Targeting ILK and β4 integrin abrogates the invasive potential of ovarian cancer

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Targeting ILK and β4 integrin abrogates the invasive potential of ovarian cancer

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At last, I have an immense amount of gratitude and appreciation towards my family for their encouragement, constant understanding, endless support, patience, and love. I deeply appreciate your sacrifices.

Sincerely,
Yoon Pyo CHOI

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ABSTRACT

Targeting ILK and β4 integrin abrogates the invasive potential of ovarian cancer

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Integrins and integrin-linked kinase (ILK) are essential to cancerous invasion because they mediate physical interactions with the extracellular matrix, and regulate oncogenic signalling pathways. The purpose of our study is to determine whether deletion of β1 and β4 integrin and ILK, alone or in combination, has antitumoral effects in ovarian cancer. Expression of β1 and β4 integrin and ILK was analyzed by immunohistochemistry in 196 ovarian cancer tissue samples. We further assessed the effects of depleting these molecules with shRNAs in ovarian cancer cells by western blot, conventional RT-PCR, cell proliferation, migration, invasion, and *in vitro* Rac1 activity assays. We also investigated effect of shRNA-mediated depletion on *in vivo* growth of ovarian cancer xenografts in nude mice. Overexpression of β4 integrin and ILK in human ovarian cancer specimens was found to correlate with tumor aggressiveness. Depletion of these targets efficiently suppressed ovarian cancer cell proliferation, migration, and invasion *in vitro* and xenograft tumor formation *in vivo*. We also demonstrated that depletion of these inhibited phosphorylation of p-Ser473 Akt and p-Thr202/Tyr204 Erk1/2, and activation of Rac1, as well as reduced expression of MMP-2 and MMP-9. By contrast, depletion of those induced caspase-3 activation. In conclusion, targeting β4 integrin combined with ILK can abrogate the invasive potential in ovarian cancer.

Key words: ovarian cancer, ILK (integrin-linked kinase), β4 integrin, combined effect, invasiveness

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I. INTRODUCTION

Ovarian cancer is the most common cause of mortality from gynecologic cancer [1]. Secondary to vague presenting symptoms and the lack of effective screening, most patients will present with advanced disease. The current standard of care for ovarian cancer therapy is surgery followed by adjuvant carboplatin and taxane-based chemotherapy [2]. Unfortunately, these protocols often do not allow for cure at initial diagnosis, and many patients will often recur and eventually die from their disease. And chemoresistance is an important hurdle in the treatment of recurrent cancer [3]. Targeted therapy has subsequently come to the forefront of research and clinical trials in an effort to overcome resistant disease and achieve improvement in patient outcomes [4].

Integrins are cell surface molecules which mediate cell-matrix and cell-cell interactions and are likely to be important for tumor cell survival and dissemination [5]. During the process of tumor progression, integrins are thought to have a major role in regulating tumor growth and metastasis [6-8]. Significant correlations between expression of the integrins and clinical stage, tumor progression, and prognosis have been found for melanoma, breast, prostate, pancreatic, ovarian, cervical, glioblastoma, non-small-cell lung, and colon cancer [9, 10]. Thus, misregulation of integrin expression and signaling has the capacity to play a major role, not only in oncogenesis, but also more importantly in tumor progression.

Integrin-mediated cell adhesion influences tumor progression and metastasis. The appropriate engagement of a cell with the surrounding extracellular matrix via integrins plays a major role in regulating cellular responses to growth and survival factors [11-13]. Recent studies demonstrated that

high levels of α4β1 and or αvβ3 were closely correlated with increased peritoneal metastasis and tumor proliferation in human ovarian cancer, respectively [14, 15]. β1 integrin expressed on metastatic ovarian cancer cells affects adhesion to the mesothelium. It has been proposed that ovarian cancer metastasis is regulated by β1 integrin binding to the fibronectin secreted by mesothelial cells [16, 17].

Some studies have shed light on the contribution of α6β4 integrin to the invasive process, which may be distinct from other integrins in many respects [18, 19]. Recent studies have also reported that high expression level of α6β4 was closely related to poor prognosis in patients with breast and bladder cancer [20, 21]. However, the progression of prostate cancer from intraepithelial neoplasia to invasive prostate carcinoma results in the loss of β4 integrin expression and the gain of alternative α6β1 integrin functions [22]. In addition, ovarian tumor cells and malignant cells derived from the ascites of the ovarian cancer patients have been shown to lack β4 integrin expression [19]. Therefore, differential expression of α6β4 integrin by normal epithelial cells and primary tumors or metastatic cells is still controversial, and seems to vary according to tumor type as well as between studies. For this reason, the role of α6 or β4 integrin subunits in modulating the phenotypic behavior of ovarian carcinoma cells has not been thoroughly investigated and is poorly understood. And the question still remains of whether β4 integrin contributes to the invasion process during ovarian cancer progression.

Meanwhile, integrin signaling is regulated by complex interactions with a number of cytosolic proteins, including integrin-linked kinase (ILK). ILK is a ubiquitously expressed protein serine/threonine kinase that was initially discovered through its interactions with the β1 and β3 integrin subunits [23, 24]. Although ILK initially named as a kinase, ILK acts as a central component of ILK-PINCH-Parvin complex at ECM adhesions mediating interactions with a large number of proteins via multiple sites including its pseudoactive site [25-28].

Now many studies have reported that ILK plays a role as an adaptor and signaling protein in various aspects of the oncogenic process through direct and indirect mechanisms during tumor progression [29, 30]. Recent reports showed that aberrant ILK mediated signaling, due to overexpression or constitutive activation of the protein, leads to pathological alterations that ultimately result in malignant progression in a range of cancers [31, 32].

ILK expression is increased in ovarian epithelial cancer relative to benign tumors and normal ovarian epithelium, correlates with increased tumor grade, and is stimulated by soluble factors in peritoneal tumor fluid through the

activation of the downstream protein kinase B/Akt pathway [33, 34]. In addition, a recent study showed that ILK directly mediated actin cytoskeletal rearrangements and cell migration and invasion through the concerted actions of phosphoinositide 3-kinase (PI3K)/Akt/Rac1 [35].

Accordingly, we hypothesized that overexpression of ILK and integrin β subunits in highly oncogenic cancer cells is related to ovarian cancer progression. We determined if targeting these molecules has antitumor effects for ovarian cancer. Furthermore, we addressed the question of whether there is a complementary and synergistic advantage when these molecules were targeted alone or in combination in highly oncogenic human ovarian cancer cells.

II. MATERIALS AND METHODS

1. Preparation of ovarian cancer patient samples for immunohistochemistry

We examined medical records and archival slides from the collection of ovarian serous adenocarcinoma of the Gynecologic Oncology Files of Yonsei University College of Medicine in Korea. One hundred ninety-six samples of ovarian serous carcinomas were isolated between 1990 and 2003 and used to create tissue microarrays with 2-mm pores in 3.8-cm × 2.2-cm × 0.5-cm frames.

The 4-μm sections were placed on silane-coated slides (Instrumedics, Inc., Hackensack, NJ, USA), deparaffinized, immersed in PBS containing 0.3% (v/v) hydrogen peroxide, and processed in a microwave oven (in 10 mM sodium citrate buffer, pH 6.5, for 15 min at 700 W). After blocking with 1% (w/v) bovine serum albumin in PBS containing 0.05% (v/v) Tween-20 for 30 min, the slides were incubated overnight at 4°C with anti-integrin β1 (7F10; mouse monoclonal, dilution 1:100; Abcam, Cambridge, UK), anti-integrin β4 (H-101; rabbit polyclonal, dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or anti-ILK (65.1; mouse monoclonal, dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoperoxidase staining was performed using the streptavidin-biotin-peroxidase complex method (LSAB universal kit, Dako, Carpenteria, CA, USA). The final reaction product was visualized with the addition of 0.03% (wt/vol) of 3,3′-diaminobenzidine tetrachloride for 5 to 20 min. Strong cytoplasmic staining and Golgi pattern dots were considered positive results. Immunostaining was graded and scored as follows: 0 signifies no staining; 1+ signifies weak, diffuse staining; and 2+ signifies strong, diffuse staining.

2. Cell lines and cell culture conditions

The TOV-112D and OV-90 cell lines were obtained from the American Type Culture Collection and cultured in a 1:1 mixture of MCDB 105 medium (Invitrogen, Carlsbad, CA, USA) and Medium 199 (Hyclone, Logan, UT, USA) containing 15% fetal bovine serum (FBS; Gibco BRL, Grand Island NY, USA), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Gibco BRL, Grand Island NY, USA). The SK-OV-3, MCF-7, and MDA-MB-231 cell lines were obtained from the Korean Cell Line Bank (Seoul, Korea) and cultured in RPMI-1640 medium (Gibco BRL, Grand Island NY, USA) containing 10% FBS (Gibco BRL, Grand Island NY, USA), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Gibco BRL, Grand Island NY, USA).

