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Snail Regulates Cell-Matrix Adhesion by Regulation of the Expression of Integrins and Basement Membrane Proteins*

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Snail, a transcriptional repressor of E-cadherin expression, plays a role in the process of epithelial-mesenchymal transition. However, the molecular basis of the role of snail in epithelial-mesenchymal transition has not been fully clarified. Here we show that the expression of snail in epithelial Madin-Darby canine kidney (MDCK) and A431 cells enhances both cell detachment and attachment. Snail did not confer resistance to anoikis induced by loss of contact but instead enhanced cell attachment to extracellular matrices such as fibronectin. This attachment was inhibited by Arg-Gly-Asp (RGD) peptides. Up-regulation of the promoter activity of integrin αV was observed in snail-expressing MDCK (MDCK/snail) cells. Snail also enhanced MDCK cell migration toward osteopontin that is a ligand for integrin $\alpha V\beta 3$. We confirmed the reduction of basement membrane proteins such as laminin (LN) $\alpha 3$, $\beta 3$, and $\gamma 2$ (laminin-5/LN-5) and of receptors for LN-5 such as integrins $\alpha 3$, $\alpha 6$, or $\beta 4$ in MDCK/snail or in snail-expressing A431 (A431/snail) cells. Nevertheless, suppression of LN-$\alpha 3$ chain by transient transfection of small interference RNAs resulted in no enhancement of cell detachment. We also found an induction of matrix metalloproteinase-3 in MDCK/snail and A431/snail cells. However, the inhibition of matrix metalloproteinase-3 showed no significant effect on the detachment of MDCK/snail cells. These results suggest that snail enhances cell detachment by multiple mechanism and leads to cell migration and reattachment at a second site, at least in part, by changing the expression of integrins in the cells.

The molecular basis of the EMT[2] process involves changes in the expression, distribution, and function of a number of proteins that play a role in extracellular matrix remodeling or in cell-cell adhesion, such as MMPs, and E-cadherins (1, 2). Down-regulation of E-cadherin expression frequently occurs during the progression of carcinomas (3, 4). Over the last few years, several transcription factors have been characterized as repressors of E-cadherin. These factors repress E-cadherin transcription through binding to the proximal E-boxes of the human or murine promoters for E-cadherin (5, 6). Among these repressors of E-cadherin, the zinc-finger factor snail induces a full EMT when overexpressed in epithelial MDCK cells leading to the acquisition of a motile/invasive phenotype (7, 8). In agreement with this role of EMT induction, snail has been found to down-regulate the expression of epithelial genes, such as occludin and claudin (9), and to induce the expression of mesenchymal and invasive genes, such as FN and MMP-9 (7, 10). The conversion of tumor cells from an epithelial to the mesenchymal phenotype is closely associated with the acquisition of metastatic potential (11). Snail expression has been detected in an increasing number of human carcinoma and melanoma cell lines (12). More importantly in terms of cell motility, snail is expressed at the invasive front of epidermoid carcinomas (8) and is associated with the invasiveness of ductal breast carcinomas and hepatocarcinomas (13, 14). However, the precise role of snail in tumor progression has not been clarified.

BMs are dynamic, thin, sheet-like structures that consist of ECM proteins, on which epithelial cells reside. Disruption of the BM itself or integrins, the receptors for BM components, contributes to the tumor process (15). BM is composed of a number of different proteins. LN, the main component of BM, is comprised of at least 15 different LN trimers. LN-5 is composed of $\alpha 3$, $\beta 3$, and $\gamma 2$ chains and is present in most epithelial BMs. LN-5 interacts with integrin $\alpha 6\beta 4$ and plays a crucial role in maintaining the stability of epithelial cells (16). Oral squamous carcinoma cells that overexpress snail are deficient in LN-5 synthesis (17). Indeed, we found the expressions of LN-5 and its receptor proteins integrin $\alpha 3$, $\alpha 6$, and $\beta 4$ were strongly suppressed in snail-expressing cells. However, cell detachment was not enhanced by inhibition of the expression of LN-5. These findings indicate that snail must affect the expressions of other molecules besides LN-5 to induce cell detachment.

