

# Metastasis suppressor 1 expression in human ovarian cancer: The impact on cellular migration and metastasis

RONG LIU<sup>1,2</sup>, TRACEY A. MARTIN<sup>1</sup>, NICOLA J. JORDAN<sup>1</sup>, FIONA RUGE<sup>1</sup>, LIN YE<sup>1</sup> and WEN G. JIANG<sup>1</sup>

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

Received April 27, 2015; Accepted May 26, 2015

DOI: 10.3892/ijo.2015.3121

**Abstract.** Metastasis suppressor 1 (MTSS1) is a potential metastasis suppressor gene involved in the regulation of cytoskeleton dynamics and subsequently in cell motility. MTSS1 expression is frequently reduced in a variety of cancer cells and tissues and this loss may account for increased invasive traits in cancer cells. The present study aimed to assess the role of MTSS1 in epithelial ovarian cancer (EOC) cells. Expression of MTSS1 in human ovarian cells was assessed at both the mRNA and protein levels using reverse transcription-PCR (RT-PCR) and immunohistochemistry, respectively. Full-length MTSS1 cDNA expression vector was used to generate MTSS1 overexpressing cells. The effect of MTSS1 overexpression on cellular functions was examined in EOC cells using a variety of *in vitro* assays. MTSS1 expression was observed both in ovarian cancer tissues and EOC cells. Over-expression of MTSS1 protein reduced the growth, invasion, adhesion and migration of EOC cell lines *in vitro*. The present study revealed that MTSS1 plays an essential inhibitory role in the development and progression of ovarian cancers. MTSS1 overexpression is intimately related to migration and metastasis, suggesting that MTSS1 is a potential prognostic marker and therapeutic molecular target in human ovarian cancer.

## Introduction

Ovarian cancer remains the most lethal of all gynaecologic malignancies in the world (1,2). According to a report of the American Cancer Society, ~22,240 new cases of ovarian cancer would be diagnosed in the United States in 2013 while nearly 14,030 of those affected would succumb to this disease (3). Epithelial ovarian cancer (EOC) accounts for 90% of ovarian cancers; it is not a single disease as there are several different histological subtypes, with serous being the most common

subtype, yet, its aetiology remains poorly understood. Most EOC cannot be detected until peritoneal or distant metastases occur, which are the major cause of high mortality in ovarian cancer. Despite cytotoxic therapy, only 30% of patients with advanced ovarian cancer survive 5 years post diagnosis. The metastatic cascade consists of a series of sequential, interrelated steps that are not yet completely understood. However, it is known that these metastatic events are modulated by many factors, including metastasis activators and suppressors. Metastasis suppressors can inhibit metastasis at any step of the metastatic cascade without blocking tumorigenicity.

Metastasis suppressor 1 (MTSS1) protein, which is also known as MIM (missing-in-metastasis) has been recently characterized as a tumor suppressor protein (4). MTSS1 is mostly expressed in normal tissues and in some non-metastatic cancer cell lines, however, its expression is significantly decreased or mostly absent in many metastatic types of cancer including metastatic bladder (5), prostate (6), kidney (7) and gastric cancer (8), suggesting that MTSS1 could function as an anti-metastatic protein. Furthermore, an inverse correlation has also been observed between MTSS1 expression and poor prognosis in breast cancer (9). These findings indicate that MTSS1 might function as a tumor suppressor and that loss of MTSS1 facilitates the development of human cancers including breast and prostate cancers. However, in contrast to its reduced expression in many human cancers, overexpression of MTSS1 has been observed in hepatocellular carcinoma (10) although its physiological significance to liver cancer remains elusive. MTSS1 is an actin and membrane binding protein. Functionally, it acts as a cytoskeletal scaffold protein that regulates cytoskeletal dynamics through interacting with many different proteins such as Rac, actin and actin-associated proteins (11-13). In addition, recent research has shown that anti-microRNA (miR182) treatment can significantly reduce the expression of miR-182 target genes including BRCA1, HMGA2 and MTSS1; downregulation of MTSS1 is strongly associated with aggressive invasion of ovarian cancer cells (14). There is a wealth of biochemical data concerning MTSS1, but the physiological roles of its various activities that regulate plasma membrane dynamics including actin monomer binding, membrane deformation and interaction with Gli transcription factors are still not fully understood.

---

**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:** MTSS1, ovarian cancer, metastasis, migration

Studies suggest that further understanding of MTSS1 expression or inactivation in different human malignancies may define it as a novel candidate to be used as a marker of primary tumors or metastasis. We sought to determine the relevance of MTSS1 in EOC and provide new insights into its biological functions and role in EOC. In the present study, expression of MTSS1 was examined in a cohort of human EOC samples and cell lines. EOC cells were forced to express this molecule by transfection with a mammalian expression plasmid containing the full sequence of MTSS1 enabling further understanding of the functional role of MTSS1 in EOC cell behaviour.

## Materials and methods

**Clinical sample collection, processing and IHC staining.** All clinical samples examined in the present 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 17 epithelial ovarian serous carcinomas, 10 samples were non-metastatic and 7 had lymph node or omentum metastases. All of the tumor samples were obtained from the primary tumor site. Diagnosis was confirmed by histopathology in all cases. All protocols were reviewed and approved by the Ethics Committee and all patients gave written informed consent.

Tissue sections (4  $\mu$ m) were prepared from formalin-fixed paraffin embedded blocks. IHC was performed using mouse anti-human MTSS1 antibody (Abnova; Caltag-Medsystems Ltd., Buckingham, UK) and the Vectastain<sup>®</sup> Elite Universal ABC kit (Vector Laboratories, Ltd., Cambridgeshire, 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 MTSS-1 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 Co., Ltd., Dorset, 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, human ovarian epithelial-mucinous carcinoma cell lines COV644 (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  $\mu$ g/ml) and amphotericin B (0.25  $\mu$ g/ml) (Sigma-Aldrich). Cells were incubated at 37°C with 95% humidity in 5% CO<sub>2</sub>.

**Construction of MTSS1 expression vectors and transfection.** The full coding sequence of MTSS1 was amplified from cDNA prepared from normal human mammary tissue RNA using the standard PCR procedure and a Master Mix with a proof-reading enzyme (sense primer, ATGGAGGCTGTGAT TGAG and antisense, CTAAGAAAAGCGAGGGG). This MTSS1 sequence was then T-A cloned into the pEF6/V5/-His-TOPO vector (Invitrogen, Paisley, UK). According to the manufacturers' protocol, the recombinant plasmid vectors were transformed into chemically competent OneShot<sup>®</sup> TOP10 *Escherichia coli* (Invitrogen) and bacteria were grown overnight on agar plates containing ampicillin (100  $\mu$ g/ml). Colonies were analysed and those carrying correct recombinant plasmids were amplified, then extracted and purified (Elute Miniprep; Sigma-Aldrich). Purified MTSS1 plasmids (10  $\mu$ g) and control plasmid vectors were then transfected into SKOV3 (300 V, 1500  $\mu$ F), COV504 (300 V, 1500  $\mu$ F) and COV644 (275 V, 100  $\mu$ F) cells (1x10<sup>6</sup>/ml) using a Gene Pulser Xcell electroporator (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). After ~2 weeks of selection with blasticidin (2-5  $\mu$ g/ml) (Melford Laboratories Ltd., Ipswich, UK), the transfectants were verified for their expression of MTSS1 mRNA and protein and successful clones were used in subsequent studies. Transfected cell cultures were maintained in medium containing 0.5  $\mu$ g/ml blasticidin.

