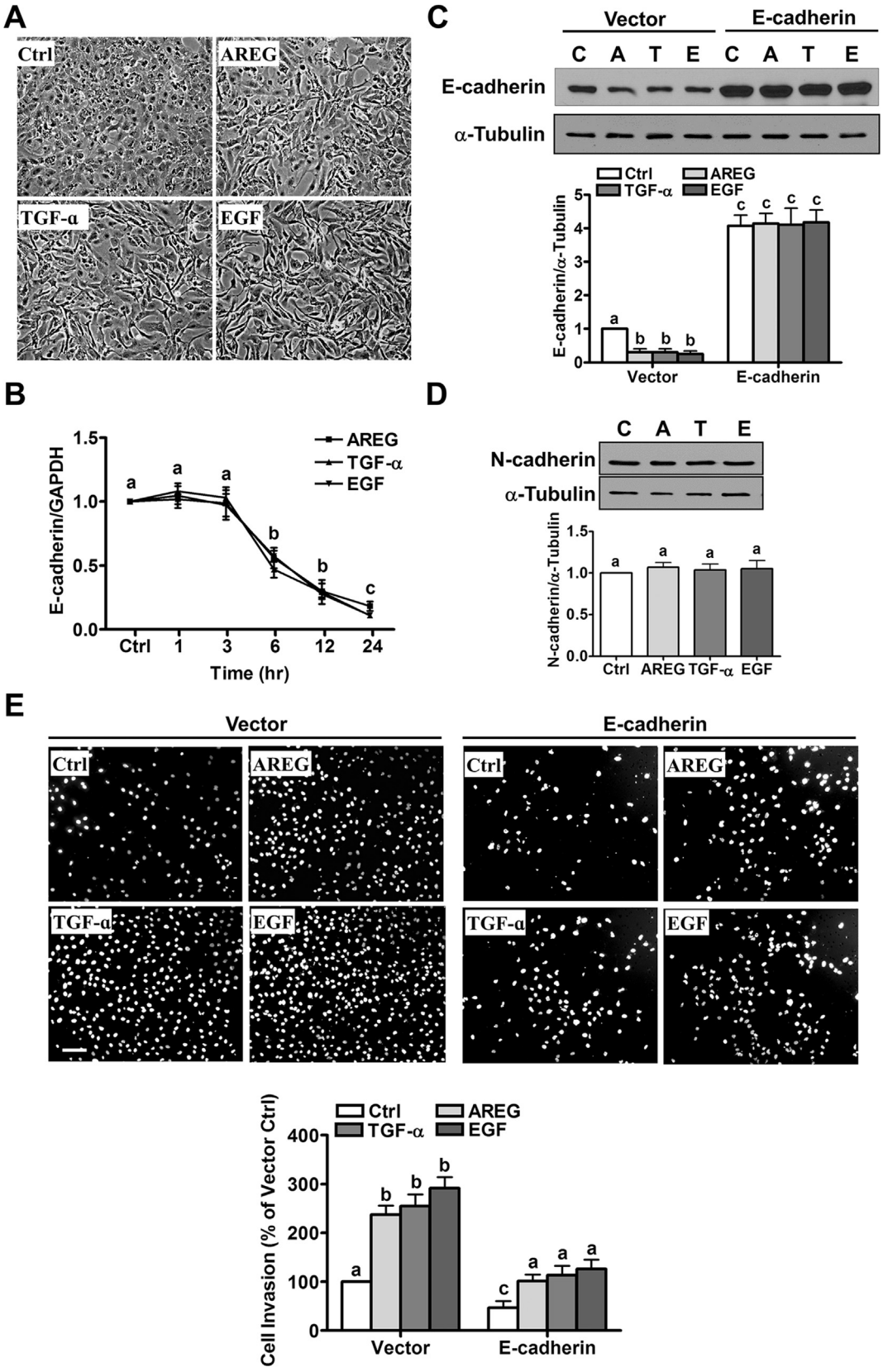

Fig. 1. Down-regulation of E-cadherin is required for TGF-α-, AREG- or EGF-induced SKOV3 ovarian cancer cell invasion. A, Cells were treated without or with 100 ng/ml TGF-α, AREG or EGF for 24 h, and cell morphology was assessed by phase contrast microscopy. B, Cells were treated with 100 ng/ml TGF-α, AREG or EGF and E-cadherin mRNA levels were analyzed at different time-points by RT-qPCR. C, Cells were transfected with empty vector (Vector) or vector encoding human E-cadherin (E-cadherin) for 24 h and then treated with 100 ng/ml TGF-α (T), AREG (A) or EGF (E) for another 24 h. E-cadherin protein levels were analyzed by Western blot. D, Cells were treated for 24 h with 100 ng/ml TGF-α (T), AREG (A) or EGF (E) and N-cadherin protein levels were analyzed by Western blot. E, Cells were transfected for 24 h with empty vector or E-cadherin vector and then treated with or without 100 ng/ml TGF-α, AREG or EGF. The cell invasiveness was examined by invasion assay. Results are expressed as the mean ± SEM from at least three independent experiments. Values without a common letter are significantly different ($p < 0.05$).

showed that E-cadherin overexpression decreased basal invasiveness and attenuated TGF-α-induced cell invasiveness (Fig. 1E). These results indicate that, as for AREG and EGF, down-regulation of E-cadherin plays a key role in TGF-α-induced ovarian cancer cell invasion.

3.2. EGFR is required for TGF-α- and AREG-induced down-regulation of E-cadherin

To examine whether EGFR is required for TGF-α- or AREG-induced down-regulation of E-cadherin, the EGFR-specific inhibitor AG1478 was used to block EGFR function. RT-qPCR and Western blot analyses showed that treatment of SKOV3 cells with AG1478 abolished the down-regulation of E-cadherin mRNA and protein by TGF-α or AREG (Fig. 2A and B). We also employed a siRNA-mediated knockdown approach to exclude possible off-target effects from pharmacological inhibition. As shown in Fig. 2C and D, treatment with EGFR siRNA significantly down-regulated endogenous EGFR mRNA and protein levels. Additionally, EGFR knockdown abolished the down-regulation of E-cadherin mRNA and protein levels by TGF-α and AREG. These results confirm that, like EGF, TGF-α and AREG suppress E-cadherin expression via EGFR.

3.3. HER2 is involved in TGF-α- and AREG-induced down-regulation of E-cadherin

HER2 has no identified ligand, however it can be activated by dimerizing with other ligand-bound ERBB family receptors [23]. We previously demonstrated that HER2 is involved in EGF-induced down-regulation of E-cadherin in ovarian cancer cells [20], though

