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Epigenetic regulation of integrin-linked kinase expression depending on adhesion of gastric carcinoma cells

Yong-Bae Kim,[1] Sung-Yul Lee,[2] Sang-Kyu Ye,[3] and Jung Weon Lee[1,2]

Cancer Research Institute, Departments of [1]Tumor Biology, [2]Molecular and Clinical Oncology, and [3]Pharmacology, College of Medicine, Seoul National University, Seoul, Korea

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Kim YB, Lee SY, Ye SK, Lee JW. Epigenetic regulation of integrin-linked kinase expression depending on adhesion of gastric carcinoma cells. *Am J Physiol Cell Physiol* 292: C857–C866, 2007. First published September 20, 2006; doi:10.1152/ajpcell.00169.2006.—Cell adhesion to the extracellular matrix (ECM) regulates gene expressions in diverse dynamic environments. However, the manner in which gene expressions are regulated by extracellular cues is largely unknown. In this study, suspended gastric carcinoma cells showed higher basal and transforming growth factor-β1 (TGFβ1)-mediated acetylations of histone 3 (H3) and Lys[9] of H3 and levels of integrin-linked kinase (ILK) mRNA and protein than did fibronectin-adherent cells did. Moreover, the insignificant acetylation and ILK expression in adherent cells were recovered by alterations of integrin signaling and actin organization, indicating a connection between cytoplasmic and nuclear changes. Higher acetylations in suspended cells were correlated with associations between Smad4, p300/CBP, and Lys[9]-acetylated H3. Meanwhile, adherent cells showed more associations between HDAC3, Ski, and MeCP2. Chromatin immunoprecipitations with anti-acetylated H3, Lys[9]-acetylated H3, or p300/CBP antibody resulted in more coprecipitated ILK promoter, correlated with enhanced ILK mRNA and protein levels, in suspended cells. Moreover, ILK expression inversely regulated cell adhesion to ECM proteins, and its overexpression enhanced cell growth in soft agar. These observations indicate that cell adhesion and/or its related molecular basis regulate epigenetic mechanisms leading to a loss of ILK transcription, which in turn regulates cell adhesion property in a feedback linkage.

cell adhesion; integrin-linked kinase; histone acetylation; gastric cancer

THE REGULATION of integrin-mediated engagements to extracellular matrix (ECM) allows cells to adhere and spread or to detach. During these processes, cells need diverse molecules to maintain cellular homeostasis. To achieve this, cells ensure that certain genes are induced or suppressed. In addition to gene regulation (4, 44), integrin-mediated cell adhesion also triggers diverse intracellular signal transductions that lead to the regulations of the activities and localizations of diverse signaling molecules and thus of cellular behaviors (8, 10, 31, 35).

Cancer cells due to multiple mutations and genomic instabilities may disseminate from a primary tumor cell body because of abnormal cell-ECM attachments and cell-cell contacts, whereas normal epithelial cells may form a monolayer attached onto ECM-enriched basement membrane until they undergo anoikis (a form of apoptosis caused by a loss of cell adhesion) to end their lives. The disseminated cancer cells survive anoikis, travel to distant sites via blood lymphoid vessels, and eventually settle down and proliferate as metastatic tumors (27, 57). During invasion through the basement membrane prior to intravasation and then settlement after extravasation, integrin-mediated adhesion of cancer cells to ECM is likely to critically affect metastatic potential (3, 22, 41, 52). In microenvironments around invasive cancer cells, growth factors and cytokines including transforming growth factor-β1 (TGFβ1) secreted by cancer cells and neighboring fibroblasts, leukocytes, and endothelial cells are known to be prevalent to remodel invasive environments and to affect the cell behaviors (55). In particular, the peritoneal dissemination and liver metastasis of gastric cancer involve roles of integrin-mediated cell adhesions (45). Integrin β1 was shown to play roles in the initial attachment of gastric cancer cells to mesothelial cells during metastasis (45). In addition, integrin α3 or α2 expressions were correlated with increased invasion or metastasis of gastric cancer to lymph nodes through increased adhesion, respectively (59). On the other hand, TGFβ1 treatment of SNU16mAd cells replated on ECM increased integrins α2 and α3 expression, focal adhesion formation, and invasion (38). Therefore, these previous studies provide evidence of the significance of integrin-mediated adhesions in gastric carcinoma metastasis. However, the mechanism of cell adhesion-dependent (with or without TGFβ1 treatment) gene regulation and its significance in gastric carcinoma cell behaviors remain largely unknown.

Gene transcription is a highly orchestrated cellular process. In addition to genetic mechanisms achieved via changes in DNA sequences, the epigenetic regulation of gene transcription defines heritable changes in gene expression that are not coded by DNA sequences. Epigenetic mechanisms involve the deacetylation or acetylation of histone tails (17–20). Open reading frame and promoter region of a specific gene may wrap a nucleosome consisting of histone octamer (two copies of H2A, H2B, H3, and H4). NH_2-terminal histone H3 and H4 tails have been shown to be targeted by various molecules with histone acetyltransferase (HAT), such as p300/CBP, or histone deacetylase (HDAC) activity. Moreover, the compactness of a nucleosome relative to neighboring nucleosomes may be altered by chromatin remodeling involving histone (de)acetylation, which enables or prohibits the approach of transcriptional machineries, consisting of transcription factors and cofactors (21, 60). Of the amino acid residues of H3, acetylation of Lys[9] is transcription-permissive, whereas its methylation is transcription-suppressive (28). Epigenetic mechanisms also involve the methylations of promoter region of a specific gene.

Address for reprint requests and other correspondence: J. W. Lee, Cancer Research Institute, Depts. of Tumor Biology and Molecular and Clinical Oncology, College of Medicine, Seoul National Univ., Seoul 110-799, Korea (e-mail: jwl@snu.ac.kr).

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CpG islands of the promoter region of a specific gene may be methylated by DNA methyltransferases, resulting in gene silencing (9). Moreover, the promoter region of a specific gene in nucleosomes may be susceptible to influence by transcription factors, such as HAT, HDAC, cofactors, and methylated DNA-binding domains (MBDs) (5, 26). Transcription-permissive protein complexes can include HATs and transcription coactivators, whereas nonpermissive or suppressive complexes can consist of HDACs and transcription co-repressors (40, 47).