3. shRNA-mediated mRNA depletion

We used the SureSilencing shRNA plasmid for human ITGB1 (KH00650G for the GFP), ITGB4 (KH00680G for the GFP), and ILK (KH00737G for the GFP), and a scrambled sequence negative control plasmid (SABiosciences, Frederick, MD, USA). The shRNA target sequences are listed in Table 1. The cells were seeded and transfected using the Attractene Transfection Reagent (QIAGEN, Valerncia, CA, USA) according to the manufacturer's protocol. At 24, 48, 72, and 96 h after transfection with the shRNA plasmids, the cells were harvested and total RNA was extracted using an RNeasy Protect Mini Kit (QIAGEN, Valerncia, CA, USA). The SuperScript III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) was used to synthesize cDNA. Polymerase chain reactions (PCRs) were performed with HotStarTaq DNA polymerase (QIAGEN, Valerncia, CA, USA). Primers, product sizes, and PCR conditions are listed in Table 2.

Table 1. shRNA target sequences

shRNA plasmids	shRNA target sequences (5'-3')
Human ITGB1 plasmid	TGTGCTCAGTCT TACTAATAA
Human ITGB4 plasmid	GGACACCATCTGCGAGATCAA
Human ILK plasmid	GCAGTACAAGGCAGACATCAA
Scrambled sequence negative control plasmid	ggaatctcattcgatgcatac

Table 2. Primers, product sizes, and PCR conditions

Molecules	Sense (5'-3')	Anti-Sense (5'-3')	Size	Tm	Conditions
ITGB1	AATTAGGC CTCTGGGC TTTACGGA	ACACTTACA GACACCAC ACTCGCA	529 bp	60°C	95°C 15 min, 26 cycles: 94°C 40 sec, Tm 1 min, 72°C 1 min, 72°C 10 min
ITGB4	TGCACCTA CAGCTACA CCATGGAA	ACTTGGTCT GCTGGAGCT TGTGTA	568 bp	60°C	95°C 15 min, 26 cycles: 94°C 40 sec, Tm 1 min, 72°C 1 min, 72°C 10 min
ILK	AAGATGG GCCAGAAT CTCAACCG T	TCGGGCAGT CATGTCCTC ATCAAT	543 bp	60°C	95°C 15 min, 26 cycles: 94°C 40 sec, Tm 1 min, 72°C 1 min, 72°C 10 min
GAPDH	AAGGTCG GAGTCAA CGGATTTG GT	AGTGATGGC ATGGACTGT GGTCAT	534 bp	60°C	95°C 15 min, 26 cycles: 94°C 40 sec, Tm 1 min, 72°C 1 min, 72°C 10 min

4. Western blot analysis

Equal amounts of cell extract were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Invitrogen, Carlsbad, CA, USA). Blots were blocked with 5% non-fat dry milk freshly dissolved in 1 × phosphate buffered saline with Tween 20 (PBS-T), and incubated for 1 h at room temperature with primary antibodies. The primary antibodies that were used were: anti-integrin β1 (M-106; rabbit polyclonal, 1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-integrin β3 (BV4; mouse monoclonal, 1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-integrin β4 (H-101; rabbit polyclonal, 1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-ILK (65.1; mouse monoclonal, 1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Cdc42 (B-8; rabbit polyclonal, 1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-RhoA (rabbit polyclonal, 1:500 dilution; Abcam, Cambridge, UK), anti-MMP-2 (mouse monoclonal, 1:500 dilution; NeoMarkers, Fremont, CA, USA), anti-MMP-9 (mouse monoclonal, 1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-AKT (rabbit monoclonal, 1:1,000 dilution; Cell Signaling, Beverly, MA, USA), anti-phospho-Akt (Ser473; rabbit polyclonal, 1:1,000 dilution; Cell Signaling, Beverly, MA, USA), anti-caspase-3 (mouse monoclonal, 1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Erk1/2 (rabbit monoclonal, 1:5,000 dilution; Db Biotech, Kosice, Slovakia), anti-phospho-p44/42 MAPK (Erk1/2; Thr202/Tyr204; rabbit monoclonal, 1:2,000 dilution; Cell Signaling, Beverly, MA, USA), and anti-GAPDH (goat polyclonal, 1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blots were probed with an enzyme (horseradish peroxidase)-linked secondary antibody in 1 × PBS-T (1:1,500–100,000) for 1 h at room temperature. Finally, chemiluminescent detection reagents were used to visualize the results. Western blot signal was analyzed by an image analysis program (Multi Gauge V3.0, FUJIFILM, Tokyo, Japan).

5. Flow cytometric analysis

The antibodies used for flow cytometry were: anti-integrin β1-PE (human CD29; mouse monoclonal; BD Pharmingen, San Diego, CA, USA), anti-integrin β3-PE (human CD61; mouse monoclonal; BD Pharmingen, San Diego, CA, USA), anti-integrin β4-PE (human CD104; rat monoclonal; BD Pharmingen, San Diego, CA, USA), and isotype control antibodies (BD Pharmingen, San Diego, CA, USA). Antibodies were added at the appropriate

dilutions and incubated for 20 min on ice in the dark. Samples were analyzed on a BD FACSAria™ cell sorter (BD Bioscience, San Jose, CA, USA).

6. Rac1 activity assay

Rac1 activation assays were performed 96 h after shRNA transfection using a Rac1 G-LISA™ Activation Assay kit (Cytoskeleton Inc. Denver, CO, USA) according to the manufacturer's instructions. We measured the level of active, GTP-loaded Rac1 protein in cell lysates by absorbance at a wavelength of 490 nm using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

7. Cell proliferation and viability analysis

Cell proliferation was measured 96 h after shRNA transfection using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. We measured sample absorbance at a wavelength of 450 nm using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

8. Cell migration and invasion assay

Cell migration and invasion were analyzed using an Oris^{TM} cell migration and invasion assay kit (Platypus Technologies, Madison, WI, USA), following the manufacturer's instructions. Briefly, 48 h post-transfection, cells were trypsinized and resuspended in culture medium at 5×10^5 cells/ml.

To perform the cell migration assay, 100 μl cell suspension (50,000 cells/well) was added to each well of Oris plate populated with silicone stoppers. After 12 h of incubation at 37°C in a 5% CO_2 incubator, the stoppers were removed from the Oris plate. Each was carefully washed with PBS to remove any unattached cells. Following a 24 h incubation (37°C, 5% CO_2), migrated cells were stained with Calcein AM and detected with a Victor™ X5 Multilabel Plate Reader (PerkinElmer Life and Analytical Sciences, Inc., Shelton, CT, USA) using 485/528 nm excitation/emission filters.

To perform the cell invasion assay, 100 μl cell suspension (50,000 cells/well) was added to each well of 4 mg/mL basement membrane extract coated Oris plate populated with the stoppers. After 12 h of incubation (37°C, 5% CO_2), the stoppers were removed from the Oris plate and each well was overlaid with 14 mg/mL basement membrane extract (+/- 10% FBS) to create a 3D environment. Following a 48-h incubation (37°C, 5% CO_2), the invaded cells were stained with Calcein AM and detected with a Victor™ X5 Multilabel

Plate Reader (PerkinElmer Life and Analytical Sciences, Inc., Shelton, CT, USA) using 485/528 nm excitation/emission filters.

9. Xenograft tumorigenicity assay

Nude mice were purchased from Central Lab Animal, Inc. (Seoul, Korea) and maintained in accordance with the institutional guidelines of Yonsei University College of Medicine. All animal studies were performed according to approved experimental protocols. Tumor cells (1×10^6 cells in 0.2 ml PBS) were injected subcutaneously in the dorsal flank of 6-week-old female nude mice. Tumors were measured every 4 days. Tumor volume was calculated with an index of the growth rate using the following equation: volume = (width + length) / 2 × width × length × 0.5236. The mice were sacrificed 60 days after inoculation of the cells, and metastatic lesions on the lungs and livers were counted macroscopically.

10. Statistical analysis

Student's *t*-test was used, and results were considered statistically significant if the null hypothesis was rejected with a *P*-value < 0.05.

III. RESULTS

1. ILK and β4 integrin are expressed in highly oncogenic human ovarian cancer cells

We evaluated the expression level of β1, β3, and β4 integrin subunits and ILK in 3 epithelial ovarian cancer cell lines of different histopathological types by western blot analysis. We showed that β1 integrin was highly expressed in all cell lines, whereas β3 and β4 integrins and ILK were differentially expressed (Fig. 1A). Integrin subunit β3 was highly expressed in TOV-112D cell. In particular, ILK and β4 integrin were highly expressed in the highly oncogenic and invasive cancer cell line, SK-OV-3. ILK (P<0.05) and β4 integrin (P<0.01) expression was also significantly augmented in SK-OV-3 cells when compared with the other ovarian cancer lines, whereas β1 and β3 integrin expression was not (Fig. 1B).