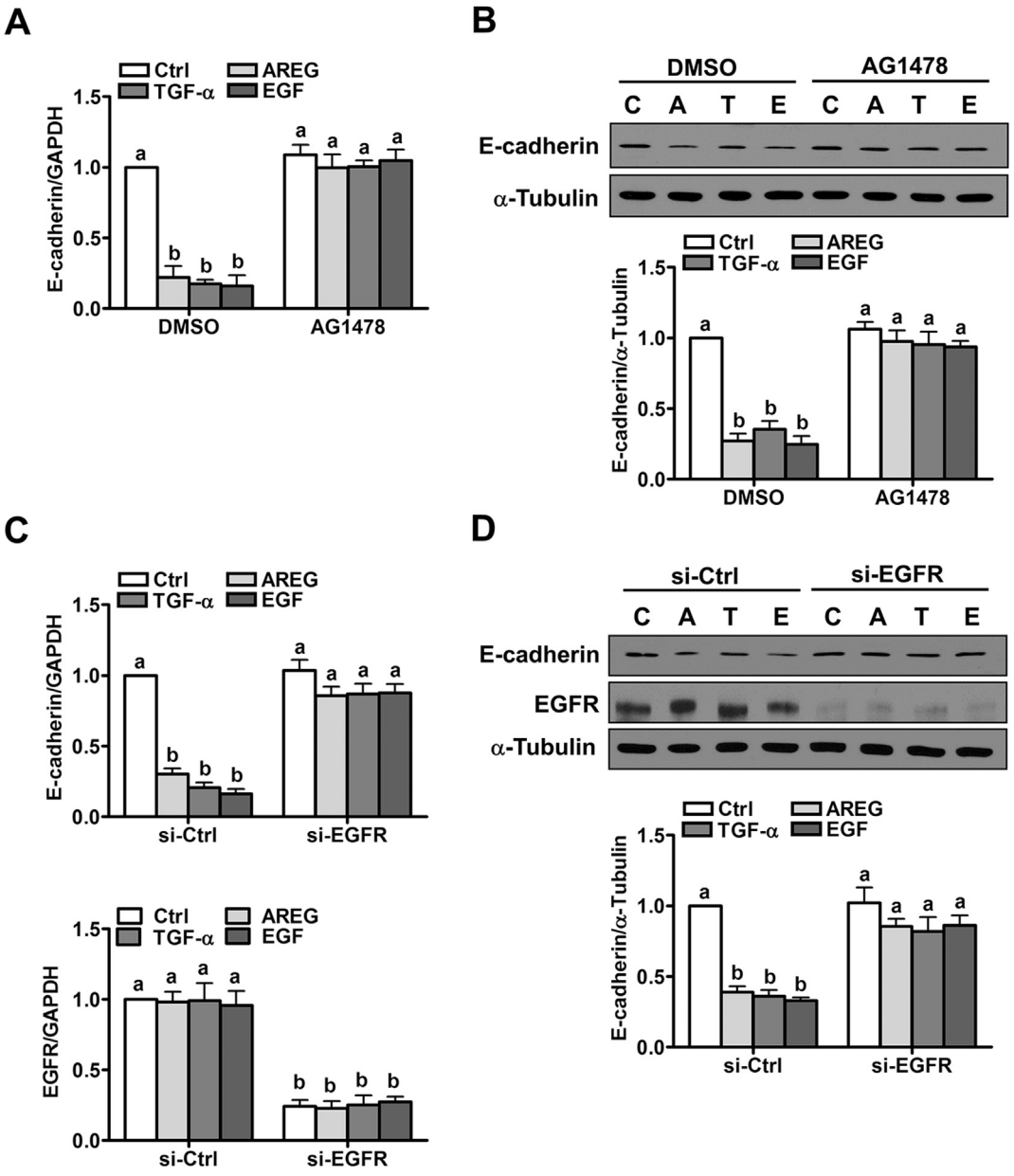

Fig. 2. EGFR is required for TGF-α-, AREG- or EGF-induced down-regulation of E-cadherin. A and B, SKOV3 cells were pretreated for 1 h with AG1478 (10 μM) and then treated with 100 ng/ml TGF-α (T), AREG (A) or EGF (E) for 24 h. E-cadherin mRNA (A) and protein (B) levels were analyzed by RT-qPCR and Western blot, respectively. C and D, Cells were transfected for 48 h with 50 nM control siRNA or EGFR siRNA and then treated with 100 ng/ml TGF-α (T), AREG (A) or EGF (E) for 24 h. E-cadherin and EGFR mRNA (C) and protein (D) levels were analyzed by RT-qPCR and Western blot, respectively. Results are expressed as the mean ± SEM from at least three independent experiments. Values without a common letter are significantly different ($p < 0.05$).

whether the same is true for TGF-α or AREG is unknown. As shown in Fig. 3A and B, treatment of SKOV3 cells with the HER2-specific inhibitor AG825 partially attenuated the down-regulation of E-cadherin mRNA and protein levels by TGF-α or AREG. Similarly, TGF-α- and AREG-induced down-regulation of E-cadherin mRNA and protein levels was attenuated by siRNA-mediated knockdown of HER2 (Fig. 3C and D). These results indicate that HER2 contributes to the suppression of E-cadherin by TGF-α and AREG in ovarian cancer cells.

