Original Article

Wnt5a Influences Viability, Migration, Adhesion, Colony Formation, E- and N-Cadherin Expression of Human Ovarian Cancer Cell Line SKOV-3

(serous ovarian cancer / Wnt5a / SKOV-3 cell line / viability / adhesion / migration / E-cadherin / N-cadherin)

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Abstract. Epithelial ovarian cancer (EOC) cells express Wnt5a, but its role in ovarian cancer progression is poorly defined. The aims of the present study were two-fold: 1) to determine the Wnt5a role in viability, apoptosis, migration, colony formation and adhesion of human serous epithelial ovarian cancer cell line SKOV-3, and 2) to assess the relationship of Wnt5a with E- and N-cadherin in high- and low-grade human serous ovarian cancer specimens. Wnt5a over-expression led to 29% increased serum-independent cell viability ($P < 0.05$) and 35% decreased caspase-3 activity ($P < 0.01$) compared to SKOV-3 cells. There was 96% ($P < 0.001$) increased cell motility in Wnt5a-transfected SKOV-3 (SKOV-3/Wnt5a) cells compared to SKOV-3, which was abrogated in the presence of JNK inhibitor. In addition, there was about 42% increased cell adhesion to Matrigel compared to SKOV-3 cells ($P < 0.001$). Colony-forming assay showed a 4.4-fold increased colony formation in SKOV-3/Wnt5a cells compared to SKOV-3 cells ($P < 0.001$). E- and N-cadherin levels were reduced by 49 % and 67 % in SKOV-3/Wnt5a cells compared to mock cells, respectively. Wnt5a and E-cadherin immunoexpression was significantly ($P < 0.001$) different in low-grade serous ovarian cancer (LGSC) and high-grade serous ovarian cancer (HGSC). In HGSC specimens, strong immunoexpression of Wnt5a was detected compared to LGSC. However, E-cadherin showed moderate immunostaining (84 %) in HGSC, whereas 100 % of LGSC specimens showed strong immunoexpression. In both groups no N-cadherin immunoexpression was detected. Moreover, Wnt5a showed a positive relationship with E-cadherin in the LGSC group ($r = 0.661$, $P = 0.027$). These results may support important roles for Wnt5a in EOC progression.

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Abbreviations: A – absorbance, BSA – bovine serum albumin, DAB – diaminobenzidine tetrahydrochloride, DMSO – dimethyl sulphoxide, EGF – epidermal growth factor, EMT – epithelial-mesenchymal transition, EOC – epithelial ovarian cancer; FBS – foetal bovine serum, Fzd – frizzled, HGSC – high-grade serous ovarian carcinoma; LGSC – low-grade serous ovarian carcinoma, MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, OSE – ovarian surface epithelium, PBS – phosphate-buffered solution, RPMI – Roswell Park Memorial Institute medium; RT – room temperature, SDS – sodium dodecyl sulphate, SKOV-3/Wnt5a – Wnt5a-transfected SKOV-3.

Introduction

Wnt molecules show a highly regulated pattern of expression and have distinct roles during development, tissue homeostasis and cancer (Klaus and Birchmeier, 2008). Wnt5a is a non-transforming Wnt protein that is implicated in cell polarity, adhesion, and motility (Kikuchi et al., 2012). Multiple and diverse functions of Wnt5a arise from the fact that Wnt5a receptor/co-receptor context dictates the downstream signalling pathways which include activating non-canonical Wnt signalling or antagonizing canonical Wnt/β-catenin signalling (Mikels and Nusse, 2006). The Wnt5a signal is mediated by orphan receptor tyrosine kinase Ror2 or by interaction with frizzled (Fzd) receptors (Mikels and Nusse, 2006). The functions of Wnt5a in human cancers are controversial and still unclear, showing paradoxical roles depending on cancer cell types (Nishita et al., 2010). Wnt5a may have a tumour-suppressing or an oncogenic effect depending on the cancer types (McDonald and

Silver, 2009). The role of Wnt5a in ovarian cancer progression is still controversial and unclear (Pukrop and Binder, 2008). Down-regulation of Wnt5a has been associated with higher tumour grade and is reported to be an independent factor indicating poor prognosis in a number of different tumour subtypes (McDonald and Silver, 2009). The tumour suppressor role for Wnt5a was reported in B lymphomas (Liang et al., 2003; Ying et al., 2007), thyroid carcinoma (Kremenevskaja et al., 2005) and colon cancer (Dejmek et al., 2005). These reports anticipated the use of Wnt5a as a therapeutic tool, as recently a synthetic peptide mimicking the properties of Wnt5a was found to inhibit cell migration in breast cancer cell lines (Säfholm et al., 2008).

On the other hand, Wnt5a expression has been described to be associated with aggressive tumour biology and poor clinical outcome. Wnt5a-increased expression has been associated with increasing tumour grade as an independent risk factor for reduced metastasis-free and overall survival in patients with melanoma skin cancer (Da Forno et al., 2008) or non-small-cell lung cancer (Huang et al., 2005). Likewise, Wnt5a was correlated with aggressiveness of gastric (Kurayoshi et al., 2006) and prostate (Yamamoto et al., 2010) cancer. Wnt5a has been reported to be associated with invasion, scattering and promotion of epithelial-mesenchymal transition (EMT) in breast cancer cell lines (Medrek et al., 2009). Previous reports showed Wnt5a expression in human ovarian cancer cell lines and EOC specimens, suggesting that Wnt5a could be involved in ovarian carcinogenesis (Matei et al., 2002; Ricken et al., 2002; Badiglian Filho et al., 2009; Bitler et al., 2011). However, these studies report a controversial role of Wnt5a in EOC, suggesting that Wnt5a is a predictor of poor prognosis for ovarian cancer (Matei et al., 2002; Badiglian Filho et al., 2009) and may play a key role in increased chemosensitivity of ovarian cancer cells to anticancer drugs (Peng et al., 2011).

