

Epithelial protein lost in neoplasm- α (EPLIN- α) is a potential prognostic marker for the progression of epithelial ovarian cancer

RONG LIU^{1,2}, TRACEY A. MARTIN¹, NICOLA J. JORDAN¹, FIONA RUGE¹, LIN YE¹ and WEN G. JIANG¹

¹Cardiff China Medical Research Collaborative, Cardiff University School of Medicine, Cardiff, CF14 4XN, UK; ²Department of Obstetrics and Gynaecology, Tongji Hospital, Tongji Medical College of Huazhong University of Science and Technology, Wuhan, Hubei 430030, P.R. China

Received October 28, 2015; Accepted January 13, 2016

DOI: 10.3892/ijo.2016.3462

Abstract. Epithelial protein lost in neoplasm- α (EPLIN- α) is a cytoskeletal protein whose expression is often lost or is aberrant in cancerous cells and tissues and whose loss is believed to be involved in aggressive phenotypes. This study examined this molecule in human epithelial ovarian tissues and investigated the cellular impact of EPLIN- α on ovarian cancer cells (EOC), SKOV3 and COV504. The expression of EPLIN- α in human ovarian tissues and EOC was assessed at both the mRNA and protein levels using reverse transcription-PCR (RT-PCR) and immunohistochemistry, respectively. *In vitro* assays for cellular matrix adhesion and migration (confirmed by an electrical cell substrate impedance sensing (ECIS) based method), invasion and cell growth were employed in order to assess the biological influence of EPLIN- α expression on EOC cells. Immunohistochemical analysis of ovarian cancer samples demonstrated that only a small number expressed EPLIN- α protein. Downregulation of EPLIN- α protein in EOC cell lines increased the growth, invasion, adhesion and migration *in vitro*. This EPLIN- α downregulation may have a prognostic value. From these data, we conclude that downregulation of EPLIN- α may be associated with poorer patient prognosis, and that this molecule appears to play a tumour suppressor role by inhibition of EOC growth and migration.

Introduction

Epithelial ovarian cancer (EOC) has the highest mortality rate among all gynaecological malignancies, metastasis is the main cause of death in patients with EOC. Epithelial protein lost in neoplasm (EPLIN), first discovered through its differential expression between normal oral epithelial cells and human papilloma virus (HPV)-immortalised oral epithelial cell lines (1), is a regulator of cytoskeletal dynamics. It has been shown to influence actin stabilization, to regulate actin turn-

over and to link the cadherin-catenin complex to F-actin (2-4). EPLIN exists as two isoforms (EPLIN- α , EPLIN- β) which arise due to transcription from two distinct promoter regions (5,6). EPLIN- α appears to play key roles in regulating actin dynamics and motility in normal cells. Cytoplasmic expression of EPLIN- α has been detected in a fibrillar pattern, similar to that of actin fibres (5). Research conducted in our laboratory has previously revealed that EPLIN- α expression was dysregulated in clinical prostate and breast cancer samples and lower EPLIN- α levels were associated with more aggressive cancer and poor survival, and that EPLIN- α may impact on the angiogenic process (7,8). It has also been suggested that the loss of EPLIN- α expression in cancerous cells may contribute to genomic instability and to the enhanced motility and invasiveness of cancer cells (2-4,7-10). All these findings indicate that EPLIN- α may act as a tumour suppressor. Recent research has shown that the special AT-rich-binding protein 2 (SATB2) plays a role as a novel regulator of osteosarcoma invasion, in part via effects on EPLIN and the cytoskeleton (11). Such findings provide conclusive evidence that reduction of EPLIN has the potential to disrupt cell-cell adhesion via disorganization of the adherens junctions, which promotes IGF1R signalling. This is followed by the attenuation of E-cadherin expression and the formation of an EMT-like phenotype (12). Whilst the importance and role of EPLIN- α in a number of cancers is beginning to become apparent, its role in EOC is currently unknown.

In this study, we investigated the clinical and biological role of EPLIN- α in human ovarian cancer. This study provides evidence that EPLIN- α knockdown in ovarian cancer cells can increase the aggressive nature of these cells.

Materials and methods

Clinical sample collection, processing and IHC staining. All clinical samples examined in this study were obtained from surgically removed ovarian tissues of inpatients in Wuhan Tongji Hospital of Huazhong University of Science and Technology (Wuhan, China) from 2013 to 2014; patients who had received pre-operative radiotherapy or chemotherapy were excluded. Immunohistochemistry was performed on 30 epithelial ovarian serous carcinomas, 15 samples were non-metastatic and 15 had lymph node or omentum metastases.

Correspondence to: Professor Wen G. Jiang, Cardiff China Medical Research Collaborative, Institute of Cancer and Genetics, Cardiff University School of Medicine, Cardiff, CF14 4XN, UK
E-mail: jiangw@cf.ac.uk

Key words: EPLIN- α , ovarian cancer

All of the tumour samples were obtained from the primary tumour site. Diagnosis was confirmed by histopathology in all cases. All protocols were reviewed and approved by the ethics committee of Wuhan Tongji Hospital, and all the patients gave their written informed consent.

Tissue sections (4 μ m) were prepared from formalin-fixed paraffin embedded blocks. IHC was performed using rabbit anti-EPLIN- α antibody (Calbiochem, Nottingham, UK) and the Vectastain[®] Elite Universal ABC kit (Vector Laboratories, Peterborough, UK). The de-paraffinized sections were rehydrated in Tris-buffered saline (TBS). Antigen retrieval was then performed by heating the samples for 20 min in a microwave in 1 mM EDTA buffer (pH 8.0). The sections were cooled and washed in tap water (10 min). Non-specific binding was blocked with 5-10% goat serum (90 min) and slides were then incubated with the primary EPLIN- α antibody (1:100 in TBS) for 1 h. Following sequential 30-min incubations with mouse biotinylated secondary and ABC complex, respectively, the target protein was visualised using freshly prepared 3,3-diaminobenzidine (DAB) (Sigma-Aldrich, Poole, UK). Slides were rinsed with water, counterstained with haematoxylin, dehydrated, cleared in xylene and mounted in DPX. Negative controls were prepared by substituting the primary antibody with TBS. The sections were then viewed under the Leica MC120 microscope, photographed and the intensity and localisation of the staining was analysed.