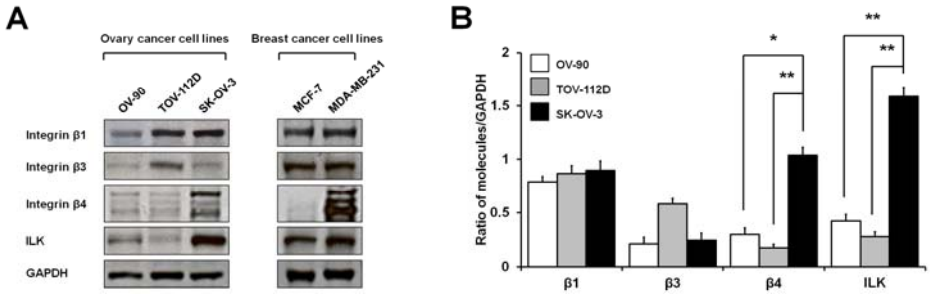

Figure 1. Expression of ILK and integrin β subunits in ovarian cancer cell lines. (A) Protein expression levels of ILK and integrin β subunits. (B) Relative expression levels are graphically represented, with GAPDH serving as a loading control. Columns, mean of 3 independent experiments; error bars, standard deviation (SD). *, P<0.05, and **, P<0.01, compared with SK-OV-3; P-values calculated using Student's t-test.

In flow cytometric analysis, subunit β1 was moderately or highly expressed in most ovarian cancer cell lines, with the positive population comprising 85–99% of the total (Fig. 2). In contrast, β3 integrin expression was low, with expression detected in only 0.5–8.2% of the cells of the ovarian cancer cell lines. In particular, β4 integrin was highly expressed in the SK-OV-3 cells (75.6% of which were positive for the integrin subunit), while it was expressed by only a small proportion (1.3–13.1%) of the cells of the other ovarian.

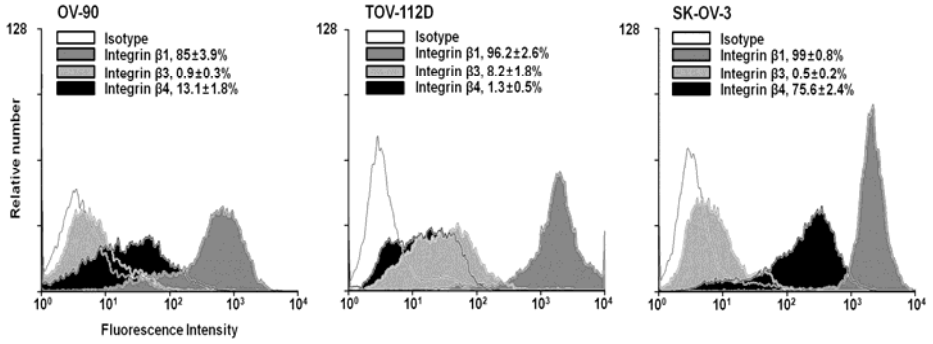

Figure 2. Analysis of cell surface expression of β1, β3, and β4 integrins in ovarian cancer cell lines. Data are represented as mean ± SD of 3 independent experiments performed in triplicate.

2. Expression of ILK and β4 integrin are related to Rac1 and Akt activation in highly oncogenic human ovarian cancer cells

To investigate Rac1 activity in 3 ovarian cancer cell lines, we performed Rac1 activity assay. Rac1 activity in SK-OV-3 cells was significantly higher than the other ovarian cancer cell lines (Fig. 3; $P<0.05$). In western blot analysis, total Akt and p-Ser473 Akt expression were also highly expressed in SK-OV-3 cells (Fig. 4). Furthermore, Rac1 activity was very similar to total Akt and p-Ser473 Akt expression in 3 ovarian cancer cell lines.

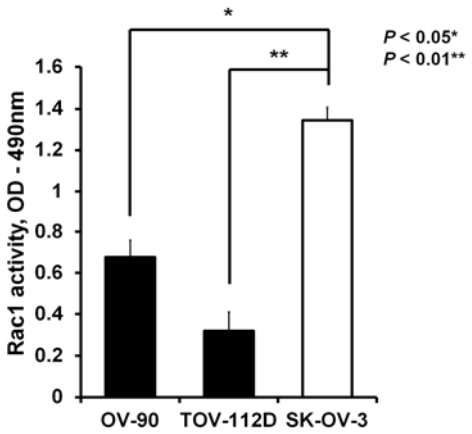

Figure 3. Analysis of Rac1 activity in ovarian cancer cell lines. Columns, mean of 3 independent experiments; error bars, standard deviation (SD). *, $P<0.05$, and **, $P<0.01$, compared with SK-OV-3; P-values calculated using Student's t-test.

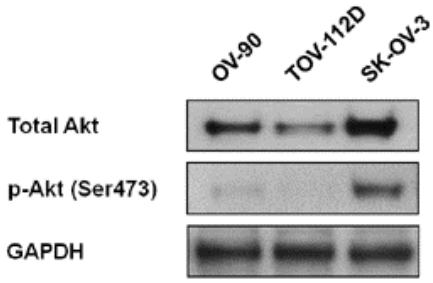

Figure 4. Expression of total Akt and p-Ser473 Akt in ovarian cancer cell lines. GAPDH served as a loading control.

3. Expression of ILK and β4 integrin are related to the expression of cell migration- and invasion-related molecules in highly oncogenic human ovarian cancer cells

To investigate the expression of cell migration- and invasion-related molecules in 3 ovarian cancer cell lines, we evaluated expression level of Cdc42, RhoA, MMP-2, and MMP-9. Using western blot analysis, we confirmed that SK-OV-3 cells expressed these molecules at a clearly higher level than the other cell lines (Fig. 5). Moreover, the expression pattern of these molecules was very similar to ILK and β4 integrin expression in SK-OV-3 cells (Fig. 1).

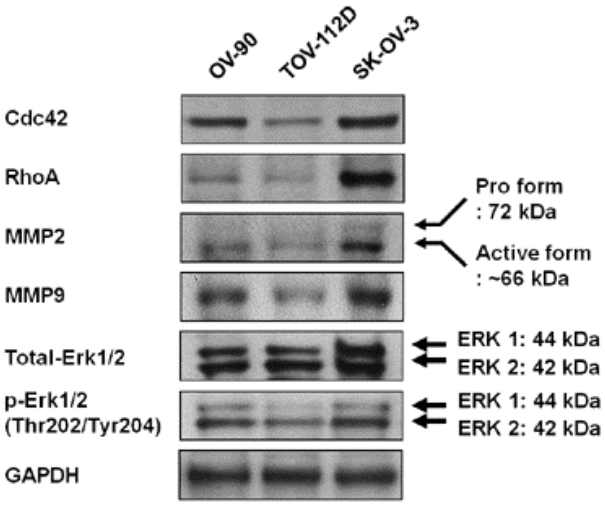

Figure 5. Expression of cell migration, invasion, and proliferation-related molecules in ovarian cancer cell lines. GAPDH served as a loading control.

4. ILK and β4 integrin were highly elevated in human ovarian cancer specimens

We performed immunohistochemistry to investigate the expression levels of β1 and β4 integrins and ILK in tissue microarrays, which consisted of 196 samples of human ovarian serous carcinomas. The percentage of ILK-positive samples at 1+ and 2+ levels was higher than those for β1 or β4 integrin (Table 3). The percentages of tumors staining positively for each protein at 1+ and 2+ levels were as follows: integrin β1 (9.18%), integrin β4 (23.47%), and ILK (33.67%). The percentage of ILK-positive samples at the 2+ level (23.47%) was also higher than those for β1 (1.02%) or β4 integrin (2.55%). Meanwhile, the percentage of β4 integrin-positive samples (20.92%) at the 1+ level was higher than β1 integrin (8.16%) or ILK (10.20%).

Table 3. Immunohistochemical analysis of β1 and β4 integrin and ILK expression in a tissue microarray of human ovarian serous carcinomas

Molecule	No. tumors; type	Staining intensity[a] (% of specimens staining positive)		
		0	1+	2+
Integrin β1	196; ovarian serous carcinomas	178	16 (8.16)	2 (1.02)
Integrin β4		150	41 (20.92)	5 (2.55)
ILK		130	20 (10.20)	46 (23.47)

[a]Immunohistochemical scoring was defined as follows: 0 = no staining; 1 = weak, diffuse staining; 2 = strong, diffuse staining.