**RNA extraction and reverse transcription PCR.** Total cellular RNA was isolated from the EOC cells using Tri Reagent according to the manufacturer's protocol (Sigma-Aldrich). RNA concentration and quality were determined through spectrophotometric measurement (NanoPhotometer; Implen GmbH, 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 GGCTG CTTTAACTCTGGTA and antisense GACTGTGGTCATG AGTCCTT) which was also used as a loading control. MTSS1 mRNA levels were assessed using primers (sense, TCAAGAA CAGATGGAAGAATGG and antisense, TGCGGTAGCGGT AATGTG). PCR was carried out in an Applied Biosystems thermo cycler using a GoTaq Green PCR reaction mix (Promega UK Ltd., Hampshire, UK). Cycling conditions were 94°C for 5 min, followed by 25-32 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<sup>®</sup> Safe (Life Technologies).

**Immunofluorescence staining.** Cells were seeded at a density of 20,000 cells/well in an 8-well chamber slide (Merck-Millipore, East Midlands, 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 MTSS1 antibody (1:100) in PBS blocking solution (Abnova; Caltag-Medsystems). 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 3x5min PBS, mounted with fluorsave (Merk-Millipore) 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 Laboratories). Protein was reduced and denatured by boiling (5 min) in Laemmli buffer (Sigma-Aldrich) and 20 µg 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 MTSS1 (1:300 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 3x5min TBS/Tween washes, protein bands were visualized using enhanced chemiluminescence (Luminata Forte; Millipore, Hertfordshire, UK) and photographed using a UVItec imager (UVItec, Inc., Cambridge, UK).

**Cell proliferation assay.** Cells were seeded into 96-well plates at a seeding density of 3,000 cells/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, washed and dried. Dye was re-solubilised in 200-µl acetic acid/well and absorbance was determined at 540 nm using an ELx800 multiplate reader (BioTek UK, Bedfordshire, UK). Each experiment was repeated at least 3 times. For each cell line, analysis compared cell number (absorbance) on day 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 (15-17). Cells were seeded into 96-well plates pre-coated with 5 µg/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 of 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 Ltd., Stonehouse, UK) with an 8.0 µm pore

size were coated with 50 µg Matrigel (BD Biosciences), dried at 55°C and rehydrated with 100-µl serum-free medium before seeding 4,000 cells/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 (18). In brief, cells were cultured to confluence in a 24-well plate before scratching the cell monolayer with a 10-µl pipette tip. The closure of the induced wound, through the migration of cells, was tracked and recorded over a 48-h period using an automated cell imaging system EVOS (Life Technologies). Using ImageJ software, the relative cell migration distance was calculated using multiple measurements of the width of wound gap after 6, 12, 24 and 48 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 ZTheta instrument and 96W1E arrays (Applied BioPhysics, Inc., Troy, NY, USA) as previously described (19). Briefly, 40,000 cells/well were added to the ECIS arrays. Impedance and resistance of the cell layer was immediately recorded for a period of up to 15 h. When confluence was reached, the monolayer in each well was electrically wounded at 2,600 µA and 6,0000 Hz for 20 sec to create a 250-µm wound/well. Impedance and resistance of the wounded cells as they migrated in the wound was then recorded for a period of up to 20 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. Differences were considered to be statistically significant at P<0.05.

## Results

**Expression of MTSS1 in human ovarian tissues and EOC cells.** HC staining of 10 sections of non-metastatic and 7 sections of metastatic epithelial cancerous ovarian growths was used to assess MTSS1 expression pattern in the clinical setting. Images are shown of both metastatic and non-metastatic samples, representing the range of staining detected (Fig. 1). Preliminary experiments showed that MTSS1 protein could also be detected in non-cancerous ovarian tissue (data not shown). In 6 out of 10 non-metastatic primary tumors very strong staining for MTSS1 was detected in epithelial cell cytoplasm (Fig. 1A). Weaker staining was present in the cytoplasm of connective tissue. In the other 4 non-metastatic samples, epithelial MTSS1 expression was either weak (n=2) (Fig. 1B) or could not be detected (n=2) (Fig. 1C). When tissue from 7 metastatic tumors was examined, MTSS1 epithelial staining

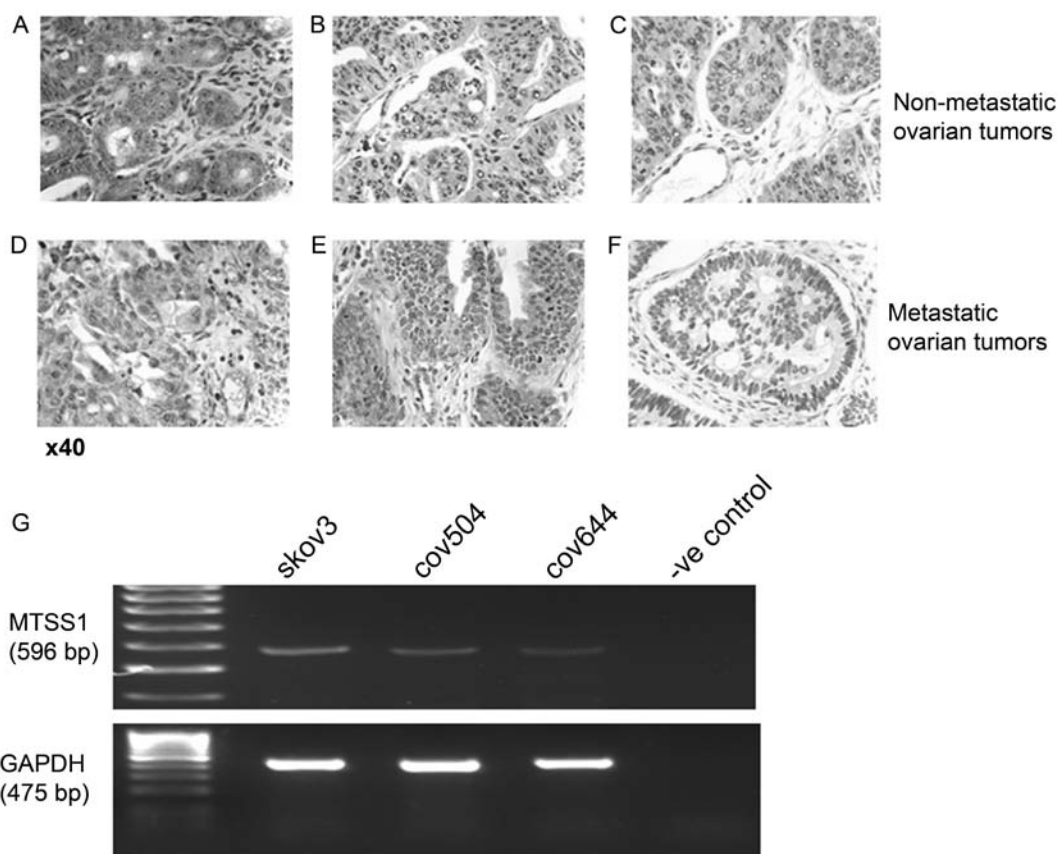


Figure 1. Expression of MTSS1 in ovarian cancer. Immunohistochemical staining of MTSS1 in human ovarian tissues. Non-metastatic ovarian tumours showing strong MTSS1 staining (brown) in epithelial cell cytoplasm (A), weak MTSS1 staining (B) and non-detectable MTSS1 staining (C). Metastatic ovarian tumors showing weaker MTSS1 staining in epithelial cell cytoplasm (D and E) and non-detectable MTSS1 staining (F). Basal expression of MTSS1 mRNA examined in ovarian cancer cell lines using RT-PCR (G). Low expression of MTSS1 (596 bp) in COV644, SKOV3 and COV504 cells (32 cycles). GAPDH (475 bp) was examined as an equal loading control (475 bp).

