EXPERIMENTAL CELL RESEARCH 313 (2007) 602–613

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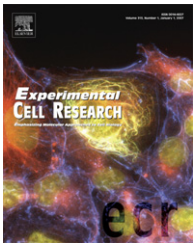

Research Article

Critical involvement of ILK in TGFβ1-stimulated invasion/migration of human ovarian cancer cells is associated with urokinase plasminogen activator system

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ARTICLE INFORMATION

Article Chronology:
Received 1 June 2006
Revised version received
2 November 2006
Accepted 3 November 2006
Available online 10 November 2006

Keywords:
Integrin-linked kinase (ILK)
Invasion
Migration
MMP
SKOV3 ovarian cancer cell line
uPA
PAI-1
TGFβ1

ABSTRACT

The present study investigated the role of integrin-linked kinase (ILK) in TGFβ1-stimulated invasion/migration of human ovarian cancer cells. We investigated TGFβ1 regulation of ILK, and effects of ILK knockdown on TGFβ1-stimulated invasion/migration and the associated proteinase systems, urokinase plasminogen activator (uPA) and matrix metalloproteinases (MMPs) in SKOV3 cells. TGFβ1 stimulated ILK kinase activity, and had no effect on ILK protein/mRNA levels. Transient transfection of an ILK-specific siRNA (ILK-H) reduced ILK protein level, mRNA level and kinase activity. ILK knockdown by ILK-H suppressed the basal and TGFβ1-stimulated invasion and migration. Further, ILK-H reduced the basal and TGFβ1-stimulated secretion of uPA, and increased the secretion of its inhibitor (PAI-1). Conversely, ILK-H did not affect TGFβ1-stimulated secretion of MMP2 and its cell-associated activator MT1-MMP. Additionally, TGFβ1 activated Smad2 phosphorylation, and this was not affected by ILK knockdown. Earlier reports indicate that Smad2 activation increased the expression of MMP2 and MT1-MMP. Thus, TGFβ1 may act through ILK-independent and Smad2-dependent signaling in regulating MMP2 and MT1-MMP in SKOV3 cells. Collectively, this study suggests that ILK serves as a key mediator in TGFβ1 regulation of uPA/PAI-1 system critical for the invasiveness of human ovarian cancer cells. And ILK is a potential target for cancer therapy.

Introduction

Ovarian cancers of majority are adenocarcinomas arising from the ovarian epithelium, and are the most common fatal gynecological malignancy [1,2]. Ovarian cancer has a high incidence of metastasis that generally remains localized within the peritoneal cavity. The metastatic capability of cancer cells is considered to be the main cause for cancer

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doi:10.1016/j.yexcr.2006.11.003

death [2]. Therefore, understanding the mechanism of the metastatic process is of great importance in developing strategies for cancer prognosis and therapy. The metastatic process involves degradation of the extracellular matrix (ECM) including interstitial stroma and basement membrane by proteinases that facilitate the detachment of tumor cells, their crossing of tissue boundaries, and invasion into adjacent or distant tissue compartments. Up to date, there are two major proteinase systems instrumental in these processes, the serine proteinase plasminogen activators (PA), and the matrix metalloproteinases (MMPs) [3,4]. PAs facilitate ECM degradation by converting the zymogen plasminogen into plasmin, which then directly degrades certain ECM proteins (*e.g.* fibronectin, vitronectin and fibrin) [5] or acts indirectly through activation of pro-MMPs into MMPs including MMP3, MMP9, MMP12 and MMP13 [6]. Besides being key enzymes in matrix remodeling, plasmin and MMPs are also implicated in the activation of cytokines and growth factors [7,8].

Several studies have shown the important role of PA in epithelial tumor progression [9]. Urokinase PA (uPA)-mediated proteolysis was consistently found to promote invasion of tumor cells [7,9]. Moreover, PA inhibitor (PAI-1) has been shown to regulate uPA activity, and is a critical factor involved in reimplantation of disseminated tumor cells and formation of a new tumor stroma at the site of metastasis [10–12]. In ovarian cancers, significant elevation of uPA and PAI-1 levels has been reported [13–15]. Furthermore, studies involving mice with specific gene disruptions for uPA and plasminogen ($uPA^{-/-}$ and $plasminogen^{-/-}$ mice) are all in agreement with the idea that uPA-catalyzed plasmin generation is rate-limiting for tumor growth, local invasion and/or formation of distant metastases [10,16]. In addition, the role of MMPs in tumor cell-mediated ECM proteolysis is also well established [3]. MMPs are zinc-dependent metalloproteinases that participate in the degradation of collagens and other ECM macromolecules [3]. Expression of MMPs, particularly MMP2 and MMP9 (type IV collagenases), has been linked to enhanced tumor invasion/metastasis in several *in vitro* and *in vivo* model systems [3]. Also, expression of MMP2 has been detected in ovarian tumors and carcinoma cell lines [17–21]. A previous report demonstrated that increased expression and activation of MMP2 are strongly associated with elevated expression level of its activator membrane-type MMP (MT1-MMP), and that it is of essence to tumor cell invasion [22]. The critical role that MT1-MMP plays in remodeling of connective tissue matrices may be related to its ability to initiate pro-MMP2 activation cascade [23]. In addition, overexpression of MT1-MMP in human cancers, melanoma, breast adenocarcinoma and glioma cells is associated with enhanced *in vitro* invasion and *in vivo* tumor growth and vascularization [24–27].

Integrin-linked kinase (ILK), a serine/threonine protein kinase, serves as an important regulator of integrin-mediated signaling [28], and modulates actin cytoskeleton [29]. ILK is implicated in the regulation of anchorage-dependent cell growth and survival, angiogenesis, and invasion/migration [30]. It has been reported that ILK expression increased in ovarian and many types of cancer [30–32]. And overexpression of ILK in epithelial cells stimulated the expression and activity of MMP2 or MMP9 [33,34]. Also, a most recent study reported that ILK mediation of osteopontin-induced expression of MMP2 and uPA conveys metastatic function in murine mammary epithelial cancer cells [35].