Integrin-linked kinase (ILK) is a Ser/Thr kinase located at integrin-enriched focal adhesions and binds to the integrin-β1 cytoplasmic tail (25). Its overexpression in rat intestinal epithelial cells (IEC) inhibits adhesion to integrin substrates (25) and causes anchorage-independent growth (51). Mammary epithelial cells overexpressing ILK were found to be resistant to anoikis or the suspension-mediated apoptosis that occurs by disruption of integrin-ECM interactions (2, 61). Recently, a protein phosphatase 2C that selectively associates with ILK and ILK-associated serine/threonine phosphatase 2C (ILKAP) was shown to inhibit ILK-mediated mitogenic signaling and anchorage-independent growth of LNCaP prostate carcinoma cells (34). In contrast, ILK expression was also correlated with an increased cell adhesion. Inhibition of ILK activity in PTEN-null PC3 prostate cancer cells reduced cell adhesion and migration presumably via the disruption of ILK/α-parvin/paxillin complex localization to focal adhesions when cells adhered to ECM (1). It was also shown that fibronectin (Fn)-adhesion-mediated ILK activation triggers the activation of PKB/Akt to promote IEC cell survival in a phosphatidylinositol 3-kinase (PI3K) activity-dependent manner (13). These studies indicate that the differential roles of ILK in cell adhesion probably depend on cell types or signaling contexts. Furthermore, the above-mentioned reports suggest that ILK expression and activity may be regulated during cell adhesion and detachment processes and that the regulation of ILK expression depending on extracellular cues may be importantly involved in metastasis. Indeed, ILK expression was previously shown to be correlated with invasion and metastasis of gastric carcinoma (29).

In this study, we mechanistically investigated the regulation of the epigenetic transcription processes leading to cell adhesion-dependent ILK suppression and their biological significance with respect to cell adhesion properties using gastric carcinoma cells.

MATERIALS AND METHODS

Cells. Gastric carcinoma cell lines including SNU16mAd (39) or colon carcinoma HT29 (ATCC) cells were maintained in RPMI-1640 containing 10% FBS in a humidified CO_2 incubator (5% CO_2 and 37°C).

Replating of cells on Fn. Cell manipulation to keep in suspension or to replate on Fn (10 μg/ml) or poly-L-lysine (10 μg/ml) in the absence of serum for 20 h was done similar to a previously reported protocol (36). Pharmacological inhibitors, such as U0126 (40 μM; LC Labs, Woburn, MA), ML9 (25 μM), cytochalasin D (1 μM or indicated concentrations), trichostatin A (TSA, 100 nM), and PP2 (10 μM; Tocris Cookson, Avonmouth, UK) were pretreated 30 min before cell replating with or without TGFβ1 (5 ng/ml). TSA treatment at a higher concentration than 500 nM did not further enhance ILK expression level, compared with at 100 nM, so that 100 nM TSA has been used in the experiments. After the 20 h incubation at 37°C in 5% CO_2, cells were collected for extract preparation, or their phase-contrast images were taken by using a digital camera-equipped microscope.

Preparation of whole cell extracts or nuclear extracts. Whole cell extracts (37) for immunoblottings or nuclear extracts for coimmunoprecipitation experiments (46) were prepared from SNU16mAd gastric or HT29 colon carcinoma cells under diverse experimental conditions, or gastric carcinoma cells transfected with either control [small interfering RNA (siRNA) against green fluorescent protein (GFP)] or ILK siRNA (Dharmacon, Chicago, IL; siGENOME SMART pool M-004499-00-0005, human ILK; NM-004517), using Lipofectamine 2000 (Invitrogen). Meanwhile, histone preparations to analyze histone modifications were prepared by acid-soluble nuclear extractions, as described previously (49). Briefly, cells under the indicated experimental conditions were washed with ice-cold PBS, pelleted, and resuspended in 1 ml of ice-cold lysis buffer (10 mM Tris·HCl, pH 6.5, 50 mM sodium bisulfite, 1% Triton X-100, 10 mM $MgCl_2$, and 8.6% sucrose), prior to homogenization. Nuclei were pelleted by a centrifugation at 1,400 *g* for 5 min, and washed three times with 1 ml of lysis buffer and then with 1 ml of Tris-EDTA solution (10 mM Tris·HCl, pH 7.4, and 13 mM EDTA). Pelleted nuclei were resuspended in 100 μl of ice-cold water. Sulfuric acid was then added to the samples to a final concentration of 0.2 M, and the mixture was then vortexed, incubated on ice for 1 h, and centrifuged at 15,000 *g* for 10 min at 4°C. The supernatant proteins were precipitated with 1 ml of acetone overnight at −20°C. The acetone-precipitated proteins were collected by a centrifugation at 15,000 *g* for 10 min at 4°C, air dried, and resuspended in 50 μl of water. Proteins in the extracts were analyzed by Western blot for histone modifications.

Western blots. Standard Western blotting and membrane stripping were done as described previously (36). The primary antibodies used include anti-acetylated H3, Lys^9-acetylated H3, Lys^9-dimethylated H3, HDACs 1 to 3 (Upstate Biotechnology, Lake Placid, NY), total H3, ILK, Smad4, Ski, methyl-CpG binding protein 2 (MeCP2), p300/CBP (Santa Cruz Biotechnology, Santa Cruz, CA), and α-tubulin (Sigma, St. Louis, MO).

Coimmunoprecipitation. The prepared extracts at an equal amount of proteins were immunoprecipitated with either anti-Smad4, p300/CBP, HDAC1, HDAC2, or HDAC3 antibody, via rocking overnight or for 4 h in a cold room. The immunoprecipitates were incubated with protein A/G-Sepharose beads (Upstate Biotechnology, Lake Placid, NY) for 2 h at 4°C, before washing four times with ice-cold PBS. Immunoprecipitated proteins were eluted from beads via boiling in the SDS-PAGE sample buffer, then immunoblotted. To avoid interference by the heavy chain of anti-HDAC3 immunoglobulin while immunoblotting to show an equal immunoprecipitation of HDAC3, another set of immunoprecipitates prepared in parallel was eluted with the nonreducing SDS-PAGE sample buffer prior to HDAC3 immunoblotting.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) was performed as explained previously (66). Briefly, SNU16mAd cells were fixed with formaldehyde for 15 min at room temperature. Soluble chromatin was immunoprecipitated with 2 μg of anti-acetylated H3, Lys^9-acetylated H3, Lys^9-dimethylated-H3, HDAC3, or p300/CBP antibody, or normal rabbit IgG (Santa Cruz Biotechnology) overnight. The same set of input DNA and 5% of purified ChIP DNA were subjected to quantitative PCR for 30 cycles, using sense (5′-ATT CTT CCC AGT CAA GCC TG-3′) and antisense (5′-TAT CAG CTC TAG GCA AAA GC-3′) ILK primers. Standard agarose gel electrophoresis was performed to visualize the PCR products.