was detected in 4 samples but the MTSS1 staining was typically weaker than the very strong epithelial staining MTSS1 detected in most of the non-metastatic samples (Fig. 1D and E). In the remaining 3 metastatic samples, MTSS1 staining could not be detected (Fig. 1F), suggesting some reduction of MTSS1 expression in metastatic compared to non-metastatic ovarian cancer.

The mRNA expression of MTSS1 was also examined in three EOC cell lines using RT-PCR. MTSS1 mRNA was expressed at relatively low levels in all cell lines, with SKOV3 cells expressing a slightly increased amount compared to the COV504 and COV644 cells (Fig. 1G).

**Overexpression of MTSS1 in EOC cells.** To investigate the impact of MTSS1 on functions of ovarian cancer cells, MTSS1 expression vectors were utilised to overexpress MTSS1. After selection using blasticidin, the expression of MTSS1 in the transfected cells was verified using RT-PCR, immunofluorescent staining and western blotting (Fig. 2). Increased expression of both mRNA (Fig. 2A) and protein (Fig. 2B) of MTSS1 was seen in SKOV3MTSS1<sup>Exp</sup>, in comparison with the controls, wild-type SKOV3<sup>WT</sup> and empty plasmid SKOV3p<sup>EF6</sup>. Overexpression of MTSS1 was also confirmed in COV504MTSS1<sup>Exp</sup> cells, in comparison with COV504<sup>WT</sup> and COV504p<sup>EF6</sup> control cells. Similarly, overexpression of MTSS1 was confirmed in COV644MTSS1<sup>Exp</sup> cells, in compar-

ison with control COV644<sup>WT</sup> and COV644p<sup>EF6</sup> control cells. Immunofluorescent staining was carried out to examine the expression and localisation of the MTSS1 in the transfected cells (Fig. 2C). MTSS1 staining (green), was predominantly associated with the cytoplasm. Control cells, both wild-type and empty vector transfectants, had weak staining intensity in all three cell lines, with the majority of the cells in the microscopic fields showing minimal staining levels. However, in the transfected cells overexpressing MTSS1 had a more intense and frequently observed staining.

**Regulation of MTSS1 expression affects the rate of cell growth of EOC cells.** The growth capacity of the EOC cells following MTSS1 overexpression was examined and compared to the wild-type and empty vector control cells using an *in vitro* cell growth assay. The growth rates of the three wild-type ovarian cell lines was notably different, with SKOV3 cells growing twice faster than ( $P < 0.05$ ) than either COV504 or COV644 cells (Fig. 3). COV644 were the slowest growing cell line. In all transfected cells, overexpression of MTSS1 protein reduced growth rate by both day 3 and day 5. In SKOV3MTSS1<sup>Exp</sup> cells, the mean cell number at day 5 was decreased by 21% (not significant) compared to pEF6 control, in COV644MTSS1<sup>Exp</sup> cell numbers significantly decreased by 36% ( $P < 0.05$ ) and in COV504MTSS1<sup>Exp</sup> cells, growth rate decreased by 50% ( $P < 0.001$ ).