3.4. TGF-α-induced down-regulation of E-cadherin is Snail-independent

The transcription factors Snail and Slug are well-characterized negative regulators of E-cadherin expression [24]. Though we have previously demonstrated that Snail and Slug are required for AREG-induced ovarian cancer cell invasion, it is not known whether they are required for TGF-α-, AREG- or EGF-induced E-cadherin down-regulation. Similar to AREG and EGF, treatment of SKOV3 cells with TGF-α induced time-dependent increases in the mRNA levels of both Snail and Slug, with maximal effects occurring after 3 h (Fig. 4A). Western blot analyses confirmed the stimulatory effects of TGF-α on Snail and Slug protein levels (Fig. 4B). Next, we used siRNA-mediated knockdown of Snail or Slug to examine their involvement in TGF-α-, AREG- or EGF-induced E-cadherin down-regulation. As shown in Fig. 4C, treatment with Snail or Slug siRNA abolished the up-regulation of Snail and Slug protein levels by TGF-α, AREG or EGF. Interestingly, TGF-α-induced reductions in E-cadherin protein levels were unaffected by Snail knockdown, whereas those of AREG and EGF were partially reversed (Fig. 4D). In contrast, Slug knockdown attenuated the suppressive effects of all

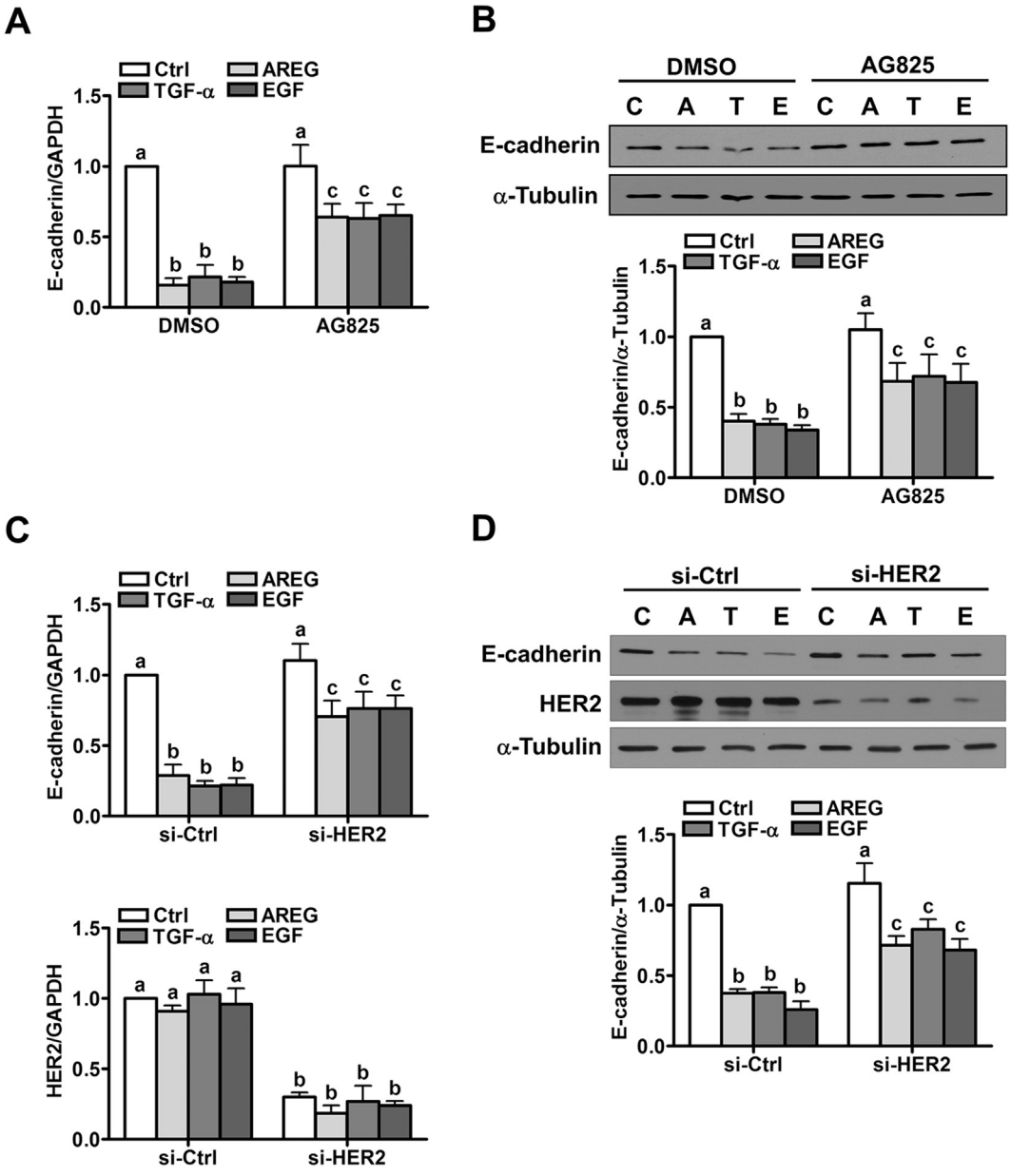

Fig. 3. HER2 is involved in the down-regulation of E-cadherin by TGF-α, AREG or EGF. A and B, SKOV3 cells were pretreated for 1 h with AG825 (10 μM) and then treated with 100 ng/ml TGF-α (T), AREG (A) or EGF (E) for 24 h. E-cadherin mRNA (A) and protein (B) levels were analyzed by RT-qPCR and Western blot, respectively. C and D, Cells were transfected for 48 h with 50 nM control siRNA or HER2 siRNA and then treated with 100 ng/ml TGF-α (T), AREG (A) or EGF (E) for 24 h. E-cadherin and HER2 mRNA (C) and protein (D) levels were analyzed by RT-qPCR and Western blot, respectively. Results are expressed as the mean ± SEM from at least three independent experiments. Values without a common letter are significantly different ($p < 0.05$).

