Abstract. Amphiregulin (AREG) is a member of the epidermal growth factor (EGF) family and is expressed in a plethora of cancers. The biological roles of AREG in the regulation of the epithelial-mesenchymal transition (EMT) in pancreatic cancer remain unclear. To investigate the expression of epidermal growth factor receptor (EGFR) and AREG in pancreatic cancer cell lines, RT-qPCR, western blot analysis, and ELISA were performed. RNAi and exogenous AREG treatment were used to alter AREG expression. Wound-healing and Transwell assays were performed to evaluate cell migration and invasion abilities. Western blot analysis and immunofluorescence staining were utilized to detect the expression of EMT markers. The protein expression of potential key factors involved in EMT, as well as those of the ERK, AKT, STAT3 and NF-κB pathways, were analysed by western blotting. The role of AREG in tumour growth in vivo was further determined using an orthotopic model of pancreatic cancer. Knockdown of AREG inhibited AsPC-1 cell migration and invasion. AREG knockdown upregulated E-cadherin but downregulated vimenterin, Snail and Slug expression in AsPC-1 cells. In addition, AREG stimulation increased cell migration, invasion and EMT in PANC-1 cells, and an NF-κB inhibitor decreased AREG-induced cell migration, invasion and EMT in PANC-1 cells. AREG stimulation increased the nuclear accumulation of NF-κB through the EGFR/ERK signalling pathway to induce EMT. Tumour growth and metastasis were decreased by AREG silencing in an orthotopic model of pancreatic cancer. AREG may play a critical role in cell migration, invasion, and EMT by activating the EGFR/ERK/NF-κB signalling pathway in pancreatic cancer cells.

Introduction
Pancreatic cancer is one of the leading causes of cancer-related mortality worldwide, and the 5-year survival rate is less than 9%; more than 50% of cases are diagnosed at an advanced stage, which partially explains the low survival rate (1). The high mortality rate among advanced pancreatic cancer patients is linked to their limited response to chemotherapy and radiotherapy. The epithelial-mesenchymal transition (EMT) phenotype is a critical mediator of drug resistance in pancreatic cancer patients (2). The activation of the EMT process has been proposed as the critical mechanism responsible for the acquisition of drug resistance in pancreatic cancer cells (3,4). Furthermore, EMT has been revealed to contribute to the invasion and metastasis of various types of carcinomas, including pancreatic cancer (5-7). However, the vital role of EMT in cancer invasion and metastasis has been recently challenged based on murine studies (8,9).

Amphiregulin (AREG) is a member of the epidermal growth factor (EGF) family and is expressed in a plethora of neoplasms, including ovarian, breast, bladder, colon, lung, major salivary glands and pancreatic cancers (10-16). AREG interacts with epidermal growth factor receptor (EGFR) to trigger numerous signalling cascades that mediate cell survival, proliferation, and motility. According to studies investigating ovarian cancer (10), and breast cancer (11), AREG plays a crucial role in cell invasion and metastasis. AREG was linked to a reduced life span in bladder cancer (12) and lung cancer patients (13). However, AREG overexpression was associated with a longer disease-free survival in colorectal cancer (14) and mucoepidermoid carcinoma patients (15). In our previous study, it was revealed that AREG expression was correlated with a poor prognosis in pancreatic cancer patients (16). To date, knowledge regarding the molecular mechanisms underlying AREG-induced migration, invasion and EMT in pancreatic cancer cells is limited.
pancreatic cancer cells is limited. The aim of this study was to
detect the molecular mechanism of AREG mediating EMT in
 pancreatic cancer cells in vitro and in vivo.