Integrin subunit β1 staining was often highly positive along the mesenchymal cell membranes predominantly around the fibrovascular core, instead of the tumor cell borders, in serous carcinoma (Fig. 6A). Staining was intense along the basement membrane between tumor cells and stroma (Fig. 6B) and frequently noted in metastatic tumor node cells (Fig. 6C). ILK was highly expressed in serous carcinoma (Fig. 7A) and present in both the cytoplasmic membrane and cytoplasm of tumor cells (Fig. 7B). It was also overexpressed along the invading front margins of tumor cells (Fig. 7C). Integrin subunit β4 was overexpressed (Fig. 8A) and accentuated in the infiltrative nests and margins of tumor nests (Fig. 8B). It was also detected in lymph node metastases without exception (Fig. 8C). None of the tissues were positive for staining with IgG1 isotype control.

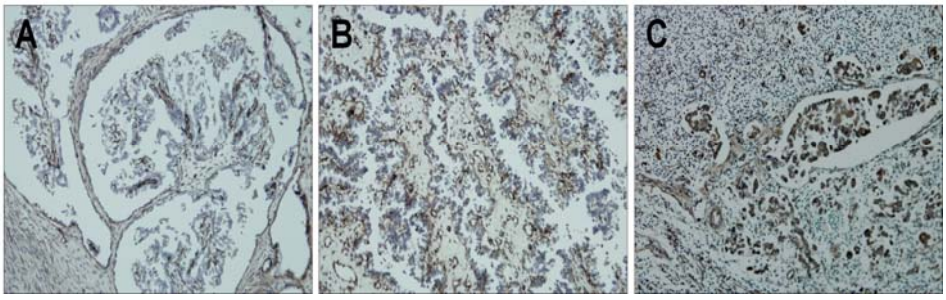

Figure 6. Immunohistochemistry of β1 integrin in human ovarian tumors. (A) Staining was mainly observed along the fibrovascular core attaching to the tumor cells. (B) Staining was intense along the basement membrane between tumor cells and stroma. (C) Notice β1 integrin positive carcinoma in the metastatic node.

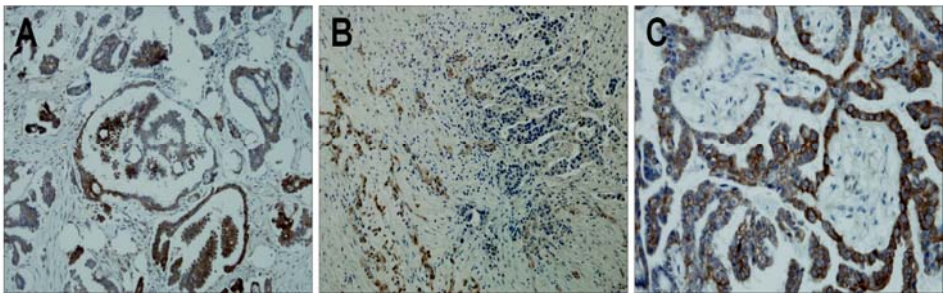

Figure 7. Immunohistochemistry of ILK in human ovarian tumors. (A) ILK was detected in the tumor cytoplasm. (B) Staining along the edge of the invasive front. (C) ILK was localized to the cytosol and cytoplasmic membrane of tumor cells.

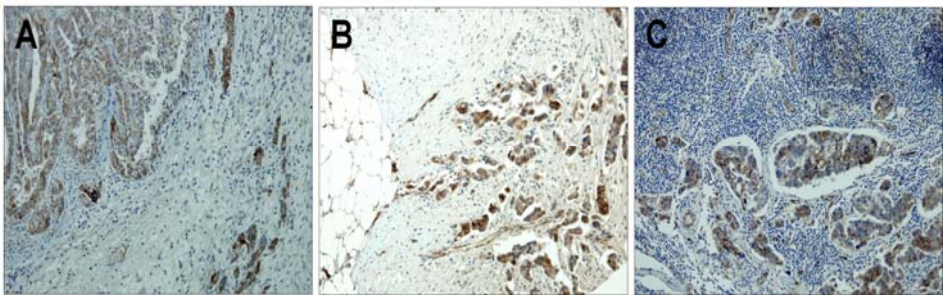

Figure 8. Immunohistochemistry of β4 integrin in human ovarian tumors. (A) Staining was intense at the edges of nests and alongside infiltrative cords. (B) Staining was strong in the infiltrative nests and cords. (C) Integrin β4 was detected in the metastatic foci.

5. Effect of ILK and integrin β4 depletion by shRNA on cell migration and invasion in human ovarian cancer cell line SK-OV-3

We measured the depletion efficiency of shRNA plasmids, alone or in combination, and confirmed that each molecule was effectively depleted when compared with mock or negative control shRNA plasmid-transfected cells. After shRNA transfection of SK-OV-3 cells, mRNA (Fig. 9) and protein expression (Fig. 10 and 11) was down-regulated by more than 80% as compared with mock or negative controls.

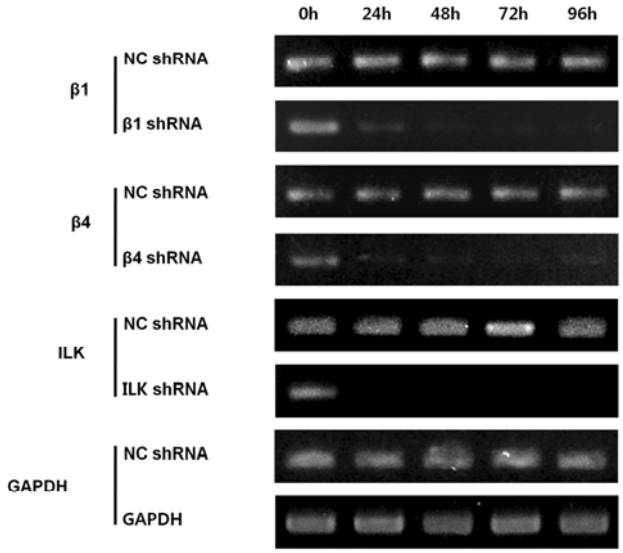

Figure 9. Reduction of mRNA by shRNA in SK-OV-3 cells at 0, 24, 48, 72, and 96 h after single shRNA transfection. All experiments were performed in triplicate.

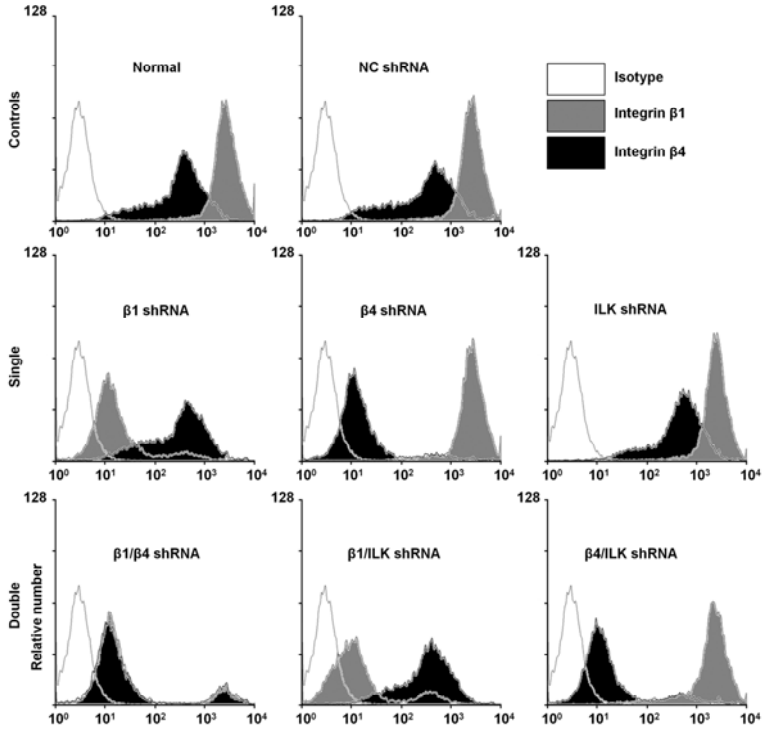

Figure 10. Inhibition of β1 and β4 integrin expression by shRNA treatment determined by flow cytometric analysis 96 h after shRNA combination transfection. All experiments were performed in triplicate.

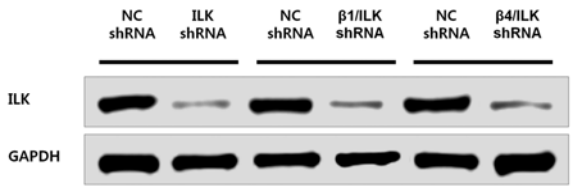

Figure 11. Inhibition of ILK expression by shRNA determined by western blot analysis 96 h after shRNA combination transfection. All experiments were performed in triplicate.

