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Nephroblastoma overexpressed gene (NOV) enhances cell motility and COX-2 upregulation of human osteosarcoma involves αvβ5 integrin, ILK and AP-1-dependent pathways

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ABSTRACT

Osteosarcoma is characterized by a high malignant and metastatic potential. Cyclooxygenase (COX)-2, the inducible isoform of prostaglandin synthase, has been implicated in tumor metastasis. Nephroblastoma overexpressed gene (NOV), also called CCN3, was regulated proliferation and differentiation of cancer cells. However, the effect of NOV on migration activity and COX-2 expression in human osteosarcoma cells is mostly unknown. Here we found that NOV increased the migration and expression of COX-2 in human osteosarcoma cells. αvβ5 monoclonal antibody (mAb), integrin-linked kinase (ILK) and Akt inhibitor reduced the NOV-enhanced the migration and COX-2 up-regulation of osteosarcoma cells. NOV stimulation increased the ILK kinase activity and phosphorylation of Akt. In addition, c-Jun siRNA also antagonized the NOV-mediated migration and COX-2 expression. Moreover, NOV enhanced the AP-1 binding activity and promoter activity. Taken together, these results suggest that the NOV acts through αvβ5 integrin to activate ILK and Akt, which in turn activates c-Jun and AP-1, resulting in the activations of COX-2 and contributing the migration of human osteosarcoma cells.

1. Introduction

Osteosarcoma is a high-grade malignant bone neoplasm that occurs primarily in children and adolescents. The principles of treatment of osteosarcoma have undergone dramatic changes in the past 20 years. Until recently, 5-year survival of 20% with surgical treatment alone was considered acceptable. This outcome suggested that 80% of the patients had pulmonary metastasis at the time of presentation [1]. Hence, chemotherapy is usually employed in an adjuvant situation to improve the prognosis and long-term survival. Recurrence usually occurs as pulmonary metastases or, less frequently, metastases to distant bones or as a local recurrence [2–4]. Thus, a novel strategy that would efficiently inhibit metastasis, especially to the lung, from the primary osteosarcoma site is highly desirable.

Decades of scrutiny into the molecular bases of cancer have largely focused on what causes oncogenic transformation and the incipient emergence of tumors [5]. The invasion of tumor cells is a complex, multistage process. To facilitate cell motility, invading cells need to change cell–cell adhesion properties, rearrange the extracellular matrix (ECM) environment, suppress anoikis and reorganize their cytoskeletons [6]. Cyclooxygenases (COXs) are the rate-limiting enzymes that catalyze the conversion of arachidonic acid to prostaglandins (PGs). COX-2 is an inducible enzyme and is activated by extracellular stimuli such as growth factors and pro-inflammatory cytokines [7]. Over-expression of COX-2 is frequently found in many types of cancer, including colon, lung, breast, pancreas, head, and neck cancers [8–10] and is usually associated with poor prognosis and short survival. Therefore, COX-2 may play a critical role in tumorigenesis, and its disruption may prevent metastasis.

NOV is a cysteine-rich protein that belongs to the CCN (Cyr61, CTGF, Nov) family of matrix cellular proteins, with developmental

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functions [11,12]. Recent studies have shown that the CCN protein family members also play important roles in tumorigenesis, including cancer cell proliferation, survival, adhesion, and invasion [13,14]. CCN proteins are mostly secreted and extracellular matrix associated and have been proposed to connect signaling pathways and facilitate cross talks between epithelium and stroma [11].

NOV molecule is widely expressed, especially in the nervous and musculoskeletal systems as well as in the blood vessels. The functions of NOV protein among these different tissues might, however, be very different. Although NOV was originally described as antiproliferative [15] and its expression was associated with differentiation and growth arrest in Wilm's tumor, chondrosarcomas and rhabdomyosarcomas [16,17], more recent data correlate NOV with increased proliferative index of 3T3 fibroblast and tissue samples of prostate and renal carcinomas [18,19]. In osteosarcoma, it is inversely associated with expression of liver/bone/kidney alkaline phosphatase isoform early marker of osteoblastic differentiation [20,21]. In Ewing's sarcoma, expression of NOV was only sporadically observed, but it was associated with a significantly higher risk of developing lung and bone metastasis [20].

Previous studies have shown that CCN1 (Cyr61) and CCN2 (CTGF) modulates cell migration and invasion in human cancer cells [22,23]. However, the effect of NOV on migration activity in human osteosarcoma cells is mostly unknown. Here we show that NOV increase migration and upregulate COX-2 expression in human osteosarcoma cells. In addition, αvβ5 integrin receptor, integrin-linked kinase (ILK), Akt and AP-1 signaling pathways were involved.

2. Materials and methods

2.1. Materials

Protein A/G beads, anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for β-actin, COX-2, p-Akt, Akt, ILK, p-c-Jun, c-Jun and the small interfering RNAs (siRNAs) against ILK, COX-2, c-Jun and control for experiments using targeted siRNA transfection (each consists of a scrambled sequence that will not lead to the specific degradation of any known cellular mRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The recombinant human NOV was purchased from PeproTech (Rocky Hill, NJ). Rabbit polyclonal antibodies specific for glycogen synthase kinase 3β (GSK3β) and p-GSK3β were purchased from Cell Signaling and Neuroscience (Danvers, MA). Mouse monoclonal antibodies specific for αvβ3 (clone LM609; MAB1976Z) and αvβ5 (clone P1F6; MAB1961Z) integrin was purchased from Chemicon (Temecula, CA). Akt inhibitor was purchased from Calbiochem (San Diego, CA). KP-392 was purchased from Kinetek Pharmaceuticals (Vancouver, Canada). The AP-1 driven luciferase plasmid was purchased from Stratagene (La Jolla, CA). The Akt (Akt K179A) dominant-negative mutant was a gift from Dr. W.M. Fu (National Taiwan University, Taipei, Taiwan). The COX-2 IPTG-induced expression plasmid, p-NLR-COX2 was a gift from Dr. M.L. Kuo (National Taiwan University). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell culture

The human osteosarcoma cell lines (U2OS, MG-63, HOS and G292) were purchased from the American Type Cell Culture Collection (Manassas, VA). The cells were maintained in RPMI-1640 medium which was supplemented with 20 mM HEPES and 10% heat-inactivated FCS, 2 mM-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C with 5% CO_2.

2.3. Migration assay

The migration assay was performed using Transwell (Costar, NY; pore size, 8-μm) in 24-well dishes. Before performing the migration assay, cells were pretreated for 30 min with different concentrations of inhibitors, including the KP392, Akt inhibitor or vehicle control (0.1% DMSO). Approximately 2×10^4 cells in 100 μl of serum-free RPMI-1640 medium were placed in the upper chamber, and 300 μl of the same medium containing NOV was placed in the lower chamber. The plates were incubated for 24 h at 37 °C in 5% CO_2, then cells were fixed in methanol for 15 min and stained with 0.05% crystal violet in PBS for 15 min. Cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were washed with PBS. Cells on the underside of the filters were examined and counted under a microscope. Each clone was plated in triplicate in each experiment, and each experiment was repeated at least three times [24].