Materials and methods

Cell culture. Four human pancreatic cancer cell lines, PANC-1
(cat. no. CRL-1469), AsPC-1 (cat. no. CRL-1682), BxPC-3
(cat. no. CRL-1687) and MIA PaCa-2 (cat. no. CRL-1420) were
purchased from American Type Culture Collection (ATCC).
The cell lines (PANC-1, AsPC-1, BxPC-3 and MIA PaCa-2) had been identified by specialized STR profiling and tested for
mycoplasma contamination. PANC-1 and MIA PaCa-2 were
maintained in Dulbeccos Modified Eagle's Medium (DMEM),
aswere PANC-1 and BxPC-2 were maintained in RPMI-1640
medium (both from Corning Incorporated). The medium was
supplemented with 10% fetal bovine serum (FBS; Gibco;
Thermo Fisher Scientific, Inc.), 1% penicillin/streptomycin and
1 mM glutamine (Invitrogen; Thermo Fisher Scientific,
Inc.). The cells were grown in 5% CO₂ at 37°C in a humidified
atmosphere.

Antibodies and reagents. EMT Antibody Sampler Kit
(cat. no. 9782), NF-κB pathway Sampler Kit (cat. no. 9936),
Phospho-EGF Receptor Antibody Sampler Kit (cat. no. 9922)
and anti-phospho-STAT3 (cat. no. 9145) antibody were
purchased from Cell Signaling Technology, Inc., except
for anti-ERK (cat. no. sc-154) and anti-phospho-ERK
(cat. no. sc-7976-R), anti-AKT (cat. no. sc-81434) and
anti-phospho-AKT (cat. no. sc-81433) antibodies (Santa Cruz Biotechnology, Inc.). The recombinant human AREG protein (cat. no. 262-AR-100) and
normal goat IgG control (cat. no. AB-108-C) were purchased from R&D Systems. PD153035 (EGFR tyrosine kinase inhib-
itor), U0126 and QNZ were dissolved in dimethyl sulphoxide (DMSO) and
were purchased from Shanghai GenePharma Co., Ltd. AREG
ELISA kit (cat. no. DAR00; R&D systems) according to the manufacturer's instructions.

RNA interference. The siRNAs targeting AREG were as follows:
AREG-Homo-390 (siAR-390), 5'-GGAUUUGAGG
GUUACCUCATT-3' (sense) and 5'-UUGAGGUAACCU
AAUUGCTT-3' (antisense); AREG-Homo-817 (siAR-817),
5'-GCAUGUUUGACAGUGUUTT-3' (sense) and 5'-AAACUACUGUCAUACUGCTT-3' (antisense); and
AREG-Homo-348 (siAR-348), 5'-UCGUUGAGCG
UAGCAATT-3' (sense) and 5'-UUGGUUACGCUUCC
AGATT-3' (antisense). The siRNA control sequences were
5'-UUUCUGGAAUGGUACUGTTT-3' (sense) and 5'-ACG
UGACAGCUGCCGAGATT-3' (antisense). All siRNAs
were purchased from Shanghai GenePharma Co., Ltd. AREG
was transfected into AsPC-1 cells in 6-well plates using
Lipofectamine RNAiMAX Reagent (Invitrogen; Thermo
Fisher Scientific, Inc.). Total protein and RNA were extracted,
and the knockdown efficiency of AREG was verified by
western blotting and RT-qPCR analyses, respectively.

Immunofluorescence. AsPC-1 cells were transfected with
AREG siRNA (siAR-390) or control siRNA (NC) for 48 h.
PANC-1 cells were treated with AREG (100 ng/ml, AREG),
or normal IgG (100 ng/ml, NC) for 48 h. A total of 2x10⁵ cells
were seeded on coverslips. After 24 h, serum-free culture
medium was removed from each well and replaced with 4% paraformaldehyde, and the plates were incubated for 15 min
at room temperature for fixation. Then, the cells were washed
with PBS three times. After blockade of non-specific epitopes
by 10% normal goat serum for 1 h at room temperature, primary antibodies against E-cadherin (1:100 dilution; cat. no. 3195;
Cell Signaling Technology, Inc.) or vimentin (1:200 dilution;
cat. no. 5741; Cell Signaling Technology, Inc.) were added.
After overnight incubation at 4°C, the primary antibody was
washed with rinsing three times with PBS, followed by incu-
bation of secondary antibodies (Alexa Fluor®488 Conjugate,
cat. no. 4412 or Alexa Fluor®594 Conjugate, cat. no. 8889, Cell
Signaling Technology, Inc., 1:1,000 dilution) for 1 h at room
temperature. The cells were stained with DAPI and observed
under a fluorescence microscope (Olympus Corp.).