Among the intraovarian regulators of ovarian function, TGFβ is the most unique one for it inhibits proliferation of many cell types, and modulates the interaction between cells and the surrounding matrix [36–38]. In tumorigenesis, TGFβ plays both positive and negative regulatory roles [38]. At early stages, TGFβ may act as a tumor suppressor when cells are still responsive to its anti-mitogenic effect. During malignant progression, TGFβ may function as a tumor promoter when cells become resistant to its growth inhibition by providing an appropriate microenvironment for tumor growth and metastasis. TGFβ thus receives considerable attention for its role in the progression of cancer formation. Enhanced expression of TGFβ is associated with various tumor types including ovarian carcinomas [38–40]. And TGFβ1 enhances the invasiveness of ovarian cancer cells [21,41,42], most likely through up-regulation of MMP [21,41,43] and uPA [43–45]. In addition, TGFβ has been shown to regulate ILK expression in kidney epithelial cells and melanoma cells [34,46]. The role of ILK in TGFβ1-stimulated ECM proteolysis and cell invasion, and their interrelationship in ovarian carcinoma remain unclear. Therefore, the purpose of this study was to investigate the involvement of ILK in TGFβ1-simulated invasion and migration of human ovarian cancer cells. We first determined the effect of TGFβ1 on ILK kinase activity and protein level. And we further investigated the effect of ILK knockdown on TGFβ1-stimulated invasion/migration and the associated proteinase systems, uPA and MMP2.

Materials and methods

Materials

The SKOV3 human ovarian carcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). Recombinant human TGFβ1 was purchased from R&D Systems Inc. (Minneapolis, MN, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Invitrogen (Grand Island, NY, USA) and PAA Laboratory GmbH (Brisbane, Australia), respectively. Followings are sources of antibodies: mouse monoclonal anti-ILK, anti-phospho-Akt(Ser473) and rabbit polyclonal anti-Smad2/3 (Upstate Biotechnology Co., Lake Placid, NY, USA), mouse monoclonal anti-MMP2 (R&D Systems Inc.), mouse monoclonal anti-uPA (Biopool AB, Ümea, Sweden), mouse monoclonal anti-PAI-1 (American Diagnostica Inc., Greenwich, CT, USA), mouse monoclonal anti-β-actin (Sigma Chemical Co., St. Louis, MO, USA), rabbit polyclonal anti-MT1-MMP catalytic domain (Chemicon International Inc., Temecula, CA, USA), rabbit polyclonal anti-phospho-Smad2(Ser465/467) (Cell Signaling, Beverly, MA, USA). Most chemicals were purchased from Sigma Chemical Co., and sources for others are indicated individually below.

Cell culture and treatment

Culture of SKOV3 cells was maintained in DMEM medium supplemented with 10% FBS at 37°C, 5% CO_2–95% air. At the

beginning of experiment, cells were plated in culture ware coated with matrigel (derived from Engelbreth–Holm–Swarm sarcoma tumors; Sigma Chemical Co.) in serum-free DMEM medium containing 0.1% lactalbumin hydrolysate. Cells were allowed to attach for 40 h, and then treated with vehicle or TGFβ1 for various time periods. At the end of incubation, total cell lysates and conditioned media were collected, and stored at −70°C until the performance of analysis.

Reverse transcription/polymerase chain reaction (RT–PCR)

Total RNA was extracted from SKOV3 cells using Trizol (Invitrogen, Grand Island, NY, USA). For RT–PCR, 1 μg of total RNA was first transcribed to cDNA using RNA PCR Kit (Promega, Madison, WI, USA), and the cDNA was then amplified for ILK and GAPDH (an internal control) on a Perkin Elmer GeneAmp PCR system 2400. For ILK, the cDNA was amplified for 40 cycles (denaturation: 95°C, 30 s; annealing: 50°C, 2 min; elongation: 72°C, 3 min) using 24-mer antisense and sense primers corresponding to the human ILK nucleotide sequence 1357–1333 (5′-CTACTTGTCCTGCATCTTCTCAAG-3′) and nucleotide sequence 1–24 (5′-ATGGACGACATTTTCACTCAGTGC-3′), respectively [28]. For GAPDH, the cDNA was amplified for 36 cycles (denaturation: 95°C, 30 s; annealing: 60°C, 1 min; elongation: 72°C, 1 min) using an antisense primer (20 mer) and an upstream sense primer (19 mer) corresponding to the human GAPDH nucleotide sequence 287–306 (GenBank™ Accession Number gi7669491): 5′-GAAGATGGTGATGGGATTTC-3′ and nucleotide sequence 81–99: 5′-GAAGGTGAAGGTCGGAGTC-3′, respectively.

Restriction fragment length polymorphism (RFLP) analysis of amplified ILK cDNA

RFLP analysis was conducted as previously described [46]. RT–PCR amplified ILK cDNA was purified using the Wizard® SV gel and PCR clean-up system (Promega, Madison, WI, USA), digested with *Bam*HI (Takara, Otsu, Shiga, Japan) or *Hinc*II (Promega, Madison, WI, USA) at 37°C for 2 h, and then subjected to electrophoresis on a 2% agarose gel containing ethidium bromide.

Transfection of small interfering RNA (siRNA)

A specific ILK siRNA duplex was synthesized by Dharmacon Inc. (Lafayette, CO, USA) based on the sequence of the human ILK gene (GenBank™ Accession Number gi3150001). Twenty-one-base sequence specifically targeting the PH domain (ILK-H) was chosen [47], and the sequence of the DNA target of ILK-H is 5′-CCUGACGAAGCUCAACGAGAAdTT-3′. And a non-specific siRNA served as a negative control was obtained from Ambion (Woodward, Austin, USA). SKOV3 cells of approximate 7×10^5 were plated in 6-cm dish coated with matrigel in 5 ml of 10% FBS containing DMEM medium, and incubated at 37°C, 5% CO_2–95% air. Approximately 20 h later, SKOV3 cells were transiently transfected with ILK-H, nonspecific siRNA or reagent vehicle of TransFast™ (Promega, Madison, WI, USA) in DMEM according to the manufacturer's protocol. About 4 h post-transfection, cell culture was gently added with a final concentration of 10% FBS containing DMEM medium. And 48 h post-transfection, the cells were replaced with serum-free medium, and treated with control vehicle or 10 ng/ml of TGFβ1 for various time periods. At the end of incubation, conditioned medium concentrates were prepared, and analyzed for the presence of uPA, PAI-1 and MMP2 by immunoblotting. Additionally, conditioned media were analyzed for gelatinase activity using zymography assay as described below. And total cell lysates were prepared, and analyzed by immunoblotting for ILK, MT1-MMP, phospho-Smad2 and Smad2 with β-actin used as an internal control.