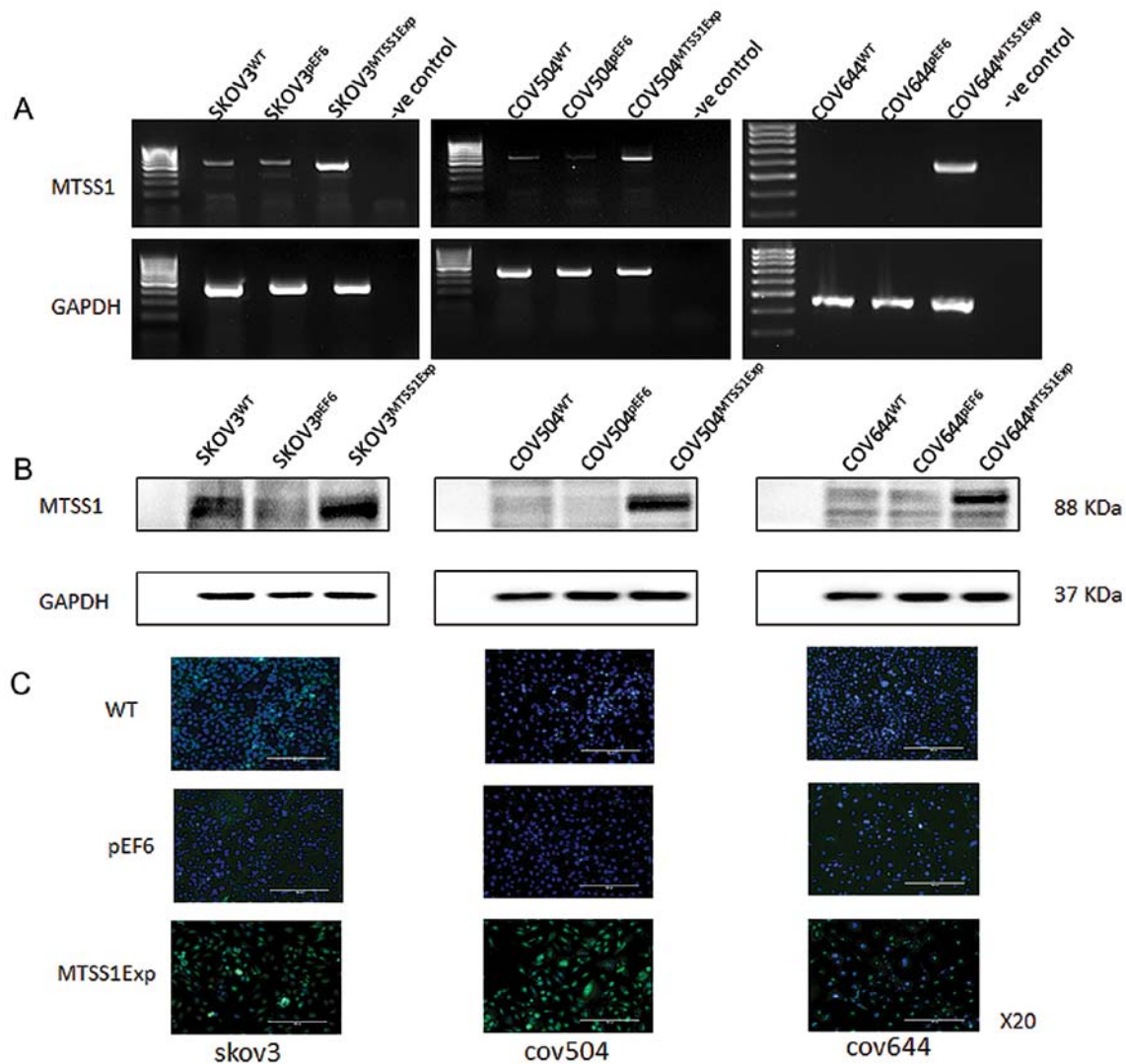


Figure 2. Overexpression of MTSS1 in transfected EOC cells. RT-PCR (32 cycles) showed increased MTSS1 mRNA transcripts in the MTSS1-transfected cells (Exp) compared to controls (WT, pEF6) in SKOV3, COV504 and COV644 cells. GAPDH (25 cycles) was examined as an equal loading control (A). Overexpression of MTSS1 protein in the same cells was verified by western blot analysis. GAPDH was used as the loading control (B). Immunofluorescence microscopy confirmed increased cytoplasmic expression of MTSS1 protein (green) in monolayers of MTSS1-transfected SKOV3, COV504 and COV644 cells (C). Bar indicates, 200  $\mu$ m.

**Effect of MTSS1 overexpression on cell-matrix adhesion in EOC cells.** The effect of MTSS1 on the ability of EOC cells to adhere to Matrigel matrix was examined (Fig. 4A-C). Overexpression of MTSS1 protein caused a significant ( $P<0.001$ ) inhibitory effect of ~40% on cell-matrix adhesion in the SKOV3 cells compared to both WT and pEF6 controls (Fig. 4A). Compared with COV504<sup>WT</sup> and COV504<sup>pEF6</sup>, the number of COV504MTSS1<sup>Exp</sup> cells that adhered was also significantly reduced ( $P<0.001$ ) by ~40% (Fig. 4B). In COV644 cells, MTSS1 overexpression significantly reduced ( $P<0.001$ ) Matrigel adhesion by 50% compared to controls. The ECIS system was also used to confirm the inhibitory effect of enhanced expression of MTSS1 on SKOV3, COV504 and COV644 cell adhesion. This was measured by change in resistance formed over the growth surface as cells attached from 0 to 5 h (Fig. 4D-E). Compared with the appropriate WT and pEF6 controls, the resistance was significantly reduced in SKOV3MTSS1<sup>Exp</sup>, COV504MTSS1<sup>Exp</sup> and COV644MTSS1<sup>Exp</sup> cells confirming that high expression of MTSS1 in ovarian cells reduced adhesive capability.

**Effect of MTSS1 overexpression on the invasion of EOC cells.** The potential biological relevance of increased MTSS1 expression was further investigated using *in vitro* invasion assays over the artificial basement membrane, Matrigel. The wild-type ovarian cell lines all had invasive capability. In a typical experiment, ~320 SKOV3 and COV644 cells invaded per membrane. COV504 cells had a slightly lower (not significant) invasive capability with 230 cells/inset (data not shown). Increased expression of MTSS1 in all of these cell lines caused a 50% reduction ( $P<0.001$ ) in basal invasion compared to the pEF6 controls (Fig. 5).

**Effect of MTSS1 on wounding/migration of EOC cells.** A cellular wounding assay was used to compare the ability of wild-type and MTSS1 overexpressing cells to migrate. SKOV3<sup>WT</sup> cells migrated slightly faster than COV504 and COV644 cells, but increased expression of MTSS1 in all three cell lines caused a marked (up to 50%) reduction in migration capability ( $P<0.001$ ).

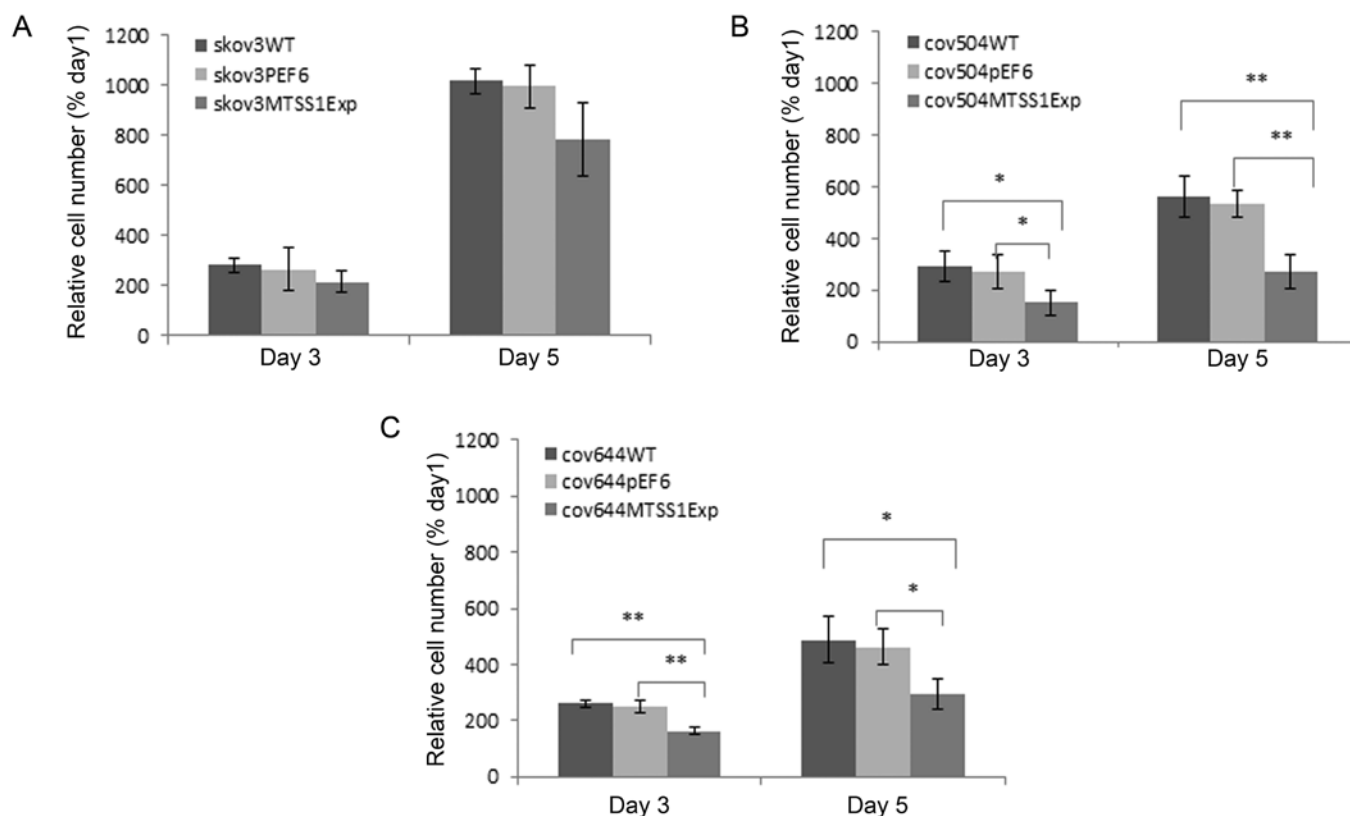


Figure 3. Effect of MTSS1 on *in vitro* growth of EOC cells. Cell numbers were measured by crystal violet absorbance after 1, 3 and 5 days growth and results expressed as % cell number on day 3 and 5 compared to day 1. (A) Shows growth after 5 days SKOV3MTSS1<sup>Exp</sup>, (B) shows that growth in COV504MTSS1<sup>Exp</sup> and (C) that growth in COV644MTSS1<sup>Exp</sup> cells was decreased in comparison with the WT and pEF6 controls. Data was analysed by paired t-test. \*P<0.05 \*\*P<0.01. The error bars represent mean  $\pm$  SD, data shown are mean of three independent experiments.