2.4. Western blot analysis

The cellular lysates were prepared as described previously [25]. Proteins were resolved on SDS-PAGE and transferred to Immobilon polyvinyldifluoride (PVDF) membranes. The blots were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit anti-human antibodies against Akt, p-Akt or p-c-Jun (1:1000) for 1 h at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1000) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY). Quantitative data were obtained using a computing densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

2.5. ILK kinase assay

ILK enzymatic activity was assayed in osteosarcoma cells lysed in Nonidet P-40 buffer (0.5% sodiumdeoxycholate, 1% Nonidet P-40, 50 mM HEPES (pH 7.4), 150 mM NaCl) as previously reported [26]. Briefly, ILK was immunoprecipitated with ILK antibody overnight at 4 °C from 250 μg of lysate. After immunoprecipitation, beads were resuspended in 30 μl of kinase buffer containing 1 μg of recombinant substrate (GSK3β fusion protein) and 200 μM cold ATP, and the reaction was carried out for 30 min at 30 °C. The phosphorylated substrate was visualized by Western blot with phospho-GSK3β antibody. Total GSK3β was detected with the appropriate antibody [27].

2.6. Transfection and reporter gene assay

Human osteosarcoma cells were co-transfected with 0.8 μg AP-1 driven luciferase plasmid, 0.4 μg β-galactosidase expression vector. Cells were grown to 80% confluent in 12 well plates and were transfected on the following day by Lipofectamine 2000 (LF2000; Invitrogen). DNA and LF2000 were premixed for 20 min and then applied to the cells. RPMI-1640 containing 20% FCS was added 4 h later. After 24 h transfection, the cells were then incubated with the indicated agents. After further 24 h incubation, the media were removed, and cells were washed once with cold PBS. To prepare lysates, 100 μl reporter lysis buffer (Promega, Madison, WI) was added to each well, and cells were scraped from dishes. The supernatant was collected after centrifugation at 11,000 × *g* for 2 min. Aliquots of cell lysates (20 μl) containing equal amounts of protein (20–30 μg) were placed into wells of an opaque black 96-well microplate. An equal volume of luciferase substrate was added to all samples, and luminescence was

measured in a microplate luminometer. The value of luciferase activity was normalized to transfection efficiency monitored by the co-transfected β-galactosidase expression vector.

2.7. Quantitative real time PCR

Total RNA was extracted from osteosarcomas by using a TRIzol kit (MDBio Inc., Taipei, Taiwan). Two μg of total RNA was reverse transcribed into cDNA by using oligo(dT) primer. The quantitative real time PCR (qPCR) analysis was carried out using Taqman® one-step PCR Master Mix (Applied Biosystems, CA). Two μl of cDNA were added per 25-μl reaction with sequence-specific primers and Taqman® probes. Sequences for all target gene primers and probes were purchased commercially (GAPDH was used as internal control) (Applied Biosystems, CA). qPCR assays were carried out in triplicate on an StepOnePlus sequence detection system. The cycling conditions were 10-min polymerase activation at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. The