ILK kinase assay

SKOV3 cells were cultured as described above, and given control vehicle or 10 ng/ml of TGFβ1 for 2 h. Cells were then lysed with RIPA buffer, and ILK kinase activity assay was performed. In brief, equivalent amounts (150 μg) of lysates were immunoprecipitated overnight at 4°C with 3 μg of mouse monoclonal anti-ILK antibody or a negative control mouse IgG (mIgG). The immune complexes were isolated with protein A/G PLUS agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA), washed three times with RIPA buffer and three times with wash buffer (50 mM HEPES, pH 7, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 200 mM Na_3VO_4 and aprotinin, leupeptin, pepstatin of 1 μg/ml each). The immunoprecipitate was determined for kinase activity using 2 μg of Akt1–GST fusion protein (Upstate Biotechnology Co.) as a substrate and 200 μM of ATP in the reaction buffer (50 mM HEPES, pH 7, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 200 mM Na_3VO_4, 200 mM NaF), and allowed to react for 30 min at 30°C. Phosphorylation of the substrate was detected by immunoblotting using anti-phospho-Akt(Ser473) antibody. Relative quantitation of ECL signal on X-ray film was analyzed using two-dimensional laser scanning densitometer (Molecular Dynamics, Sunnyvale, CA, USA).

Immunoblot analysis

SKOV3 cells were cultured in 6-cm or 10-cm dish (Falcon Labware, Lincoln Park, NJ, USA), and treated with control or 10 ng/ml of TGFβ1 in serum-free medium for various time periods. At the end of incubation, conditioned media were collected, and concentrated and desalted using microconcentrators (mol wt cut-off, 10 kDa, Millipore Corporation, Billerica, MA, USA). The concentrate was then lyophilized, resuspended in Laemmli SDS sample buffer, and analyzed by immunoblotting. The cells were washed with ice-cold PBS and then extracted with lysis buffer (Radioimmunoprecipitation assay (RIPA) buffer containing 2 mM PMSF, 2 mM Na_3VO_4, 2 mM NaF, and aprotinin, leupeptin and pepstatin of 2 μg/ml each). Concentrated conditioned medium samples (10-ml equivalent each) and cell lysates (40–75 μg protein each) were analyzed by SDS–polyacrylamide gel and electroblotting as previously described [21]. Specific signals were detected using an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech UK Limited, Little Chalfont, Buckinghamshire, England) according to the manufacturer's protocol. Relative quantitation of ECL signal on X-ray film was analyzed using two-dimensional laser scanning densitometer.

Cell invasion assay

The invasive activity of the tumor cells was determined using an *in vitro* assay as previously described [21] with slight modifications. Briefly, an insert well with 8-μm pore size polycarbonate filter (Millipore Corporation) was pre-coated with 50 μg protein of matrigel (Sigma Chemical Co.). Forty-eight hours post-transfection with ILK-H, nonspecific siRNA or transfection reagent vehicle, SKOV3 cells of approximate 5×10^4 cells were inoculated into each insert well, and incubated for 2 h at 37°C, 5% CO_2–95% air before the beginning of treatment. Cells were then given control vehicle or 10 ng/ml of TGFβ1. After an incubation period of 72 h, the filters were fixed in 1% glutaraldehyde (Merck KGaA, Danmstadt, Germany) for 30 min, stained with 0.1% crystal violet for 2 h, and then washed with water. Cells remaining on the inner surface of the filter were removed with a cotton swab. Invasive cells adhering to the under surface of the filter were counted using light microscope.

Cell migration assay

Forty-eight hours post-transfection with ILK-H or transfection reagent vehicle, confluent SKOV3 cells were wounded by manually scraping the cells with a 200-μl micropipette tip. The cell culture was then replaced with fresh serum-free medium, and treated with control or 10 ng/ml of TGFβ1 for 48 h. Wound closure was monitored at various time points by photographing taken under phase contrast microscope (40×). And the degree of migration was determined by relative gap distance defined as the ratio of gap distance at 48 h to that at 0 h.

Zymography

Gelatin zymography was performed as previously described [48]. In brief, medium samples were electrophoresed on 7.5% SDS–polyacrylamide gels (14 cm×10 cm) containing 0.1% gelatin obtained from porcine skin (Sigma). Equal volume of each conditioned medium sample was analyzed for gelatinase