Cell lines and culture conditions. Human ovarian epithelial-serous carcinoma cell lines SKOV3 and COV504 (ECACC, European Collection of Animal Cell Culture, Salisbury, UK) were routinely maintained in DMEM-F12 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μ g/ml) and amphotericin B (0.25 μ g/ml) (Sigma). Cells were incubated at 37°C with 95% humidity in 5% CO₂.

Generation of EPLIN- α ribozyme transgenes. Anti-Eplin- α hammerhead ribozyme transgenes were synthesised and cloned into pEF6/V5-His-TOPO plasmid vector as described in previous studies (13-15). Purified EPLIN- α plasmids (8 μ g) and control plasmid vectors were then transfected into SKOV3 and COV504 cells (300 V, 1500 μ F), (1x10⁶/ml) using a Gene Pulser X cell electroporator (Bio-Rad Laboratories, Hemel Hempstead, UK). Transfected cells underwent selection for ~2 weeks with blasticidin (5 μ g/ml) (Melford Laboratories, Ipswich, UK). Positively transfected cell cultures were maintained in medium containing 0.5 μ g/ml blasticidin. The transfectants were verified for their expression of Eplin- α mRNA and protein, and the successful clones were used in subsequent studies.

RNA extraction and reverse transcription PCR. Total cellular RNA was isolated from the EOC cells using Tri-reagent according to the manufacturer's instructions (Sigma-Aldrich). RNA concentration and quality were determined through spectrophotometric measurement (NanoPhotometer, Implen, Munich, Germany). RNA (500 ng) was reverse transcribed into cDNA using an Applied Biosystems high capacity reverse transcription kit (Life Technologies, Paisley, UK). DNA quality was verified using GAPDH PCR (sense, GGCTGCTTT TAACTCTGGTA; antisense, GACTGTGGTCATGAGTC

CTT) which was also used as a loading control. Eplin- α mRNA levels were assessed using primers (sense, AAGCA AAAATGAAAACCTAAG; antisense, GACACCCACCTTAG CAATAG). PCR was carried out in an Applied Biosystems thermocycler using a Go Taq green PCR reaction mix (Promega UK, Southampton, UK). Cycling conditions were 94°C for 5 min, followed by 28 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. This was followed by a final 7-min extension period at 72°C. The products were visualized on 2% agarose gel stained with SYBR Safe (Life Technologies).

Immunofluorescence staining. Cells were seeded at a density of 20,000 cells per well in an 8-well chamber slide (Merck-Millipore, UK). Following an overnight incubation, the medium was aspirated and the cells were fixed in 4% formalin (4°C, 20 min). Following fixation, the cells were rehydrated in phosphate-buffered saline (PBS) for 20 min at room temperature before being permeabilised for 5 min in a 0.1% Triton in PBS. Non-specific binding was blocked by 1-h incubation in phosphate-buffered saline (PBS) containing 5-10% goat serum. Cells were incubated for 1 h with Eplin- α antibody (1:100) in PBS blocking solution (Calbiochem). Slides were washed 3x5 min in PBS then incubated on a shaker platform in the dark for 1 h with FITC conjugated anti-mouse secondary antibody (Insight Biotechnology Ltd., Middlesex, UK) and 1:1,000 DAPI (Roche, Hertfordshire, UK). Slides were finally washed 3x5 min PBS, mounted with Fluorsave (Merck-Millipore, UK) and visualised using an EVOS fluorescence auto imaging system (Life Technologies).

Western blot analysis. Cell lines were grown to 70% confluence, monolayers were washed with PBS and lysed in ice cold lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EGTA, 1% Triton X-100 pH 7.5) supplemented with protease inhibitor cocktail (Roche). Lysates were clarified by centrifugation (12,000 rpm, 15 min, 4°C) and the protein concentrations in the supernatants were determined using the DC Protein Assay kit (Bio-Rad, Hemel Hempstead, UK). Protein was reduced and denatured by boiling (5 min) in Laemmli buffer (Sigma-Aldrich) and 20 μ g protein samples were resolved by SDS-PAGE and transferred onto nitrocellulose membrane (GE Healthcare Life Sciences, Buckinghamshire, UK). After blocking for 1 h in 5% skimmed milk (TBS/Tween: 140 mM NaCl; 50 mM Tris, 0.05% Tween pH 7.4), blots were incubated overnight at 4°C with primary antibodies Eplin- α (1:500 prepared in TBS/Tween/1% milk) and GAPDH (1:1,000 in TBS/Tween/1% milk) (Santa Cruz Biotechnology, Heidelberg, Germany) was used as a loading control. Blots were washed with TBS/Tween and bound antibodies were detected after 1-h incubation (room temperature) with appropriate horseradish peroxidase-conjugated secondary antibody (1:1,000, Sigma-Aldrich). Following 3x5 min TBS/Tween washes, protein bands were visualized using enhanced chemiluminescence (Luminata Forte, Millipore, Herefordshire, UK), and photographed using a UVITech imager (UVITech, Inc., Cambridge, UK).

Cell proliferation assay. Cells were seeded into 96-well plates at a seeding density of 3,000 cells per well with 12 replicates/experiment. Cells were fixed with 4% formalin after 1, 3 and 5 days growth. Fixed cells were stained with 0.5% crystal violet,