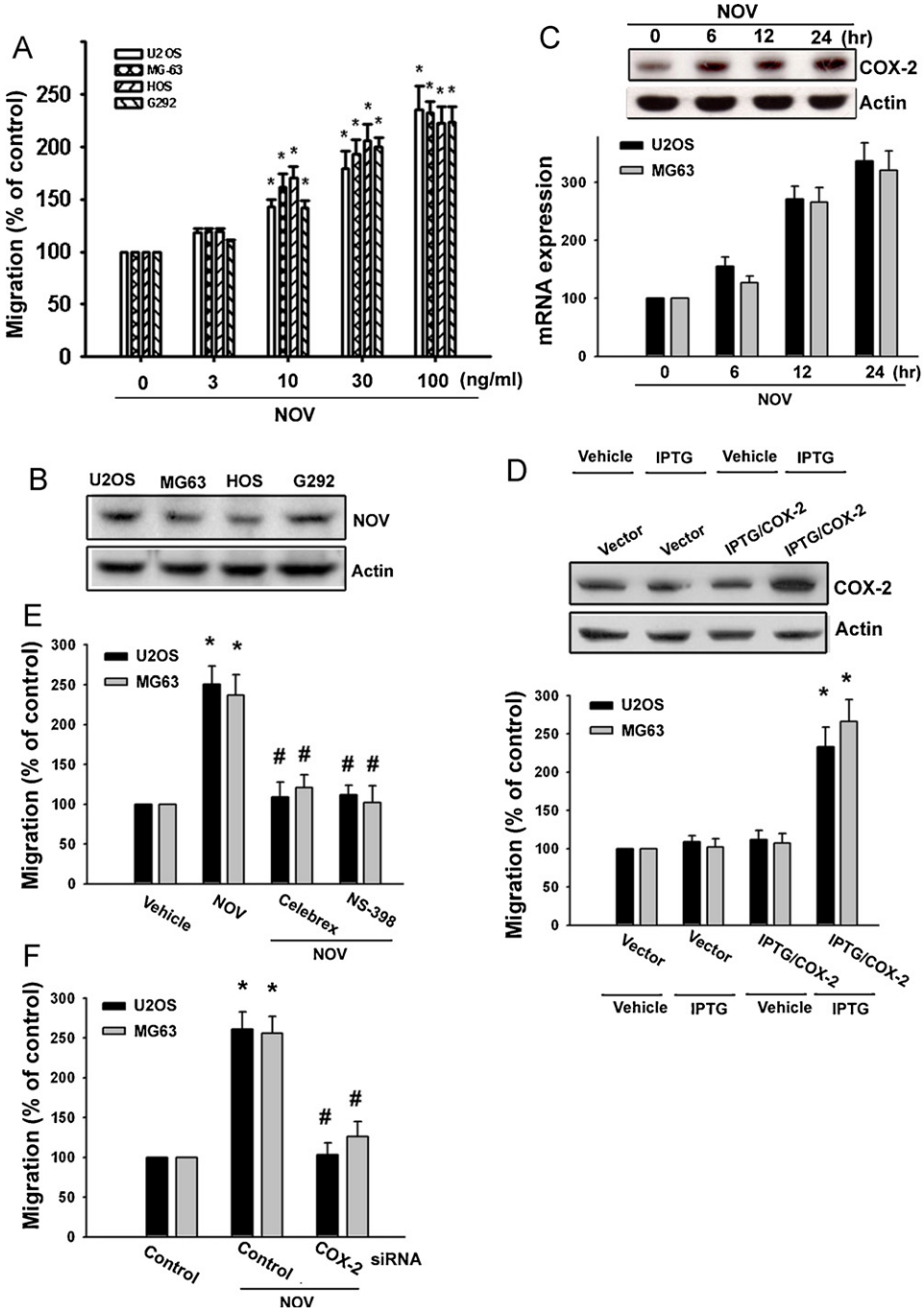

Fig. 1. NOV induced migration and COX-2 expression in human osteosarcoma cells. (A) Cells were incubated with various concentrations of NOV, and *in vitro* migration activities measured with the Transwell after 24 h ($n = 5$). (B) The NOV expression from osteosarcoma cell lines by using Western blot ($n = 4$). (C) Cells were incubated with NOV (100 ng/ml) for indicated time intervals, and COX-2 expression was examined by Western blot and qPCR ($n = 4$). (D) Cells were transfected with IPTG/COX-2 expression plasmid or control vector for 24 h followed by stimulation with IPTG (5 mM) for 24 h, the COX-2 expression and migration activity were determined by Western blot and Transwell ($n = 4$). (E) Cells were and pretreated with celebrex (10 μM) or NS-398 (20 μM) for 30 min followed by stimulation with NOV, and *in vitro* migration was measured with the Transwell after 24 h ($n = 4$). (F) Cells were transfected with COX-2 siRNA for 24 h followed by stimulation with NOV, and *in vitro* migration measured with the Transwell after 24 h ($n = 4$). Results are expressed as the mean ± S.E. *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with NOV-treated group.

threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted C_T).

2.8. Electrophoretic mobility shift assay (EMSA)

EMSA was performed by using EMSA 'gel shift' kit (Panomics, Fremont, CA) according to the manufacturer's protocol. Oligonucleotides corresponding to canonical human COX-2 promoter AP-1 binding sequence (5′- AGAAACAGTCATTTC-3′) was used. Cells nuclear extract (3 μg) was incubated with poly d(I–C) at room temperature for 5 min. The nuclear extract was then incubated with biotin-labeled probes and the incubated at RT for 30 min. After electrophoresis on a 6% polyacrylamide gel, the samples on gel were transferred onto a presoaked immobilon-Ny+ membrane (Millipore, Billerica, MA, USA). The membrane was baked at 80 °C for 1 h, crosslinked in an oven for 3 min, and then developed by adding the blocking buffer and streptavidin-HRP conjugate and then subjected to Western blot analysis.

2.9. Statistics

The values given are means ± S.E.M. The significance of difference between the experimental groups and controls was assessed by Student's t test. The difference is significant if the p value is <0.05.

3. Results

3.1. NOV-directed osteosarcoma cells migration through COX-2 upregulation

Previous studies have shown that NOV regulates cell migration and invasion in human cancer cells [28,29]. However, the effect of NOV in migration of osteosarcoma is mostly unknown. NOV-triggered migration in osteosarcoma cells was examined by using the Transwell assay. NOV directed human osteosarcoma cells (U2OS, MG-63, HOS and G292 cells) migration (Fig. 1A). On the other hand, NOV did not induce proliferation and apoptosis of osteosarcoma cells (Supplementary Fig. S1). However, treatment of

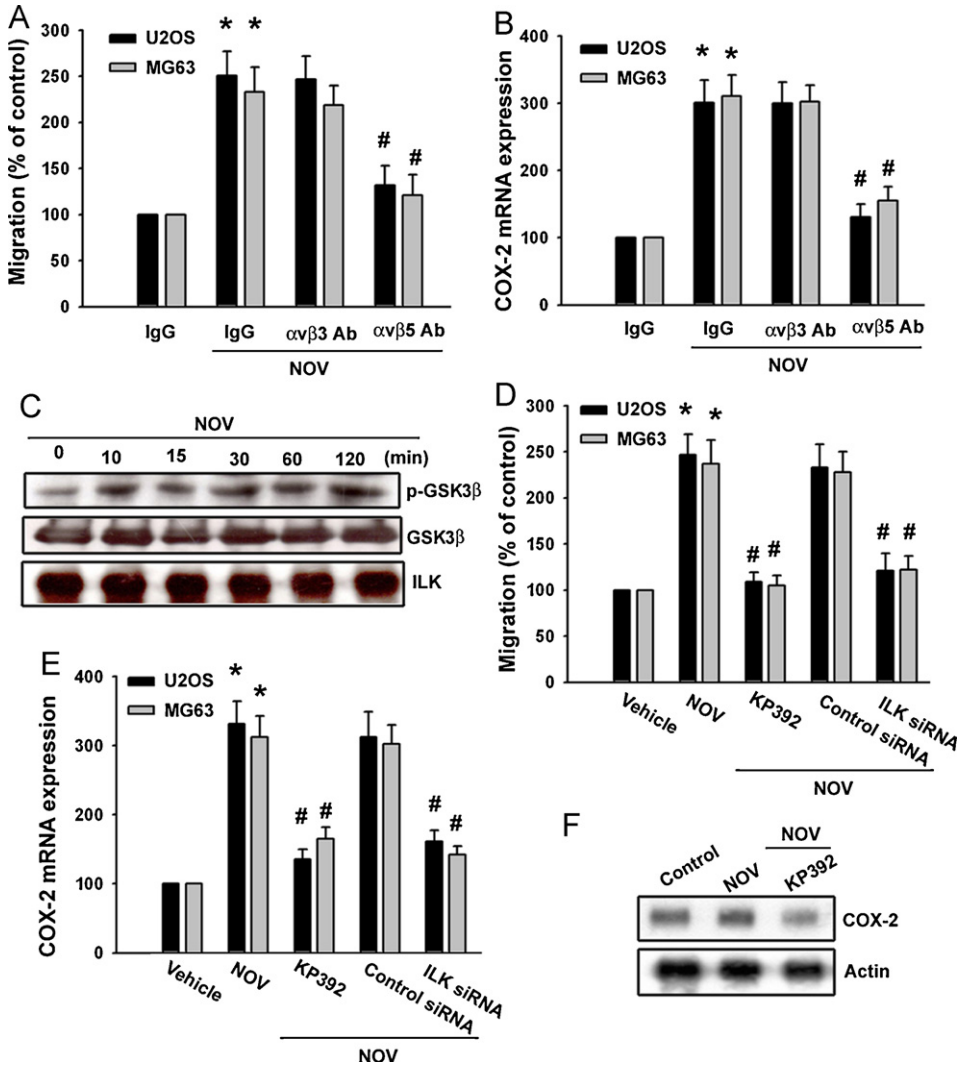

Fig. 2. NOV induced osteosarcoma cell migration through αvβ5 integrin. (A and B) Cells were and pretreated with αvβ3 and αvβ5 monoclonal antibody (10 μg/ml) for 30 min followed by stimulation with NOV. The cell migration and COX-2 expression were measured with the Transwell and qPCR ($n = 4$). (C) MG63 cells were exposed to NOV for indicated time intervals, and cell lysates were immunoprecipitated with an antibody specific for ILK. Immunoprecipitated proteins were separated by SDS-PAGE and immunoblotted with anti-pGSK3β or GSK3β ($n = 4$). (D–F) Cells were and pretreated with KP-392 (10 μM) for 30 min or transfected with ILK siRNA for 24 h followed by stimulation with NOV. The cell migration and COX-2 expression were measured with the Transwell, qPCR and Western blot ($n = 4$). Results are expressed as the mean ± S.E. $^{*}p < 0.05$ compared with control; $^{\#}p < 0.05$ compared with NOV-treated group.