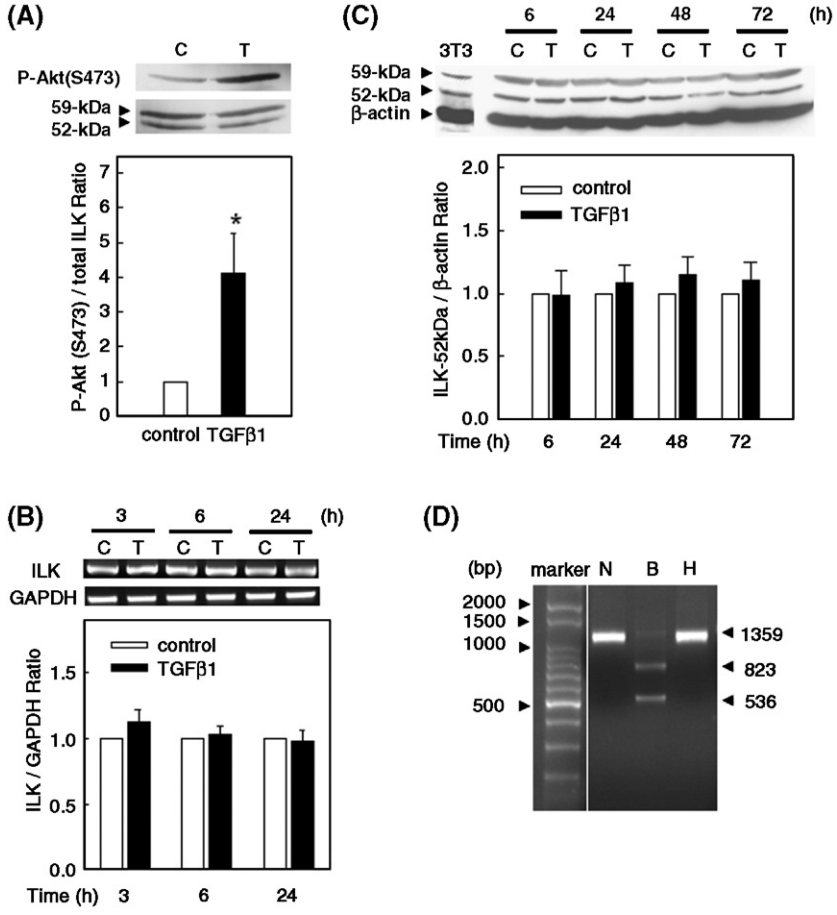


Fig. 1 – Effect of TGFβ1 on ILK in ovarian cancer cells. SKOV3 cells were treated with control vehicle or 10 ng/ml of TGFβ1 (A) for 2 h to determine ILK kinase activity by kinase activity assay, (C) for 6 to 72 h to determine ILK protein level by immunoblotting, with β-actin used as an internal control. 3T3 cell lysate was included as a positive control of ILK antibody, and (B) for 3 to 24 h to determine ILK mRNA level by RT–PCR, with GAPDH used as an internal control. (D) RT–PCR/RFLP analysis to determine the expression of ILK isoforms in SKOV3 cells. ILK-1 and ILK, 2 respectively, contains a *Bam*HI and a *Hinc*II restriction site. Each bar represents the mean (±SEM) relative density of three to five independent experiments. Relative density was calculated by setting the mean density of the control value as one. Data were analyzed using Student's paired *t*-test. *Significant difference compared to the control ($P<0.05$). C, control; T, TGFβ1; N, Non-digested; B, *Bam*HI; H, *Hinc*II.

activity, and β-actin protein level in cell lysate was used as an internal control. Electrophoresis was performed in 192 mM glycine, 25 mM Tris, pH 8.0, and 0.1% SDS at 20 mA/gel during the separation period. At the end of electrophoresis, gels were washed in 2.5% Triton X-100 for approximately 1 h with a change of solution, and in reaction buffer (50 mM Tris–HCl, pH 8.0 containing 5 mM $CaCl_2$ and 0.02% NaN_3) for 30 min. Gels were incubated in reaction buffer at 37°C for about 40 h, then stained with 0.25% Coomassie brilliant blue R-250 in 10% acetic acid–30% ethanol, and destained in the same solution without dye. Quantification of gelatinase was achieved by computerized image analysis using two-dimensional laser scanning densitometer.

Statistics

Quantitative data were analyzed by ANOVA and Duncan's multiple range test at a significance level of 0.05 using the general linear model of the SAS program (SAS Institute Inc., Cary, NC, USA). Differences between two treatment groups were analyzed using the Student's *t*-test at a significance level of 0.05.

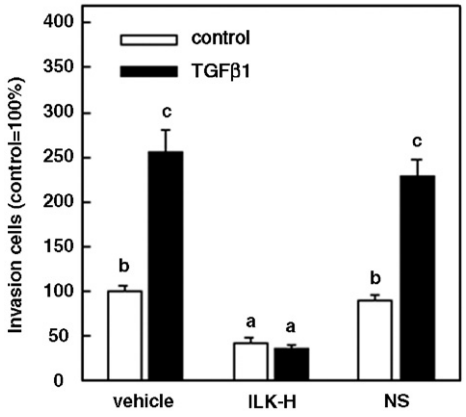


Fig. 3 – Effect of ILK knockdown on invasive behavior of human ovarian cancer cells. SKOV3 cells were transiently transfected with reagent vehicle, 100 nM of ILK siRNA (ILK-H) or a nonspecific siRNA (NS). About 48 h post-transfection, invasion assays were performed. Cell cultures were replaced with serum-free medium and treated with control vehicle or 10 ng/ml of TGFβ1 for an additional 72 h. Each bar represents relative mean (±SEM) percentage of cell number using vehicle control value as 100%, *n*=9–12 per treatment group. Data were analyzed using analysis of variance and multiple range test. Different lower-case letters indicate significant differences among groups (*P*<0.05).

Results

Effect of TGFβ1 on ILK in human ovarian cancer cells

We first examined whether TGFβ1 affects ILK kinase activity and mRNA and protein levels in ovarian cancer SKOV3 cells. ILK kinase activity assay was performed by immunoprecipitation of ILK from cell lysate, and then using Akt–GST fusion protein as a substrate. Fig. 1A shows that TGFβ1 treatment for 2 h significantly increased ILK kinase activity. TGFβ1 treatment had no significant effect on ILK mRNA and protein levels in SKOV3 cells (Figs. 1B and C).