To evaluate the direct effect of β1 and β4 integrin and ILK shRNA-mediated depletion, alone or in combination, on cell migration and invasion process, we performed cell migration and cell invasion assays in SK-OV-3 cells. The data showed that the effects of silencing these targets alone or in combination with shRNAs on cell migration and invasion were very similar. The most effective combination treatment consisted of the depletion of β4 integrin/ILK, which decreased cell migration (Fig. 12) and invasion (Fig. 13) by 71.4% and 67.6%, respectively, compared with negative control shRNA-transfected cells (P<0.05). The most effective single treatment consisted of the depletion of ILK, which decreased cell migration (Fig. 12) and invasion (Fig. 13) by 54.3% and 54.8%, respectively, compared with negative control shRNA-transfected cells (P<0.05). In addition, of the combination treatments, only depletion of β4 integrin/ILK produced statistically significant differences in cell migration (P=0.034) and invasion (P=0.028) when compared with ILK shRNA single depletion.

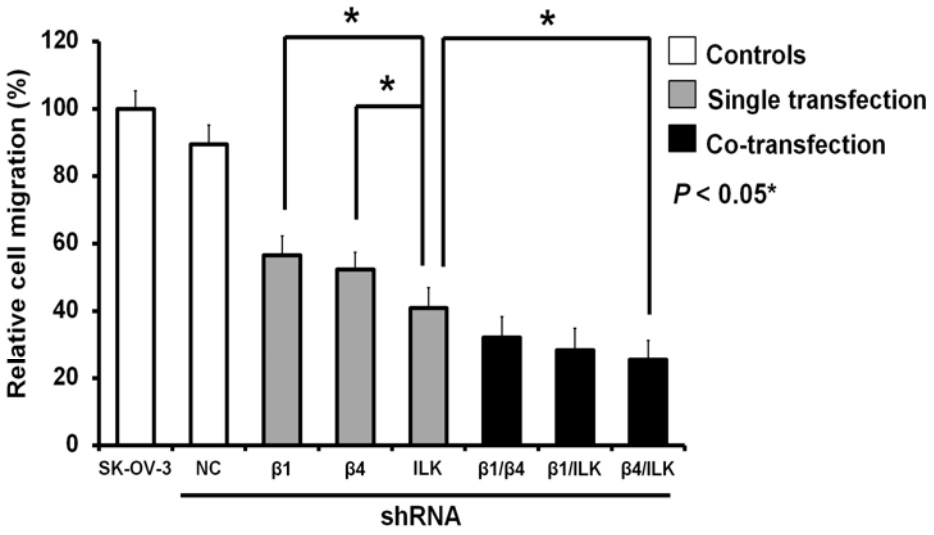

Figure 12. Effect of shRNA-mediated depletion on cell migration in SK-OV-3 cells. Cells were trypsinized and resuspended in culture medium at 48 h post-transfection. Cell migration assays were carried out 24 h after plating. Columns, means from 3 independent experiments, each performed in triplicate; error bars, SD. *, P<0.05, compared with ILK shRNA; P-values calculated using Student's t-test.

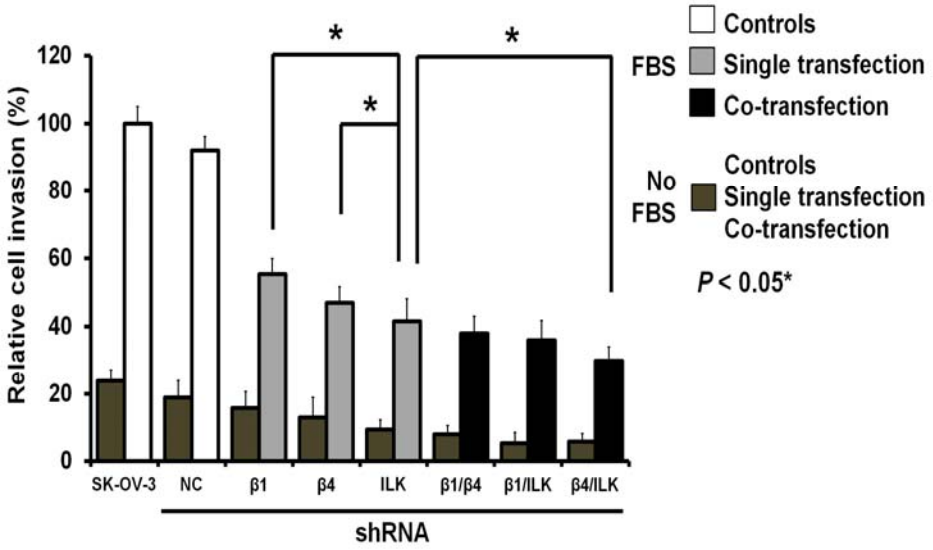

Figure 13. Effect of shRNA-mediated depletion on cell invasion in SK-OV-3 cells. Cells were trypsinized and resuspended in culture medium at 48 h post-transfection. Cell invasion assays were carried out 48 h after plating. Columns, means from 3 independent experiments, each performed in triplicate; error bars, SD. *, $P<0.05$, compared with ILK shRNA; *P*-values calculated using Student's *t*-test.

6. Effect of ILK and β4 integrin depletion by shRNA on cell migration- and invasion-related molecules in human ovarian cancer cell line SK-OV-3

We performed western blot analysis to evaluate the effect of shRNAs, alone or in combination, on cell migration- and invasion-related molecules in SK-OV-3 cells. The shRNA treatment-induced reduction of expression of the cell migration-related molecules, Cdc42 and RhoA (Fig 14), and the cell invasion-related molecules, MMP-2 and MMP-9 (Fig. 15), showed a very similar pattern to the results for cell migration (Fig. 12) and invasion assays (Fig. 13). The most effective treatment was combination depletion of β4 integrin/ILK, and the most effective single depletion treatment was that of ILK. However, there were no differences between the effects of ILK depletion and the other combination treatments on cell migration- and invasion-related molecules.

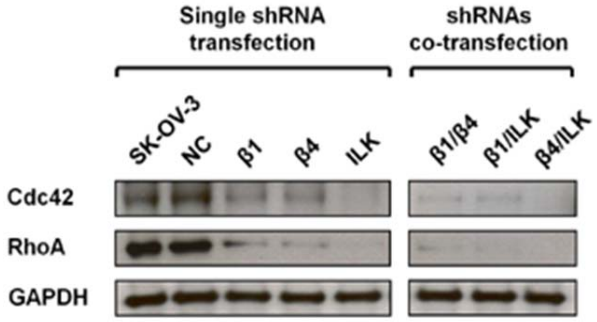

Figure 14. Effect of shRNA-mediated depletion on cell migration-related molecules in SK-OV-3 cells. Expression of Cdc42 and RhoA at 72 h after transfection. GAPDH served as a loading control.

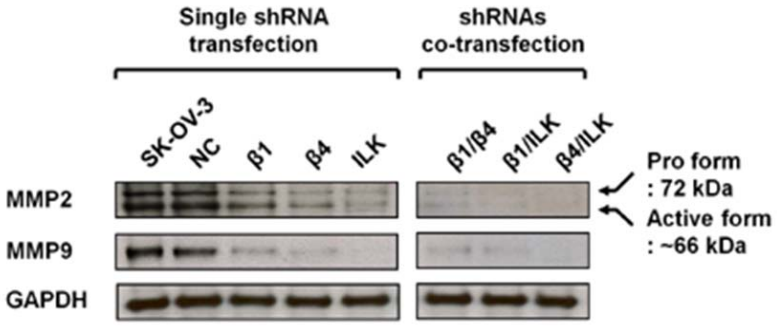

Figure 15. Effect of shRNA-mediated depletion on cell invasion-related molecules in SK-OV-3 cells. Expression of MMP-2 and MMP-9 at 96 h after transfection. GAPDH served as a loading control.

7. Effect of ILK and β4 integrin depletion by shRNA on PI3K/Akt/Rac1 cascade in human ovarian cancer cell line SK-OV-3

To investigate the effect of shRNAs on Rac1 activity, we performed a Rac1 activity assay. The inhibition of Rac1 activity by shRNA showed a very similar pattern to the results for cell migration (Fig. 12) and invasion assays (Fig. 13). The most effective treatment consisted of combination depletion of β4 integrin/ILK, which decreased Rac1 activity by 73.5%, compared with negative control shRNA transfection. The most effective single depletion treatment was that of ILK, which decreased Rac1 activity by 62.7%, compared with negative control shRNA transfection (Fig. 16; $P<0.05$). In addition, of the combination depletions, only β4 integrin/ILK depletion reduced Rac1 activity statistically significantly more than ILK shRNA single depletion treatment ($P=0.037$).