Western blot analysis. Western blot analysis was performed
as previously described (17). Dilution rates for all primary and
secondary antibodies were 1:1,000 and 1:10,000, respec-
tively. The blots were probed with ECL Plus Western Blotting
Detection kit (Pierce Biotechnology; Thermo Fisher Scientific,
Inc.). The densities of the bands were scanned and analysed
using ImageJ software (version 1.48v; National Institutes of
Health).

ELISA. The secretion level of AREG was assessed using an
AREG ELISA kit (cat. no. DAR00; R&D systems) using ImageJ software (version 1.48v; National Institutes of
Health).
on the Transwell membrane were fixed and stained (by 4% paraformaldehyde and 0.1% crystal violet, respectively, for 15 min each at room temperature). Finally, the number of cells was counted in five random fields (magnification, ×100; BX53 upright microscope; Olympus Corp.).

Wound-healing assay. PANC-1 cells were treated with AREG (100 ng/ml) alone (AREG), AREG combined with QNZ (20 µM, AREG+QNZ), or normal IgG (100 ng/ml, NC) for 48 h. A total of 5x10⁵ cells were seeded in a 6-well plate and then, an incision of <1 cm was performed to expose the pancreas. AsPC-1 cells (2x10⁶) were injected into the tail of seven-week-old female athymic nude mice (BALB/c nu/nu) were randomly assigned to 2 groups. Six mice with LV-AREG cells were distributed to the experimental group, while the other 6 mice with LV-con cells were in the control group. In detail, the mice were anaesthetized and placed in a supine position; the abdomen was disinfected with 70% alcohol, and then, an incision of <1 cm was performed to expose the pancreas. AsPC-1 cells (2x10⁶) were injected into the tail of the pancreas, and the wound was sutured. The mice were anaesthetized again and weighed twice weekly. The mice were sacrificed by CO₂ asphyxiation on the 39th day after the injection. The pancreas was removed, and the tumours were weighed. The stomach, spleen, liver, intestine and kidney were examined for the presence of metastases. This study was conducted with the approval of the Institutional Animal Care and Use Committee of Peking Union Medical College Hospital and was in accordance with the National Policy on Use of Laboratory Animals.

Statistical analysis. All data were sorted and analysed using SPSS software for Windows, version 17.0 (SPSS, Inc.). The results are expressed as the mean ± SD. Significance was evaluated by performing Student's t-tests or one-way analysis of variance (ANOVA) with Tukey's post hoc test. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

EGFR and AREG expression in pancreatic cancer cells. To investigate EGFR and AREG expression in pancreatic cancer cell lines, RT-qPCR, western blot analysis and ELISA were performed. These analyses revealed that EGFR expression was significantly high in the AsPC-1 cells and BxPC-3 cells compared with that in MIA PaCa-2 cells (Fig. 1A and B). AREG expression was significantly high in the AsPC-1 cells, BxPC-3 cells and MIA PaCa-2 cells, compared with that in PANC-1 cells (Fig. 1C and D). AsPC-1 (high EGFR expression and high AREG expression) and PANC-1 cells (high EGFR expression and low AREG expression) were selected for the subsequent loss-of-function and gain-of-function experiments. To characterize the biological effects of AREG, AREG siRNAs were used to suppress the endogenous expression of AREG in AsPC-1 cells. Three different AREG siRNAs and 1 control siRNA were used. The results revealed that siAR-390 was most effective in inhibiting AREG mRNA (65% reduction) and protein (64% reduction) expression in AsPC-1 cells (Fig. S1). Therefore, siAR-390 was used in the subsequent experiments.