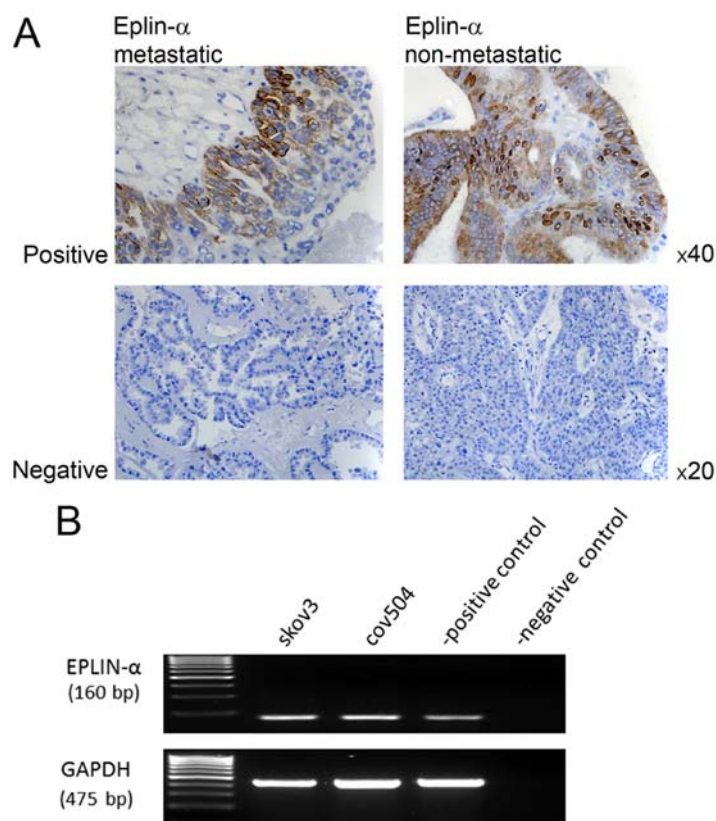


Figure 1. Expression of EPLIN- α in ovarian cancer. Immunohistochemical staining of EPLIN- α in human ovarian tissues (A). Basal expression of EPLIN- α mRNA examined in ovarian cancer cell lines using RT-PCR (B). High expression of EPLIN- α (160 bp) in SKOV3 and COV504 cells (28 cycles). GAPDH (475 bp) was used as an equal loading control. The negative control was sterile water and the positive control CDNA from the normal prostate cell line PZHPV7.

washed and dried. Dye was re-solubilised in 200 μ l acetic acid/well and absorbance was determined at 540 nm using an ELx800 multi-plate reader (BioTek UK, Bedfordshire, UK). Each experiment was repeated at least 3 times. For each cell line, analysis compared cell number (absorbance) on days 3 and 5 relative to day 1.

Cell adhesion assay. Cell-matrix adhesion was examined using an *in vitro* Matrigel adhesion assay adapted from a previously described method (16-18). Cells were seeded into 96-well plates pre-coated with 5 μ g/well Matrigel basement membrane matrix (BD Biosciences, Oxford, UK). After 40 min of incubation (37°C) the cells were washed with PBS to remove unbound cells. The remaining adherent cells were fixed with 4% formalin, stained with 0.5% crystal violet, visualized under a microscope (x20) and cell number counted per field of view. Four counts were made from each 6 replicate wells and results were expressed as mean cell number/well. Each experiment was repeated 3 times.

Cell invasion assay. Cell invasive capability was examined using an *in vitro* Matrigel invasion assay. Transwell inserts (Greiner Bio-One, Stonehouse, UK) with 8.0- μ m pore size were coated with 50 μ g Matrigel (BD Biosciences), dried at 55°C and rehydrated with 100 μ l serum-free medium before seeding 4,000 cells per insert. After 48 h of incubation at 37°C, non-invasive cells and Matrigel were removed from the inside of the inserts with a cotton swab. Cells that had invaded to the underside of the insert were fixed (4% formalin), stained with

0.5% crystal violet and washed. Cell invasion was quantitated by counting the cell number in 4 fields of view (x20 magnification). Data were analysed as mean cell number per field of view for 3 independent experiments with 3 replicates per experiment. Results were confirmed by incubating the stained inserts in 10% acetic acid. Absorbance of solubilized crystal violet was determined at 540 nm.

Migration assay. A cellular wounding assay was used to study directional cell migration *in vitro* as previously described (19). In brief, cells were cultured to confluence in a 24-well plate before scratching the cell monolayer with a 10- μ l pipette tip. The closure of the induced wound, through the migration of cells, was tracked and recorded over a 36-h period using an automated cell imaging system EVOS (Life Technologies). Using ImageJ software, the relative distance cells migrated was calculated using multiple measurements of the width of wound gap after 12, 24 and 36 h compared to 0 h.

Electric cell-substrate impedance sensing (ECIS)-based attachment and migration assay. Cell attachment and migration were further studied using an ECIS Z-Theta instrument and 96W1E arrays (Applied Biophysics, Inc., NY, USA) as previously described (7). Briefly, 40,000 cells per well were added to the ECIS arrays. Impedance and resistance of the cell layer was immediately recorded for a period of ≤ 5 h. When confluence was reached, the monolayer in each well was electrically wounded at 2,600 μ A and 6,000 Hz for 20 sec to create a 250- μ m wound per well. Impedance and resistance