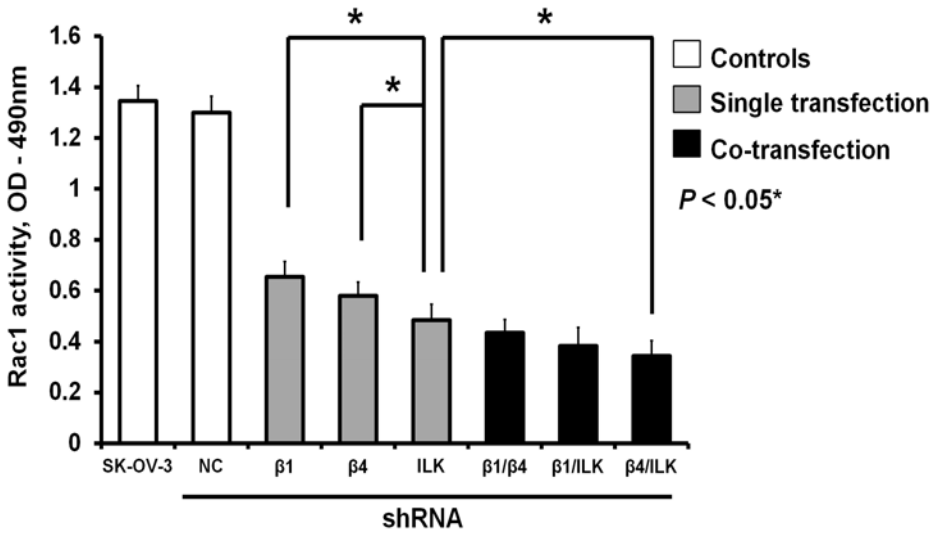

Figure 16. Effect of shRNA-mediated depletion on *in vitro* Rac1 activity in SK-OV-3 cells. Rac1 activity assays were were carried out 96 h post-transfection. Columns, means from 3 independent experiments, each performed in triplicate; error bars, SD. *, $P<0.05$, compared with ILK shRNA; *P*-values calculated using Student's *t*-test.

8. Effect of ILK and β4 integrin depletion by shRNA on Akt and caspase-3 activation in human ovarian cancer cell line SK-OV-3

Using western blot analysis, the data showed that all shRNA treatments effectively decreased p-Ser473 Akt, which activates the PI3K/Akt pathway, compared with mock or negative control shRNA plasmid transfection, in SK-OV-3 cells, whereas total Akt levels were not affected by shRNA treatment (Fig. 17). Meanwhile, inhibition of p-Ser473 Akt showed a very similar pattern to cell migration (Fig. 14) and invasion-related molecules (Fig. 15). In addition, down-regulation of ILK and β4 integrin enhanced apoptotic signaling through the activation of caspase-3. Therefore, the inhibition of ILK and β4 integrin leads to activation of caspase-3 and inhibition of p-Ser473 Akt. Consequently, it is possible to cause cell apoptosis by enhancing apoptotic signaling by blocking the PI3K/Akt/Rac1 cascade in ovarian cancer cells.

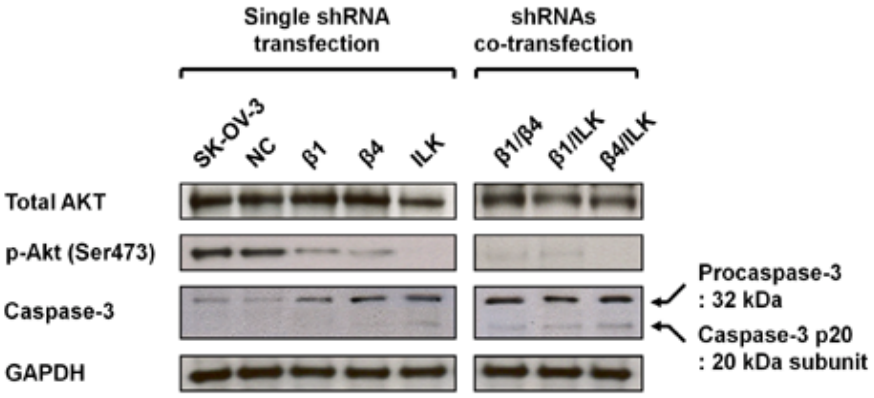

Figure 17. Effect of shRNA-mediated depletion on Akt and caspase-3 activation in SK-OV-3 cells. Expression of Akt, p-Ser473 Akt, and caspase-3 were examined 96 h after transfection. GAPDH served as a loading control.

9. Effect of ILK and integrin β4 depletion by shRNA on cell proliferation in human ovarian cancer cell line SK-OV-3

We performed cell proliferation assays and observed a significant reduction in proliferation of shRNA-transfected cells. The reduction of proliferation by single shRNA treatment showed a very similar pattern to the results for cell migration (Fig. 12) and invasion assays (Fig. 13), whereas the reduction pattern of combination treatments differed slightly from the single treatments (Fig. 18). The most effective treatment consisted of combination depletion of β4 integrin/ILK, which decreased cell proliferation by 70.1%, compared with negative control shRNA transfection. The most effective single depletion treatment was that of ILK, which decreased cell proliferation by 55.3%, compared with negative control shRNA transfection (Fig. 6A-a; $P<0.05$). Meanwhile, single depletion of β1 integrin ($P=0.025$) and combination depletions of β1/β4 integrin ($P=0.023$) and β4 integrin/ILK ($P=0.037$) each reduced cell proliferation statistically significantly more than ILK shRNA single depletion, whereas other treatments did not.

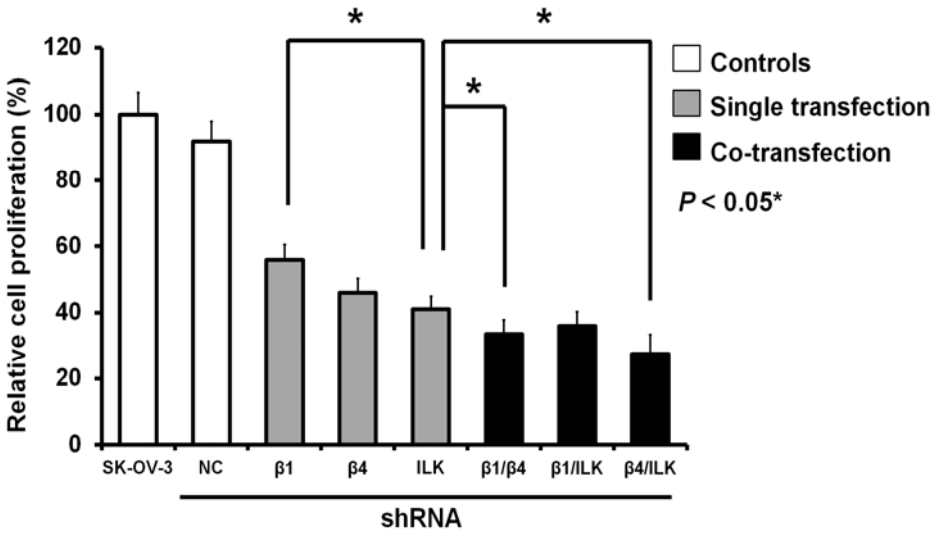

Figure 18. Effect of shRNA-mediated depletion on cell proliferation in SK-OV-3 cells. cell proliferation assays were carried out 96 h post-transfection. Columns, means from 3 independent experiments, each performed in triplicate; error bars, SD. *, $P<0.05$, compared with ILK shRNA; *P*-values calculated using Student's *t*-test.

10. Effect of ILK and integrin β4 depletion by shRNA on Erk1/2 activation in human ovarian cancer cell line SK-OV-3

By western blot analysis, we showed that all shRNA treatments effectively decreased p-Thr202/Tyr204 Erk1/2 expression compared with mock or negative control shRNA plasmid transfection in SK-OV-3 cells, whereas total Erk1/2 expression was not affected by shRNA treatment (Fig. 19). The inhibition pattern of p-Thr202/Tyr204 Erk1/2 followed a very similar pattern to the inhibition of cell proliferation (Fig. 18). The most effective treatment consisted of combination depletion of β4 integrin/ILK, and the most effective single depletion treatment was that of ILK.

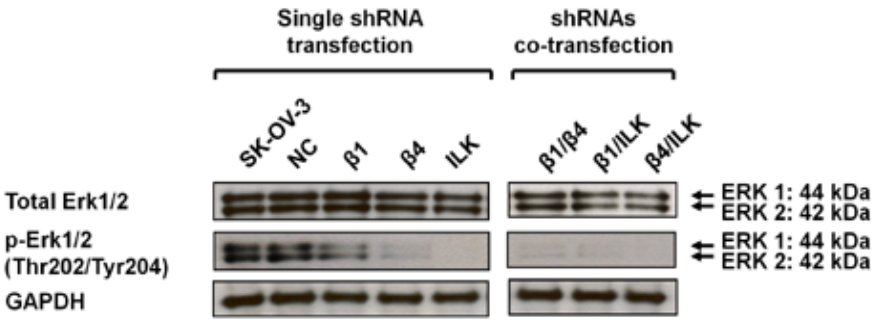

Figure 19. Effect of shRNA-mediated depletion on Erk1/2 activation in SK-OV-3 cells. Expression of Erk1/2 and p-Thr202/Tyr204 Erk1/2 was examined 96 h after transfection. GAPDH served as a loading control.