osteoblastic cells (MC3T3-E1) with NOV (100 ng/ml) did not induce migration activity (data not shown). We next examined human osteosarcoma cell lines for expression of the NOV using Western blot. We found that human osteosarcoma cell lines expressed the protein level of NOV (Fig. 1B). Previous study has shown that COX-2 mediated cell motility in human cancer cells [30,31]. We therefore, hypothesized that COX-2 may be involved in NOV-mediated osteosarcoma migration. Treatment of osteosarcoma with NOV enhanced mRNA and protein expression of COX-2 (Fig. 1C). We used an IPTG-inducible COX-2 gene expression vector to examine the role of COX-2 in osteosarcoma cells. U2OS cells were transfected with IPTG-inducible COX-2 gene expression vector or a control vector, and then IPTG (5 mM) was added for 24 h. Using Western blot analysis, we found that IPTG induced COX-2 expression (Fig. 1D; upper panel). Furthermore, over-expression of COX-2 enhanced cell migration in osteosarcoma cells (Fig. 1D; lower panel). To confirm COX-2 mediated NOV-induced cell migration, the COX-2 specific inhibitors (Celebrex and NS-398) were used. Celebrex and NS-398 reduced NOV-enhanced cell migration (Fig. 1E). In addition, transfection of cells with COX-2 siRNA also reduced NOV-increased cell migration (Fig. 1F). These data suggest that NOV-induced cancer migration may occur via up-regulation of the COX-2.

3.2. NOV-directed osteosarcoma cell migration through αvβ5 integrin

Previous study has shown that CCN affects cells migration through binding to cell surface integrin receptors [22]. We therefore, hypothesized that integrin-signaling pathway may be involved in NOV-directed migration of osteosarcoma cells. Pretreatment of cells for 30 min with anti-αvβ5 but not αvβ3 monoclonal antibody (mAb) markedly inhibited the NOV-induced cancer migration (Fig. 2A). On the other hand, pretreatment of cells with αvβ5 but not αvβ3 mAb reduced NOV-mediated COX-2 expression (Fig. 2B). These data suggest that NOV-induced cancer migration may occur via activation of the αvβ5 integrin.

3.3. ILK and Akt signaling pathways are involved in the NOV-mediated COX-2 upregulation and migration of osteosarcoma

ILK has been shown to be capable of regulating integrin-mediated signaling [32]. We directly measured the ILK kinase activity in response to NOV stimulation by the immunoprecipitation of ILK from lysates. Fig. 2C shows that NOV exposure in osteosarcoma time-dependently increased ILK kinase activity, assessing the phosphorylation of the recombinant GSK3β on Ser9. To further explore whether ILK is involved in NOV-induced cell migration and COX-2 expression, ILK inhibitor KP-392 or ILK siRNA were used. As shown in Fig. 2D–F, pretreatment of osteosarcoma with KP-392 inhibited NOV-induced cell migration and COX-2 expression. Transfection of osteosarcoma with ILK siRNA also antagonized the potentiating effect of NOV (Fig. 2D and E). Therefore, ILK plays an important role in NOV-induced cell migration and COX-2 expression. It has been reported that ILK is an upstream regulator of the phosphorylation of Akt on Ser473 [33], we then examined whether NOV stimulation also enhances the association of ILK with Akt. Stimulation of osteosarcoma cells with NOV increased Akt phosphorylation time dependently (Fig. 3A). Pretreatment of cells for 30 min with Akt inhibitor inhibited the NOV-induced cell migration and COX-2 expression (Fig. 3B–D). Transfection of cells with Akt mutant also reduced NOV-mediated cell migration and COX-2 expression (Fig. 3B and C). Taken together, these results indicate that the ILK and Akt pathways are involved in NOV-induced migration activity and COX-2 up-regulation in human osteosarcoma cells.

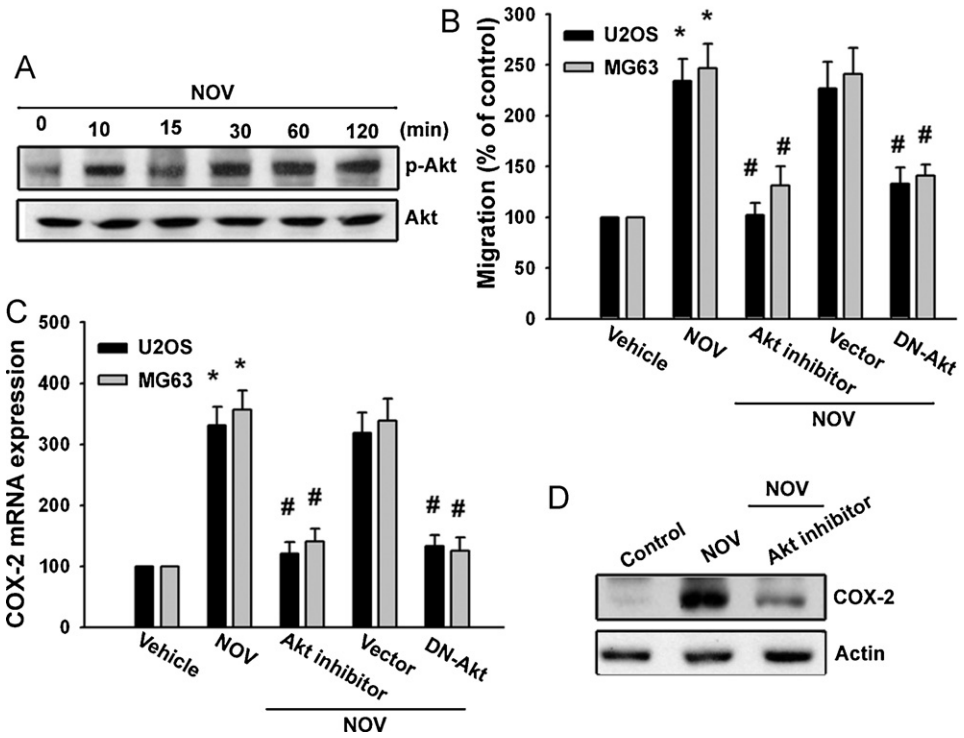

Fig. 3. Akt is involved in NOV-mediated migration and COX-2 expression in osteosarcoma. (A) MG63 cells were incubated with NOV for indicated time intervals, and p-Akt expression was determined by Western blot ($n = 4$). (B–D) Cells were and pretreated with Akt inhibitor (10 μM) for 30 min or transfected with Akt mutant for 24 h followed by stimulation with NOV. The cell migration and COX-2 expression were measured with the Transwell, qPCR and Western blot ($n = 4$). Results are expressed as the mean ± S.E. $^{*}p < 0.05$ compared with control; $^{\#}p < 0.05$ compared with NOV-treated group.