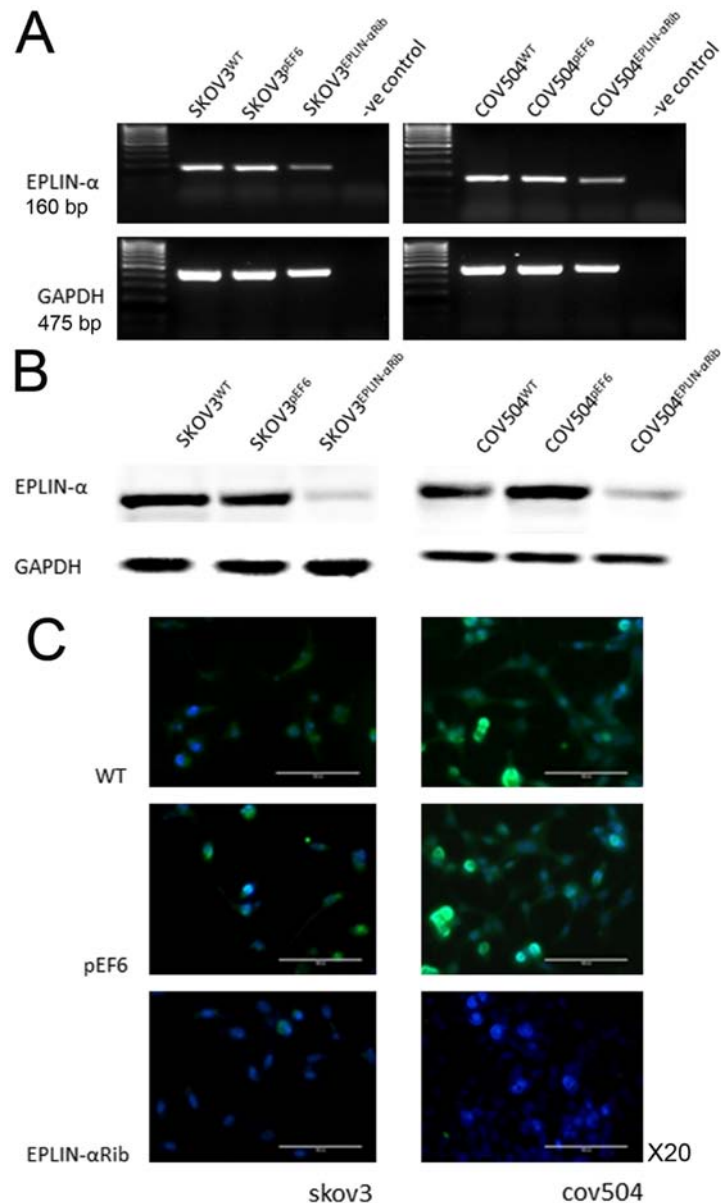


Figure 2. Knock-down of EPLIN- α in transfected EOC cells. RT-PCR (28 cycles) showed decreased EPLIN- α mRNA transcripts in the EPLIN- α -transfected cells (Rib) compared to controls (WT, pEF6) in SKOV3 and COV504 cells. GAPDH (25 cycles) was examined as an equal loading control (A). Knock-down of EPLIN- α protein in the same cells was verified by western blot analysis. GAPDH was used as the loading control (B). Immunofluorescence microscopy confirmed reduce cytoplasmic expression of EPLIN- α protein (green cytoplasmic staining) in monolayers of EPLIN- α -transfected SKOV3 and COV504 cells (blue, DAPI nuclear stain) (C). Bar, 100 μ m.

of the wounded cells as they migrated in the wound was then recorded for a period of up to 10 h. Data were analysed using the ECIS software, supplied by the manufacturer.

Statistical analysis. All statistical analysis was performed using the paired t-test for normally distributed data (data were tested for normal distribution before further statistical analyses were carried out). Differences were considered to be statistically significant at $p < 0.05$.

Results

Expression of Eplin- α in human ovarian tissues and EOC cells. IHC staining of 15 sections of non-metastatic and 15 sections of metastatic epithelial cancerous ovarian growths was used to

assess Eplin- α expression pattern in the clinical setting. Eplin- α expression was detected in 6/30 tissue samples examined; 2/15 non-metastatic tumours and 4/15 metastatic tumours were localised in epithelial cell cytoplasm. Where staining was observed there was no difference in the intensity of the stain between non-metastatic and metastatic samples. Images are shown of both metastatic and non-metastatic samples, representing the range of staining detected (Fig. 1A).

The mRNA expression of Eplin- α was also examined in two EOC cell lines using RT-PCR. Eplin- α mRNA was expressed at relatively high levels in all cell lines (Fig. 1B).

Knock-down of Eplin- α in EOC cells. To investigate the impact of Eplin- α on a range of behavioural functions of ovarian cancer cells, Eplin- α hammerhead ribozyme transgenes were

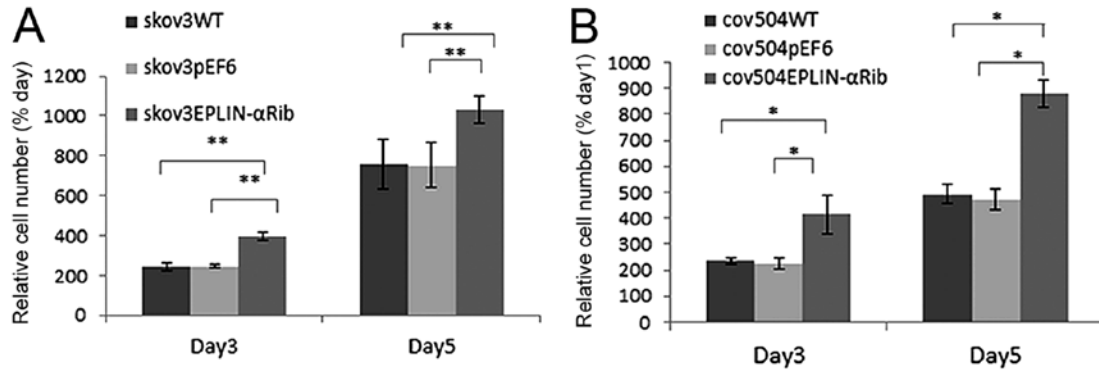


Figure 3. Effect of EPLIN- α on *in vitro* growth of EOC cells. Cell numbers were measured by crystal violet absorbance after 1, 3 and 5 days growth and results expressed as % cell number on days 3 and 5 compared to day 1. After 5 days, growth in SKOV3^{Epln- α Rib}, COV504^{Epln- α Rib} cells was increased in comparison with the WT and pEF6 controls. Data were analysed by paired t-test. * $p < 0.05$ ** $p < 0.01$. The error bars represent mean \pm SD, data shown is the mean of three independent experiments.