The ability of increased MTSS1 expression to reduce 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. 6D-F). Measurements taken 10 h post-wound also showed that the migration capacity of SKOV3MTSS1<sup>Exp</sup>, COV504MTSS1<sup>Exp</sup> and COV644MTSS1<sup>Exp</sup> were markedly reduced ( $P<0.001$ ) in comparison with wild-type and pEF6 control cells (Fig. 6D-F).

## Discussion

Over the past decade, prognosis for patients with EOC has improved little, with the cancer recurring in 70-80% of the patients who ultimately succumb to the disease (20). There are a number of genetic and epigenetic changes that lead to transformation of ovarian epithelial cells into tumor cells (21). 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. Although many of these tumorigenesis mechanisms remain largely unknown, over-expression of certain oncoproteins (22) or downregulation of tumor suppressor proteins (23) has been demonstrated to play important roles in the process of tumor growth and metastasis.

Downregulation of the potential tumor suppressor MTSS1, has been observed in a number of human cancer types and complete loss of MTSS1 can be associated with poorly differentiated metastatic tumors and with poor survival rates (9).

However, the available data are controversial and whether or not MTSS1 serves as a metastasis suppressor has not been clearly defined. To date, the role of MTSS1 in ovarian cancer remains largely unknown. To the best of our knowledge, the present study is the first to have examined the staining pattern of MTSS1 in human EOC tissues and to test the impact of MTSS1 on the growth, adhesion, invasion and migration of EOC cells by genetically manipulating the expression of MTSS1.

The present study determined whether there was a relationship between MTSS1 protein expression and the aggressiveness of clinical ovarian cancer. Conclusions were limited by the relatively small sample size used, but immunohistochemical analysis clearly demonstrated that the samples we examined expressed a range of MTSS1 protein. We detected relatively high levels of MTSS1 in non-cancerous ovarian tissue and in some metastatic and non-metastatic tumors. However, typically the MTSS1 staining in samples from tumors that had metastasised was weaker than that detected in non-metastatic cancers. We also detected a higher percentage (40%) of completely MTSS1 negative metastatic compared to non-metastatic (20%) samples. Due to the relatively low number of clinical samples included in our study, the results did not enable statistical analysis. It has been previously reported that MTSS1 can be lost in aggressive tumors in a number of tumor types including bladder (5), prostate (6), kidney (7) and gastric (8) cancer. However, as seen in hepatocellular carcinoma (10) and currently in our ovarian tumor samples, MTSS1

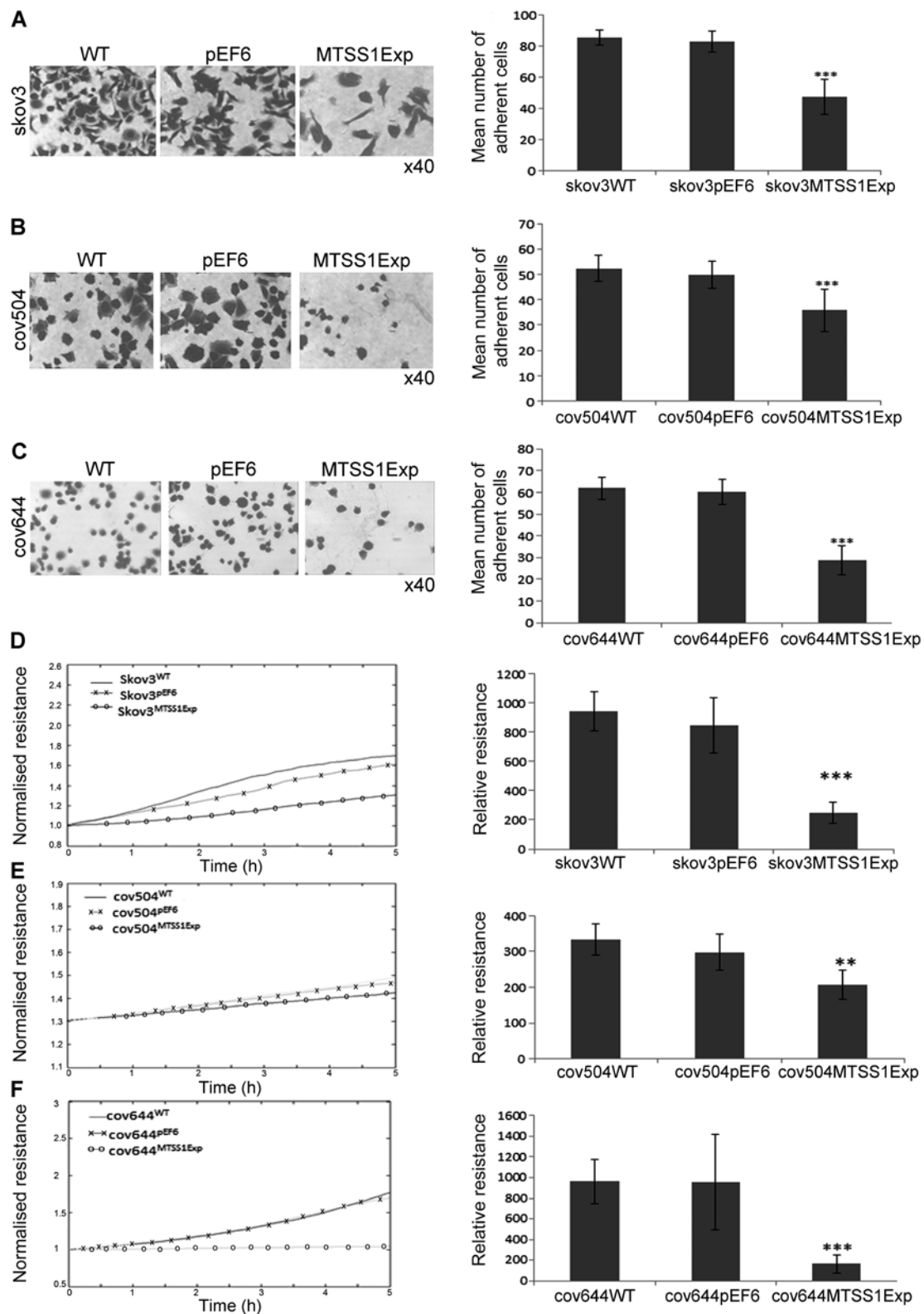


Figure 4. Effect of MTSS1 on adhesion of EOC cells *in vitro*. Effect of MTSS1 increased expression on rapid (40 min) cell adhesion to Matrigel basement membrane. MTSS1 overexpression significantly reduced (A) SKOV3MTSS1<sup>Exp</sup> (B) COV504MTSS1<sup>Exp</sup> and (C) COV644MTSS1<sup>Exp</sup> cell adhesion compared to pEF6 and WT controls. Data were analysed by paired t-test (MTSS1<sup>Exp</sup> vs. pEF6 control). \*\*\*P<0.001. The error bars represent mean cell count/well  $\pm$  SD. Image is a representative field of view. Data shown are mean of three independent experiments. The (D) SKOV3MTSS1<sup>Exp</sup>, (E) COV504MTSS1<sup>Exp</sup> and (F) COV644MTSS1<sup>Exp</sup> cells which overexpressed MTSS1 showed a markedly reduced 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<0.01; \*\*\*P<0.001 comparing MTSS1<sup>Exp</sup> vs. empty plasmid control cells.