RT-PCR. Total RNAs from cells suspended or replated on Fn for 20 h, as explained above, were extracted using TRIzol reagent (GIBCO-BRL), as instructed by the manufacturer. cDNAs were synthesized from 1 μg of total RNA using MMLV reverse transcriptase (Invitrogen) and 250 ng of random hexamers. ILK cDNA was amplified by quantitative PCR using sense (5′-^{791}AAG GTG CTG AAG GTT CGA GA^{810}-3′) and antisense (5′-^{955}ATA CGG CAT CCA GTG TGT GA^{936}-3′) primers. Reactions were performed in 20

μl under the following conditions: 94°C for 5 min; then 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and finally 10 min at 72°C. GAPDH was used as an internal control. Standard agarose gel electrophoresis was performed to visualize the PCR products.

Cell adhesion assay. Cell adhesion analyses were performed as previously described (32). Cells were transiently transfected either with pRc/CMV control or pRc/CMV-ILK13 plasmid (ILK WT; a gift from Dr. R. L. Juliano, Department of Pharmacology, University of North Carolina, Chapel Hill, NC), or either control (siRNA against GFP) or ILK siRNA (Dharmacon; siGENOME SMART pool M-004499-00-0005, human ILK; NM-004517), using Lipofectamine 2000 (Invitrogen). Two days later, cells were replated on Fn-precoated (10 μg/ml) or collagen I-precoated (10 μg/ml) 96-well plates and incubated at 37°C and 5% CO_2 for 20 h. Washing, fixation, and crystal violet staining of adherent cells were performed as explained previously (32). After staining and washing, we evaluated the degrees of staining at 564 nm using a microplate reader, to determine relative cell adhesion (defined as adhesion under a specified condition subtracted from adhesion on BSA-precoated condition). Five wells were handled in parallel, and the middle three values were averaged. Data shown (means ± SD) are representative of three different assays.

Soft agar assay. Two days after transfection of cells with pRc/CMV control or pRc/CMV-ILK13 plasmid, cells were collected and counted. Cells in culture medium containing 10% FBS and 0.3% agar (DNA grade, Sigma) at 10^5 or 10^6 cells per 60-mm dish were plated onto an already hardened underlayer (0.7% agar medium with 10% FBS). The plates were then incubated for 27 days in a 5% CO_2 incubator at 37°C, with replenishment of medium containing 400 μg/ml G418 and 10% FBS every other day. Colonies were imaged using a digital camera-equipped microscope.

RESULTS

Cell adhesion status- and TGFβ1-dependent histone acetylation and ILK expression. To study roles of signal transduction by extracellular cues in the regulation of epigenetic gene transcription, we first examined the effects of cell adhesion status in the absence or presence of TGFβ1 treatment as an extracellular stimulus on H3 acetylation. Trypsinized SNU16mAd cells were suspended and rolled over for 45 min to nullify basal signaling activity (30), then either kept in suspension (with rolling over at 60 rpm) or replated on Fn-precoated dishes with or without TGFβ1 treatment for 20 h in a serum-free condition, before nuclear extracts preparations. These experimental conditions did not cause any significant cell death (data not shown). Compared with suspended gastric carcinoma SNU16mAd cells, cells replated on Fn-precoated dishes showed lower H3 and Lys^9 of H3 (Lys^9-H3) acetylations (Fig. 1*A*, *lanes 1* and *3*). Furthermore, extracellular stimulation with a multifunctional cytokine TGFβ1 caused more acetylations of H3 and Lys^9-H3 only in suspended cells, compared with those in Fn-adherent cells (Fig. 1*A*, *lanes 2* and 4). Since these cells adhere very slowly, we found that cell adherence longer than 8 h incubation onto any type of ECM including Fn was required for preparation of enough cell extracts. Keeping cells in suspension or replating them on Fn for 8 or 12 h also resulted in the differential acetylation pattern, and this difference was accentuated after incubating cells for 20 h (33). However, the difference did not correlate with the expression levels of HDACs or a HAT, p300/CBP (Fig. 1*A*). These observations suggest that signaling activities triggered by integrin-mediated adhesion and extracellular TGFβ1 stimulation may regulate H3 and Lys^9-H3 acetylations in gastric carcinoma SNU16mAd cells and that these probably lead to alterations on gene transcriptions.

We next examined differential H3 and Lys^9-H3 acetylations with a respect to cell adhesion status in the absence or presence of TSA, a potent HDACs inhibitor. Interestingly, TGFβ1 treatment enhanced the acetylations in TSA-untreated suspended cells, but not any further in TSA-treated suspended cells, although TSA treatment substantially enhanced these acetylations in suspended cells (Fig. 1*B*, left 4 lanes). Further-

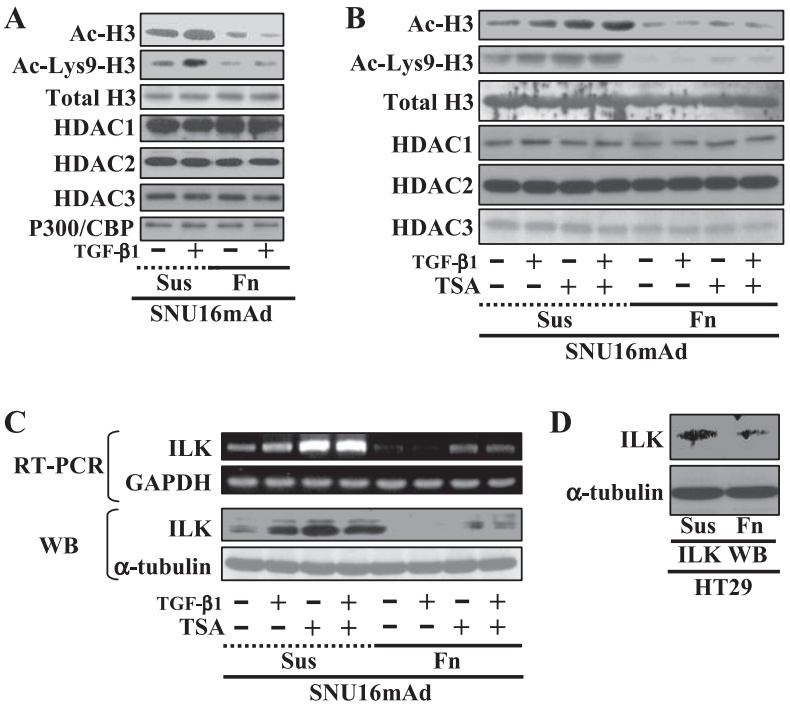

Fig. 1. Basal and transforming growth factor-β1 (TGFβ1)-mediated histone 3 (H3) acetylation and integrin-linked kinase (ILK) expression were differentially regulated in a cell adhesion status-dependent manner. SNU16mAd (*A–C*) or HT29 (*D*) cells were either maintained in suspension (Sus) or replated on fibronectin-precoated (Fn, 10 μg/ml) dishes. After incubation for 20 h with ("+") or without ("−") TGFβ1 (5 ng/ml) in the presence of vehicle (DMSO) or trichostatin A (TSA, 100 nM) pretreatment, nuclear extracts (*A* and *B*), RNA (*top* of *C*), or whole cell lysates (*bottom* of *C* and *D*) were prepared and immunoblotted (WB, Western blot) using antibodies against the indicated molecules or subjected to RT-PCR using ILK primers, as explained in MATERIALS AND METHODS. Data are representative of several independent experiments. Ac-H3, acetylated H3; Ac-Lys9-H3, Lys^9-acetylated H3; HDAC, histone deacetylase.