On the other hand, a recent study reports that the loss of Wnt5a predicts poor outcome in EOC patients and suppresses growth of EOC cells by triggering cellular senescence (Bitler et al., 2011). In the normal human ovarian surface epithelium (OSE), N-cadherin is highly expressed and E-cadherin is not expressed, but in the epithelium of cortical inclusion cysts, the most common origin of primary ovarian tumour masses, E-cadherin is highly expressed (Sundfeldt et al., 1997). *In vitro* experiments have shown that over-expression of E-cadherin in human immortalized OSE cells initiated adherens junction formation, and regulated OSE cell morphology and migration (Wu et al., 2008). Analysis of cell lines derived from human OSE demonstrates an indirect correlation between E-cadherin expression and the invasive capacity of these cells *in vitro* (Patel et al., 2003). Moreover, an *in vivo* model demonstrated that knockdown expression of E-cadherin by siRNA in ovarian cancer cells promoted cancer metastasis via integrin α5 up-regulation (Sawada et al., 2008). It has been proposed that a switch in the expression of cadherin subtypes may be a dynamic process that allows carcinoma cells to detach from the primary tumour, invade into the underlying stroma and endothelium, or metastasize to specific secondary sites through the establishment of novel homophilic interactions (Patel et al., 2003). Thus, alterations in cadherin expression levels have been associated with the development of ovarian cancer. However, the role of E-cadherin in ovarian cancer progression is still controversial. The loss of E-cadherin itself may contribute to dysregulation of PI3K/Akt signalling and Rho GTPase activation to promote tumour proliferation and invasion in human ovarian cancer cells (Lau et al., 2013). It is well known that Wnt signalling regulates expression of the E-cadherin repressor Snail (Yook et al., 2005); however, the possible association of Wnt5a with E- and N-cadherin in ovarian cancer remains to be determined.

The aims of the present study were two-fold: 1) to determine the role of Wnt5a in the viability, apoptosis, migration, colony formation and adhesion of human serous epithelial ovarian cancer cell line SKOV-3 by using Wnt5a over-expressing cells; 2) to assess the possible relation of Wnt5a with E- and N-cadherin in high- and low-grade human serous ovarian cancer specimens.

Material and Methods

Cell culture and establishment of Wnt5a stably expressing clones

SKOV-3 cells were a kind gift from Dr. A. H. Zarnani (Avicenna Research Center, Tehran, Iran). Cells were grown in Roswell Park Memorial Institute medium (RPMI)-1640 supplemented with 10% foetal bovine serum (FBS) and pencillin/streptomycin antibiotics obtained from Life Technologies GmbH (Darmstadt, Germany) at 37 °C in 5% CO_2 atmosphere under 90–95% humidity. The pcDNA3.1(+)-Wnt5a plasmid encoding the full-length human Wnt5a was obtained from Origene Technologies Inc. (Rockwill, MD). The coding region was cloned and inserted into pIRES2-EGFP plasmid (Clontech, Mountain View, CA). The sequences of the plasmid were verified by DNA sequencing. SKOV-3 cells were transfected with pIRES2-EGFP-Wnt5a or pIRES2-EGFP using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the protocol of the manufacturer. Stably transfected clones were selected 24 h later by adding the selection reagent G418 (500 mg/ml; Sigma-Aldrich, Munich, Germany). Selection was continued for 14 days, with the medium being refreshed every other day. Transfectants with vector pIRES2-EGFP--Wnt5a and pIRES2-EGFP were termed SKOV3/Wnt5a and mock cells, respectively. Non-transfected cells were termed SKOV-3. The stable clones were then evaluated for Wnt5a expression by Western blot analysis.

Cell survival assay

Cell survival was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromi-

de (MTT) assay. SKOV-3/Wnt5a, mock and SKOV-3 cells were seeded at 8000 cells/wells in 96-well plates in serum-free medium; after 24 h or 48 h, 10 µl MTT solution was added to each well followed by incubation at 37 °C for 3 h, then the medium was removed and formazan crystals were revealed by adding 100 µl dimethyl sulphoxide (DMSO) to each well, followed by gentle 10 min shaking at room temperature (RT), and the absorbance (A) value was measured at 570 nm with an ELISA reader (Awareness Technology Inc, Palm City, FL). Individual samples were analysed in quadruplicate against a background of blank wells. Cell survival from three independent experiments was expressed as percentage (A value of transfected cells / SKOV-3 cells ×100).

Caspase-3 activity assay

The levels of an apoptotic marker, cleaved caspase-3 (active form), was measured in cell lysates using a colorimetric assay kit (GenScript, Piscataway, NJ) according to the manufacturer's instructions. Briefly, SKOV-3/Wnt5a, mock and SKOV-3 cells at 80% confluence were pelleted and suspended in 50 µl cold lysis buffer, 0.5 µl DTT (1 M) + 5µl PMSF (100 mM), and incubated on ice for 1 h with intermittent vortexing. After centrifugation (2000 × *g*) at 4 °C for 1 min, supernatants were removed and protein concentration was determined in a new tube by performing the Bradford assay. Protein amount of 100–200 µg was mixed with 50 µl of reaction buffer, 0.5 µl DTT (1 M), 0.25 µl PMSF (100 mM) and 5 µl caspase-3 substrate (2 mM), incubated at 37 °C for 4 h, and the A value of each sample was determined at 400 nm with an ELISA reader. Results were analysed in duplicate against a background of blank wells. Results from two experiments were expressed as percentage (A value of transfected cells / SKOV-3 cells × 100).

Colony forming assay

SKOV-3/Wnt5a, mock and SKOV-3 cells (2,400 cells/well) were plated in 6-well plates in order to obtain isolated cells for 14 days. Colonies were fixed and stained with 0.1% crystal violet and counted under an inverted microscope (Zeiss, Jena, Germany) using the standard definition that a colony consists of 50 or more cells. Photographs were taken with a digital camera (AxioCam, Zeiss).