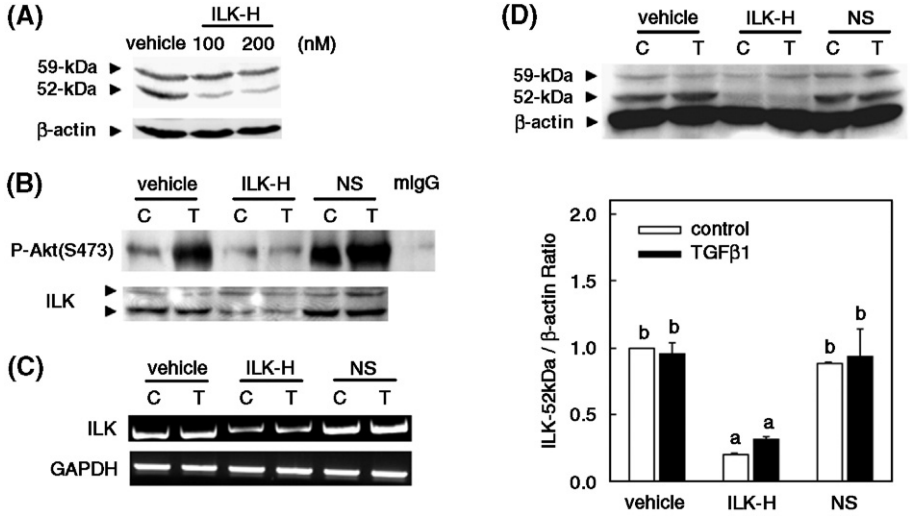
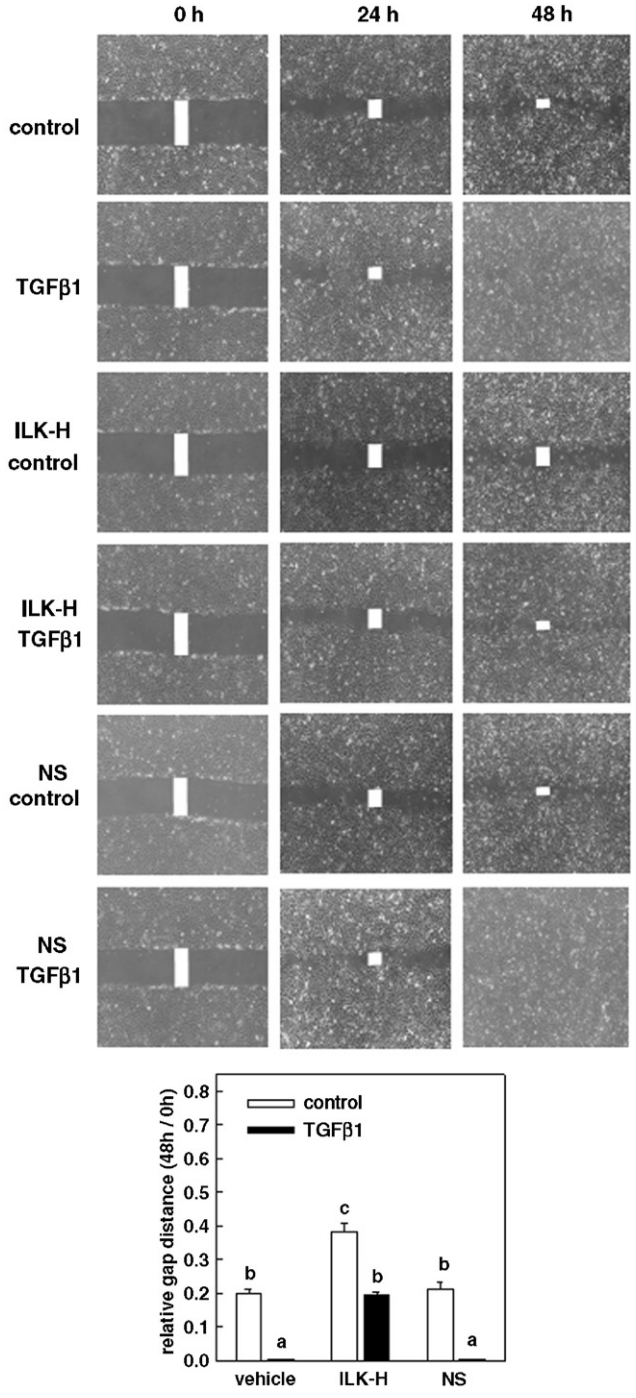


Fig. 2 – ILK knockdown by small interference RNA in ovarian cancer cells. SKOV3 cells were transiently transfected with reagent vehicle, 100 nM of ILK siRNA (ILK-H) or a nonspecific siRNA (NS). About 48 h post-transfection, (A) cell lysates were prepared to determine the ILK protein level by immunoblotting. In addition, cells were treated with control vehicle or 10 ng/ml of TGFβ1 for 2 h to determine (B) ILK kinase activity by immunoprecipitation and kinase activity assay, and for 48 h to determine (C) ILK mRNA level by RT–PCR, and (D) ILK protein level by immunoblotting. Relative density of 52-kDa ILK protein band was calculated by setting the mean density of the vehicle control value as one. Each bar represents the mean (±SEM) relative density of five independent experiments. Data were analyzed using analysis of variance and multiple range test. Different lower-case letters indicate significant differences among groups (*P*<0.05). C, control; T, TGFβ1.

ILK is constitutively expressed in SKOV3 cells, and there were two immunoreactive ILK bands of approximate 52 kDa and 59 kDa (Fig. 1C). Previous studies also demonstrated the existence of two ILK protein bands in cell lines including CHO and NIH 3T3, and human ovarian tumors [32,49]. The expression of ILK isoforms was determined using restriction fragment length polymorphism (RFLP) analysis of RT–PCR amplified cDNA: a unique *Bam*HI restriction site in the ILK-1 cDNA (generating two fragments of 536 and 823 bp) and a unique *Hinc*II restriction site in the ILK-2 cDNA (giving rise to two fragments of 411 and 948 bp) [46]. As shown in Fig. 1D, SKOV3 cells predominantly expressed ILK-1.



Effect of ILK knockdown on invasive and migratory behavior of human ovarian cancer cells

To determine whether ILK is essential for TGFβ1-stimulated invasion and migration of SKOV3 cells, double-stranded small interfering RNA (siRNA) was used to decrease the expression of endogenous ILK. Transient transfection of an ILK-specific siRNA (ILK-H), but not the nonspecific siRNA, for 48 h resulted in substantial knockdown of the immunoreactive 52-kDa ILK protein but not the 59-kDa one in SKOV3 cells (Fig. 2A). We then immunoprecipitated ILK from cell lysates of 2-h treatment cultures and assayed for the kinase activity. ILK-H, but not the nonspecific siRNA suppressed the basal and TGFβ1-stimulated ILK kinase activity, and this is mainly attributed to the decrease of ILK protein level (Fig. 2B). Also, a negative control using mouse IgG exhibited no obvious ILK kinase activity (Fig. 2B). Additionally, the knockdown of ILK was maintained 4 days after transfection, and there is no difference of ILK mRNA and protein level between control and the respective TGFβ1-treated group (Figs. 2C and D). Further, the potential involvement of ILK in TGFβ1-stimulated invasion of SKOV3 cells through reconstituted basement membrane was analyzed using matrigel-coated invasion chambers. SKOV3 cells were transiently transfected with ILK-H, a nonspecific siRNA (a negative control) or reagent vehicle for 48 h, cells were then treated with control vehicle or TGFβ1 for an additional 72 h. ILK-H, but not the nonspecific siRNA significantly suppressed the basal and TGFβ1-stimulated invasion of SKOV3 cells (Fig. 3). In addition, ILK-H attenuated the basal and TGFβ1-stimulated cell migration observed at 48 h after TGFβ1 treatment, while nonspecific siRNA had no effect (Fig. 4). Together, these data show that ILK is specifically involved in the basal and TGFβ1-stimulated invasion and migration of SKOV3 cells. The molecular mechanism through which TGFβ1 promotes ovarian cancer cell invasion and migration was further studied.

Effect of ILK knockdown on the invasion-associated proteinase systems in human ovarian cancer cells

To determine the effect of ILK knockdown on the invasion-associated proteinases, uPA and MMP systems were examined.