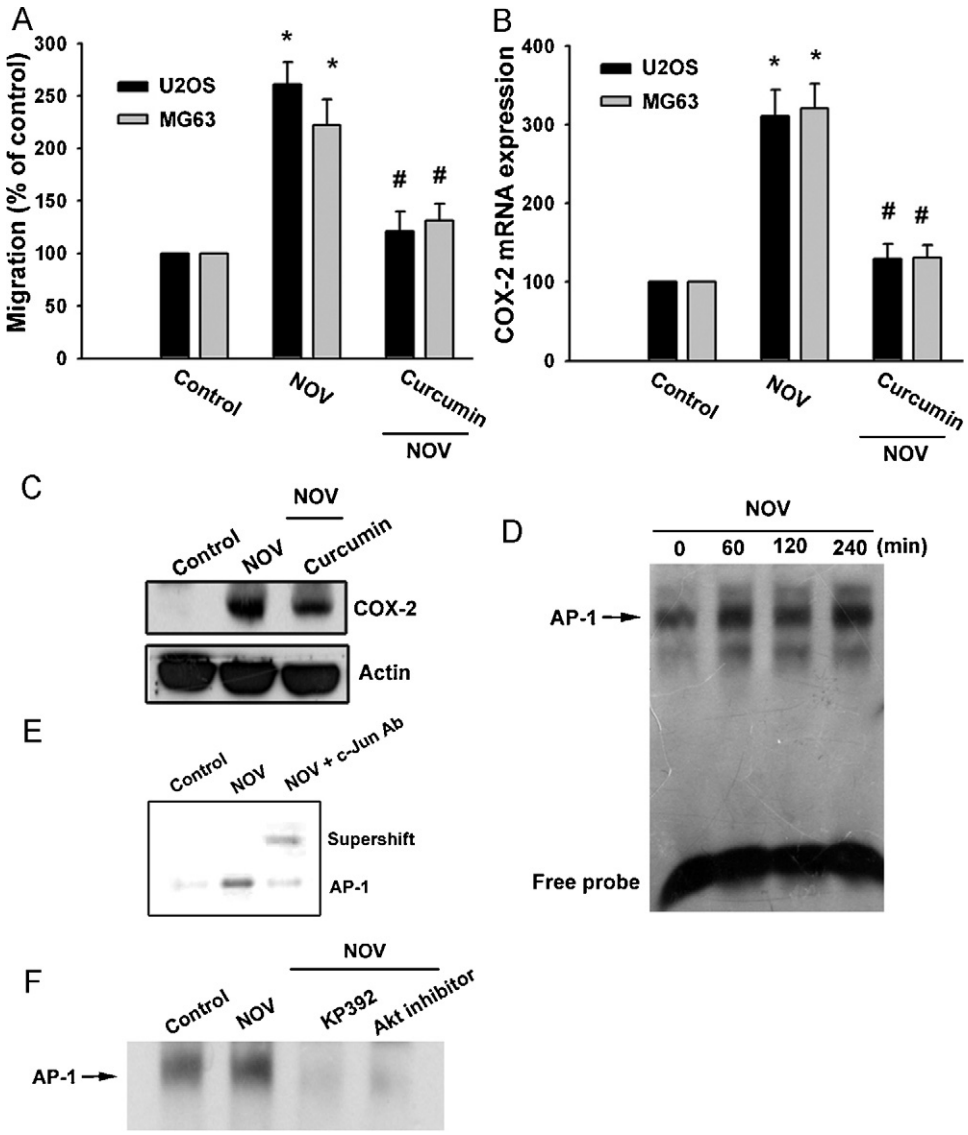

Fig. 4. AP-1 is involved in NOV-mediated migration in human osteosarcoma. (A–C) Cells were and pretreated with curcumin (10 μM) for 30 min followed by stimulation with NOV. The cell migration and COX-2 expression were measured with the Transwell, qPCR and Western blot ($n = 5$). (D and F) MG63 cells were exposed to NOV for indicated time intervals or pretreated for 30 min with KP392 or Akt inhibitor followed by stimulation with NOV for 240 min, and electrophoretic mobility shift assay was performed as described in Section 2 ($n = 4$). (E) Nuclear extracts from MG63 cells exposed to NOV for 120 min, and c-Jun binding to AP-1 element was examined by EMSA analysis. Preincubation of c-Jun antibody (Ab, 5 μg) with nuclear extracts isolated from cells exposed to NOV induced the supershift of AP-1 DNA complex. Results are expressed as the mean ± S.E. $^{*}p < 0.05$ compared with control; $^{\#}p < 0.05$ compared with NOV-treated group.

3.4. Involvement of AP-1 in NOV-induced cell migration and COX-2 expression

AP-1 plays a critical role in COX-2 expression [34]. To examine the role of the AP-1 binding site in NOV-mediated migration and COX-2 expression, an AP-1 inhibitor (curcumin) was used. Pretreatment of cells with curcumin inhibited NOV-enhanced cell migration and COX-2 expression (Fig. 4A–C). AP-1 activation was further evaluated by analyzing the electrophoretic mobility shift assay, as well as by the phosphorylation of c-Jun. Stimulation of cells with NOV resulted activation of AP-1 specific DNA–protein complex formation (Fig. 4D). To identify the specific subunit involved in the formation of the AP-1 complex, supershift assay was performed using antibody specific for anti-c-Jun. Incubation of nuclear extracts with anti-c-Jun antibody increased supershift of AP-1 DNA–protein complex (Fig. 4E). Pretreatment of cells with KP392 and Akt inhibitor reduced NOV-mediated AP-1-specific DNA–protein complex formation (Fig. 4F). Transfection of cells with c-Jun siRNA suppressed the cell migration and COX-2 expression (Fig. 5A–C). Treatment of cells with NOV resulted in a marked phosphorylation of c-Jun (Fig. 5D). To directly determine AP-1 activation after NOV treatment, cells were transiently transfected with AP-1 driven luciferase as an indicator of AP-1 activation. As shown in Fig. 5E, NOV treatment of cells for 24 h increased in AP-1 driven luciferase activity. In addition, αvβ5 mAb, KP392 and Akt inhibitor antagonized the NOV-induced AP-1 driven luciferase activity (Fig. 5E). Co-transfection of cells with ILK siRNA, Akt mutant and c-Jun siRNA also reduced NOV-increased AP-1 driven luciferase activity (Fig. 5F). Taken together, these data suggest that activation of αvβ5 integrin, ILK, Akt and c-Jun are required for NOV-induced AP-1 activation in human osteosarcoma cells.