Cell adhesion assay

A 96-well plastic plate was coated with 4 µg/well Matrigel (BD Bioscience, Bedford, MA) at 4 °C overnight. After washing wells twice with phosphate-buffered solution (PBS), non-specific binding sites were blocked by incubating wells with 0.2% bovine serum albumin (BSA) for 2 h at RT followed by three times washing in PBS, then the plate was used for adhesion assay. Cells at 20×10^3 were seeded to each well in triplicate and incubated for 15 or 30 min at 37 °C, washed two times with PBS and fixed with 4% paraformaldehyde (Sigma Aldrich) for 10 min at RT, stained with a solution containing 0.5% crystal violet, 2% ethanol and 40% methanol in PBS for 20 min and destained with distilled water. Then, 100 µl sodium dodecyl sulphate (SDS) 1% was added to each well followed by gentle 10 min shaking at RT and the A value was measured at 600 nm with an ELISA reader, analysed in quadruplicate against a background of blank wells. Cell adhesion from three independent experiments was expressed as percentage (A value of transfected cells / SKOV-3 cells ×100).

Migration and invasion assay

Cells were resuspended at a density of 2.5×10^5/ml in RPMI-1640. One hundred µl of the cell suspension was added to the upper chamber of an 8-µm pore size Transwell insert (Costar, Corning, NY). RPMI-1640 culture solution (650 µl) containing 10% FBS was added to the lower chamber of each well and incubated for 20 h at 37 °C. Non-migratory cells on the upper surface of the membrane were removed and cells were fixed with 4% paraformaldehyde in PBS, stained in 0.5% crystal violet and membranes were mounted on a microscope slide. Migrated cells were counted in 10 random fields. Invasion assays were carried out in a manner similar to migration assay. Transwell inserts with 8-µm pores (Costar) were coated with 100 µl Matrigel, which was diluted 1 : 40 in ice-cold RPMI-1640, and allowed to gel at 37 °C. Sub-confluent cell cultures were detached as described above, resuspended in RPMI-1640, and 2.5×10^4 cells were seeded in the upper chamber. Culture plates were incubated for 20 h at 37 °C and the cells were then fixed, stained and counted as described above. Percent of invasion was expressed as: number of invaded cells/number of migrated cells ×100. Invasion and migration indices were expressed relative to SKOV-3 cells as 100 %. All the experiments were carried out three times and the results were expressed as mean ± SD.

Scratch wound healing assay

The amount of 800×10^3 cells (SKOV-3/Wnt5a; mock, SKOV-3) was seeded in a 6-well plate, allowed to attach and reach confluence, and a scratch was made through the confluent monolayer using a sterile pipette tip. Cells were treated with 10 µM JNK inhibitor (anthra[1,9-cd]pyrazol-6(2H)-one, 1,9-pyrazoloanthrone, SP600125, SAPK Inhibitor II, Calbiochem, Gibbstown, NJ) 1 h prior to cell scratching. Photographs of cells invading the scratch were taken at 12 h, 24 h and 48 h after scratching. SKOV-3/Wnt5a, mock and SKOV-3 cells were assayed in three independent experiments.

Western blotting and immunoprecipitation

Sources of antibodies and concentrations used were as follows: mouse monoclonal anti-human Wnt5a antibody (1 : 1500), rabbit polyclonal anti-human E-cadherin antibody (1 : 20,000), rabbit polyclonal anti-human N-cadherin antibody (1 : 1000) were obtained from Abcam (Cambridge, UK). GAPDH antibody (1 : 1000, Abcam) was used as internal control. Cells were grown to 80% confluence and then harvested on ice using cell

lysis buffer: 150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris, pH 8.0. Cells were sonicated and centrifuged at 10,000 × *g* for 10 min. The supernatant was quantitated using the Bradford protein quantitation assay (Merck, Darmstadt, Germany). Wnt5a and N-cadherin were detected by using 5 and 40 µg of each lysate that was run on SDS-PAGE 10% Tris-glycine and transferred onto PVDF membrane. E-cadherin in the cell lysate was immunoprecipitated by using ProtA/G magbeads (GenScript), separated on SDS-PAGE 8% Tris-glycine, followed by Western blotting. The membranes were probed with anti-mouse IgG or anti-rabbit-IgG antibodies and bands were visualized by using the ECL system (Millipore, Billerica, MA). Bands were quantified by densitometric analysis, using AlphaEaseFC software (Miami, FL). Results were expressed relative to SKOV-3 cells set as arbitrary value = 1.0.

Immunohistochemistry

Ovarian specimens were obtained from surgeries performed between 1990 to 2012 at Tehran University Women's Hospital. All samples were examined by two independent and experienced gynaecological pathologists for histological diagnosis and grade. The patients (age = 31–68, median = 52) were divided into two groups: high-grade serous ovarian cancer (HGSC, N = 49) and low-grade serous ovarian cancer (LGSC, N = 11). Serous ovarian carcinoma specimens were sectioned (5 µm) and mounted on Vectabond (Vector Laboratories Ltd., Peterbrough, UK)-coated slides. Sections were deparaffinized in three changes of xylene and rehydrated in a graded series of ethanol finishing in distilled water. For antigen retrieval, slides were placed in 0.01 M citrate-buffer, pH 6.0, and heated for 20 min at 90 °C. Endogenous peroxidases were quenched by incubating with 3% H_2O_2 in 10% methanol for 10 min at room temperature. Subsequently, non-specific binding sites were blocked with 4% BSA for one hour; then sections were incubated overnight at 4 °C with the following antibodies obtained from Abcam: mouse monoclonal anti-human Wnt5a antibody (1 : 500), rabbit monoclonal anti-human E-cadherin (1 : 300) and rabbit polyclonal anti-human N-cadherin (1 : 500) diluted in 4% BSA + 0.1% Triton X 100. Immunoreactivities were visualized by exposing the cells to HRP LSAB reagents (Dako France SAS, Trappes Za Du Buisson La Couldre, France) and revealed with diaminobenzidine tetrahydrochloride (DAB, Vector Laboratories Ltd.). The sections were counterstained with haematoxylin, rinsed and mounted with Vectamount (Vector Laboratories Ltd.). The samples were analysed semi-quantitatively as 0 (0–10 % positive tumour cells), 1+ (11–50 % positive tumour cells), 2+ (< 50 % positive tumour cells).