more, these differential acetylation patterns by TGFβ1 treatment or TSA treatment in suspended cells were correlated with ILK mRNA (Fig. 1*C*, *top*) and protein levels (Fig. 1*C*, *bottom*). The lack of TGFβ1-enhanced acetylation and ILK expression in the presence of TSA cotreatment in suspended cells (Fig. 1, *B* and *C*, *lanes 3* and *4*) may indicate that the TGFβ1-mediated regulation of ILK expression adopts biochemical processes that could be completely achieved by HDACs inhibition. TSA treatment of Fn-adherent cells resulted in a slight recovery, if any, in the acetylation levels (Fig. 1*B*) and a partial recovery in ILK transcription and expression independent of TGFβ1 treatment (Fig. 1*C*). The degree of recovered H3 acetylations even after TSA treatment was minimally detected, indicating that concomitant decreases in deacetylation and methylation might also be involved in the TSA-mediated recovery of ILK expression in Fn-adherent cells. This differential ILK expression depending on cell adhesion status also appeared valid in HT29 colon carcinoma cells (Fig. 1*D*).

Cell adhesion-mediated intracellular signaling activity is involved in the regulation of H3 and Lys^9-H3 acetylations and ILK expression levels. Next, we investigated whether H3 and Lys^9-H3 acetylations are suppressed by the intracellular signaling activity triggered by integrin-mediated cell adhesion with or without TGFβ1 treatment. SNU16mAd cells were replated on Fn or poly-L-lysine in the absence or presence of TGFβ1 for 20 h, and nuclear or whole cell extracts were then prepared. As was shown above, basal and TGFβ1-mediated H3 and Lys^9-H3 acetylations in Fn-adherent cells were insignificant or minimal (Figs. 1 and 2*A*). Therefore, we tried to determine whether pharmacological inhibition of certain intracellular signaling molecules would recover minimally suppressed acetylations in adherent cells. Since activations of MEK/Erk1/2, c-Src family kinase, and focal adhesion kinase (FAK) and actin reorganization are triggered upon cell adhesion to ECM substrates (8, 10, 31, 35), we have pretreated cells with diverse reagents including inhibitors of these molecules prior to keeping cells in suspension or replated on Fn and consequent analysis of histone acetylations. Among inhibitors we tested, pretreatment of cells with U0126 (an MEK inhibitor), PP2 (c-Src family kinases inhibitor), or cytochalasin D [a reagent that inactivates FAK (6) and disrupts actin organization] recovered the acetylations, although PP2 pretreatment recovered Lys^9-H3 more than H3 acetylation in Fn-adherent cells (Fig. 2*A*). Furthermore, pretreatment with these reagents also induced ILK expression (Fig. 2*B*, *left* and *middle*) even in Fn-adherent cells. In addition, these pretreatments resulted in round cell shapes without significant detachments (Fig. 2*C*), with recapitulation of the suspended round cells with a higher Lys^9-H3 acetylation and ILK expression. Suspended or Fn-adherent cells (without TGFβ1 treatment) also showed round shapes without any significant cytotoxic effects by these pretreatments (data not shown). However, these recoveries in acetylation and ILK expression did not occur when cells were replated on poly-L-lysine, to which cells adhere through electrical charge interactions; TGFβ1 treatment of cells on poly-L-lysine with or without cytochalasin D pretreatment did not increase but rather reduced Lys^9-H3 acetylation and ILK expression, unlike on Fn (Fig. 2, *A* and *B*, *right*). In addition, ML9 treatment that would inhibit intracellular contractility reduced or increased the acetylation in suspended or adherent cells, respectively (Fig. 2*D*, see DISCUSSION). These observations indicate that changes in cytoplasmic signaling activity and actin organization mediated by integrin-ECM interaction and/or TGFβ1 treatment may lead to alterations on H3 and Lys^9-H3 acetylations and thereby on nuclear biochemical processes of ILK gene transcription.

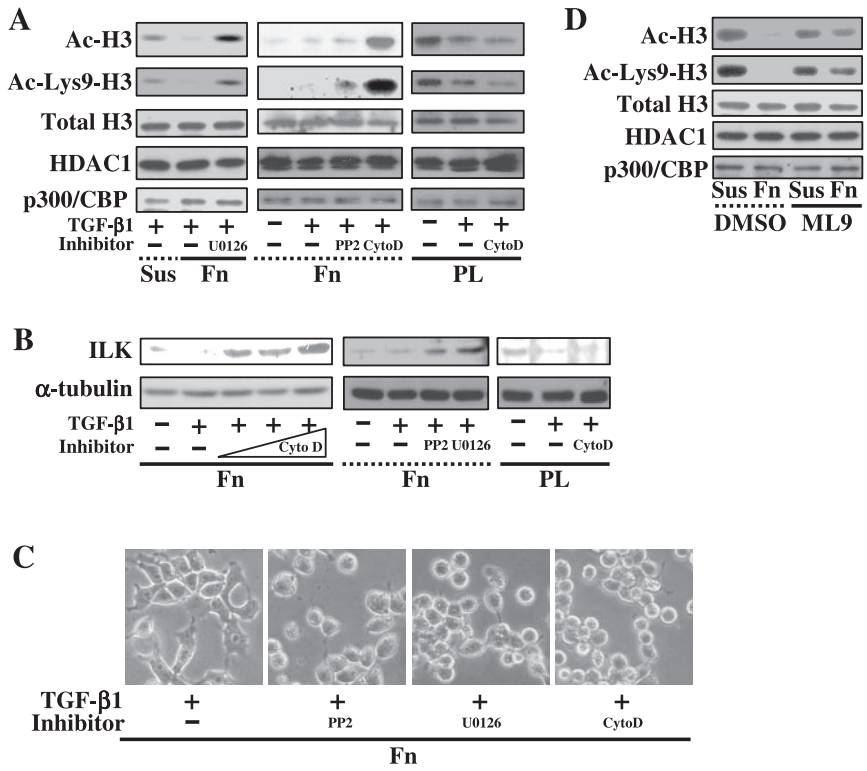

Fig. 2. Minimal (Lys^9)-H3 acetylation and ILK expression in adherent cells depend on adhesion-mediated intracellular signaling activities. *A* and *B*: SNU16mAd cells were kept in suspension (Sus) or replated onto fibronectin (10 μg/ml, Fn) or poly-L-lysine (10 μg/ml, PL) with or without TGFβ1 (5 ng/ml). The cells were was pretreated with DMSO vehicle or pharmacological inhibitor [the MLCK inhibitor, ML9, at 25 μM; the MEK inhibitor, U0126, at 20 μM; the c-Src family kinases inhibitor, PP2, at 10 μM; or cytochalasin D (CytoD) at 1.0 μM in *A* and *C* but at 0.1, 0.5, and 1.0 μM in *B*] 30 min before the replating for 20 h at 37°C. Nuclear extracts (*A* and *D*) or whole cell lysates (*B*) were then prepared for immunoblotting against the indicated molecules, or cell images were taken (*C*). Data shown are representative of 3 isolated experiments.