Fig. 4 – Effect of ILK knockdown on migration behavior of human ovarian cancer cells. SKOV3 cells were transiently transfected with reagent vehicle, ILK siRNA (ILK-H) or a nonspecific siRNA (NS). About 48 h post-transfection, migration assays were performed using scratch wound assay. Cell cultures were then replaced with serum-free medium, and treated with control vehicle or 10 ng/ml of TGFβ1. Wound closure was monitored by microscopy at 0, 24 and 48 h after treatment. The white line insert is an indicator of the distance of wound gap. And the degree of migration was determined by relative gap distance defined as the ratio of gap distance at 48 h to that at 0 h. Each bar represents the mean (±SEM) relative gap distance of three independent experiments. Data were analyzed using analysis of variance and multiple range test. Different lower-case letters indicate significant differences among groups ($P<0.05$).

Both uPA and its native inhibitor (PAI-1) mainly are of secretory form, we thus measured their protein levels in culture conditioned media by immunoblotting analysis. TGFβ1 treatment for 48 h dramatically up-regulated the secretion of uPA in SKOV3 cells, and ILK knockdown by ILK-H reduced the basal and TGFβ1-stimulated secretion of uPA while nonspecific siRNA had no obvious effect (Fig. 5A). PAI-1 exists in different molecular sizes that depend on the degree of glycosylation, and higher molecular weight forms are also suggested to be uPA/PAI-1 complexes [50,51]. And active forms of PAI-1 are documented to be within 54 kDa and 33 kDa [51]. This study shows that PAI-1 predominantly exists in two major forms of approximate 52 kDa and 42 kDa in SKOV3 cells (Fig. 5B). TGFβ1 treatment dramatically increased the secretion of 52-kDa and 42-kDa PAI-1 (Fig. 5B). And ILK-H further increased the TGFβ1-stimulated secretion of 52-kDa and 42-kDa PAI-1 and an additional PAI-1 immunoreactive band of approximate 70 kDa (Fig. 5B). Thus, ILK knockdown by ILK-H reduced the secretion of uPA, as well as increased TGFβ1-stimulated secretion of PAI-1. Together, our data demonstrate that TGFβ1 increased the mean ratio of uPA to PAI-1, and ILK knockdown decreased such ratio (control, 1.0; TGFβ1, 2.69; ILK-H control, 0.45; and ILK-H plus TGFβ1, 0.45). Here only the ratios of uPA to 42-kDa PAI-1 were calculated due to the weak intensity of 52-kDa PAI-1 in controls.

We then determined the effect of ILK knockdown on the invasion-associated MMP2 in SKOV3 cells. MMP2 mainly is of secretory form, we therefore measured MMP2 gelatinase activity and protein level in cell culture conditioned media using zymography and immunoblotting analyses. Consistent with our previous study [21], TGFβ1 stimulated the secretion of a 68-kDa gelatinase characterized as MMP2 (Fig. 6). And, ILK knockdown by ILK-H had no significant effect on the basal or TGFβ1-stimulated secretion of MMP2 gelatinase activity and protein level (Fig. 6). MT1-MMP, a cell-associated activator of MMP2, was also examined. MT1-MMP predominantly exists in three major forms in SKOV3 cells, estimated 63 kDa, 60 kDa and 50 kDa (Fig. 7). It has been reported that MT1-MMP exists in different molecular masses, membrane-associated inactive form of about 60 kDa, and active forms of about 57 kDa and 44 kDa [52]. TGFβ1 increased the MT1-MMP protein levels of all three forms in SKOV3 cells (Fig. 7). And similar to that of MMP2, ILK-H had no effect on MT1-MMP protein level (Fig. 7).

It is well known that TGFβ1 signaling is mediated in part by Smad proteins, and Smad2 activation was reported to increase the expression of MMP2 and MT1-MMP [53,54]. Thus, it is important to evaluate whether ILK knockdown by ILK-H affects TGFβ1-stimulated phosphorylation of Smad2. Here we show that SKOV3 cells treated with TGFβ1 increased Smad2 phosphorylation from 15 min, and maintained until 24 h (Fig. 8). The antibody used recognizes phosphorylation at Ser465 and Ser467 of Smad2 which are direct targets of the TGFβ type-I receptor kinase [55]. ILK knockdown had no effect on the basal and TGFβ1-induced phosphorylation of Smad2, and Smad2 protein

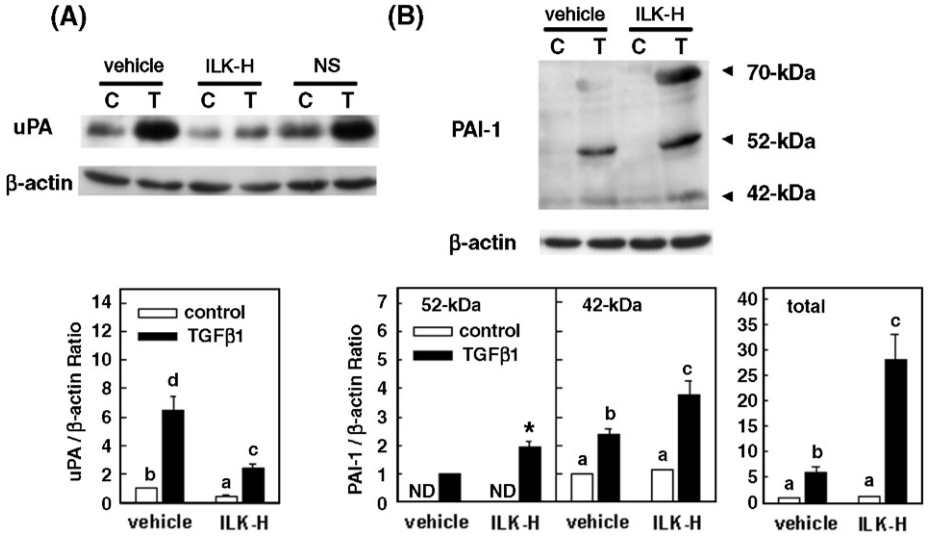


Fig. 5 – Effect of ILK knockdown on the secretion of uPA and PAI-1 in human ovarian cancer cells. SKOV3 cells were transiently transfected with reagent vehicle, ILK siRNA (ILK-H) or a nonspecific siRNA (NS). About 48 h post-transfection, cells were treated with control vehicle or 10 ng/ml of TGFβ1 for an additional 48 h. At the end of culture, conditioned media of 10 ml each were concentrated prior to immunoblotting analysis of (A) uPA and (B) PAI-1. And cell lysates were prepared for immunoblotting analysis of β-actin used as an internal control indicating similar amount of cells in each experimental group. Individual density of 52- and 42-kDa PAI-1 band, as well as total density of three PAI-1 bands was quantified. Each bar represents the mean (±SEM) relative density of three independent experiments. Relative density was calculated by setting the density of vehicle control value of uPA and vehicle TGFβ1-treated value of PAI-1 as one. Data were analyzed using analysis of variance and multiple range test. Different lower-case letters indicate significant differences among groups ($P<0.05$). *Significant difference compared to the respective vehicle group ($P<0.05$). C, control; T, TGFβ1; ND, non-detectable.