11. *In vivo* xenograft model of human ovarian cancer cells

By monitoring the tumor growth rate of each group of xenografts for 60 days, we concluded that down-regulation of β4 integrin and ILK strongly suppressed tumorigenesis in nude mice (Fig. 20 and Table 4). Gross tumor size was also lower in those mice that received combination treatment compared with those that received shRNA control treatment (Fig. 21). In addition, these results were consistent with the result of cell proliferation assays (Fig. 11A). Particularly, tumor growth rate after single depletion of β1 integrin (P=0.019) and combination depletions of β1/β4 integrin (P=0.022) and β4 integrin/ILK (P=0.0058) caused statistically significantly augmented inhibition of cell proliferation, compared with ILK shRNA single depletion treatment (Fig. 20).

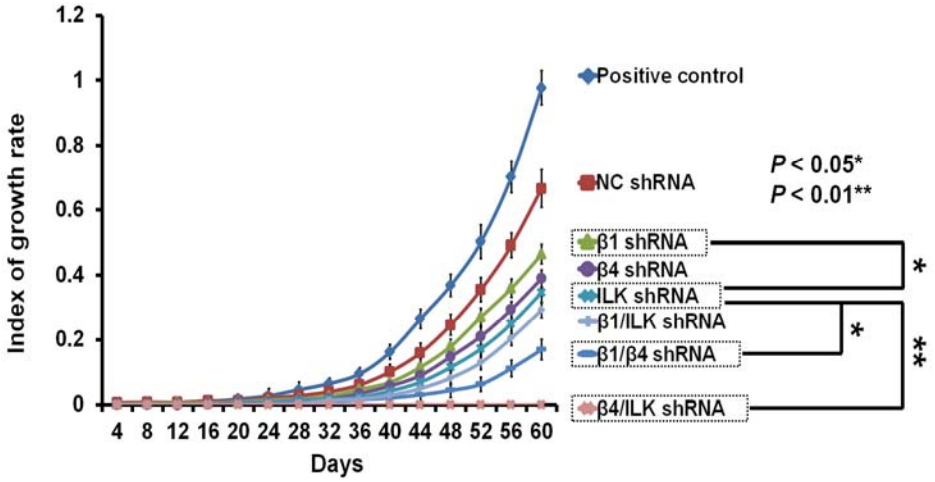

Figure 20. Effect of shRNA-mediated depletion on *in vivo* growth of ovarian cancer xenografts in nude mice. Index of the growth rate. SK-OV-3 cells (1×10^6 per mouse) were injected subcutaneously in both sides of the dorsal flanks of female nude mice 48 h after transfection. Tumors were measured every 4 days for 60 days. Columns, means from 3 independent experiments, each performed in triplicate; error bars, SD. *, $P<0.05$, and **, $P<0.01$, compared with ILK shRNA; *P*-values calculated using Student's *t*-test.

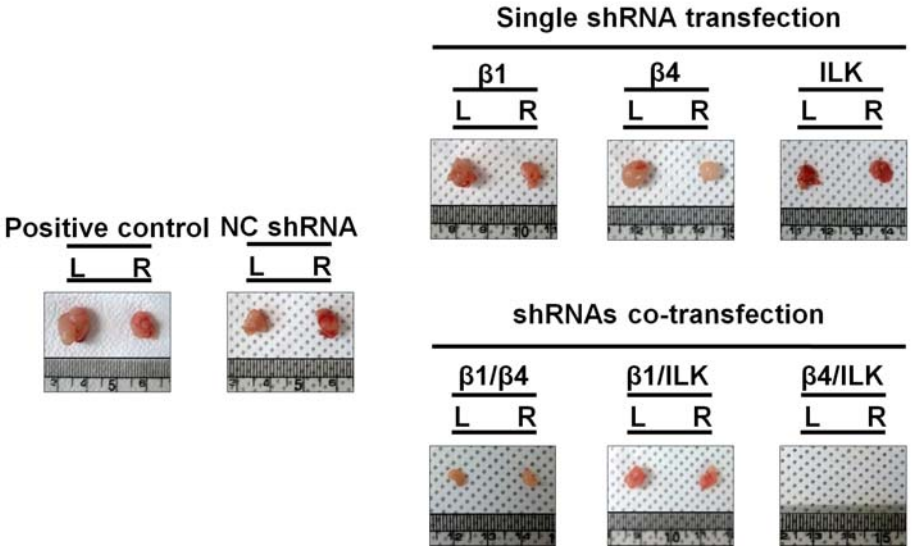

Figure 21. Images of the tumors excised from the nude mice subcutaneously injected with control or shRNA-transfected cells. The mice were sacrificed 60 days after inoculation of the cells.

Table 4. Tumor growth in nude mice

	Groups	Incidence[a]	Latency (d)[b]	Growth rate (min–max)[c]
Control	Positive control	3/3 mice	4, 4, 4	0.0051–0.9776
	Negative control shRNA	2/3 mice	4, 4	0.0029–0.6665
Single transfection	β1 integrin shRNA	1/3 mice	4	0.0021–0.4648
	β4 integrin shRNA	1/3 mice	16	0.0014–0.3901
	ILK shRNA	1/3 mice	24	0.0014–0.3466
Co-transfection	β1 and β4 integrin shRNA	1/3 mice	24	0.0007–0.1692
	β1 integrin and ILK shRNA	1/3 mice	24	0.0014–0.2921
	β4 Integrin and ILK shRNA	0/3 mice	ND	ND

[a]Number of mice with tumors/the number of mice injected.

[b]Tumor latency refers to the interval (days) from injection to the first detection of a palpable tumor.

[c]Index of growth rate: volume (cm^3); the equation as described in the Materials and Methods section. Abbreviation: ND, not determined.

IV. DISCUSSION

Here we investigated the potential of targeting integrins and ILK as combination therapy for the treatment of highly aggressive ovarian cancer. We present clear evidence that depletion of these targets, alone or in combination, suppresses ovarian cancer cell proliferation, migration, and invasion *in vitro* and xenograft tumor formation *in vivo*. Thus, we demonstrate that combined targeting of β4 integrin and ILK has potent and effective inhibitory effects on ovarian cancer progression.

Integrins synergize with growth factor pathways to enhance their activity as well as induce ligand-independent trans-activation of other integrins or tyrosine kinase growth factor receptors [6-9]. ILK can bind β1 and β3 integrin and play a role as an adaptor [23, 24]. ILK also coordinates signal transduction from the extracellular matrix and growth factors [29-32]. In addition, integrin-specific signals overlap and integrate with the action of receptor tyrosine kinases (RTKs) and ultimately affect various phases of tumor development by cooperating with signaling molecules that function downstream of integrins and RTKs, such as focal adhesion kinase (FAK), Src, and Shc [36, 37].

Recent studies have reported that the direction of adhesive signaling by the integrin β cytoplasmic domain is possible to specify [38, 39]. The integrin β1 subunit promotes random migration, whereas the β3 integrin promotes persistent migration in the same epithelial cell background. Although most integrin β subunits usually bind the SH3 domain of Src, β3 integrin most dominantly binds it and regulates cell spreading during tumor progression [40, 41]. In particular, some studies demonstrated that self-association of the β4 integrin cytoplasmic domains can initiate intracellular signaling events independently of ligand binding and may promote anchorage-independent survival [12, 42]. This is supported by the relationship between high expression levels of α6β4 integrin and the poor prognosis for patients with various cancers [43]. In addition, signaling by the α6β4 integrin proceeds through Src family kinase (SFK)-mediated phosphorylation of the large and unique cytoplasmic tail of β4, recruitment of Shc, and activation of Ras [44-46] and PI3K [47]. Activation of EGF receptor (EGF-R) and Ron RTKs also enhances phosphorylation of β4 integrin, causing disruption of hemidesmosomes and increased epithelial cell migration [48, 49]. Therefore, deregulation of α6β4-RTK co-signaling contributes to tumor progression.

Meanwhile we previously documented the possibility of CD24 as an ovarian cancer stem cell (CSC) marker in our previous studies [50, 51]. We further

reported that CD24 expression in ovarian cancer can promote cell invasion, induce significant amounts of EMT-associated markers, and increase SDF-1-mediated chemotactic migration through upregulation of CXCR4 protein. In addition, CD24 expression in ovarian cancer enhanced cell adhesion to fibronectin through the activation of β1 integrin [52]. A recent study also reported that CD24 indirectly stimulates cell adhesion to fibronectin, collagens I and IV, and laminin through the activation of α3β1and α4β1 integrin activity [53]. Based on these findings, $CD24^+$ cells in ovarian cancer are highly likely to be involved in tumor progression, drug resistance, and clonal asynchronous evolution that affect intratumoral heterogeneity. In addition, specific integrin expression in $CD24^+$ subpopulation could play crucial roles in cell invasion, migration, and adhesion to the extracellular matrix. Accordingly, targeting CD24 may be useful as a part of novel therapeutic strategies, based on CSCs to treat ovarian cancer.