can also frequently be overexpressed in primary tumors. It has been suggested that in primary tumor formation, high

MTSS1 expression may initially be selectively beneficial by increasing plasma membrane EGFR expression, resulting in



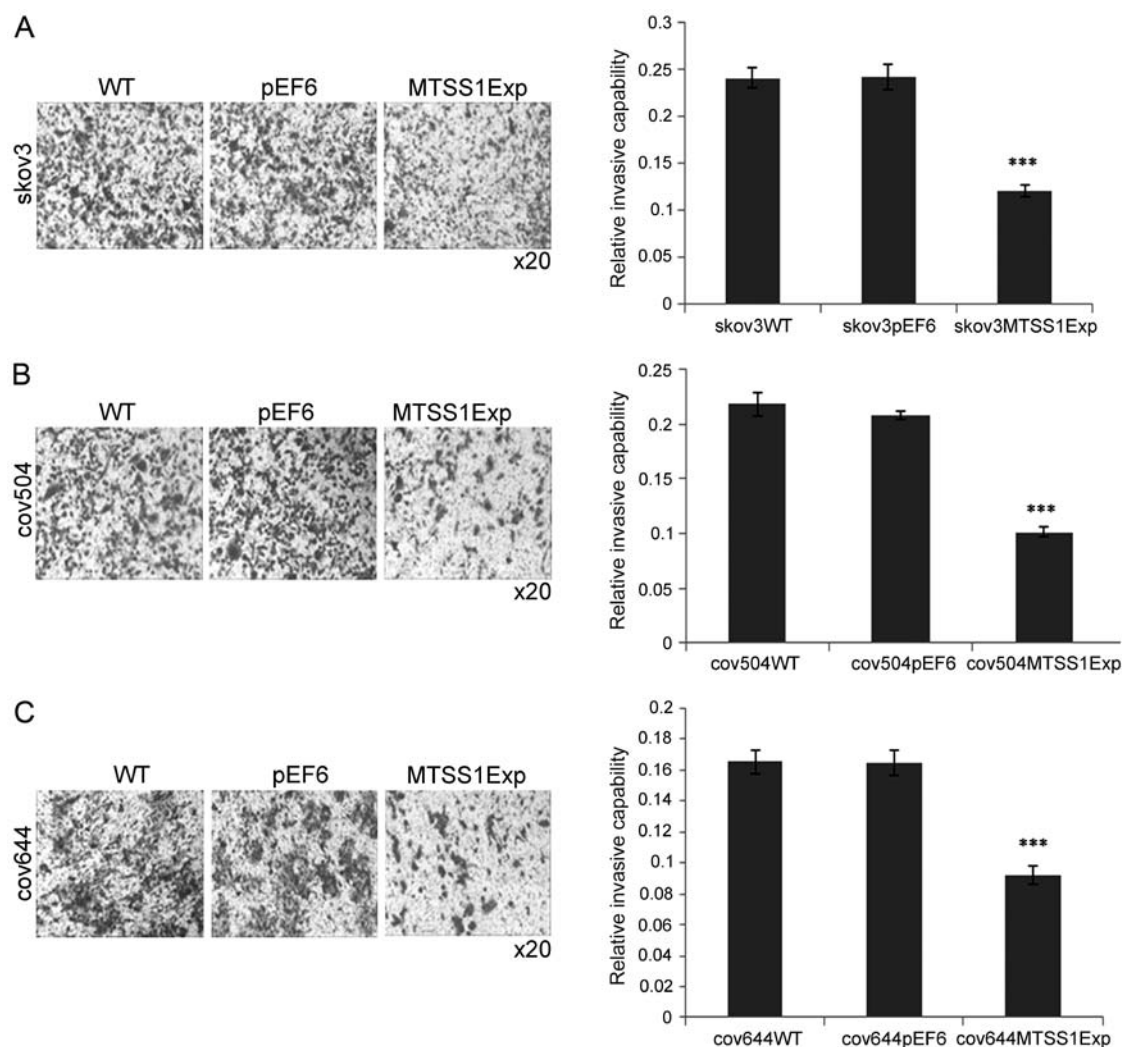


Figure 5. Effect of MTSS1 overexpression on the invasion of EOC cells. Cell invasion through Matrigel coated barrier was quantitated after fixing and staining invaded cells. Invasion capability was reduced in (A) SKOV3MTSS1<sup>Exp</sup>, (B) COV504MTSS1<sup>Exp</sup> and (C) COV644MTSS1<sup>Exp</sup> cells. Four fields of view/insert were photographed and counted. Images shown are from a typical field of view (x20 magnification). Data shown are mean  $\pm$  SD cell number/membrane from 3 independent experiments. T-test compared MTSS1<sup>Exp</sup> vs. the controls for each cell line. \*\*\*P<0.001.

increased EGF signalling, Erk1/2 activation and cancer proliferation and survival (25). As cell density increases with tumor growth, MTSS1 may switch to the inhibitory tumor suppressor role for which it is better known; when it inhibits EGFR and Akt signalling and cells retain the epithelial-like morphology consistent with an anti-metastatic role (25). In some tumor types, including breast and oesophageal cancer, MTSS1 may be considered a suitable biomarker for tumor progression, with high MTSS1 being associated with favourable prognosis and reduced MTSS1 a poorer outcome (9,26). How MTSS1 can be overexpressed in early tumor formation and lost in later metastatic stages remains unclear, although methylation has been considered as one mechanism used to silence the gene expression (28). Our clinical results show that although there is a trend for reduced MTSS1 expression in metastatic tumors, MTSS1 expression is variable between individuals which may reflect a range of differences in ovarian cancer etiology or disease stages when samples were taken. Conclusive results can only be obtained if a larger cohort is used in the study.