4. Discussion

Osteosarcoma is a debilitating, though not always fatal, high-grade malignant bone neoplasm that targets children and adolescents. The chemotherapies are not fully effective, and as a

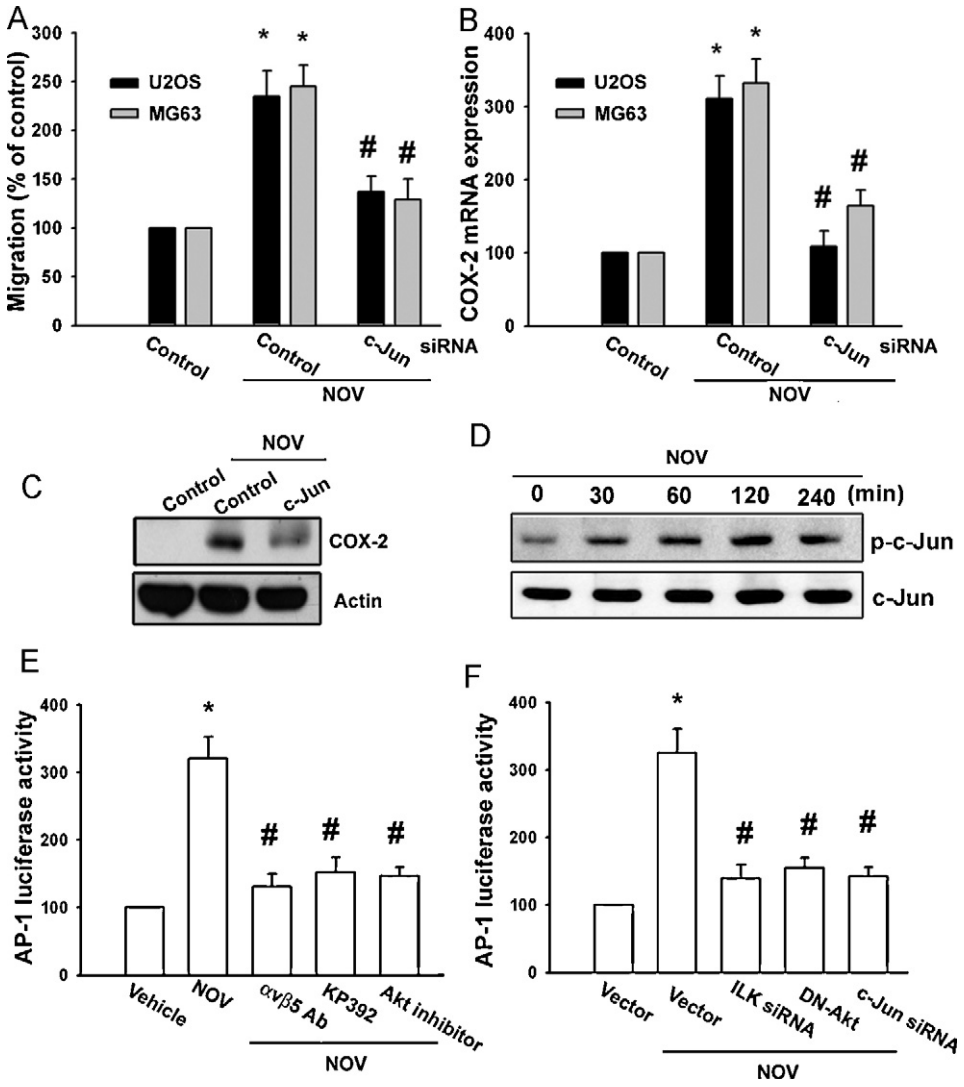

Fig. 5. Integrin/ILK/Akt pathway is mediated NOV-induced AP-1 activation. (A–C) Cells were transfected with c-Jun siRNA for 24 h followed by stimulation with NOV. The cell migration and COX-2 expression were measured with the Transwell, qPCR and Western blot ($n = 5$). (D) MG63 cells were incubated with NOV for indicated time intervals, and p-c-Jun expression was determined by Western blot ($n = 4$). (E and F) MG63 cells were pretreated with αvβ5 mAb, KP392 and Akt inhibitor for 30 min or co-transfected with ILK siRNA, Akt mutant and, c-Jun siRNA before exposure to NOV. The AP-1 driven luciferase activity was measured, and the results were normalized to the β-galactosidase activity and expressed as the mean ± S.E. for three independent experiments performed in triplicate. Results are expressed as the mean ± S.E. $^{*}p < 0.05$ compared with control; $^{\#}p < 0.05$ compared with NOV-treated group.

result, 20% of all patients die due to metastasis of osteosarcoma cells to the lungs [3]. Therefore, it is important to develop effective adjuvant therapy for preventing osteosarcoma metastasis. We hypothesized that NOV and its receptor would help to direct the migration of osteosarcoma cells. We found that NOV increased cell migration in human osteosarcoma cell lines. However, NOV induces only a 2.5-fold increases in migration of osteosarcoma cells. In our previous study, we found a maximum 3-fold increase in migration of chondrosarcoma cells induced by another chemokine (SDF-1) [35]. Therefore, the maximum increase in migration of osteosarcoma cells is ∼2- to 3-fold. One of the mechanisms underlying NOV directed migration was transcriptional upregulagtion of COX-2 and activation of αvβ5 integrin, ILK, Akt and AP-1 pathways. Due to the presented data we recommend the COX-2 selective inhibitor may possible treatment regime for the prevention of osteosarcoma metastasis.

COX-2 is a pleiotropic enzyme that mediates many physiological functions such as inhibition of cell apoptosis, augmentation of angiogenesis, and increased cell motility. It has been reported that the expression of COX-2 is associated with a metastatic phenotype of human cancer cells [30,31]. In this study, we found that overexpression COX-2 induced cell motility of osteosarcoma. Treatment of cells with NOV increased COX-2 expression in human osteosarcoma. On the other hand, COX-2 inhibitors antagonized NOV-reduced cell motility. In addition, the inhibition of NOV-induced COX-2 protein expression with siRNA significantly reversed NOV-inhibited migration. Besides, NOV also increased PGD2, PGI2 and PGE2 production in osteosarcoma cells (Supplementary Fig. S2). Therefore, COX-2-producted PGs may be the NOV-responsive mediator, and lead to enhance cancer migration and metastasis.