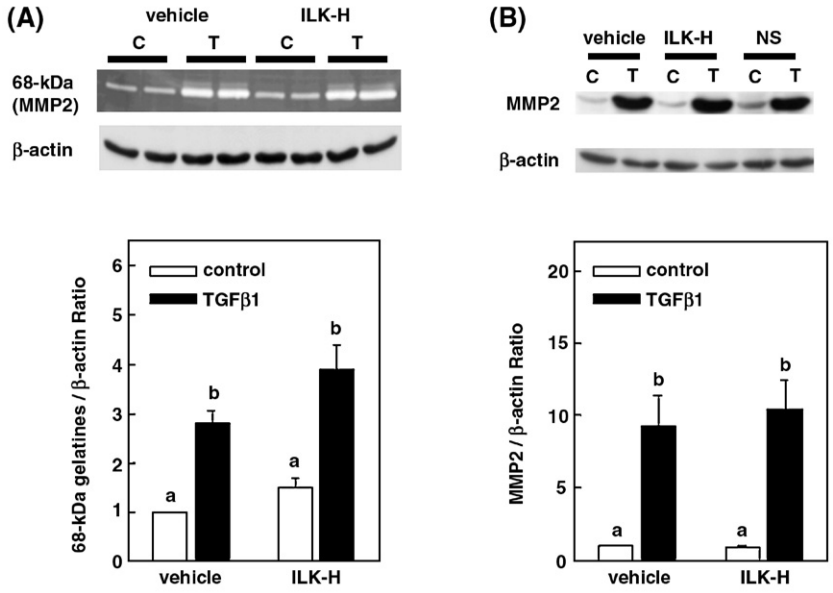


Fig. 6 – Effect of ILK knockdown on the MMP2 gelatinase activity and protein level in human ovarian cancer cells. SKOV3 cells were transiently transfected with reagent vehicle, ILK siRNA (ILK-H) or a nonspecific siRNA (NS). About 48 h post-transfection, cells were treated with control vehicle or 10 ng/ml of TGFβ1 for an additional 48 h. At the end of culture, conditioned media were collected and analyzed for (A) gelatinase activity by gelatin zymography and (B) concentrated conditioned media were analyzed for MMP2 by immunoblotting. Cell lysates were analyzed by immunoblotting for β-actin used as an internal control. For 68-kDa gelatinase activity, each bar represents the mean (±SEM) relative density of four independent experiments. For MMP2 protein level, each bar represents the mean (±SEM) relative density of three independent experiments. Relative density was calculated by setting the mean density of the vehicle control value as one. Data were analyzed using analysis of variance and multiple range test. Different lower-case letters indicate significant differences among groups ($P<0.05$). C, control; T, TGFβ1.

levels appear to be similar in all groups (Fig. 8). In addition, the extent of TGFβ1-induced Smad2 phosphorylation was similar at all time points observed (15 min, 30 min, 1 h and 24 h) (data not shown). These data suggest that TGFβ1-induced Smad2 phosphorylation activation is independent of ILK signaling.

Discussion

TGFβ1 has been implicated to enhance the invasiveness of ovarian cancer cells, most likely through up-regulation of MMPs [21,41,43] and uPA [43–45]. The present study demonstrates for the first time that ILK is critically involved in basal and TGFβ1-stimulated invasion and migration of human ovarian epithelial cancer cells, and this may be partly attributed to up-regulation of uPA and PAI-1 but not MMP2 and MT1-MMP as evidenced by the following findings. First, TGFβ1 significantly stimulated ILK kinase activity in SKOV3 cells (Fig. 1A), and had no effect on ILK mRNA and protein level (Figs. 1B and C). We also showed that SKOV3 predominantly expressed ILK-1 but not ILK-2 (Fig. 1D). Therefore, this is consistent with an earlier study showing that TGFβ1 increased the expression of ILK-2 but not ILK-1 in melanoma cells [46]. Moreover, knockdown of ILK by specific siRNA (Fig. 2) attenuated the basal and TGFβ1-stimulated invasion and migration of SKOV3 cells (Figs. 3 and 4). And this is associated with a decrease in uPA to PAI-1 ratio (as evidenced from a decrease in uPA level and an increase in PAI-1 level, Fig. 5), but not associated with any significant change in MMP2 and MT1-MMP levels (Figs. 6 and 7).

Several lines of evidence suggest that PI3K-ILK signaling plays an important role in TGFβ1-stimulated cancer invasion. TGFβ1 increased PI3K kinase activity in human ovarian cancer HRA cells, and inhibition of PI3K kinase activity or knockdown of PI3K expression suppressed TGFβ1-stimulated cell invasion *in vitro* and *in vivo* [56]. And constitutively activated PI3K could stimulate ILK kinase activity [57]. Our unpublished results show that pretreatment with wortmannin (a PI3K inhibitor) reduced the TGFβ1-stimulated increase in uPA level, and have no effect on TGFβ1-stimulated increase in MMP2 level, suggesting that TGFβ1 may regulate ILK activity partly through PI3K. Additionally, ILK is involved in the promotion of cell invasion and migration [30,58,59]. It has been reported that expression of uPA and PAI-1 increased with advanced stage of ovarian cancers [60–63]. And overexpression of active wild-type PAI-1 alone reduced invasion and migration in human ovarian and breast cancer cell lines [64]. Furthermore, this study demonstrates that TGFβ1 stimulated the invasion and migration of ovarian cancer cells (Figs. 3 and 4), as well as increased uPA to PAI-1 ratio (Fig. 5). The critical role of ILK in the regulation of uPA is supported by