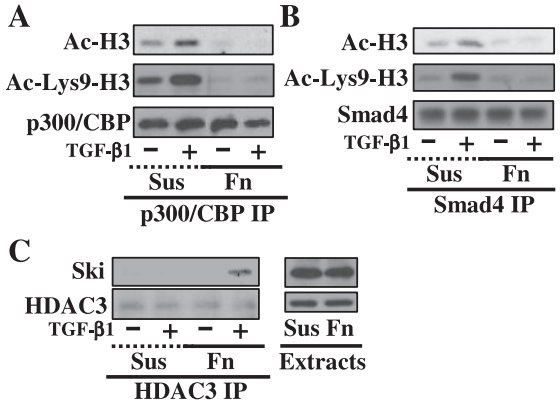

Fig. 3. Differential H3 acetylations were correlated with formations of different transcription complexes. Nuclear extracts were prepared from SNU16mAd cells under the indicated conditions, as described in Fig. 1. Nuclear extracts at an equal amount of proteins were immunoprecipitated either with anti-p300/CBP (*A*), anti-Smad4 (*B*), or anti-HDAC3 antibody (*C*). The immunoprecipitates (IP) or extracts were immunoblotted with the antibody against Ac-H3, Ac-Lys^9-H3, p300/CBP, Smad4, or the TGFβ1-mediated transcription cosuppressor Ski. Data are representative of 3 independent experiments.

Differential H3 and Lys^9-H3 acetylations appeared to result from formation of different protein complexes in a cell adhesion status-dependent manner. We then hypothesized that these higher acetylations of H3 and Lys^9-H3 in suspended cells might be at least partially due to differential complex formations involving modified histones and transcription cofactors. To test this hypothesis, we performed coimmunoprecipitations to examine protein associations in nuclear extracts prepared from suspended or Fn-adherent cells with or without TGFβ1. p300/CBP immunoprecipitates of the nuclear extracts from suspended, but not from Fn-adherent, SNU16mAd cells coprecipitated acetylated H3 and Lys^9-H3 (Fig. 3*A*, *lanes 1* and *3*). In addition, TGFβ1 treatment of suspended, but not of Fn-adherent, cells enhanced the association between p300/CBP and acetylated H3 and Lys^9-H3 (Fig. 3*A*, *lanes 2* and *4*). Next, we examined the associations between acetylated H3 or Lys^9-H3 and Smad4, a TGFβ1-dependent transcription coactivator. Acetylated H3 and Lys^9-H3 were coimmunoprecipitated with Smad4 more in suspended than in Fn-adherent cells (Fig. 3*B*). Moreover, this coimmunoprecipitation was obvious in the presence of TGFβ1 treatment but not under TGFβ1-untreated basal conditions, as was expected. On the other hand, an association between HDAC3 and Ski, a TGFβ1-mediated transcription cosuppressor, was observed in TGFβ1-treated adherent cells (Fig. 3*C*). Therefore, there appeared to form a protein complex nonpermissive for transcription in Fn-adherent cells but a transcription-permissive complex in suspended cells. These observations indicate that extracellular cues, such as integrin engagement and TGFβ1 treatment, could regulate H3 and Lys^9-H3 acetylations and ILK mRNA and protein levels, at least partially, via formation of different protein complexes consisting of transcription cofactors and (Lys^9)-acetylated H3, in a cell adhesion status-dependent manner.

Minimal H3 and Lys^9-H3 acetylations in Fn-adherent cells were correlated with the association between HDAC3 and MeCP2. DNA methylation in the promoter region of a specific gene causes gene silencing and is functionally connected with histone modifications (5, 26). This connection may be possible through associations between HDACs and MBDs. To test this possibility, HDACs immunoprecipitates from nuclear extracts prepared from suspended or Fn-adherent cells were immunoblotted for MBDs including MeCP2 and MBD1–MBD3, or HDACs. Interestingly, basal and TGFβ1-mediated MeCP2 associations with HDAC3, but not with HDAC1 or HDAC2, were higher in Fn-adherent than in suspended cells (Fig. 4, data not shown). Therefore, the increased association between MeCP2 and HDAC3 in adherent cells may also contribute to ILK gene suppression.

Epigenetic suppression of ILK expression upon adhesion to Fn. As shown in Figs. 1 and 2, H3 and Lys^9-H3 acetylations were minimally downregulated in adherent cells, and these minimal acetylations were correlated with an insignificant ILK transcription and expression. Next we performed ChIPs to determine how ILK promoter regions associates with transcription factors upon replating of cells onto Fn. When chromatins prepared from suspended or Fn-adherent cells were immunoprecipitated with anti-acetylated H3 or Lys^9-H3 antibody, ILK promoter was coimmunoprecipitated only in suspended cells (Fig. 5*A*). Meanwhile, more ILK promoter was coimmunoprecipitated with Lys^9-dimethylated-H3 nonpermissive for gene transcriptions in Fn-adherent cells (Fig. 5*A*). ChIP with anti-HDAC3 recovered more ILK promoter region also in Fn-adherent than in suspended cells (Fig. 5*B*). Furthermore, ChIP with anti-p300/CBP resulted in a greater association between p300/CBP and ILK promoter in suspended rather than in Fn-adherent cells (Fig. 5*B*). Moreover, this suppressive ChIP pattern in adherent cells correlated with lower ILK mRNA (Fig. 5*C*) and protein levels (Fig. 5*D*). These observations indicate that ILK promoter region is closely associated with transcription-permissive (Lys^9)-acetylated H3 and cofactors in suspended cells only, and with nonpermissive Lys^9-dimethylated H3 and HDAC3 in Fn-adherent cells, indicating an epigenetic suppression of ILK in adherent SNU16mAd cells.