Statistical analysis

Normality of nominal variables was analysed by using the Kolmogorov-Smirnov test. For *in vitro* studies, the results were analysed by using *t*-test. Comparative analysis of Wnt5a, E- and N-cadherin immunoexpression between HGSC and LGSC groups was performed by using the Kruskal-Wallis test. The associations between these molecules in each group were analysed by Pearson's χ^2 test. All experimental data were analysed using a statistical software package SPSS 19.0 (SPSS Inc., Chicago, IL), $P < 0.05$ was considered statistically significant.

Results

Increased survival with concomitant reduced caspase-3 activity in SKOV-3/Wnt5a cells

The levels of Wnt5a expression were significantly higher in SKOV-3/Wnt5a cells (2.3 ± 0.2) compared to SKOV-3 (1.0 ± 0.3) and mock (0.8 ± 0.4) cells ($P < 0.001$) (Fig. 1A, B). Serum-independent cell viability was increased in SKOV-3/Wnt5a cells compared to SKOV-3 and mock cells by 29 % and 25 %, respectively ($P < 0.05$) (Fig. 2A), which was corroborated by 35% and 33% decreased caspase-3 activity in SKOV-3/Wnt5a cells compared to SKOV-3 and mock cells, respectively ($P < 0.01$) (Fig. 2B). Cell cycle analysis did

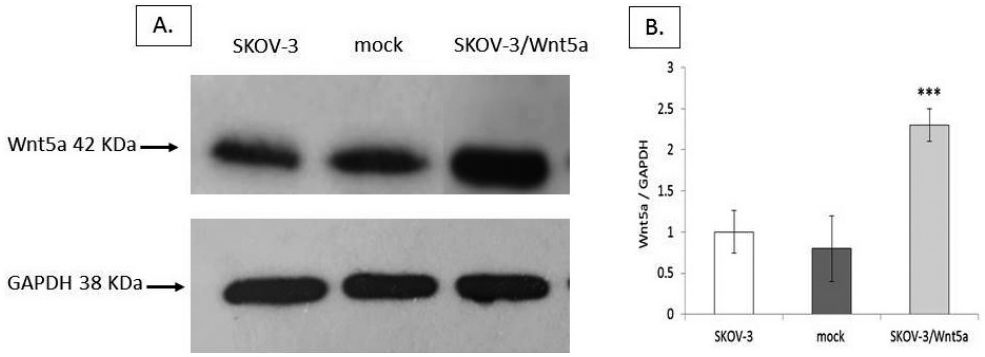

Fig. 1. Analysis of Wnt5a expression in Wnt5a/SKOV-3, mock and SKOV-3 cells
A. Higher expression in Wnt5a/SKOV-3 cells compared to mock and SKOV-3 cells. Western blot represents one of three independent experiments. **B.** Normalized values (mean ± SD) from three independent Western blots for Wnt5a were also shown (densitometric analysis was done by using AlphaEaseFC software), GAPDH levels were used as internal control. ***: $P < 0.001$ compared to SKOV-3

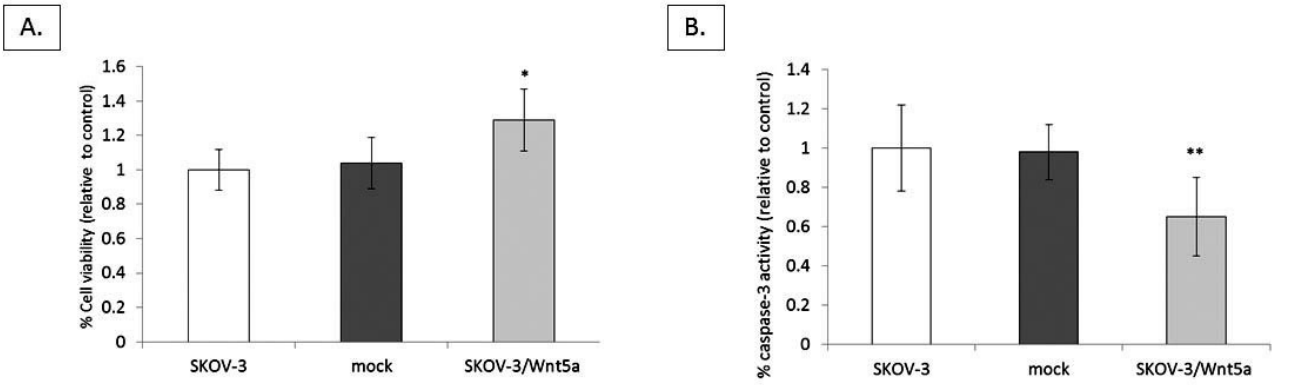

Fig. 2. Cell viability and caspase-3 activity in Wnt5a over-expressing cells
A. Cell survival was assessed by using the MTT assay, after 48 h in SKOV-3/Wnt5a, mock and SKOV-3 cells in serum-free medium. **B.** Cells were incubated for 48 h and apoptosis was assessed by using colorimetric caspase-3 activity assay. Quantification is reported as percent viability/caspase-3 activity in relation to SKOV-3 as a control group (100 %). Data are reported as the mean ± SD from at least three independent experiments.
*: $P < 0.05$; **: $P < 0.01$ compared to SKOV-3 as a control

not show any significant difference between SKOV-3/Wnt5a and mock or SKOV-3 cells (data not shown).

Increased colony formation and adherence of SKOV-3/Wnt5a cells

In the next step, based on increased survival and decreased caspase-3 activity in SKOV-3/Wnt5a, it was interesting to assess the reproductive viability of isolated SKOV-3/Wnt5a cells compared to mock and SKOV-3 cells by performing a clonogenic assay. There was 2.25--fold and 4.40-fold higher number of colonies in SKOV-3/Wnt5a cells compared to mock and SKOV-3, respectively ($P < 0.01$ and $P < 0.001$, respectively) (Fig. 3A, B).