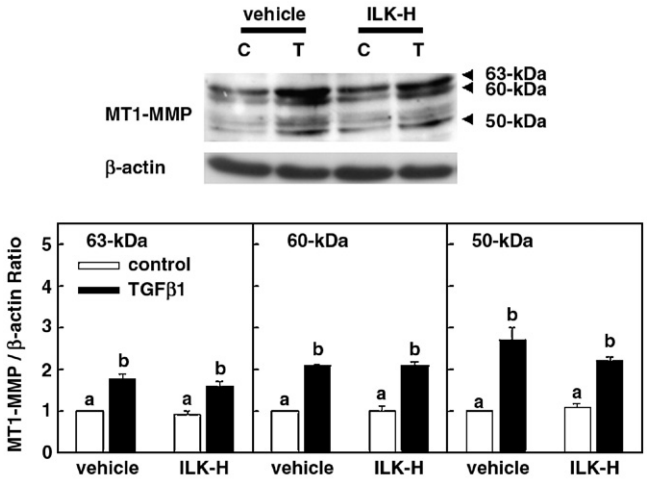


Fig. 7 – Effect of ILK knockdown on the MT1-MMP protein level in human ovarian cancer cells. SKOV3 cells were transiently transfected with reagent vehicle or ILK siRNA (ILK-H). About 48 h post-transfection, cells were treated with control vehicle or 10 ng/ml of TGFβ1 for an additional 48 h. At the end of culture, cell lysates were prepared for immunoblotting analysis of MT1-MMP with β-actin used as an internal control. Each bar represents the mean (±SEM) relative density of three independent experiments. Relative density was calculated by setting the density of the vehicle control value as one. Data were analyzed using analysis of variance and multiple range test. Different lower-case letters indicate significant differences among groups ($P<0.05$). C, control; T, TGFβ1.

a most recent study and our present work. Overexpression of ILK did not affect uPA expression in mammary epithelial 4T1 cells; however, knockdown of ILK activity decreased uPA expression [35]. This study further shows that knockdown of ILK decreased uPA to PAI-1 ratio in ovarian cancer SKOV3 cells as evidenced by a decrease in basal and TGFβ1-stimulated uPA protein level and an increase in TGFβ1-stimulated PAI-1 protein level (Fig. 5). Together, these studies implicate that the increased potential of ovarian cancer invasiveness is critically attributed to the net balance between uPA and its native inhibitor PAI-1 in response to TGFβ1, and ILK serves as a key signaling mediator in such process.

Our previous and present studies demonstrate that TGFβ1 stimulated the secretion of MMP2 [21] and increased cell-associated MT1-MMP level (Figs. 6 and 7) in human ovarian cancer SKOV3 cells. Further, MMP inhibitor BB3103 abrogated the TGFβ1-stimulated invasive behavior of SKOV3 cells [21]. The role of ILK in the regulation of MMP2 varies depending on the cell type, and may also depend on the cell context. In renal tubular epithelial cells, overexpression of ILK increased MMP2 activity and protein level, but not MMP9 activity [34]. And in intestinal epithelial cells, overexpression of ILK only increased MMP9 activity but not MMP2 activity [33]. Also, overexpression of ILK did not affect MMP2 expression in mammary epithelial 4T1 cells; however, knockdown of ILK activity decreased MMP2 expression [35]. Our present study demonstrates that knockdown of ILK had no significant effect on the basal or TGFβ1-stimulated secretion of MMP2 gelatinase activity and protein level, and MT1-MMP level in SKOV3 cells (Figs. 6 and 7). The present study also shows that TGFβ1 elevated the phosphorylation activation of Smad2, and this was not affected by knockdown of ILK (Fig. 8). Smad2 activation was reported to increase the expression of MMP2 and MT1-MMP [53,54]. And TGFβ1-increased MMP2 expression was Smad2-dependent in human proximal-tubule epithelial cells [65]. Whereas Smad3 was reported to mediate the TGFβ1 inhibition of the expression of MMP1 and MMP12 [66–68], and upregulation of MMP13 [68]. Together, these results indicate that MMP is involved in TGFβ1-stimulated invasion in ovarian cancer SKOV3 cells, and TGFβ1 may act through ILK-independent and Smad2-dependent signaling in regulating MMP2 and MT1-MMP in SKOV3 cells.

In conclusion, this study further suggests that ILK may serve as a key signaling mediator in TGFβ1 regulation of uPA/PAI-1 system critical for TGFβ1-stimulated invasiveness of human ovarian cancer cells. Therapeutic strategies target to ILK may thus be of potential for cancer treatment.

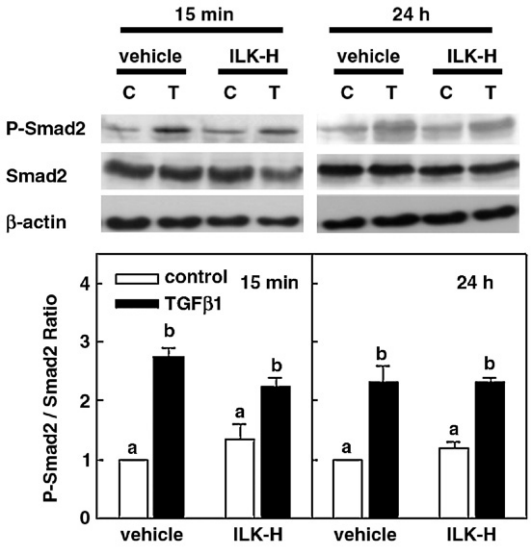


Fig. 8 – Effect of ILK knockdown on the phosphorylation of Smad2 in human ovarian cancer cells. SKOV3 cells were transiently transfected with reagent vehicle or ILK siRNA (ILK-H). About 48 h post-transfection, cells were treated with control vehicle or 10 ng/ml of TGFβ1 for an additional 15 min, 30 min, 1 h and 24 h. At the end of culture, cell lysates were prepared for immunoblotting analysis of P-Smad2 and Smad2 with β-actin used as an internal control. Representative immunoblots of 15-min and 24-h cell lysate samples were shown. Each bar represents the mean (±SD) relative density of two independent experiments. Relative density was calculated by setting the density of the vehicle control value as one. Data were analyzed using analysis of variance and multiple range test. Different lower-case letters indicate significant differences among groups ($P<0.05$). C, control; T, TGFβ1.

Acknowledgments

This study was supported by grants from National Science Council of Taiwan NSC92-2320-B-010-040 (to J-J H), and NSC93-2320-B-001-043 (to M-T L).

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