ILK expression affects cell adhesion and anchorage-independent growth. ILK has been shown to be critically involved in integrin-mediated signal transduction to regulate diverse cellular functions (24, 50, 63), including adhesion and anchorage-independent growth (25, 34, 51), although its effects are controversial (see Introduction and DISCUSSION). Thus, we asked how ILK expression regulates cell adhesion in this system. To answer this question, we examined the effects of altered ILK expression on cell adhesion. Two days after the wild type ILK cDNA or control plasmid transfection of SNU16mAd cells, the

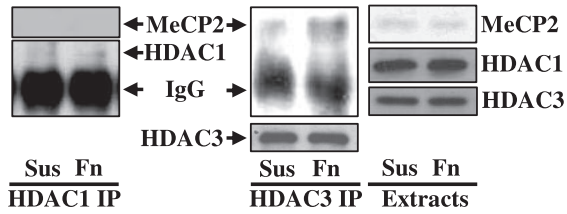

Fig. 4. Preferential association between HDAC3 and MeCP2 in Fn-adherent cells. Nuclear extracts at an equal amount of proteins prepared from SNU16mAd cells, as explained in Fig. 1, were immunoprecipitated with anti-HDAC1 or anti-HDAC3 antibody. The immunoprecipitates were immunoblotted with the antibody against either HDACs or methyl CpG binding protein 2 (MeCP2). The nuclear extracts were also immunoblotted with either anti-MeCP2 or anti-HDACs to indicate equal expressions of them both in suspended and adherent cells. Data are representative of 3 independent experiments.

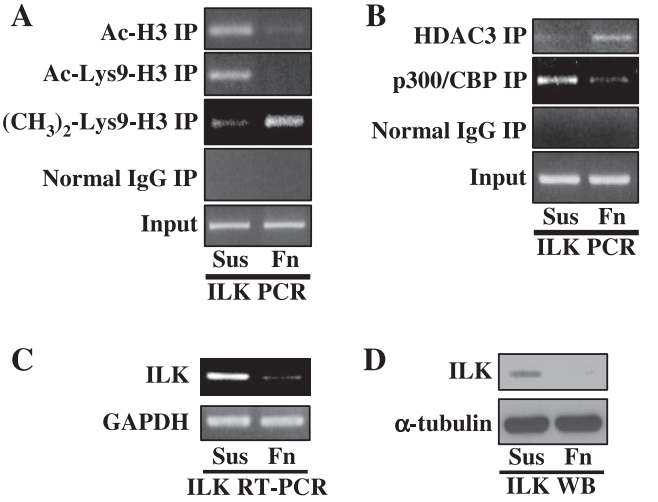

Fig. 5. Association of ILK promoter region with acetylated H3 in suspended cells. SNU16mAd cells were either kept in suspension or replated onto fibronectin (10 μg/ml) for 20 h prior to preparation of either soluble chromatin for chromatin immunoprecipitation (ChIP) (*A* and *B*), RNA for RT-PCR using ILK primers (*C*), or whole cell lysates for ILK and α-tubulin immunoblots (*D*), as explained in the MATERIALS AND METHODS. ChIPs were performed with the indicated antibody or normal IgG (*A* and *B*). The same set of input DNA in *A* and *B* that did not undergo the ChIPs also was shown in parallel. Data are representative of 3 isolated experiments. $(CH_3)_2$-Lys^9-H3, Lys^9-dimethylated H3.

cells were subjected to adhesion assays. ILK-overexpressing cells showed lower adhesions on collagen I or Fn than cells transfected with the control plasmid did (Fig. 6*A*). Next, we tried to examine if a knock-down of ILK by siRNA against human ILK increases cell adhesion. The transfection of ILK siRNA successfully downregulated ILK expressions in diverse gastric carcinoma cell variants, including SNU16, integrin-α5-expressing SNU16 (SNU16α5), and integrin-α5-expressing SNU620 (SNU620α5) (Fig. 6*B*). Originally SNU16 and SNU620 are anchorage-independently growing and SNU16α5 and SNU620α5 are their stable transfectants with human integrin-α5 (a typical Fn-binding receptor) to be anchorage-independent on collagen I but adherent on Fn (32). In addition to adherent SNU16mAd cells, anchorage-independent cell variants became significantly more adhesive onto collagen I upon ILK suppression (Fig. 6*C*, *left*). In addition, adhesions of the cell variants also onto Fn were increased by ILK suppression (Fig. 6*C*, *right*). In addition, soft agar assays were performed at two different cell densities to ensure anchorage-independent growth. Being correlated with the reduced adhesion of ILK-overexpressing cells, ILK expression resulted in more colonies in soft agar than control plasmid-transfected cells did (Fig. 6*D*). These observations indicate the presence of a bidirectional regulatory linkage between ILK gene expression and gastric cancer cell adhesion.

Altogether, the observations of this study suggest that the signaling activities stimulated by extracellular cues differentially regulate the epigenetic transcription activity of ILK gene, through differential H3 and Lys^9-H3 acetylations, transcription cofactor associations, and accessibilities of acetylated H3 to transcription cofactors and ILK promoter regions, and that the epigenetic regulation of ILK expression and cell adhesion appear to regulate each other (Fig. 7).