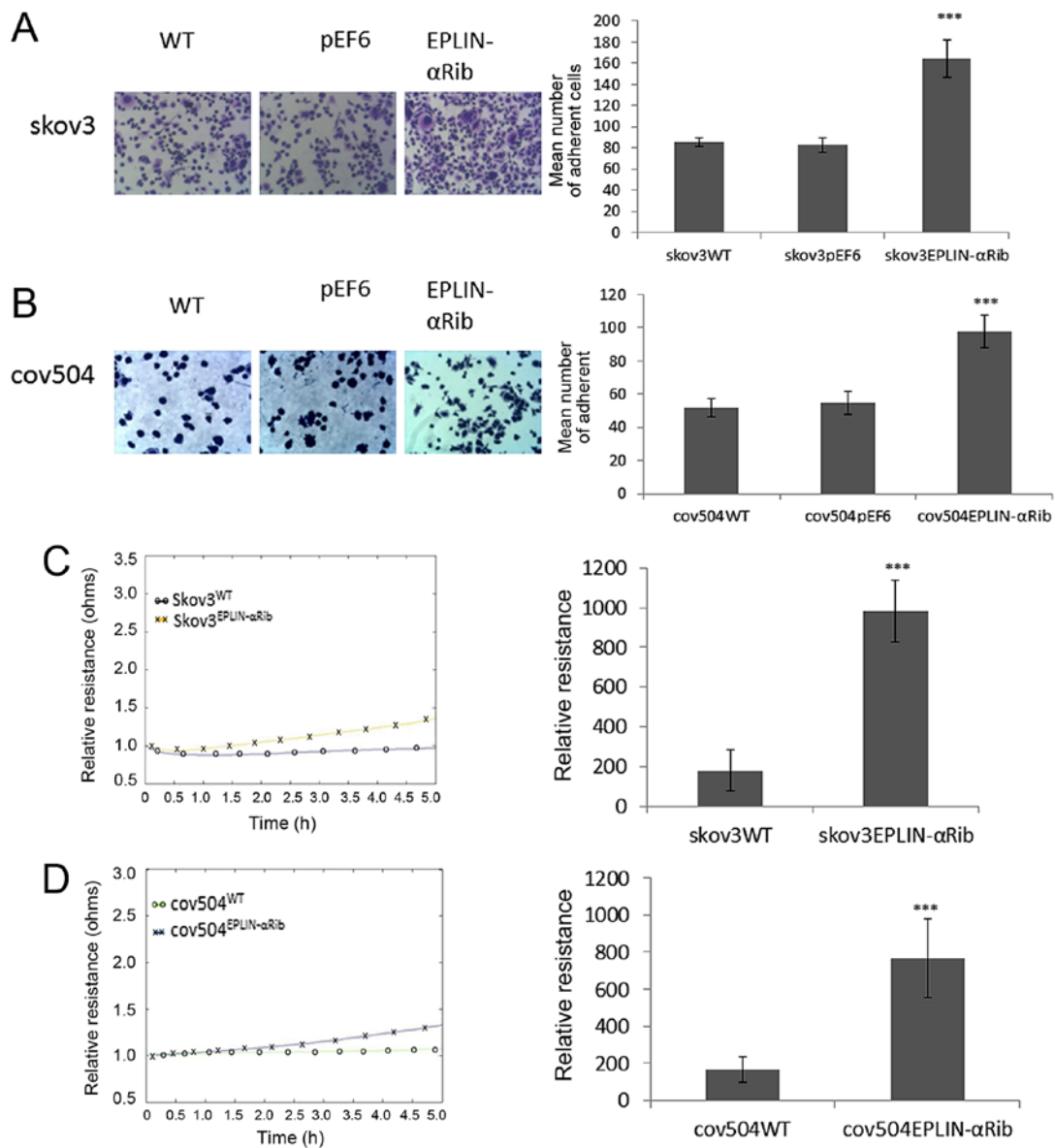


Figure 4. Effect of Epln- α on adhesion of EOC cells *in vitro*. Effect of Epln- α decreased expression on rapid (40 min) cell adhesion to Matrigel basement membrane. Knock-down of Epln- α expression significantly increased SKOV3^{Epln- α Rib} (A) COV504^{Epln- α Rib} (B) cell adhesion compared to pEF6 and WT controls. Data were analysed by paired t-test (Epln- α ^{pEF6} control). *** $p < 0.001$. The error bars represent mean cell count/well \pm SD. Images are representative fields of view. Data shown are the mean of three independent experiments. The SKOV3^{Epln- α Rib} (C), COV504^{Epln- α Rib} (D) cells with knocked down Epln- α showed a markedly increased attachment using an ECIS model to measure resistance over 5 h. Data show relative change in resistance from 0 to 5 h for each cell line and are mean of 3 independent experiments \pm SD. *** $p < 0.001$ comparing Epln- α Rib versus WT control cells.

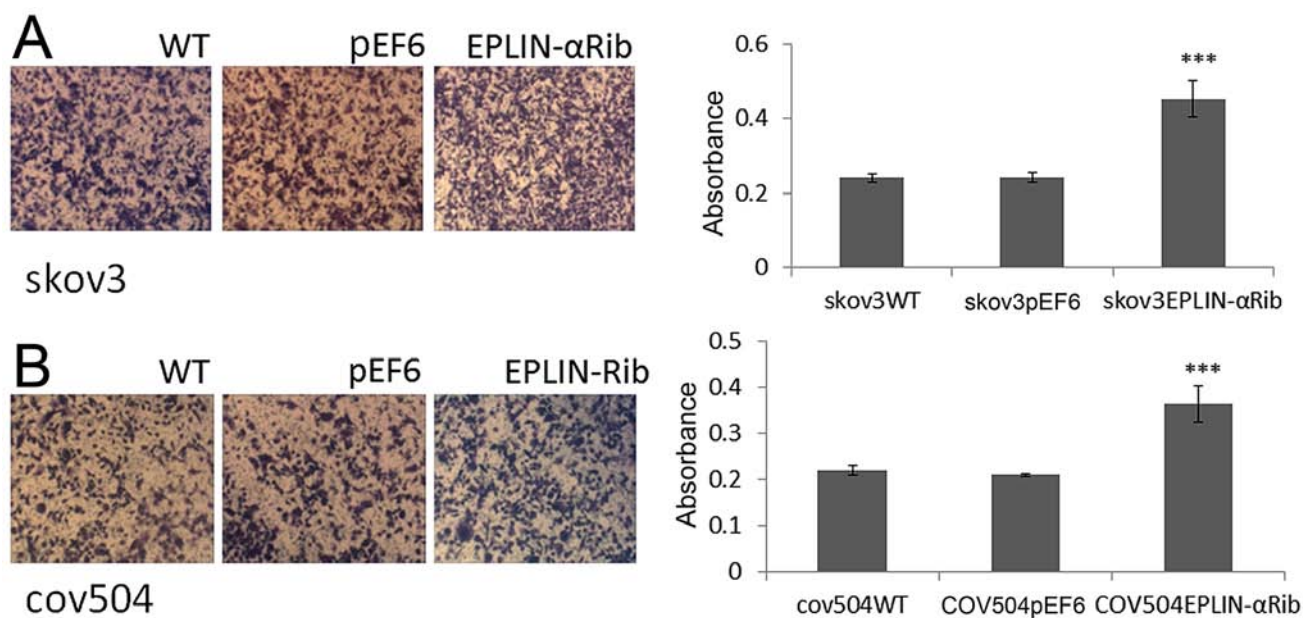


Figure 5. Effect of Eplin- α decreased-expression on the invasion of EOC cells. Cell invasion through Matrigel coated barrier was quantitated after fixing and staining invaded cells. Invasion capability was increased in SKOV3^{Eplin- α Rib} (A) and COV504^{Eplin- α Rib} (B) cells. Four fields of view/insert were photographed and counted. Images shown are from a typical field of view (x4 magnification). Data shown are the mean \pm SD cell number/membrane from 3 independent experiments. t-test compared Eplin- α Rib versus the controls for each cell line. *** p <0.001.