In this study, we investigated the potential of targeting integrins and ILK as combination therapy for highly aggressive ovarian cancer. Our data showed that targeting ILK alone and ILK in combination with β4 integrin effectively inhibited the PI3K/Akt/Rac1 cascade in invasive human ovarian cancer cell line SK-OV-3 by blocking the activation of Akt and Rac1 in *in vitro* and *in vivo* assays. In particular, our data discloses for the first time an unanticipated role and a synergistic effect for the combination of ILK with β4 integrin as an anticancer target in ovarian cancer, which elevates this integrin from candidate to culprit status in tumor progression. Thus, we showed that the results obtained by ILK depletion, alone or in combination with β1 and β4 integrin, could be due to the activation of different signaling pathways through ILK and integrin β subunits.

As a result, ILK directly induces the phosphorylation of Akt and the activation of Rac1, and their activation plays a crucial role in tumor progression via the PI3K/Akt/Rac1 cascade (Fig. 22A). Integrin subunit β4 also directly activates the PI3K/Akt cascade via SFK/Shc, and its activation affects tumor progression (Fig. 22B). On the contrary, β1 and β3 integrins indirectly affect tumor progression through the activation of PI3K/Akt/Rac1 via FAK/Src/Shc (Fig. 22C). Therefore, considering the direct effects on the oncogenic process through the PI3K/Akt/Rac1 pathway, the most effective target for ovarian cancer treatment is ILK. Moreover, it was not surprising that the combination depletion of ILK and β4 integrin was the most effective combination treatment, because the combination depletion of ILK and β4 integrin can generate a synergistic effect and boost the antitumoral effect by co-inhibiting the PI3K/Akt/Rac1 cascade.

Consequently, implicit in these findings is the idea that combined targeting of ILK/β4 integrin in the oncogenic process adds therapeutic value to experimental approaches aimed at interfering with cancer growth and progression in solid tumors.

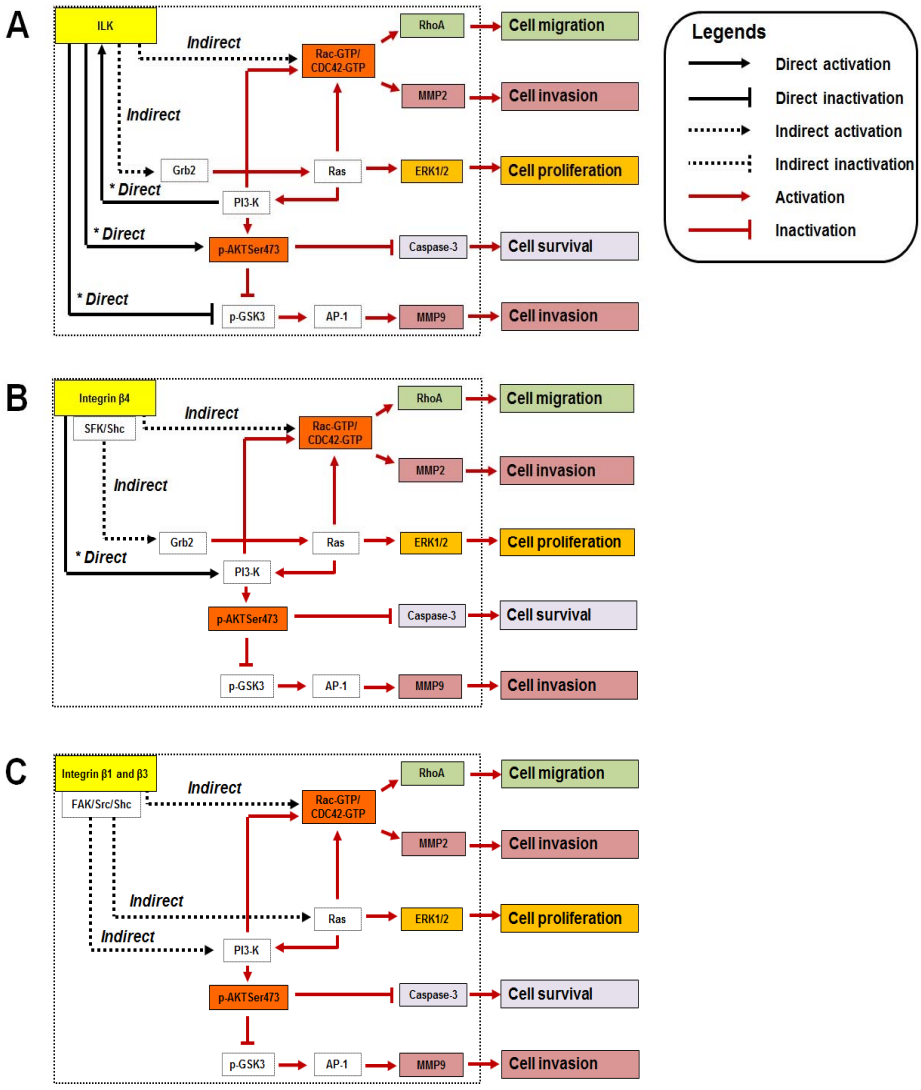

Figure 22. ILK and β4 integrin signaling promotes tumor progression by activating oncogenic signals. (A) ILK signaling pathways, (B) β4 integrin signaling pathways, and (C) β1 integrin signaling pathways. *Note:* This figure was summarized and modified from reference [29].

V. CONCLUSION

Combination targeted therapy has recently emerged as an effective new generation of therapeutics. In fact, molecular targeted therapies have been implicated in cancer cell survival, angiogenesis, invasion, and resistance to chemotherapy. In addition, integrin-targeted and combination therapy is possible to apply to various premalignant cancers that overexpress integrins.

Herein, we may provide a clue to the use of integrin-targeted therapy for ovarian cancer and demonstrate the benefit of combination therapy for aggressive ovarian cancer. We investigated the potential of targeting ILK and integrins as combination therapy for highly aggressive ovarian cancer. Finally, we found that single targeting of ILK and combined targeting of ILK/β4 integrin has potent inhibitory effects on ovarian cancer progression. Therefore, combined ILK/β4 integrin targeted therapy is a new, effective strategy for ovarian cancer treatment. Future studies must elucidate the factors responsible for tumor susceptibility to these inhibitors. Furthermore, a further elucidation of the mechanisms by β4 integrin expression will help in our understanding of the invasion and metastasis of ovarian cancer cells.

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ABSTRACT (IN KOREAN)

인테그린 연관 인산화효소와 베타4 인테그린의 표적화를 통한 난소암의 침윤성 억제효과

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인테그린과 인테그린 연관 인산화효소는 세포외 기질과 물리적 상호작용을 매개하고 종양형성의 신호전달을 조절한다. 본 연구의 목적은 베타1, 베타4 인테그린과 인테그린 연관 인산화효소를 단독 혹은 이들을 조합한 결실이 난소암에서 항종양 효과를 보이는가를 결정하는 것이다. 베타1, 베타4 인테그린과 인테그린 연관 인산화 효소의 발현을 면역화학조직법을 이용해 196개의 난소암 환자 조직 샘플에서 확인하였고, 또한 난소암 세포주들에 shRNA를 처리하여 이런 표적 분자들에 대한 결실효과를 면역발색법, 역전사 중합효소연쇄반응법, 세포증식 분석법, 세포이동 분석법, RAC1 활성 분석법, 이종이식 종양형성능 분석법에 의해서 평가하였다. 사람 난소암 검체들에서 베타4 인테그린과 인테그린 연관 인산화효소의 과발현이 종양 공격성과 상관관계가 있음을 밝혀냈다. 또한 이런 표적들의 결실이 난소암의 세포증식, 세포이동, 세포침윤, 이종이식 종양형성능을 효과적으로 억제함을 확인하였다. 그리고 인테그린 연관 인산화효소 단독 혹은 베타4 인테그린/인테그린 연관 인산화효소를 조합한 결실이 하위 신호전달 표적들인 Akt (p-Ser 473), Erk1/2 (p-Thr202/Tyr204)의 인산화와 Rac1 활성을 억제하였고, MMP-2, MMP-9의 발현을 감소시켰으며, 이와 반대로 Caspase-3의 발현을 증가시킴을 확인하였다. 결론적으로 인테그린 연관 인산화효소와 베타4 인테그린을 표적으로 하는 것은 난소암에서 잠재성 종양형성과 침윤성을 감소시킬 수 있다.

핵심되는 말: 난소암, 인테그린 연관 인산화효소, 베타4 인테그린, 병용효과, 침윤성

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