Changes in cell adhesion contribute to the disease development and metastasis, and we therefore sought to determine the role of Wnt5a in cell-matrix adhesion. To this aim Matrigel, which mainly consists of laminin,

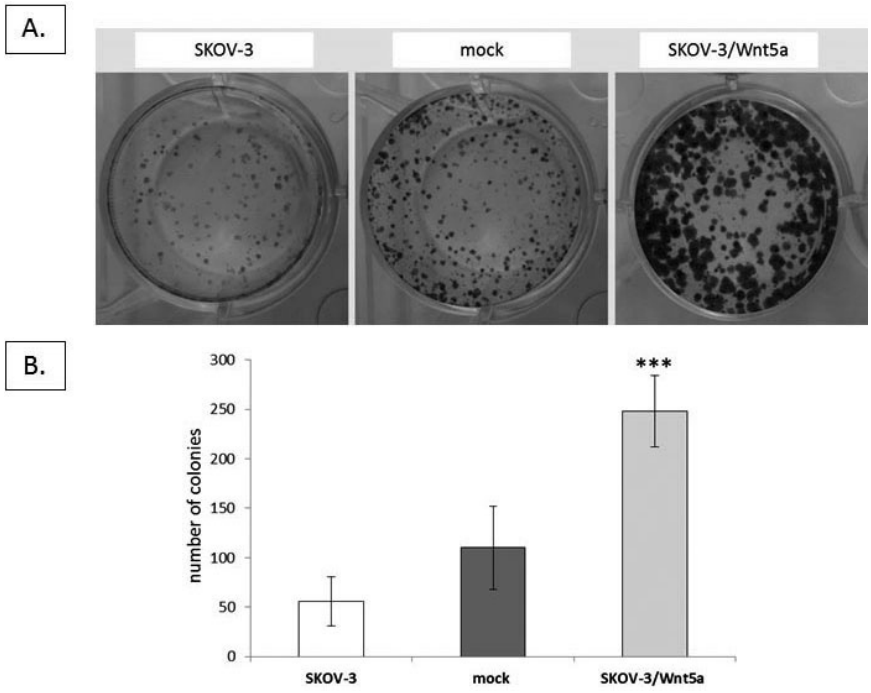

Fig. 3. Colony formation of Wnt5a-transfected *versus* non-transfected SKOV-3 cells
A. Morphology of SKOV-3/Wnt5a, mock and SKOV-3 colonies. Few and small colonies were rare and only small clusters were present in SKOV-3 cells and their size increased in mock cells, while numerous and large sized colonies were present in SKOV-3/Wnt5a cells. Cells were stained with crystal violet. **B.** Quantification of colonies is reported as percent growth in relation to SKOV-3 (100% growth). Data are reported as the mean ± SD obtained from two independent experiments performed in duplicate under the same conditions.
***$P < 0.001$ compared to SKOV-3 as a control

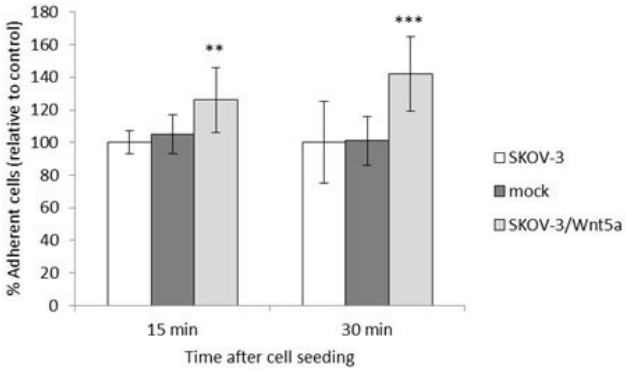

Fig. 4. Substrate-dependent cell adhesion assay
SKOV-3/Wnt5a, mock and SKOV-3 cells were plated onto wells coated with Matrigel for 15 or 30 min, then washed four times. Data (mean ± SD, N = 3) were presented as the percentage of remaining adherent cells that was estimated relative to SKOV-3.
**P < 0.01; ***P < 0.001

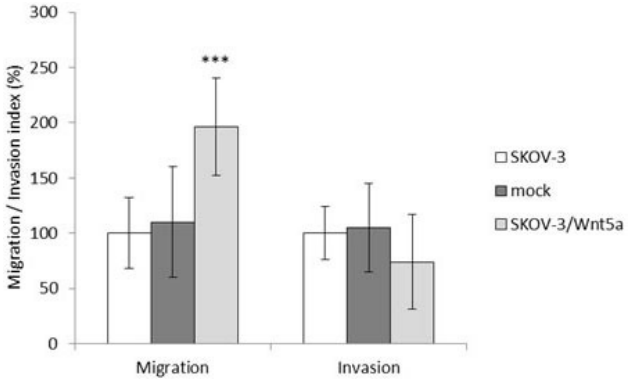

Fig. 5. In vitro migration and invasion assay
Highly significantly increased migration index was seen in SKOV-3/Wnt5a cells versus mock and SKOV-3 cells. There were no significant differences in the invasion index between SKOV-3/Wnt5a cells versus mock and SKOV-3 cells. Cells were counted under a light microscope in 10 random views.
***P < 0.001 (mean ± SD, N = 3)