utilised to knock down Eplin- α . After selection using blasticidin, the reduced expression of Eplin- α in the transfected cells was verified using RT-PCR, immunofluorescent staining and western blotting (Fig. 2). Decreased expression of both Eplin- α mRNA (\sim 50%) (Fig. 2A) and protein (\sim 80%) (Fig. 2B) was seen in SKOV3^{Eplin- α Rib}, in comparison with the controls (wild-type SKOV3^{WT} and empty plasmid SKOV3^{pEF6}). Knock-down of Eplin- α mRNA (\sim 40%) and protein (\sim 80%) was also confirmed in COV504^{Eplin- α Rib} cells, in comparison with the COV504^{WT} and COV504^{pEF6} control cells. Immunofluorescent staining was carried out to examine the expression and localisation of the Eplin- α in the transfected cells (Fig. 2C). Eplin- α staining (green), was predominantly associated with the cytoplasm. Control cells, both wild-type and empty vector transfectants, had strong staining intensity in two EOC cell lines, with the majority of the cells showing more intense and frequently observed staining. However, in the transfected knocked down cells Eplin- α had minimal staining levels.

Regulation of Eplin- α expression affects the rate of cell growth of EOC cells. The growth capacity of the EOC cells following Eplin- α knock-down was examined and compared to the wild-type and empty vector control cells using an *in vitro* cell growth assay (Fig. 3). In all transfected cells, knock-down of Eplin- α protein increased growth rate by both day 3 and day 5. In SKOV3^{Eplin- α Rib} cells, the mean cell number at day 5 was increased by 36% (p <0.01) compared to pEF6 control, in COV504^{Eplin- α Rib} cells, growth rate increased by 46% (p <0.05) (Fig. 3).

Effect of knocked down Eplin- α on cell-matrix adhesion in EOC cells. The effect of Eplin- α on the ability of EOC cells to adhere to Matrigel matrix was examined (Fig. 4A and B). Knock-down of Eplin- α protein caused a significant (p <0.001) increase of \sim 40% on cell-matrix adhesion in the SKOV3

cells compared to both WT and pEF6 controls (Fig. 4A). Compared with COV504^{WT} and COV504^{pEF6}, the number of COV504^{Eplin- α Rib} cells that adhered was also significantly increased (p <0.001) by \sim 40% (Fig. 4B). The ECIS system was also used to confirm the accelerative effect of reduced expression of Eplin- α on SKOV3, COV504 cell adhesion. This was measured by change in resistance formed over the growth surface as cells attached from 0 to 5 h (Fig. 4C and D). Compared with the appropriate WT and pEF6 controls, the resistance was significantly increased in SKOV3^{Eplin- α Rib} and COV504^{Eplin- α Rib} cells, confirming that low expression of Eplin- α in ovarian cells increased the adhesive capability.

Effect of knocked down Eplin- α on the invasion of EOC cells. The potential biological relevance of reduced Eplin- α expression was further investigated using *in vitro* invasion assays over the artificial basement membrane, Matrigel. Reduced expression of Eplin- α in all of these cell lines caused a 50% increase (p <0.001) in basal invasion compared to both WT and pEF6 controls (Fig. 5).

Effect of Eplin- α on wounding/migration of EOC cells. A cellular wounding assay (Fig. 6A and B) was used to compare the ability of wild-type and knock-down Eplin- α cells to migrate. Reduced expression of Eplin- α in the two EOC cell lines caused a marked (\leq 30%) increase in migration capability compared to the WT control (p <0.05). The reduced ability of Eplin- α expression to increase ovarian cell motility was also confirmed by measuring the ability of cell lines to recover from an electrical wound generated using the ECIS system (Fig. 6C and D). Measurements taken 10 h post-wound also showed that the migration capacity of SKOV3^{Eplin- α Rib} and COV504^{Eplin- α Rib} were markedly increased (p <0.001) in comparison with wild-type control cells.