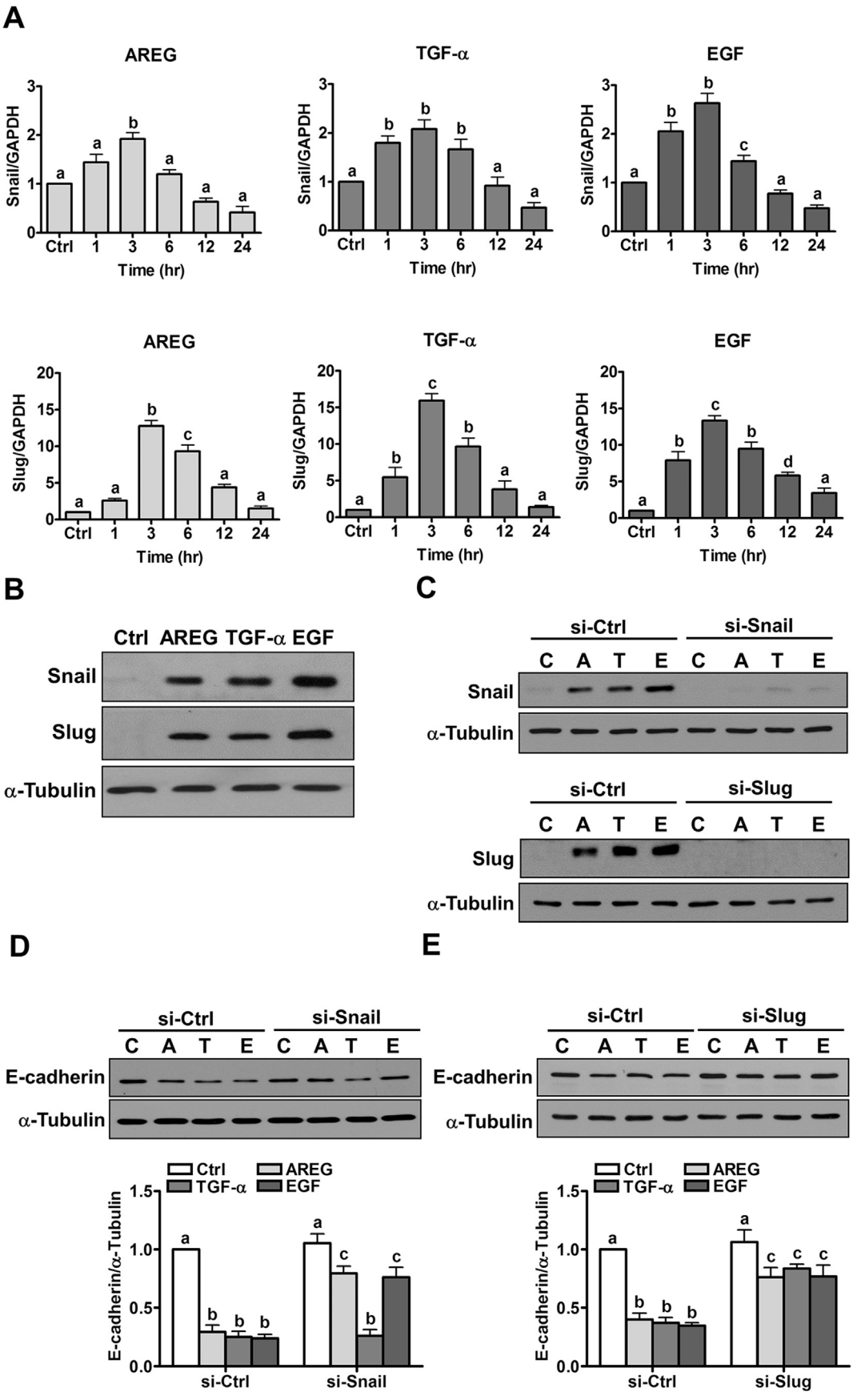

Fig. 4. Snail and Slug are differentially involved in TGF-α-, AREG- or EGF-induced down-regulation of E-cadherin. A, SKOV3 cells were treated for varying amounts of time with 100 ng/ml TGF-α, AREG or EGF and RT-qPCR was used to measure Snail and Slug mRNA levels. B, Cells were treated for 3 h with 100 ng/ml TGF-α, AREG or EGF and Snail and Slug protein levels were analyzed by Western blot. C, Cells were transfected for 48 h with 50 nM control siRNA, Snail siRNA or Slug siRNA and then treated with 100 ng/ml TGF-α (T), AREG (A) or EGF (E) for 3 h. Snail and Slug protein levels were analyzed by Western blot. D and E, Cells were transfected for 48 h with 50 nM control siRNA, Snail siRNA (D) or Slug siRNA (E) and then treated with 100 ng/ml TGF-α (T), AREG (A) or EGF (E) for 24 h. E-cadherin protein levels were analyzed by Western blot. Results are expressed as the mean ± SEM from at least three independent experiments. Values without a common letter are significantly different ($p < 0.05$).

three EGFR ligands on E-cadherin protein levels (Fig. 4E). These results indicate that the down-regulation of E-cadherin by TGF-α in ovarian cancer cells differs from that of AREG and EGF in that it is Snail-independent.

4. Discussion

EGFR signaling is known to regulate various cellular functions that play important roles in modulating multiple developmental, physiological and pathological processes [7,25]. In mice, *Egfr* knockout results in embryonic to perinatal lethality due to pleiotropic abnormalities [26–28]. However, mice deficient in *Tgf-α*, *Areg*, *Egf* or all three ligands combined are viable [29]. Differences in viability between receptor and ligand knockout may result from ERBB family receptor heterodimerization, where other ERBB ligands can compensate for the loss of these three *Egfr* ligands and maintain *Egfr* activity. Moreover, *Tgf-α*, *Areg* and *Egf* knockout mice each exhibit different developmental defects, which indicate a variety of potentially unique biological functions for these EGFR ligands. Here, we focused only on the effects of TGF-α, AREG and EGF on E-cadherin and cancer cell invasion, but whether these three ligands utilize common or divergent molecular mechanisms to regulate other biological functions will be an interesting question for future investigation.