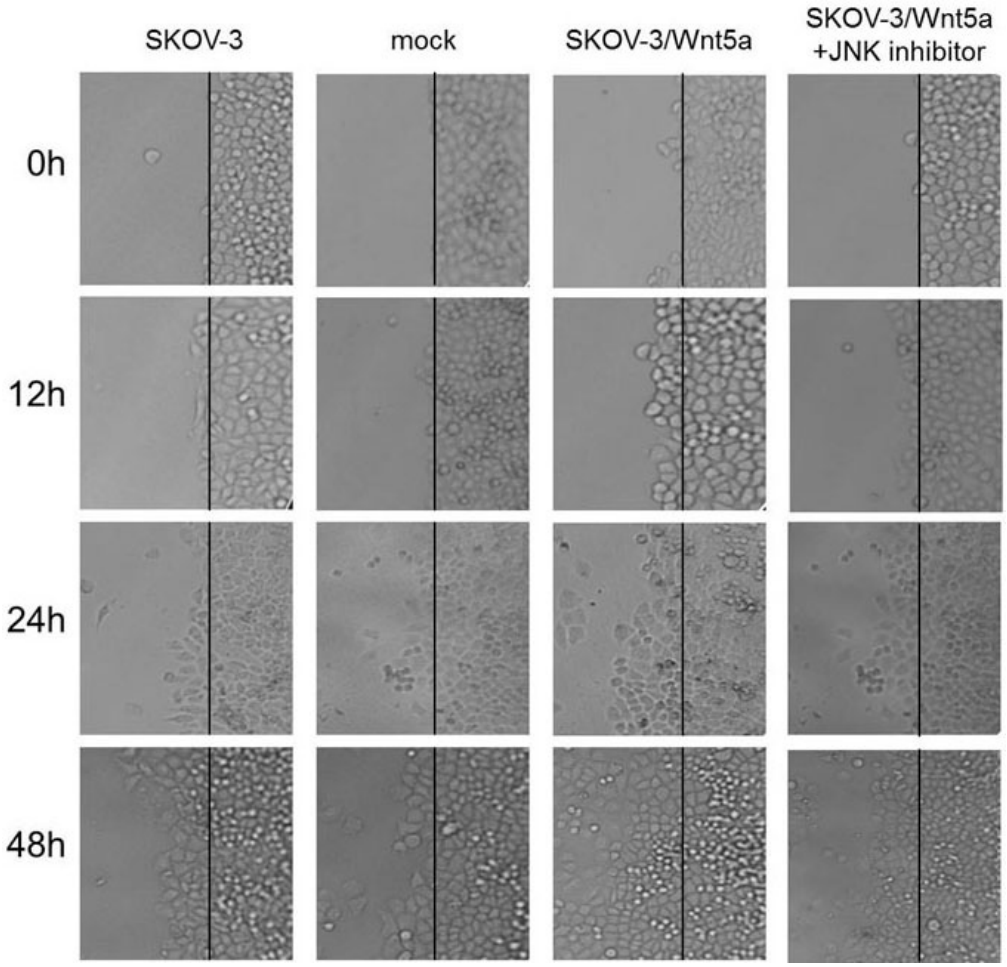

Fig. 6. Scratch wound healing assay
Monolayer was wounded by manual scratch with a pipette tip and maintained at 37 ºC in medium with 1% FBS. Cells were treated with 10 μM JNK inhibitor (SP600125) 1 h prior to cell scratching. Black line indicates the wound edge. Photographs (100 x magnification) were taken at 0, 12, 24 and 48 h after scratching and were representative of two independent experiments. Cell motility was increased in SKOV-3/Wnt5a cells compared to mock and SKOV-3 cells, while it was abrogated in the presence of JNK inhibitor.

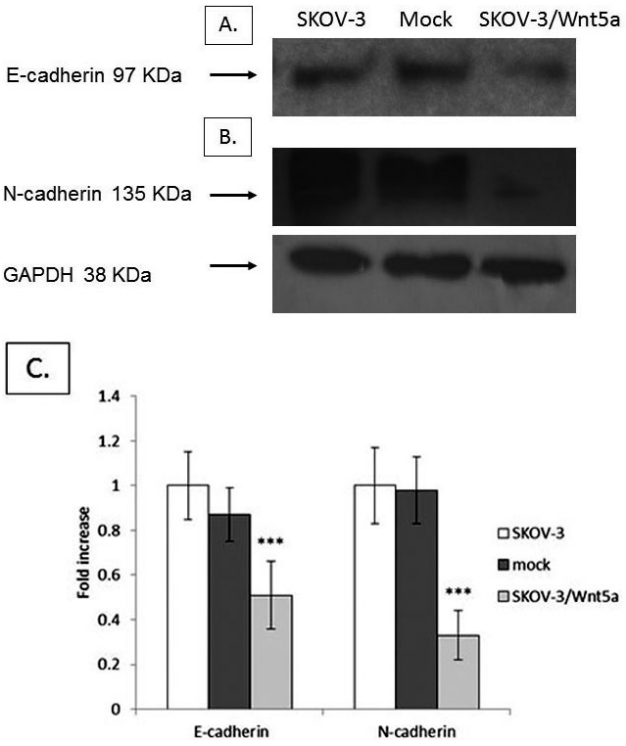

Fig. 7. Analysis of E- and N-cadherin expression
A. Decreased E-cadherin expression in SKOV-3/Wnt5a compared to SKOV-3 and mock cells as revealed by immunoprecipitation (N = 3). **B.** Decreased N-cadherin expression in SKOV-3/Wnt5a compared to SKOV-3 and mock cells as revealed by Western blot analysis (N = 3). **C**. Normalized values (mean ± SD) from three independent experiments. ***P < 0.001 compared to mock or untransfected (SKOV-3) cells

collagen type IV and proteoglycans, was used. There was 21% ($P < 0.01$) and 41% ($P < 0.001$) increased percentage of SKOV-3/Wnt5a cells attached to Matrigel compared to mock or SKOV-3 cells after 15 and 30 min, respectively (Fig. 4).

Increased cell motility of SKOV-3/Wnt5a, which was abrogated in the presence of JNK inhibitor

Cell-matrix adhesion mediates cell migration, as Wnt5a plays an important role in different subtypes of cancer cell migration, but its role in ovarian cancer remains unknown. Here, we found that the migration index was increased almost 1.9-fold in the SKOV-3/Wnt5a cells compared to mock and SKOV-3 cells ($P < 0.001$) (Fig. 5). Although the invasion index in SKOV-3/Wnt5a cells was reduced compared to mock and SKOV-3 cells, this decrease was not significant (Fig. 5). Moreover, the wound healing assay showed increased migration in SKOV-3/Wnt5a cells compared to mock and SKOV-3 cells after 12 h and the wound was closed after 48 h (Fig. 6). There was decreased cell migration in the presence of JNK inhibitor (Fig. 6, last column), showing that Wnt5a-induced cell migration could be mediated by the JNK pathway.