AREG facilitates the migration and invasion of pancreatic cancer cells. Transwell assays were performed to monitor whether AREG could affect pancreatic cancer cell migration and invasion. The results revealed a nearly 50% reduction in cell migration and 47% reduction in cell invasion in AREG siRNA-transfected AsPC-1 cells (P<0.05 and P<0.01, Fig. 2A). Knockdown of AREG significantly decreased the migration ability of BxPC-3 cells (P<0.01, Fig. S2). To confirm the involvement of AREG in pancreatic cancer cell migration and invasion, recombinant human AREG treatment was used. Exposure to exogenous AREG (100 ng/ml) significantly promoted the migration and invasion of the PANC-1 cells (P<0.05 and P<0.01, Fig. 2B). It was revealed that AREG was expressed in activated pancreatic stellate cells (PSCs), and co-culture with PSCs significantly increased the migration and invasion of PANC-1 cells (Fig. S3). The results revealed that AREG facilitated the migration and invasion of pancreatic cancer cells.

AREG promotes EMT in pancreatic cancer cells. E-cadherin and ZO-1 expression was upregulated, however, vimentin, β-catenin, Snail, Slug, and ZEB-1 levels were downregulated in the AREG siRNA-transfected AsPC-1 cells (Fig. 3A). Knockdown of AREG upregulated the expression of E-cadherin and downregulated the expression of β-catenin and vimentin in BxPC-3 cells (Fig. S2). As anticipated, these proteins displayed the opposite expression profiles in the AREG-treated (100 ng/ml) PANC-1 cells, whereas ZEB-1 expression exhibited no evident change (Fig. 3B). The changes in the expression of E-cadherin and vimentin induced by AREG were also examined by immunofluorescence. The results revealed the upregulated expression of E-cadherin and the downregulated expression of vimentin in AREG siRNA-transfected AsPC-1 cells. In contrast, the downregulated expression of E-cadherin and the upregulated expression of vimentin were observed in AREG-treated PANC-1 cells (Fig. 3C).
AREG mediates EMT in pancreatic cancer cells via EGFR/ERK/NF-κB

AREG activates the EGFR/ERK signalling pathway in pancreatic cancer cells. To further explore the upstream signalling that may be responsible for AREG-induced EMT, the phosphorylation of EGFR, ERK, AKT and STAT3 was evaluated by performing western blot analyses. The phosphorylation levels of EGFR, ERK, AKT and STAT3 levels were...
downregulated in the AREG siRNA-transfected AsPC-1 cells (Fig. 4A). As anticipated, the exogenous treatment of AREG upregulated the phosphorylation levels of EGFR, ERK, AKT and STAT3 to varying degrees in PANC-1 cells. It was revealed that the small molecule inhibitors of EGFR (PD153035) suppressed the AREG-induced ERK, AKT and STAT3 phosphorylation (Fig. 4B). The expression levels of total EGFR, ERK, AKT, and STAT3 were not significantly altered in pancreatic cancer...
cells (Fig. 4A and B). The results revealed a significant decrease in phospho-ERK in siRNA-transfected AsPC-1 cells (P<0.01) and a significant increase in phospho-ERK in exogenous AREG-treated PANC-1 cells (P<0.05). To further characterize the effect of AREG on ERK activation, ERK phosphorylation was examined at various time-points after AREG stimulation; the peak phosphorylated ERK level occurred 30 min after AREG stimulation (Fig. 4C). The small molecule inhibitor of MEK1/2 (U0126) significantly reduced the phosphorylation level of ERK in exogenous AREG-treated PANC-1 cells.
Inhibition of the NF-κB signalling pathway suppresses AREG-induced cell migration, invasion and EMT in pancreatic cancer cells. Previous studies have suggested that NF-κB plays an essential role in the induction and maintenance of EMT (19,20). We proposed that the role of AREG-induced pancreatic cancer cell migration, invasion and EMT may be through regulation of the NF-κB signalling pathway. To evaluate this possibility, the nuclear accumulation level of NF-κB/p65 and the phosphorylation level of IκBα were analysed by western blot analysis. It was revealed that the nuclear accumulation level of NF-κB/p65 and the phosphorylation level of IκBα were significantly decreased in AREG siRNA-transfected AsPC-1 cells compared with the siRNA control group (P<0.05, Fig. 5A). In contrast, the nuclear accumulation level of NF-κB/p65 and the phosphorylation level of IκBα were significantly increased in AREG-treated PANC-1 cells. U0126 attenuated the AREG-promoted nuclear accumulation of NF-κB/p65 in PANC-1 cells (P<0.05, Fig. 5B). Furthermore, the role of the NF-κB signalling pathway in AREG-induced cell migration, invasion and EMT in pancreatic cancer cells was investigated using the selective NF-κB inhibitor QNZ. PANC-1 cells were pre-treated with 20 µM QNZ for 1 h, followed