Though HER2 is a member of the ERBB family, it does not bind EGF-like ligands and functions instead as a heterodimerization partner for other ligand-bound ERBB family receptors [23]. Amplification or overexpression of HER2 is frequent in many types of human cancers, including ovarian cancer [30]. HER2 has been shown to potentiate EGFR-induced signaling through the formation of heterodimers with EGFR [31]. In ovarian cancer, treatment with pertuzumab, a HER2 dimerization inhibitor, induces anti-tumor activity in xenograft models [32]. In addition, we have shown that HER2 is involved in EGF-induced E-cadherin down-regulation and ovarian cancer cell invasion [20], though whether it is involved in the corresponding effects of TGF-α and AREG remains unknown. Our study is the first to show that blocking HER2 activity, either pharmacologically or by knockdown, partially inhibits the suppressive effects of TGF-α and AREG on E-cadherin expression. Together with our previous results, these findings indicate that a subset of TGF-α-, AREG- and EGF-induced effects involve HER2, and that simultaneous EGFR and HER2 inhibition may be necessary to produce optimal therapeutic effects in ovarian cancers driven by elevated EGF-family-ERBB signaling.

Although Snail and Slug are well-known transcriptional repressors of E-cadherin [33,34], whether they function redundantly or differentially is not fully understood. Previous studies have shown that the binding affinity of Snail for the mouse E-cadherin promoter is higher than that of Slug [34], and that Slug overexpression does not affect E-cadherin expression in rat bladder cancer cells [35]. In human breast cancer, while both Snail and Slug can repress E-cadherin expression *in vitro*, only Slug expression correlated with E-cadherin down-regulation *in vivo* [36]. Likewise, expression of Slug but not Snail was negatively correlated with E-cadherin expression in human bladder cancer [37]. These results indicate that the repressive effects of Snail and Slug on E-cadherin expression are variable, and may be cell-type or context dependent. Though we have previously demonstrated that TGF-α, AREG and EGF can induce Slug and/or Snail expression and E-cadherin down-regulation in human ovarian cancer cells [18,21], the direct involvement of Snail and/or Slug in the effects of these ligands on E-cadherin has never been demonstrated. Interestingly, our results indicate that although these ligands have comparable effects on E-cadherin, they use common and divergent molecular mediators to achieve these effects. Specifically, while both Snail and Slug are involved in AREG- and EGF-induced E-cadherin down-regulation, the effects of TGF-α on E-cadherin do not involve Snail. In addition to Snail and Slug, other transcription factors have been shown to repress E-cadherin expression, such as Twist and ZEB1 [38]. We have previously demonstrated that AREG can up-regulate ZEB1 in ovarian cancer cells, however it was not required for AREG-induced cell invasion [21]. Future studies will be required to compare the effects of TGF-α, AREG and EGF on Twist and ZEB1 expression, and to examine their roles in the effects of these ligands on E-cadherin expression and ovarian cancer cell invasion.

In summary, this study demonstrates that TGF-α induces ovarian cancer cell invasion by down-regulating E-cadherin. Similar to EGF, TGF-α- and AREG-induced E-cadherin down-regulation involves both EGFR and HER2. However, whereas the effects of TGF-α on E-cadherin are mediated by Slug, but not Snail, both Snail and Slug mediate the effects of AREG and EGF on E-cadherin. This study shows that TGF-α uses common and divergent molecular mediators to regulate E-cadherin expression and cell invasion.

Disclosure statement

The authors have nothing to disclose.

Grant support

This work was supported by an operating grant from the Canadian Institutes of Health Research to P.C.K.L.

Conflict of interest

None.

Acknowledgments

This work was supported by grants from the Canadian Institutes of Health Research to P.C.K.L. X.Q. and Q. F. are supported by the China Scholarship Council.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.180.

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