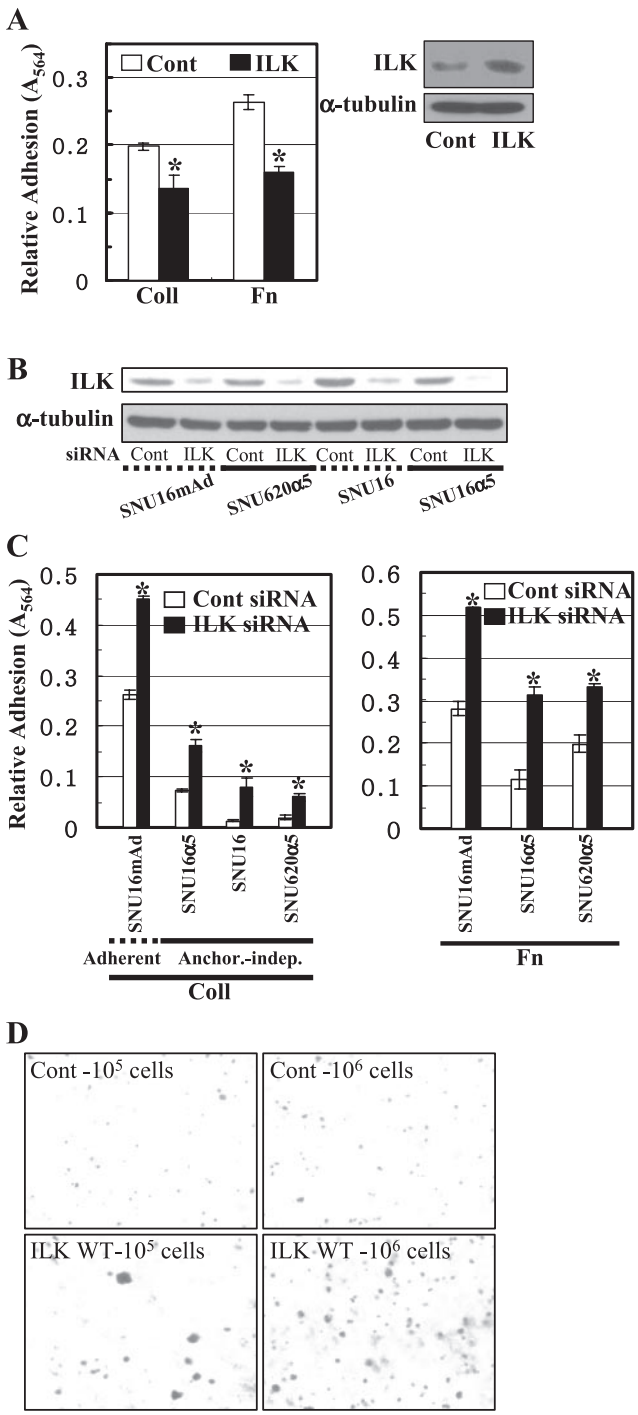

Fig. 6. ILK expression levels inversely correlated with cell adhesion properties. *A*: SNU16mAd cells were transfected with a control pRc/CMV (Cont) or pRc/CMV-wild-type ILK plasmid (ILK), and 48 h after this transfection cells were subjected to cell adhesion assays on collagen-precoated (Coll) or fibronectin-precoated (Fn) 96-well plates (10 μg/ml, *left*), as described in MATERIALS AND METHODS. The overexpression of ILK was confirmed by ILK immunoblotting (*right*). Data are representative of 3 independent experiments. *B* and *C*: diverse gastric carcinoma cell variants were transfected with control small interfering RNA (siRNA; against green fluorescent protein) or human ILK siRNA prior to immunoblotting against ILK and α-tubulin (*B*) or adhesion assays (*C*), as explained in MATERIALS AND METHODS. *D*: 2 days after transfection as in *A*, cells were subjected to soft agar assays at two different densities, as explained in MATERIALS AND METHODS.Each condition examined was independently duplicated, and after incubation in a humidified CO_2 incubator for 27 days, colonies in 5 independent areas in each dish were imaged. Representative images for each condition are shown. $^*P < 0.05$ for statistical significance via Student's *t*-tests.

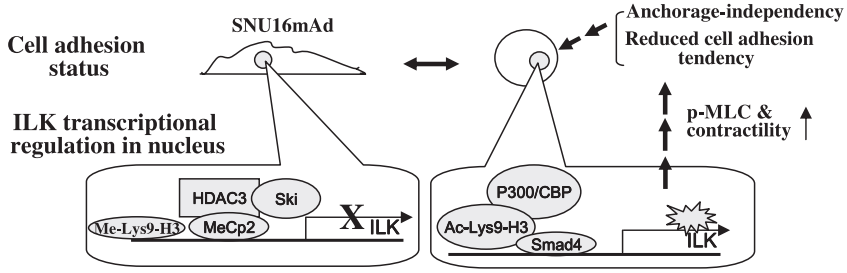

Fig. 7. Working model of a bidirectional regulatory linkage between cell adhesion and ILK expression. H3 acetylation in suspended gastric cancer cells is significantly higher, compared with that in Fn-adherent cells, presumably due to suspension-enhanced contractility and thereby a reduced HDACs activity (33). On treating suspended cells with TGFβ1, transcription cofactors favorable to ILK expression including p300/CBP, Smad4, and (Lys^9)-acetylated H3 associate together, locate to the ILK promoter region, and facilitate ILK transcription. However, in the case of Fn-adherent cells, ILK promoter region is occupied with protein complexes consisting of Lys^9-methylated H3 (Me-Lys^9-H3), Ski, MeCP2, and HDAC3, which may be nonpermissive for ILK expression. On the other hand, up- or downregulation of ILK expression levels caused decreased or increased cell adhesion on extracellular matrices, respectively, and its overexpression allowed anchorage-independent growth. Therefore, the epigenetic ILK expression and adhesion properties of gastric carcinoma cells appear to affect each other through a bidirectional linkage.

DISCUSSION

Recently epigenetic gene regulation has been intensively studied for cancer therapeutic purposes. However, how it is regulated by extracellular cues-mediated signal transduction is largely unknown. In this study, we explored the effects of signal transduction by cell adhesion and extracellular TGFβ1 stimulation on epigenetic regulation of ILK expression. Therefore, implication of the observations would be interesting, since ILK expression levels were shown to be critically involved in (gastric) cancer invasion and metastasis (29, 50).

Cell adhesion-mediated epigenetic suppression of ILK. This study reveals that basal and TGFβ1-mediated H3 and Lys^9-H3 acetylations are preferentially higher in suspended than in Fn-adherent cells. The minimal H3 and Lys^9-H3 acetylations in Fn-adherent cells may be caused by higher HDACs activities which result from an adhesion/spreading-mediated reduction in contractility (33, 53). Inhibition of intracellular signaling molecules (i.e., c-Src family kinases, FAK, and MEK/Erks) and of actin polymerization recovered H3 and Lys^9-H3 acetylations from the minimal levels in adherent cells. These inhibitions also resulted in a round morphology, reminiscent of suspended cells. Therefore, suspended or round-shaped cells caused by the inhibition of intracellular signaling molecules and actin polymerization have increased intracellular contractility (33, 53), which could in turn downregulate HDACs activity (33) and increase the acetylations. In addition, we observed that suspended SNU16mAd cells with TGFβ1 treatment appeared to have more associations between (Lys^9)-acetylated H3, p300/CBP, and Smad4 to assemble a transcription-permissive machinery, whereas Fn-adherent cells with TGFβ1 treatment had more associations between HDAC3, MeCP2, and Ski to assemble a suppressive machinery, at the ILK promoter region (Fig. 7). Although the present study does not rule out the roles of other transcription regulatory components in ILK gene regulation during cell adhesion or detachment, these observations indicate that DNA methylation, histone deacetylation, and transcriptional regulation are linked one another. Cell adhesion status-dependent transcriptional regulation in gastric carcinoma cells appears to be quite specific for ILK or related genes, since TM4SF5 (transmembrane 4, L6 family member 5) was more expressed in the adherent cells (data not shown). In addition to ILK, our microarray experiments and RT-PCR approaches showed that *ROCK1* and *PINCH2* genes were more preferentially transcribed in suspended than in Fn-adherent cells (Ref. 33 and data not shown). ROCK1 is a contractility-regulatory kinase that phosphorylates myosin light chain (MLC), with leads to an increase in intracellular contractility (58); PINCH2 binds ILK to regulate ILK-dependent cell adhesion and spreading and to protect ILK from degradation (56).