ILK, a potential candidate signaling molecule, has been shown to be capable of regulating integrin-mediated signaling [32]. ILK can interact with the cytoplasmic domain of β-integrin subunits and is activated by both integrin activation as well as growth factors and is an upstream regulator of Akt [36]. The current study showed that NOV stimulation increased kinase activity of ILK. Treatment with the ILK inhibitor of KP-392 inhibited NOV-induced cell migration and COX-2 expression. Furthermore, the ILK siRNA also antagonized the NOV-mediated potentiation of migration and

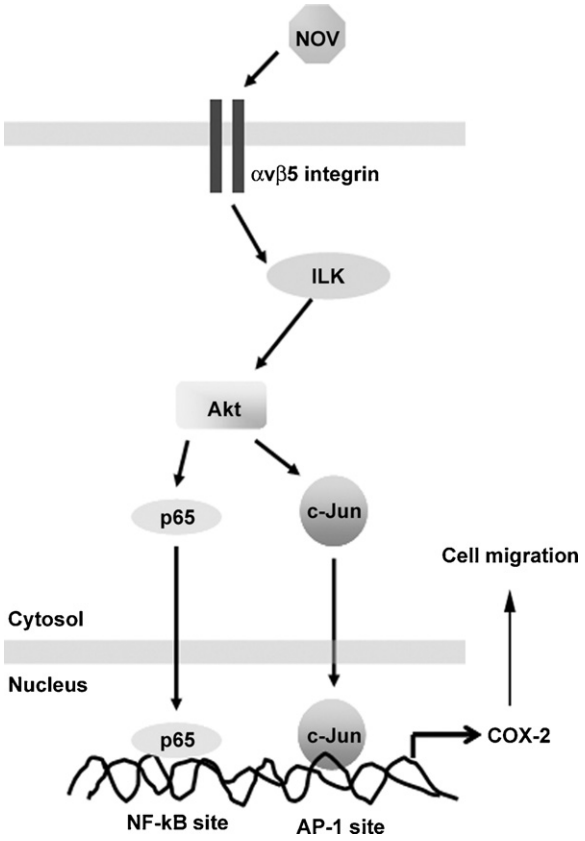

Fig. 6. Schematic diagram of the signaling pathways involved in NOV-induced cell migration and COX-2 expression in osteosarcoma cells. NOV and αvβ5 integrin interaction activates ILK and Akt pathways, which in turn induces AP-1/NF-κB activation, which leads to COX-2 expression and increases the migration of human osteosarcoma cells.

COX-2 expression. Therefore, ILK activation is involved in NOV-induced cell migration and COX-2 expression in osteosarcoma. ILK possibly regulated the cell function by promoting the phosphorylation of Akt on Ser473 [37]. Our results demonstrate that pretreatment of osteosarcoma with Akt inhibitor antagonized the increase of cell migration and COX-2 expression under NOV stimulation. This was further confirmed by the result that the dominant negative mutant of Akt inhibited the enhancement of cell migration and COX-2 expression under NOV stimulation. Here we also found that the cytoplasmic kinase Akt was activated by NOV stimulation in osteosarcoma. These effects implicate the involvement of ILK and Akt activation in NOV-mediated induction of cell migration and COX-2 expression.

It has been reported that chemokines induced COX-2 expression through AP-1 dependent pathway [34,38]. The results of this study show that AP-1 activation contributes to NOV-induced migration and COX-2 expression in osteosarcoma cells. The AP-1 sequence binds to members of the Jun and Fos families of transcription factors. These nuclear proteins interact with the AP-1 site as Jun homodimers or Jun-Fos heterodimers formed by protein dimerization through their leucine zipper motifs. The results of this study show that NOV induced c-Jun phosphorylation. In addition, c-Jun siRNA abolished the NOV-induced cell migration and COX-2 expression in osteosarcoma cells. Furthermore, NOV also increased the AP-1 specific DNA–protein complex formation. The AP-1 specific DNA–protein complex formation was attenuated by KP392 and Akt inhibitor. On the other hand, KP392, Akt inhibitor and curcumin or ILK siRNA and Akt mutant reduced NOV-mediated AP-1 promoter activity. These results indicate that NOV might act through the ILK, Akt, c-Jun, and AP-1 pathway to induce COX-2 activation in human osteosarcoma cells. NF-κB is reported to be the main transcriptional factor involved in COX-2 mRNA transcription [39]. We also found that stimulation of cells with NOV increased the accumulation of p65 in the nucleus (Supplementary Fig. S3). Therefore, NF-κB also participate the NOV-mediated migration and COX-2 expression in human osteosarcoma.

Due to the prognosis of patients with osteosarcoma distant metastasis is generally considered as very poor. Thus, preventing human osteosarcoma metastasis is an important issue nowadays. Our study presents that NOV increases the expression of COX-2 via αvβ5 integrin, ILK, Akt, c-Jun/p65, and AP-1/NFκB-dependent pathway and increasing migration of human osteosarcoma cells (Fig. 6). Furthermore, the discovery of NOV-mediated pathway helps us to understand the mechanism of human osteosarcoma metastasis and may help us to develop effective therapy in the future.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.12.005.

References

[1] Ek ET, Choong PF. The role of high-dose therapy and autologous stem cell transplantation for pediatric bone and soft tissue sarcomas. Expert Rev Anticancer Ther 2006;6:225–37.
[2] Kempf-Bielack B, Bielack SS, Jurgens H, Branscheid D, Berdel WE, Exner GU, et al. Osteosarcoma relapse after combined modality therapy: an analysis of unselected patients in the Cooperative Osteosarcoma Study Group (COSS). J Clin Oncol 2005;23:559–68.
[3] Arndt CA, Crist WM. Common musculoskeletal tumors of childhood and adolescence. N Engl J Med 1999;341:342–52.
[4] Bacci G, Ferrari S, Longhi A, Perin S, Forni C, Fabbri N, et al. Pattern of relapse in patients with osteosarcoma of the extremities treated with neoadjuvant chemotherapy. Eur J Cancer 2001;37:32–8.
[5] Caswell PT, Vadrevu S, Norman JC. Integrins: masters and slaves of endocytic transport. Nat Rev Mol Cell Biol 2009;10:843–53.
[6] Tsuji T, Ibaragi S, Hu GF. Epithelial-mesenchymal transition and cell cooperativity in metastasis. Cancer Res 2009;69:7135–9.
[7] Turini ME, DuBois RN. Cyclooxygenase-2: a therapeutic target. Annu Rev Med 2002;53:35–57.
[8] Sano H, Kawahito Y, Wilder RL, Hashiramoto A, Mukai S, Asai K, et al. Expression of cyclooxygenase-1 and -2 in human colorectal cancer. Cancer Res 1995;55: 3785–9.
[9] Hida T, Yatabe Y, Achiwa H, Muramatsu H, Kozaki K, Nakamura S, et al. Increased expression of cyclooxygenase 2 occurs frequently in human lung cancers, specifically in adenocarcinomas. Cancer Res 1998;58:3761–4.
[10] Hwang D, Scollard D, Byrne J, Levine E. Expression of cyclooxygenase-1 and cyclooxygenase-2 in human breast cancer. J Natl Cancer Inst 1998;90:455–60.
[11] Holbourn KP, Acharya KR, Perbal B. The CCN family of proteins: structure–function relationships. Trends Biochem Sci 2008;33:461–73.
[12] Perbal B. NOV (nephroblastoma overexpressed) and the CCN family of genes: structural and functional issues. Mol Pathol 2001;54:57–79.
[13] Kleer CG, Zhang Y, Pan Q, Merajver SD, WISP3. (CCN6) is a secreted tumor-suppressor protein that modulates IGF signaling in inflammatory breast cancer. Neoplasia 2004;6:179–85.
[14] Kleer CG, Zhang Y, Pan Q, van Golen KL, Wu ZF, Livant D, et al. WISP3 is a novel tumor suppressor gene of inflammatory breast cancer. Oncogene 2002;21: 3172–80.
[15] Joliot V, Martinerie C, Dambrine G, Plassiart G, Brisac M, Crochet J, et al. Proviral rearrangements and overexpression of a new cellular gene (nov) in myeloblastosis-associated virus type 1-induced nephroblastomas. Mol Cell Biol 1992;12:10–21.
[16] Chevalier G, Yeger H, Martinerie C, Laurent M, Alami J, Schofield PN, et al. novH: differential expression in developing kidney and Wilm's tumors. Am J Pathol 1998;152:1563–75.
[17] Yu C, Le AT, Yeger H, Perbal B, Alman BA. NOV (CCN3) regulation in the growth plate and CCN family member expression in cartilage neoplasia. J Pathol 2003;201:609–15.