Table I. Incidence of metastasis in a pancreatic cancer orthotopic model.

<table>
<thead>
<tr>
<th>Metastatic sites</th>
<th>LV-AREG (n=5)</th>
<th>LV-con (n=6)</th>
</tr>
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<tbody>
<tr>
<td>Liver</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Spleen</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Intestine</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Stomach</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Kidney</td>
<td>1</td>
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AREG, amphiregulin.
by incubation with 100 ng/ml AREG for 48 h. Based on the wound-healing assay, QNZ significantly inhibited the migratory ability of PANC-1 cells induced by AREG at 72 h (P<0.01, Fig. 5C). Transwell assay results revealed that QNZ abolished the invasive properties of PANC-1 cells induced by AREG (P<0.01, Fig. 5D). According to the western blot analysis, pre-treatment of PANC-1 cells with QNZ resulted in the upregulation of E-cadherin and ZO-1 and the down-regulation of vimentin, Slug, and Snail (Fig. 3B). All of the aforementioned results indicated that inhibition of the NF-κB signalling pathway suppressed AREG-induced cell migration, invasion and EMT in pancreatic cancer cells.
Orthotopic model of pancreatic cancer. The survival rate in our model was 91.7%; one mouse in the LV-AREG group died the day after the injection. All mice in the LV-AREG and LV-con groups exhibited a tumour at the site of the injection of cells into the pancreas tail. The incidence of regional and distant metastasis was lower in the LV-AREG group compared with the LV-con group (Table I; Fig. 6A). Macroscopically, the tumours appeared as firm, white, irregular masses. There had been a downward trend in tumour growth with AREG silencing between the LV-AREG group and LV-con groups (P>0.05, Fig. 6B). H&E staining confirmed pancreatic tumour development in mice (Fig. 6C).

Discussion
Pancreatic cancer is one of the leading causes of cancer-related mortality worldwide. The drug-resistant characteristics of pancreatic cancer cells result in their partial responses to traditional cytotoxic chemotherapy. The EMT phenotype is emerging as an important feature of drug-resistant pancreatic cancer cells (2-4). EMT modifies the adhesion molecules expressed by the epithelial cells, allowing the cells to adopt migratory and invasive behaviours (21).

AREG gene overexpression has been observed in a wide variety of human cancer tissues (22). Willmarth et al (11) reported that AREG upregulated numerous genes involved in cell motility and invasion in the human mammary epithelial cell line MCF10A. Liu et al (23) revealed that AREG promoted cancer cell motility in osteosarcoma and upregulated the expression of ICAM-1 through the EGFR/PI3K/Akt/NF-κB signalling pathway. Loss-of-function and gain-of-function experiments have revealed that AREG modulates the cell migration and invasion of pancreatic cancer cells in vitro. In an orthotopic model of pancreatic cancer, AREG silencing was revealed to be related to smaller tumour sizes and reduced metastatic ability. The present data are in line with the results of previous studies, suggesting that AREG plays a critical role in the motility and metastasis of pancreatic cancer cells.