TGFβ1-mediated ILK expression through epigenetic biochemical processes in suspended cells. When TGFβ1-mediated effects were compared with TSA-mediated effects in suspended cells, it appeared that TSA treatment alone caused more H3 and Lys^9-H3 acetylations and ILK mRNA and protein levels (at least ≥2-fold) than mediated by TGFβ1 alone. In addition, TGFβ1 treatment did not cause any further enhancement of the effects in the presence of TSA-mediated HDACs inhibition, possibly indicating that TGFβ1-mediated ILK transcription is controlled by the HDACs activities at the experimental conditions (where cells were treated with 5 ng/ml TGFβ1 for 8~20 h). However, it is still possible that ILK may be induced nonepigenetically under different treatment periods and/or doses of TGFβ1. Although TGFβ1 signaling activates RhoA GTPases for actin reorganization and intracellular contractility (42), the molecular basis by which TGFβ1 transduces signals leading to acetylations of H3 and Lys^9-H3 and to formation of the transcriptional machinery complexes including HDACs is currently unknown. However, it was previously shown that TGFβ1 causes the acetylation of Ets1, which was further enhanced by TSA-mediated HDACs inhibition (11). In addition, TGFβ1 inhibited lipopolysaccharide-induced NF-κB recruitment to the interleukin-6 gene promoter in IEC through blockade of histone acetylation (23). These previous studies and the current study suggest that TGFβ1 may be involved in epigenetic transcriptional regulation, via affecting *1*) acetylations of proteins including transcription cofactors and/or histones, *2*) inductions of transcription cofactors, and/or *3*) formations of differential transcriptional complexes with specific gene promoter(s).

ILK expression and cell adhesion. In the present study using gastric carcinoma cells, we observed that SNU16mAd cells epigenetically suppress ILK expression on adhesion. We also observed that ILK expression levels regulated adhesion on ECMs in an inverse manner and that its overexpression caused

anchorage-independent growth. Therefore, the epigenetic ILK expression and adhesion property of gastric carcinoma cells appear to affect each other, through a bidirectional regulatory linkage.

Then how does gastric carcinoma cell adhesion epigenetically regulate ILK expression? Based on the observations from this study, epigenetic ILK suppression upon adhesion appeared to involve cell adhesion-dependent signaling activities and biochemical processes, such as a reduced contractility and an increased HDACs activity upon cell adhesion (33), histone modification(s), and nonpermissive transcriptional machinery formation at ILK promoter region, as explained above. Observations with inhibition of integrin-mediated signaling molecules and actin cytoskeleton, which caused a round cellular shape and a concomitant recovery in the acetylation of (Lys^9)-H3 and ILK expression from their minimal levels in adherent cells, may indicate a contractility-dependent reduction in HDACs activity, especially in round-shaped cells, in a manner reminiscent of that reported in suspended spherical cells (33). Similarly, treatment with ML9, an MLC kinase (MLCK) inhibitor, caused a partial recovery in the acetylations of H3 and Lys^9-H3 from their minimal levels in adherent cells and a partial reduction in their acetylations in suspended cells. This indicates that the effects of ML9 treatment on HDACs activities in suspended or adherent cells can be stimulatory or inhibitory, respectively, as was shown by a previous study (33), although ML9 is known to inhibit MLCK (which phosphorylates MLC) and thereby intracellular contractility upon treatment. This may be possible because MLCK controls the global contractility of suspended spherical cells, but only the locally peripheral contractility of Fn-adherent cells, based on a previous report that MLCK controls peripheral contractility, whereas ROCK controls actin polymerization and thus contractility inwardly distal from cellular peripheries (58). Therefore, it may be likely that the inverse relationship between a higher cellular contractility and a lower HDACs activity is correlated with higher H3 acetylations in suspended or round cells. However, adherent and well-spread cells may involve more than just lower acetylations of histones by virtue of a higher HDACs activity (33), such as, higher methylation and deacetylation levels of histones to suppress epigenetic transcriptional activity, since the correlation between (Lys^9)-H3 acetylation (Fig. 1*B*) and ILK expression (Fig. 1*C*) was not greatly impressive.

How could ILK expression affect cell adhesion properties? It was previously reported that ILK also mediates the phosphorylation of MLC Thr^{18}/Ser^{19} (16, 62) and the phosphorylation/inhibition of MLC phosphatase (MLCP) (15). The phosphorylation of MLC causes an increased cellular contractility, which causes a retraction of the cellular morphology for round shapes (33, 53, 54). Intensive retraction may cause a reduced adhesion or cell detachment. It appears that the correlation between ILK expression and cell adhesion may depend on cell types and the signaling contexts. It has been shown that ILK is transiently activated upon adhesion of IEC to Fn and that its activation leads to activation of the PI3K/Akt survival pathway (13). In addition, inhibition of PI3K-dependent ILK activity in PTEN-null PC3 prostate cancer cells disrupted localization of ILK/α-parvin/paxillin complex to focal adhesions with leading to decreased cell adhesion and migration (1). However, ILK overexpression in IEC cells inhibited adhesion to integrin substrates (25) and caused anchorage-independent growth (51). Mouse and human mammary epithelial cells overexpressing ILK were found to be resistant to the suspension-mediated apoptosis or anoikis that occurs by disruption of integrin-ECM interactions (2, 61). It was thus suggested that a loss of ILK expression or activity might lead to anchorage-independent growth relevant to metastasis, when other signaling molecules such as PTEN and PI3K are dysregulated (12).

Cell adhesion and gastric cancer. Integrin-ECM engagement triggers integrin clustering and activation leading to intracellular signal transduction for actin reorganization and contractility (14, 48) and regulates gene expression as required (7). Actin cytoskeleton reorganized by cell adhesion was suggested to evoke further architectural changes via an association with the nuclear matrix, which may cause alterations on transcriptional machinery complex formation to regulate gene transcription (7). It was shown that albumin gene expression required ECM-rendered transcription-permissive histone remodeling (43). Meanwhile, this current study shows that ILK in suspended or round-shaped SNU16mAd cells (with a higher contractility) was highly elevated and that this was further enhanced by TGFβ1 treatment, although this expression was suppressed as cells adhered. TGFβ1 treatment of SNU16mAd cells was shown to enhance integrins-α2 and -α3 expression and then invasion through a three-dimensional Matrigel layer in a previous study (38). Interestingly, ILK expression was found to correlate with the invasion and metastasis of gastric carcinoma (29). In addition, integrins (e.g., β1, α2, and α3) were shown to play important roles in gastric cancer metastasis (45, 59, 64, 65), as explained in introduction. Thus, these current and previous studies may suggest that ILK expression levels in round shaped cells within a three-dimensional Matrigel may facilitate turnover of adhesion to ECM and enhance invasive potentials. Therefore, the observations of the present study may provide mechanistic clues for ILK-targeted therapeutic approaches against gastric carcinoma metastasis.

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