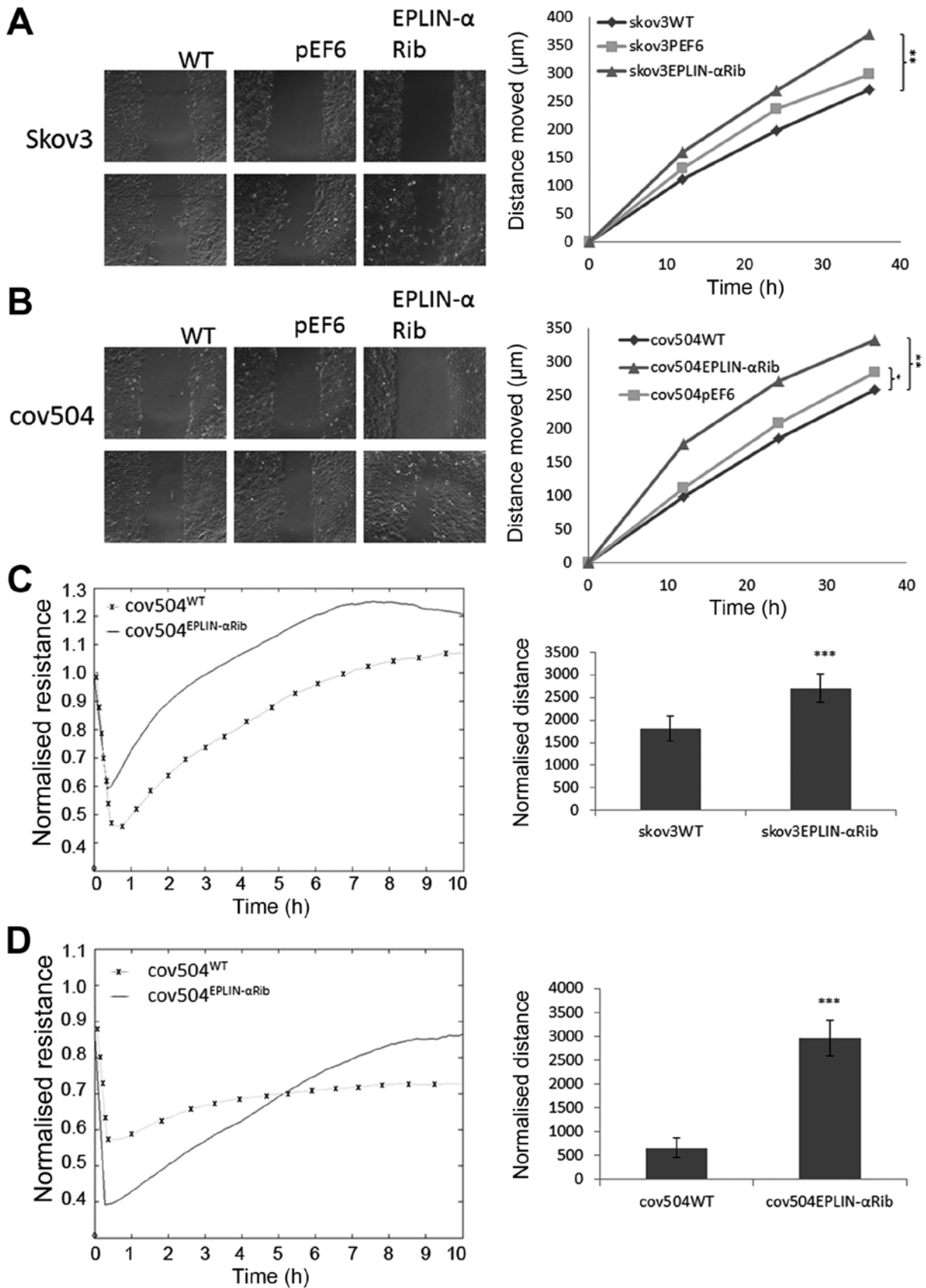


Figure 6. Effect of Eplin-α expression on migration of EOC cells. Confluent monolayers of EOC cells were scratched and the distance moved by cells to cover the wound was measured after 12, 24 and 36 h and compared to time 0. After 36 h, SKOV3 (A) and COV504 (B) cells with knockdown of Eplin-α protein expression all showed significantly increased migration compared to WT or pEF6 controls (t-test *p<0.05 or **p<0.01). Images shown are taken at 0 and 36 h are from representative experiment. The data shown are from 3 independent experiments. ECIS confirmed the increased migratory capability of SKOV3^{Eplin-αRib} (C) and COV504^{Eplin-αRib} (D) cells compared to the appropriate empty vector control cells. At time 0 the confluent monolayer of cells was electrically wounded and the impedance changes were recorded over 10 h as an indication of how rapidly the cells migrated to cover the wound. Data show relative change in resistance from 0 to 10 h for each cell line and are the mean of 3 independent experiments ± SD. ***p<0.001 comparing Eplin-αRib versus control cells.

Discussion

Uncontrolled tumour cell proliferation and robust neovascularization are prominent features of aggressive ovarian cancers, distant metastasis being a key factor in the poor prognosis associated with this cancer. Although great efforts in anti-ovarian cancer therapy have been made in the past decades, the 5-year survival rates for ovarian cancer patients are still poor, and effective drugs to cure ovarian cancer patients are absent. To improve the prognosis, assessment and treatment of EOC patients, it is crucial that we identify the key molecular regulators of tumorigenesis and understand the key molecular pathways involved. However, many of these tumorigenesis mechanisms remain largely unknown.

Eplin- α (epithelial protein lost in neoplasm) has previously been found to localise with actin stress fibres and plays an important role in regulating actin dynamics and linking the catenin-cadherin complex to F-actin (2-4), suggesting a key role for this molecule in regulating cellular motility. It has been previously reported that Eplin- α has been found to be downregulated in a number of oral, breast, esophageal and prostate (5,7,8,20) cancer cell lines compared to their normal counterparts. Previous studies from our laboratories have provided data supporting a tumour/metastasis suppressive role for EPLIN- α , where enhanced levels of EPLIN- α can negatively impact on key metastatic and angiogenic traits *in vitro* and *in vivo* (7,8,21). Our study aimed to determine whether there was a relationship between Eplin- α protein expression and the aggressiveness of clinical ovarian cancer. Immunohistochemical analysis demonstrated that only a small number of ovarian cancer tissues expressed Eplin- α protein, but conclusions were limited by the relatively small sample size used. The majority of tumours, both metastatic and non-metastatic, were negative for Eplin- α (80%), and in the tissues where positive staining was seen there appeared to be no distinct difference in the localisation and intensity of stain. Due to the relatively small number of clinical samples included in our study, the results did not enable statistical analysis. In some tumour types, including breast and esophageal cancer, EPLIN- α may be considered a suitable biomarker for tumour progression, with high EPLIN- α being associated with favourable prognosis and reduced EPLIN- α a poorer outcome (7,20). EPLIN- α expression is variable between individuals which may reflect a range of differences in ovarian cancer aetiology or disease stages when samples were taken. Conclusive results can only be obtained if a larger cohort is studied.

A relatively small number of *in vitro* studies have reported in which the function of EPLIN- α is characterized. These studies with a panel of human cell lines have shown EPLIN- α is differentially expressed with an inverse correlation between cell differentiation, invasive capability and EPLIN- α expression (5,7,8,20), for example, MDA-MB-231 cells, considered highly invasive, were negative for Eplin- α expression (7). Our present investigation showed that the ovarian tumour cell lines, SKOV3 and COV504, which both demonstrated reasonable (but not high) invasive capability, also expressed a relatively high level of EPLIN- α mRNA and protein suggesting other factors in addition to EPLIN- α expression may also be involved in regulating invasion. However, cellular function tests did demonstrate that the presence of EPLIN- α was related to the inhibition of the

ovarian cancer cell aggressiveness. Knock-down of EPLIN- α expression resulted in an increase in the *in vitro* growth rate of SKOV3 and COV504 cells during the 3 and 5-day incubation periods ($p < 0.01$, $p < 0.05$). Knock-down of EPLIN- α expression also impacted cell-matrix adhesion significantly decreasing it compared to that of pEF6 control cells.

We have demonstrated here that knock-down of EPLIN- α expression resulted in a strong increase in ovarian cancer cell line growth, adhesion, invasion and migration, in comparison with control cells. The inhibitory effect of EPLIN- α on ovarian cancer cell growth is in agreement with the findings in breast, prostate, esophageal and endothelial cell lines (7,8,20,21). Although the precise molecular mechanisms by which EPLIN- α inhibits tumour growth remains unknown, research studies provide conclusive evidence that reduction of EPLIN has the potential to disrupt cell-cell adhesion via disorganization of the adherens junctions, which promotes IGF1R signalling. This is followed by the attenuation of E-cadherin expression and an EMT-like phenotype (12). Further studies are required to reveal the exact molecular mechanisms and signalling pathways through which EPLIN- α modulates cancer cell migration and invasion.