EMT may be a key process for the acquisition of capabilities required for metastasis (7,21). The functional decrease in E-cadherin is among the hallmarks of EMT. AREG has been reported to stimulate ovarian cancer cell invasion by downregulating the expression level of E-cadherin (10). AREG was revealed to markedly decrease E-cadherin expression in psoriatic lesions from both AREG-transgenic mice and individuals with psoriasis (24). Consistent with these studies, in the present study, knockdown of AREG upregulated the expression of E-cadherin and downregulated the expression of β-catenin and vimentin in AsPC-1 and BxPC-3 cells. The loss of cell polarity complexes and adhesion complexes leads to the failure to maintain epithelial cell polarity, induces EMT and enhances cell invasive potential (25). Kleeff et al have revealed that ZO-1 was significantly increased in pancreatic cancer (26). In the present study, the inhibition of AREG induced ZO-1 expression in pancreatic cancer cells. Transcriptional repression mediated by factors from the Snail, ZEB and basic helix-loop-helix families is a basic mechanism of the dynamic silencing of the E-cadherin promoter CDH1 (27). Snail was revealed to suppress the expression of E-cadherin and induce the expression of the mesenchymal markers fibronectin and ZEB-1 in tumour cells (28,29). The knockdown of Slug in glioblastoma cells decreased invasion and increased survival in a mouse intracranial human glioblastoma transplantation model (30). Consistently, in the present study, AREG increased the levels of Snail and Slug. Thus, AREG may be involved in EMT in pancreatic cancer cells.

Numerous cellular pathways have been implicated in the regulation of the EMT process in pancreatic cancer (6,31). The present results revealed that AREG induced the activation of the ERK, AKT and STAT3 pathways, although the activation of the ERK pathway was the most marked. NF-κB is constitutively active in different cancers. It was revealed that AREG induced the activation of IκB and NF-κB in pancreatic cancer cells. The pharmacological inhibition of EGFR or ERK attenuated the effects of the AREG-induced nuclear accumulation of NF-κB. Thus, the EGFR/ERK/NF-κB axis was likely involved in AREG-mediated pancreatic cancer cell migration, invasion and EMT.

The stroma plays a dynamic role in tumour cell proliferation, invasion and metastasis in pancreatic cancer (32). PSCs play a critical role in the tumour desmoplasticity of pancreatic cancer (33,34). PSCs were revealed to promote EMT in pancreatic cancer cells and facilitate BxPC-3 cell invasion (35,36). The expression of AREG by PSCs and its roles in the tumour microenvironment remains elusive. In the present study, it was revealed that AREG was expressed in activated PSCs, and co-culture with PSCs significantly increased the migration and invasion of PANC-1 cells. However, using an anti-human AREG-neutralizing antibody did not decrease the migration and invasion abilities of PANC-1 cells, indicating that AREG was not the main inducer of PANC-1 cell migration in PSC secretions. In future research, the role of how AREG affects the biological characteristics of PSCs requires further thorough investigation. The animal experiment was originally designed to verify whether knockdown of AREG would affect tumorigenicity in vivo. Although there was a trend in reduced tumour burden with AREG silencing, no significant differences were revealed between the LV-AREG group and LV-con groups (P>0.05). In a future experimental design, appropriate increasing of the sample size of animal experiments will be considered in order to obtain more sample sizes for subsequent experiments and render the research results more complete.

In conclusion, the EGFR ligand AREG plays an important role in NF-κB-mediated migration and invasion as well as EMT in pancreatic cancer cells by activating the EGFR/ERK/NF-κB signalling pathway (Fig. 7). AREG may be a promising target for the treatment of pancreatic cancer.

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Availability of data and material

The datasets used during and/or analysed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

LiW was mainly responsible for the research and writing the manuscript. LiWi assisted in selecting the specimens and LiW was mainly responsible for the research and writing the manuscript. LilW assisted in selecting the specimens and LiW was mainly responsible for the research and writing the manuscript. ZL conceived and performed the manuscript revisions. ZZ contributed to the immunofluorescence and Transwell assays and the orthotopic model of pancreatic cancer. JL assisted with the western blot assays and ELISA. ZZ performed the statistical analyses. HW supplied the technical analysis and performed the manuscript revisions. ZL conceived and designed the whole study. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated.

Ethics approval and consent to participate

The present study was conducted with the approval of the Institutional Animal Care and Use Committee of Peking Union Medical College Hospital and was in accordance with the National Policy on Use of Laboratory Animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