A relatively small number of *in vitro* studies have been previously reported in which the function of MTSS1 is char-

acterized. Our studies have shown that 3 different ovarian tumor cell lines, SKOV3, COV504 and COV644, which expressed a reasonable (but not high) invasive capability, all expressed a similar low level of MTSS1 mRNA and protein. Cellular function tests further demonstrated that the presence of MTSS1 was related to the inhibition of the ovarian cancer cell aggressiveness. Previous studies with a panel of human cell lines have shown MTSS1 is differentially expressed with an inverse correlation between cell differentiation, invasive capability and MTSS1 expression (9,25,26). These results were not without exceptions as some cell lines, like the breast cancer cell ZR75.1, considered non-invasive, were negative for MTSS1 expression suggesting other factors in addition to MTSS1 are involved in regulating invasion (9). We have demonstrated here that MTSS1 overexpression resulted in a dramatic reduction in ovarian cancer cell line growth, adhesion, invasion and migration, in comparison to control cells. The inhibitory effect of MTSS1 on ovarian cancer cell growth is in agreement with the findings in kidney, bladder, oesophageal, breast and prostate cell lines (7,9,26,27,29). Although the precise molecular mechanisms by which MTSS1 inhibits



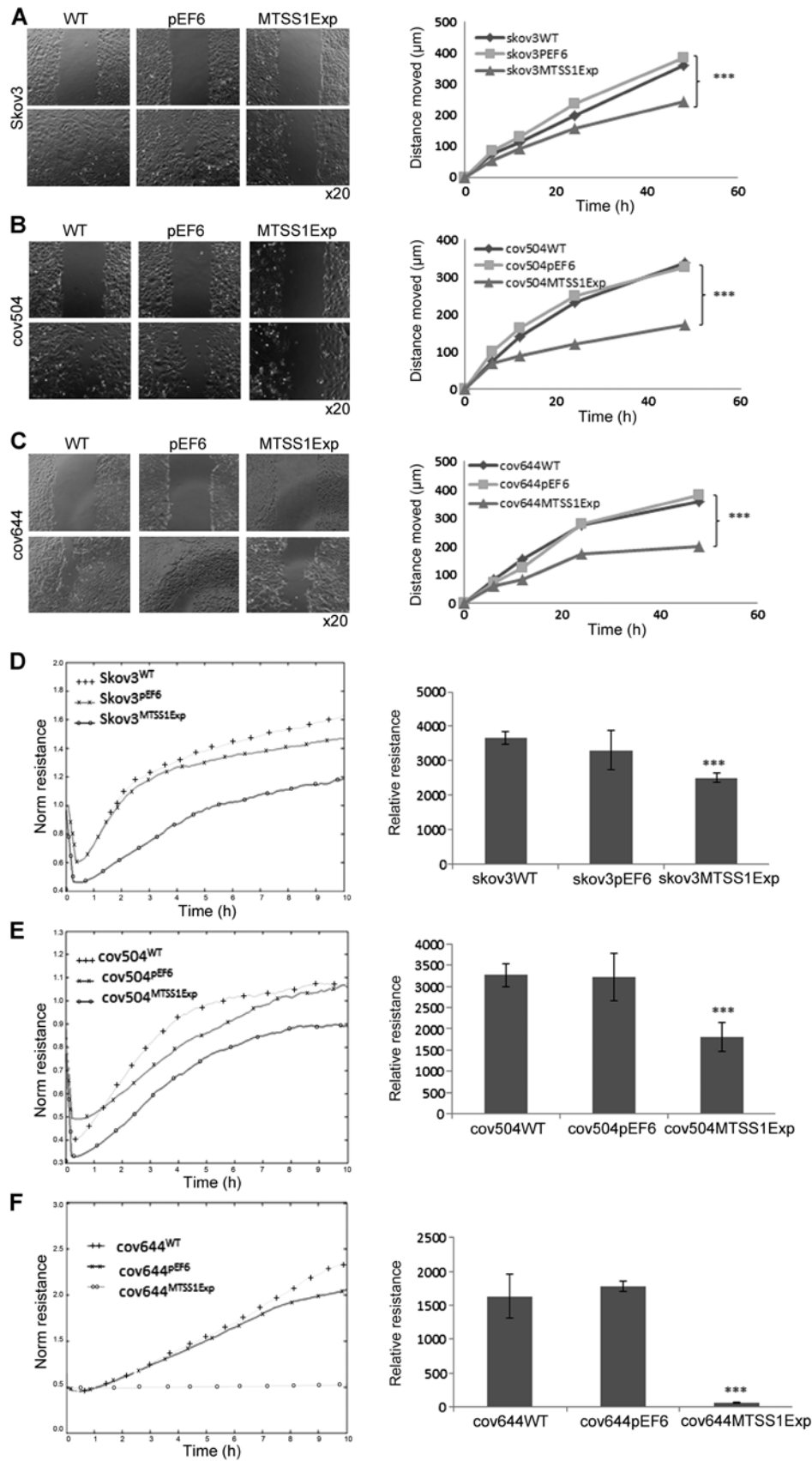


Figure 6. Effect of MTSS1 overexpression on migration of EOC cells. Confluent monolayers of EOC cells were scratched and the distance moved by cells to cover the wound was measured after 6, 12, 24 and 48 h and compared to time 0. After 48 h, (A) SKOV3, (B) COV504 and (C) COV644 cells which overexpressed the MTSS1 protein all showed significantly reduced migration compared to WT or pEF6 controls (T-test \*\*\* $P < 0.001$ ). Images shown are taken at 0 and 48 h are from representative experiment. The data shown are from 3 independent experiments. ECIS confirmed the reduced migratory capability of (D) SKOV3MTSS1<sup>Exp</sup>, (E) COV504MTSS1<sup>Exp</sup> and (F) COV644MTSS1<sup>Exp</sup> 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 mean of 3 independent experiments  $\pm$  SD. \*\*\* $P < 0.001$  comparing MTSS1<sup>Exp</sup> vs. control cells.

tumor growth remains unknown, at high cell density MTSS1 has been shown to dampen EFG signalling which inhibits proliferation in epithelial-like layers (25). MTSS1 is a member of a growing family of cytoskeletal components that associate with transcription factors to affect nuclear signaling. MTSS1 is known to behave as a sonic hedgehog (Shh) responsive gene and may use this pathway to modulate responses in cancer growth and development (30,31).

MTSS1 overexpression resulting in dramatic inhibition of tumor cell adhesion and migration and invasion over Matrigel has also been previously reported in the ovarian cell line SKOV3 (14) as well as in breast and prostate cell lines (9,27). There are, however, differences between cancer types, because overexpression of MTSS1 in bladder cancer cell lines has been reported to inhibit growth and adhesion but have no effect on invasion or migration (29). In contrast, in breast cancer, increased MTSS1 expression inhibits invasion but has no effect of cell adhesion (9). Cytoskeletal rearrangement is a critical event in cell motility. MTSS1 is known to act as a cytoskeletal scaffold protein where it regulates plasma membrane dynamics and actin filaments in a complex fashion. The detailed mechanism of the MTSS1 effect on cell motility remains to be further defined. MTSS1 protein appears to control the formation of lamellipodia, membrane ruffles and filopodia and enhance changes in cell shape (32). In contrast, MTSS1 also promotes cell-cell junction assembly through recruiting small GTPase and actin, which drives junction maintenance. Therefore, loss of MTSS1 in cancers may lead to the loss of junction stability, which ultimately promotes EMT and metastasis (24). Furthermore, MTSS1 is known to negatively regulate the epidermal growth factor signaling to suppress metastasis (25). Further studies are required to reveal the exact molecular mechanisms and signaling pathways through which MTSS1 modulates cancer cell migration and invasion.