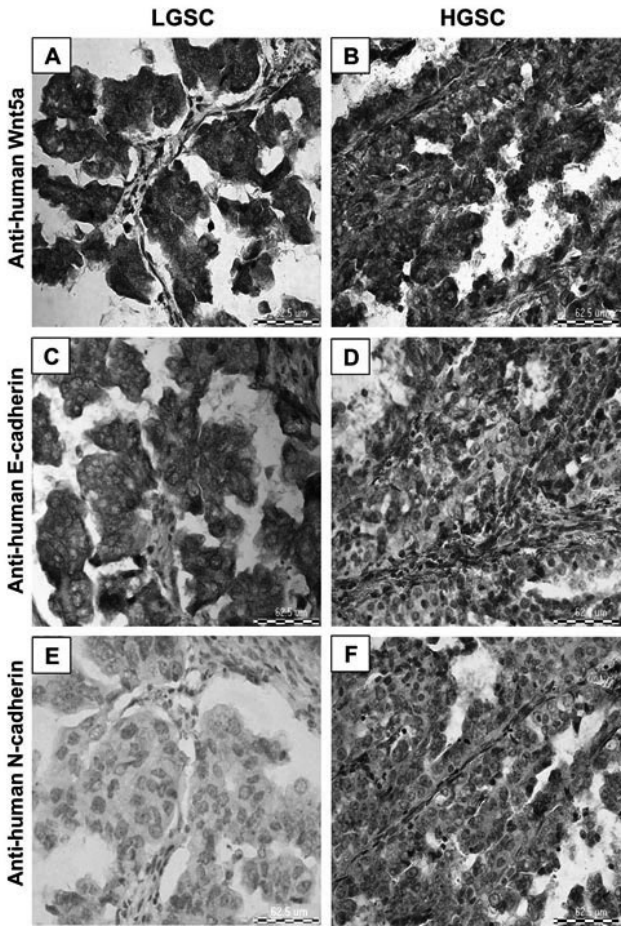

Fig. 8. Immunostaining of Wnt5a, E- and N-cadherin in human serous ovarian cancer specimens
A and **B**: Wnt5a immunoexpression; **C** and **D**: E-cadherin immunoexpression; **E** and **F**: N-cadherin immunoexpression. LGSC: low-grade serous cancer (N = 11); HGSC: high-grade serous cancer (N = 49)

Decreased expression of E- and N-cadherin in Wnt5a/SKOV-3

Cell-cell adhesion maintains epithelial tissues and can facilitate directed migration. Thus, we sought to determine a possible alteration of E- and N-cadherin expression levels in Wnt5a/SKOV-3 cells compared to mock and SKOV-3 cells. Immunoprecipitation of E-cadherin showed lower expression in SKOV-3/Wnt5a compared to SKOV-3 and mock cells (Fig. 7A). Furthermore, Western blot analysis showed decreased expression of N-cadherin in SKOV-3/Wnt5a compared to SKOV-3 and mock cells (Fig. 7B). E- and N-cadherin levels were reduced by 36 % and 65 % and by 49 % and 67 % compared to mock cells, respectively ($P < 0.001$) (Fig. 7C).

Immunoexpression of Wnt5a in HGSC and LGSC specimens and its relationship with E- and N-cadherin

Here, we sought first to determine the levels of Wnt5a expression in HGSC and LGSC and second, to assess

Table 1. Immunostaining intensity of Wnt5a, E- and N-cadherin in HGSC and LGSC specimens

Wnt5a	$\chi^2 = 25.877$, P = 0.001
E-cadherin	$\chi^2 = 16.830$, P = 0.001
N-cadherin	$\chi^2 = 0.413$, NS

P value < 0.05 was considered significant by using Kruskal-Wallis test analysis.

Table 3. Relationship between Wnt5a, E- and N-cadherin immunoexpression in HGSC and LGSC groups

		E-cadherin	N-cadherin
HGSC	Wnt5a	r = 0.253, NS	r = –0.019, NS
	E-cadherin		r = –0.208, NS
LGSC	Wnt5a	r = 0.661, P = 0.027	r = 0.413, NS
	E-cadherin		r = 0.443, NS

P value < 0.05 was considered significant by using Pearson test analysis.

the correlation between Wnt5a and E- or N-cadherin. Higher expression of Wnt5a was immunoexpressed in HGSC compared to LGSC (Fig. 8A, B and Table 1). Both groups expressed E-cadherin (Fig. 8C, D); however, E-cadherin immunostaining was variable in HGSC, showing 84% moderate expression and 10% strong expression versus 100% strong staining in LGSC (Table 1). There was no N-cadherin immunoreactivity in HGSC or LGSC specimens (Fig, 8E, F and Table 1). The levels of Wnt5a and E-cadherin immunostaining were significantly different between HGSC and LGSC (P < 0.001, Table 2). Interestingly, there was a positive correlation between Wnt5a and E-cadherin immunoexpression (r = 0.661, P = 0.027) in LGSC but not in HGSC specimens (Table 3).

Discussion

Here, we showed for the first time that Wnt5a affects serum-independent cell survival via anti-apoptotic effect. Moreover, this study demonstrated increased colony formation, substrate-dependent adhesion and JNK-dependent cell motility with concomitant decrease of E- and N-cadherin expression levels in the Wnt5a/SKOV-3 cell line. In addition, immunohistochemical analysis of Wnt5a, E- and N-cadherin in serous ovarian cancer specimens showed a significant difference between HGSC and LGSC in Wnt5a and E-cadherin immunostaining. Interestingly, there was a positive correlation between Wnt5a and E-cadherin immunoexpression in LGSC.

Conflicting data exists regarding the Wnt5a role in ovarian cancer. Some studies reported its role in ovarian cancer progression and increased chemoresistance (Badiglian Filho et al., 2009; Peng et al., 2011). In contrast, a recent study showed that Wnt5a suppresses growth of EOC cells by triggering cellular senescence, and the loss of Wnt5a may predict poor outcome in EOC patients (Bitler et al., 2011). It should be noted that Wnt5a signals via different intracellular pathways mediated by its interaction with orphan tyrosine receptor Ror2 or with frizzled receptors (Mikels and Nusse, 2006). Thus it seems that tumour suppressor or promotor role of Wnt5a highly depends on the receptor context.