Our studies suggest that decreased expression of EPLIN- α is seen in cancerous tissue where it may be associated with a poorer prognosis making EPLIN- α loss a potential prognostic marker. As far as the authors are aware, this is the first study examining the expression of EPLIN- α in human EOC tissue and reporting the effect of reduced EPLIN- α expression on EOC cell line behaviour.

In conclusion, this study shows that the knock-down of EPLIN- α protein can increase the aggressiveness of human ovarian cancer, furthermore, it suggests that preventing EPLIN- α degradation, or partially restoring EPLIN- α expression, could be a possible novel strategy to treat aggressive ovarian cancer growth and metastasis. These data clearly indicate that EPLIN- α may potentially have use as a prognostic indicator and that the molecule may act as a protective factor in patients with EOC.

Acknowledgements

The authors are grateful to Cancer Research Wales and the Albert Hung Foundation for their support and funding of this study. Dr Rong Liu is a recipient of Cardiff University China Medical Scholarship.

References

1. Chang DD, Park NH, Denny CT, Nelson SF and Pe M: Characterization of transformation related genes in oral cancer cells. *Oncogene* 16: 1921-1930, 1998.
2. Song Y, Maul RS, Gerbin CS and Chang DD: Inhibition of anchorage-independent growth of transformed NIH3T3 cells by epithelial protein lost in neoplasm (EPLIN) requires localization of EPLIN to actin cytoskeleton. *Mol Biol Cell* 13: 1408-1416, 2002.
3. Maul RS, Song Y, Amann KJ, Gerbin SC, Pollard TD and Chang DD: EPLIN regulates actin dynamics by cross-linking and stabilizing filaments. *J Cell Biol* 160: 399-407, 2003.
4. Abe K and Takeichi M: EPLIN mediates linkage of the cadherin catenin complex to F-actin and stabilizes the circumferential actin belt. *Proc Natl Acad Sci USA* 105: 13-19, 2008.
5. Maul RS and Chang DD: EPLIN, epithelial protein lost in neoplasm. *Oncogene* 18: 7838-7841, 1999.

6. Chen S, Maul RS, Kim HR and Chang DD: Characterization of the human EPLIN (epithelial protein lost in neoplasm) gene reveals distinct promoters for the two EPLIN isoforms. *Gene* 248: 69-76, 2000.
7. Jiang WG, Martin TA, Lewis-Russell JM, Douglas-Jones A, Ye L and Mansel RE: Eplin-alpha expression in human breast cancer, the impact on cellular migration and clinical outcome. *Mol Cancer* 7: 71-80, 2008.
8. Sanders AJ, Martin TA, Ye L, Mason MD and Jiang WG: EPLIN is a negative regulator of prostate cancer growth and invasion. *J Urol* 186: 295-301, 2011.
9. Zhang S, Wang X, Osunkoya AO, Iqbal S, Wang Y, Chen Z, Müller S, Chen Z, Jossou S, Coleman IM, *et al*: EPLIN downregulation promotes epithelial-mesenchymal transition in prostate cancer cells and correlates with clinical lymph node metastasis. *Oncogene* 30: 4941-4952, 2011.
10. Han MY, Kosako H, Watanabe T and Hattori S: Extracellular signal-regulated kinase/mitogen-activated protein kinase regulates actin organization and cell motility by phosphorylating the actin cross-linking protein EPLIN. *Mol Cell Biol* 27: 8190-8204, 2007.
11. Seong BKA, Lau J, Adderley T, Kee L, Chaukos D, Pienkowska M, Malkin D, Thorner P and Irwin MS: SATB2 enhances migration and invasion in osteosarcoma by regulating genes involved in cytoskeletal organization. *Oncogene* 34: 3582-3592, 2015.
12. Steder M, Alla V, Meier C, Spitschak A, Pahnke J, Fürst K, Kowtharapu BS, Engelmann D, Petigk J, Egberts F, *et al*: DNP73 exerts function in metastasis initiation by disconnecting the inhibitory role of EPLIN on IGF1R-AKT/STAT3 signaling. *Cancer Cell* 24: 512-527, 2013.
13. Jiang WG, Watkins G, Douglas-Jones A, Mokbel K, Mansel RE and Fodstad O: Expression of Com-1/P8 in human breast cancer and its relevance to clinical outcome and ER status. *Int J Cancer* 117: 730-737, 2005.
14. Jiang WG, Davies G and Fodstad O: Com-1/P8 in oestrogen regulated growth of breast cancer cells, the ER-beta connection. *Biochem Biophys Res Commun* 330: 253-262, 2005.
15. Jiang WG, Davies G, Martin TA, Kynaston H, Mason MD and Fodstad O: Com-1/p8 acts as a putative tumour suppressor in prostate cancer. *Int J Mol Med* 18: 981-986, 2006.
16. Jiang WG, Davies G, Martin TA, Parr C, Watkins G, Mason MD and Mansel RE: Expression of membrane type-1 matrix metalloproteinase, MT1-MMP in human breast cancer and its impact on invasiveness of breast cancer cells. *Int J Mol Med* 17: 583-590, 2006.
17. Jiang WG, Davies G, Martin TA, Parr C, Watkins G, Mason MD, Mokbel K and Mansel RE: Targeting matrilysin and its impact on tumor growth in vivo: The potential implications in breast cancer therapy. *Clin Cancer Res* 11: 6012-6019, 2005.
18. Jiang WG, Hiscox S, Singhrao SK, Nakamura T, Puntis MC and Hallett MB: Inhibition of HGF/SF-induced membrane ruffling and cell motility by transient elevation of cytosolic free Ca²⁺. *Exp Cell Res* 220: 424-433, 1995.
19. Jiang WG, Hiscox S, Hallett MB, Horrobin DF, Scott C and Puntis MCA: Inhibition of invasion and motility of human colon cancer cells by gamma linolenic acid. *Br J Cancer* 71: 744-752, 1995.
20. Liu Y, Sanders AJ, Zhang L and Jiang WG: EPLIN-Alpha expression in human oesophageal cancer and its impact on cellular aggressiveness and clinical outcome. *Anticancer Res* 32: 1283-1289, 2012.
21. Sanders AJ, Ye L, Mason MD and Jiang WG: The impact of EPLIN α (Epithelial protein lost in neoplasm) on endothelial cells, angiogenesis and tumorigenesis. *Angiogenesis* 13: 317-326, 2010.