In summary, the present study shows that the overexpression of the MTSS1 protein can suppress the aggressiveness of human ovarian cancer. Our work further suggests that preventing MTSS1 degradation, or partially restoring MTSS1 expression, could be a possible novel strategy to treat aggressive ovarian cancer growth and metastasis.

## Acknowledgements

The authors are grateful to Cancer Research Wales and the Albert Hung Foundation for their support and funding of the present study.

## References

- Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. *CA Cancer J Clin* 61: 69-90, 2011.
- Siegel R, Naishadham D and Jemal A: Cancer statistics, 2012. *CA Cancer J Clin* 62: 10-29, 2012.
- Siegel R, Naishadham D and Jemal A: Cancer statistics, 2013. *CA Cancer J Clin* 63: 11-30, 2013.
- Lee YG, Macoska JA, Korenchuk S and Pienta KJ: MIM, a potential metastasis suppressor gene in bladder cancer. *Neoplasia* 4: 291-294, 2002.
- Nixdorf S, Grimm MO, Loberg R, Marreiros A, Russell PJ, Pienta KJ and Jackson P: Expression and regulation of MIM (Missing In Metastasis), a novel putative metastasis suppressor gene, and MIM-B, in bladder cancer cell lines. *Cancer Lett* 215: 209-220, 2004.
- Loberg RD, Neeley CK, Adam-Day LL, Fridman Y, St John LN, Nixdorf S, Jackson P, Kalikin LM and Pienta KJ: Differential expression analysis of MIM (MTSS1) splice variants and a functional role of MIM in prostate cancer cell biology. *Int J Oncol* 26: 1699-1705, 2005.
- Du P, Ye L, Li H, Yang Y and Jiang WG: The tumour suppressive role of metastasis suppressor-1, MTSS1, in human kidney cancer, a possible connection with the SHH pathway. *J Exp Ther Oncol* 10: 91-99, 2012.
- Liu K, Wang G, Ding H, Chen Y, Yu G and Wang J: Downregulation of metastasis suppressor 1 (MTSS1) is associated with nodal metastasis and poor outcome in Chinese patients with gastric cancer. *BMC Cancer* 10: 428, 2010.
- Parr C and Jiang WG: Metastasis suppressor 1 (MTSS1) demonstrates prognostic value and anti-metastatic properties in breast cancer. *Eur J Cancer* 45: 1673-1683, 2009.
- Ma S, Guan XY, Lee TK and Chan KW: Clinicopathological significance of missing in metastasis B expression in hepatocellular carcinoma. *Hum Pathol* 38: 1201-1206, 2007.
- Yamagishi A, Masuda M, Ohki T, Onishi H and Mochizuki N: A novel actin bundling/filopodium-forming domain conserved in insulin receptor tyrosine kinase substrate p53 and missing in metastasis protein. *J Biol Chem* 279: 14929-14936, 2004.
- Mattila PK, Salminen M, Yamashiro T and Lappalainen P: Mouse MIM, a tissue-specific regulator of cytoskeletal dynamics, interacts with ATP-actin monomers through its C-terminal WH2 domain. *J Biol Chem* 278: 8452-8459, 2003.
- Woodings JA, Sharp SJ and Machesky LM: MIM-B, a putative metastasis suppressor protein, binds to actin and to protein tyrosine phosphatase delta. *Biochem J* 371: 463-471, 2003.
- Liu Z, Liu J, Segura MF, Shao C, Lee P, Gong Y, Hernando E and Wei JJ: MiR182 overexpression in tumorigenesis of high-grade ovarian papillary serous carcinoma. *J Pathol* 228: 204-215, 2012.
- 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.
- 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.
- 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  $Ca^{2+}$ . *Exp Cell Res* 220: 424-433, 1995.
- 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.
- 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.
- Onda T and Yoshikawa H: Neoadjuvant chemotherapy for advanced ovarian cancer: Overview of outcomes and unanswered questions. *Expert Rev Anticancer Ther* 11: 1053-1067, 2011.
- Kurman RJ, Visvanathan K, Roden R, Wu TC and Shih IeM: Early detection and treatment of ovarian cancer: Shifting from early stage to minimal volume of disease based on a new model of carcinogenesis. *Am J Obstet Gynecol* 198: 351-356, 2008.
- Yang JL, Ow KT, Russell PJ, Ham JM and Crowe PJ: Higher expression of oncoproteins c-myc, c-erb B-2/neu, PCNA, and p53 in metastasizing colorectal cancer than in nonmetastasizing tumors. *Ann Surg Oncol* 3: 574-579, 1996.
- Goncharuk VN, del-Rosario A, Kren L, Anwar S, Sheehan CE, Carlson JA and Ross JS: Co-downregulation of PTEN, KAI-1, and nm23-H1 tumor/metastasis suppressor proteins in non-small cell lung cancer. *Ann Diagn Pathol* 8: 6-16, 2004.
- Mustafa N, Martin TA and Jiang WG: Metastasis tumour suppressor-1 and the aggressiveness of prostate cancer cells. *Exp Ther Med* 2: 157-162, 2011.
- Dawson JC, Timpson P, Kalna G and Machesky LM: Mtss1 regulates epidermal growth factor signaling in head and neck squamous carcinoma cells. *Oncogene* 31: 1781-1793, 2012.
- Xie F, Ye L, Chen J, Wu N, Zhang Z, Yang Y, Zhang L and Jiang WG: The impact of metastasis suppressor-1, MTSS1, on oesophageal squamous cell carcinoma and its clinical significance. *J Transl Med* 9: 95, 2011.

27. Dawson JC, Bruche S, Spence HJ, Braga VM and Machesky LM: Mtss1 promotes cell-cell junction assembly and stability through the small GTPase Rac1. *PLoS One* 7: e31141, 2012.
28. Fan H, Chen L, Zhang F, Quan Y, Su X, Qiu X, Zhao Z, Kong KL, Dong S, Song Y, Chan THM and Guan XY: MTSS1, a novel target of DNA methyltransferase 3B, functions as a tumor suppressor in hepatocellular carcinoma. *Oncogene* 31: 2298-2308, 2012.
29. Du P, Ye L, Ruge F, Yang Y and Jiang WG: Metastasis suppressor-1, MTSS1, acts as a putative tumour suppressor in human bladder cancer. *Anticancer Res* 31: 3205-3212, 2011.
30. Callahan CA, Ofstad T, Horng L, Wang JK, Zhen HH, Coulombe PA and Oro AE: MIM/BEG4, a Sonic hedgehog-responsive gene that potentiates Gli-dependent transcription. *Genes Dev* 18: 2724-2729, 2004.
31. Xie F, Ye L, Ta M, Zhang L and Jiang WG: MTSS1: A multi-functional protein and its role in cancer invasion and metastasis. *Front Biosci (Schol Ed)* 3: 621-631, 2011.
32. Zhong J, Shaik S, Wan L, Tron AE, Wang Z, Sun L, Inuzuka H and Wei W: SCF  $\beta$ -TRCP targets MTSS1 for ubiquitination-mediated destruction to regulate cancer cell proliferation and migration. *Oncotarget* 4: 2339-2353, 2013.