The present study showed increased cell viability and also higher reproductive ability revealed by clonogenic assay in Wnt5a/SKOV-3 cells compared to SKOV-3 and mock cells. This observation corroborates the recent report of Peng et al. (2011), demonstrating increased chemoresistance of over-expressed Wnt5a SKOV-3 cells. Other studies showed the Wnt5a anti-apoptotic effect in colon and pancreatic cancer cells (Bordonaro et al., 2011; Griesman et al., 2013). These reports agree with our present finding demonstrating an anti-apoptotic effect of Wnt5a mediated by inhibition of caspase-3 activity.

It is well known that Wnt5a may be implicated in migration of normal and cancer cells (Nishita et al., 2010) and mediates migration of different types of cancer cells such as human epidermoid carcinoma (Ren et al., 2011), melanoma (Jenei et al., 2009) as well as non-melanoma skin cancer (Pourreyron et al., 2012), gastric cancer (Liu et al., 2013), ewing cell sarcoma (Jin et al., 2012), and breast cancer cells (Zhu et al., 2012). This is the first study demonstrating increased cell motility of an ovarian cancer cell line under the influence of Wnt5a. In addition, the cell-cell contact was reduced in SKOV-3/Wnt5a cells as E- and N-cadherin levels were reduced, which may facilitate cell migration. This finding agrees with another study reporting that loss of E-cadherin by over-expression of Wnt5a contributes to tumour growth and invasion in ovarian cancer (Lau et al., 2013). Our data could suggest that Wnt5a could play a role in EMT of ovarian cancer. Further study with specific EMT markers may help to understand a possible relation between Wnt5a and this process.

Wnt5a-dependent cell invasion has been reported in prostate cancer (Yamamoto et al., 2010), melanoma

Table 2. Differences in Wnt5a, E- and N-cadherin immunoexpression between HGSC and LGSC groups

Histotype	**HGSC (N = 49)**			**LGSC (N = 11)**		
Immunostaining intensity	**0**	**1+**	**2+**	**0**	**1+**	**2+**
Wnt5a	–	–	49 (100)	–	11 (100)	–
E-cadherin	3 (6)	41 (84)	5 (10)	–	–	11 (100)
N-cadherin	49 (100)	–	–	11 (100)	–	–

Number in parentheses represents percentage.

(Jenei et al., 2009) and glioma (Kamino et al., 2011). Our results did not reveal a significant Wnt5a effect on cell invasion. It should be noted that the Wnt5a/Ror2 interaction may play an important role in cell migration and invasion (Nishita et al., 2010). Further analysis of Ror2 expression in ovarian cancer cell lines helps us to understand the molecular pathway of Wnt5a-mediating migration/invasion signalling in ovarian cancer. It has been demonstrated that Wnt5a signalling mediated by the Ror2 pathway increases melanoma cell adhesion (O'Connell et al., 2010) with consequent Wnt5a-induced integrin expression (Dissanayake et al., 2007). Correspondingly, the present study showed increased adhesion of SKOV-3/Wnt5a cells, and further integrin expression profile analysis may be interesting.

Higher immunoexpression intensity of Wnt5a was detected in HGSC compared to LGSC, suggesting that Wnt5a may have an oncogenic role in serous ovarian cancer corroborated by previous reports (Matei et al., 2002; Badiglian Filho et al., 2009).

OSE has a mesodermal origin and presents both epithelial and mesenchymal characteristics with little or no E-cadherin expression (Sundfeldt, 2003). In contrast to most carcinomas which show E-cadherin down-regulation, a unique feature of primary ovarian cancers is the gain of epithelial features characterized by an increase in E-cadherin expression (Sundfeldt et al., 1997; Fujioka et al., 2001; Sundfeldt, 2003; Hudson et al., 2008). Furthermore, Elloul et al. (2010) reported that E-cadherin expression is up-regulated in ovarian carcinoma effusions compared to corresponding primary tumours. The presence of E-cadherin has been reported in metaplastic OSE cells that resemble the more complex epithelia of the oviduct, endometrium and endocervix, and in primary EOC and in micro-metastasis from such tumours (Auersperg et al., 1999). Reddy et al. (2005) showed that in ovarian cancer development, E-cadherin expression was high in benign, borderline, and malignant ovarian carcinomas irrespective of the degree of differentiation, whereas normal ovarian samples did not express E-cadherin. Establishment of high E-cadherin expression in an ovarian cancer cell line led to the activation of Akt and MAP kinase pathways with further ligand-independent activation of epidermal growth factor (EGF) receptor (Reddy et al., 2005). Higher E-cadherin expression has been associated with increased proliferation and higher survival of ovarian cancer cells (Reddy et al., 2005). This study reports a significantly higher E-cadherin immunostaining in LGSC compared to HGSC, corresponding to previous findings claiming that positive E-cadherin staining is decreased in stage III/IV versus stage I/II tumours (Imai et al., 2004) and negative E-cadherin is predictive of poor overall survival (Daraï et al., 1997; Faleiro-Rodrigues et al., 2004). One of the most interesting results in this study was the relationship found between Wnt5a and E-cadherin immunoexpression in LGSC specimens, as there was moderate Wnt5a expression and strong E-cadherin immunostaining in LGSC and vice versa for HGSC, predicting overall poor survival rate in HGSC.

In contrast to other epithelia, OSE epithelial integrity is maintained primarily by N-cadherin, which is consistent with the epithelial-mesenchymal phenotype of this tissue (Patel et al., 2003). N-cadherin has been detected in benign and borderline tumours as well as malignant effusions (Daraï et al., 1998). One study showed that one-third of serous and endometroid tumours (N = 147) expressed moderate N-cadherin immunostaining (Peralta Soler et al., 1997). However, we were not able to detect N-cadherin immunoexpression, which may be due to the small sample size of this study. Questions raised from this study remain to be investigated further by analysing signal transduction pathways including the Wnt5a/Ror2 or Wnt5a/Fzds, and additional use of dominant-negative and/or RNA interference approaches will help to determine the signalling pathways via which Wnt5a influences survival, adhesion and EMT in ovarian cancer cells.

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