[18] Liu C, Liu XJ, Crowe PD, Kelner GS, Fan J, Barry G, et al. Nephroblastoma overexpressed gene (NOV) codes for a growth factor that induces protein tyrosine phosphorylation. Gene 1999;238:471–8.
[19] Maillard M, Cadot B, Ball RY, Sethia K, Edwards DR, Perbal B, et al. Differential expression of the ccn3 (nov) proto-oncogene in human prostate cell lines and tissues. Mol Pathol 2001;54:275–80.
[20] Manara MC, Perbal B, Benini S, Strammiello R, Cerisano V, Perdichizzi S, et al. The expression of ccn3(nov) gene in musculoskeletal tumors. Am J Pathol 2002;160:849–59.
[21] Stein GS, Lian JB, Owen TA. Relationship of cell growth to the regulation of tissue-specific gene expression during osteoblast differentiation. FASEB J 1990;4:3111–23.
[22] Tan TW, Yang WH, Lin YT, Hsu SF, Li TM, Kao ST, et al. Cyr61 increases migration and MMP-13 expression via alphavbeta3 integrin, FAK, ERK and AP-1-dependent pathway in human chondrosarcoma cells. Carcinogenesis 2009;30:258–68.
[23] Tan TW, Lai CH, Huang CY, Yang WH, Chen HT, Hsu HC, et al. CTGF enhances migration and MMP-13 up-regulation via alphavbeta3 integrin, FAK, ERK, and NF-kappaB-dependent pathway in human chondrosarcoma cells. J Cell Biochem 2009;107:345–56.
[24] Chuang JY, Yang WH, Chen HT, Huang CY, Tan TW, Lin YT, et al. CCL5/CCR5 axis promotes the motility of human oral cancer cells. J Cell Physiol 2009;220: 418–26.
[25] Chiu YC, Lin CY, Chen CP, Huang KC, Tong KM, Tzeng CY, et al. Peptidoglycan enhances IL-6 production in human synovial fibroblasts via TLR2 receptor, focal adhesion kinase, Akt, and AP-1- dependent pathway. J Immunol 2009;183:2785–92.
[26] Tseng WP, Yang SN, Lai CH, Tang CH. Hypoxia induces BMP-2 expression via ILK, Akt, mTOR, and HIF-1 pathways in osteoblasts. J Cell Physiol 2010;223:810–8.
[27] Tang CH, Lu DY, Tan TW, Fu WM, Yang RS. Ultrasound induces hypoxia-inducible factor-1 activation and inducible nitric-oxide synthase expression through the integrin/integrin-linked kinase/Akt/mammalian target of rapamycin pathway in osteoblasts. J Biol Chem 2007;282:25406–15.
[28] Benini S, Perbal B, Zambelli D, Colombo MP, Manara MC, Serra M, et al. In Ewing's sarcoma CCN3(NOV) inhibits proliferation while promoting migration and invasion of the same cell type. Oncogene 2005;24:4349–61.
[29] Sin WC, Bechberger JF, Rushlow WJ, Naus CC. Dose-dependent differential upregulation of CCN1/Cyr61 and CCN3/NOV by the gap junction protein Connexin43 in glioma cells. J Cell Biochem 2008;103:1772–82.
[30] Yang SF, Chen MK, Hsieh YS, Chung TT, Hsieh YH, Lin CW, et al. PGE2/EP1 signaling pathway enhances ICAM-1 expression and cell motility in oral cancer cells. J Biol Chem 2010.
[31] Liu JF, Fong YC, Chang CS, Huang CY, Chen HT, Yang WH, et al. Cyclooxygenase-2 enhances alpha2beta1 integrin expression and cell migration via EP1 dependent signaling pathway in human chondrosarcoma cells. Mol Cancer 2010;9:43.
[32] Hannigan GE, Leung-Hagesteijn C, Fitz-Gibbon L, Coppolino MG, Radeva G, Filmus J, et al. Regulation of cell adhesion and anchorage-dependent growth by a new beta 1-integrin-linked protein kinase. Nature 1996;379:91–6.
[33] Troussard AA, Mawji NM, Ong C, Mui A, St-Arnaud R, Dedhar S. Conditional knock-out of integrin-linked kinase demonstrates an essential role in protein kinase B/Akt activation. J Biol Chem 2003;278:22374–8.
[34] Lu DY, Leung YM, Huang SM, Wong KL. Bradykinin-induced cell migration and COX-2 production mediated by the bradykinin B1 receptor in glioma cells. J Cell Biochem 2010;110:141–50.
[35] Huang CY, Lee CY, Chen MY, Yang WH, Chen YH, Chang CH, et al. Stromal cell-derived factor-1/CXCR4 enhanced motility of human osteosarcoma cells involves MEK1/2, ERK and NF-kappaB-dependent pathways. J Cell Physiol 2009;221:204–12.
[36] Wu C, Dedhar S. Integrin-linked kinase (ILK) and its interactors: a new paradigm for the coupling of extracellular matrix to actin cytoskeleton and signaling complexes. J Cell Biol 2001;155:505–10.
[37] Persad S, Attwell S, Gray V, Mawji N, Deng JT, Leung D, et al. Regulation of protein kinase B/Akt-serine 473 phosphorylation by integrin-linked kinase: critical roles for kinase activity and amino acids arginine 211 and serine 343. J Biol Chem 2001;276:27462–9.
[38] Chien MH, Ku CC, Johansson G, Chen MW, Hsiao M, Su JL, et al. Vascular endothelial growth factor-C (VEGF-C) promotes angiogenesis by induction of COX-2 in leukemic cells via the VEGF-R3/JNK/AP-1 pathway. Carcinogenesis 2009;30:2005–13.
[39] Yeoh A, Gibson R, Yeoh E, Bowen J, Stringer A, Giam K, et al. Radiation therapy-induced mucositis: relationships between fractionated radiation, NF-kappaB, COX-1, and COX-2. Cancer Treat Rev 2006;32:645–51.