Collagen IV is also a major component of BM. It has been suggested that Collagen IV $\alpha 5/\alpha 6$ chains might protect against rapid cancer progression, as it has been shown that the normal

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[2] The abbreviations used are: EMT, epithelial-mesenchymal transition; ECM, extracellular matrix; LN, laminin; Col IV, collagen IV; FN, fibronectin; BM, basement membrane; MDCK, Madin-Darby canine kidney; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; BSA, bovine serum albumin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT, reverse transcription; siRNA, small interference RNA; poly-HEMA, poly-2-hydroxyethyl methacrylate; LDH, lactate dehydrogenase; CMV, cytomegalovirus; FL, firefly luciferase; RL, *Renilla* luciferase; PI, propidium iodide; CM, conditioned medium; DC, deoxycholic acid.

production and assembly of BM is disrupted during malignant cancer progression (18).

Integrins, heterodimeric transmembrane receptor complexes, interact with specific ECM proteins and thereby transmit signals to the cells. Enhanced expression of integrin $\alpha V\beta 3$, in either a solid tumor or a melanoma, has been shown to correlate with tumor malignancy and with enhanced tumor cell growth and invasion (19, 20). Furthermore, enhanced activity of integrin $\alpha V\beta 3$ leads to increased tumor cell migration and adhesion to bone matrix protein (21).

Although genetic alterations in cancerous cells may vary in different types of metastatic cancers, all of these cancers share the characteristic of dissemination to multiple distant organs (22, 23). Typically, these aggressive cells detach from the site of origin, move across tissue boundaries, eventually extravasate from these vessels, and then colonize new sites. Although numerous studies have examined cell migration and invasion in snail-expressing cells, a role for snail expression in the regulation of cell attachment or detachment has not been described. This is the first report that demonstrates that snail enhances cell detachment from and attachment to ECM proteins at least in part by regulating the expression of integrins and BM proteins.

EXPERIMENTAL PROCEDURES

Cells and Transfection—MDCK and A431 were grown in DMEM supplemented with 10% fetal calf serum (FCS). MDCK and A431 cells were transfected with 10 μg of HA-tagged human snail expression vector or control vector by a calcium phosphate method, as described previously (10), and were designated MDCK/snail, MDCK/neo, A431/snail, and A431/neo cells, respectively. We mainly used dog MDCK cells in this series of experiments, because we believe the cells show typical normal epithelial cell phenotype. However, because of the limited commercial availability of antibodies that recognize canine proteins, we also used human A431 cells. The mouse osteoblast cell line, MC3T3-E1 (kindly provided by Dr. Ohnishi, Kagoshima University), was maintained in α-minimal essential medium (Sigma) supplemented with 10% FCS containing antibiotics. To initiate osteoblast differentiation, confluent MC3T3-E1 cells were treated with 50 μg/ml ascorbic acid (Wako, Osaka, Japan) and 5 mM glycerophosphate (Merck, Darmstadt, Germany). After 4 weeks of treatment, the conditioned medium of these cells was collected every 4–5 days (24).

Antibodies and Reagents—Mouse monoclonal antibodies against E-cadherin, integrins αV, $\alpha 3$, $\alpha 5$, and $\beta 4$, and LN-$\beta 3$ were purchased from BD Biosciences (Lexington, KY). Mouse anti-human integrin $\alpha V\beta 3$ antibody (LM609) was purchased from Chemicon (Temecula, CA). Mouse monoclonal antibody against vinculin was purchased from Sigma. Rat monoclonal antibody against HA was purchased from Roche Applied Science (Mannheim, Germany). Human fibronectin was purchased from BD Biosciences Labware (Bedford, MA). Human collagen IV was purchased from Chemicon. Human collagen I was purchased from Collaborative Biomedical products (Bedford, MA). Mouse osteopontin was purchased from R&D systems (Minneapolis, MN). Human LN-5 was purchased from Oriental Yeast Co. (Nagahama, Japan). FN-related peptides (GRGDSP and GRGESP) were purchased from Takara (Otsu, Japan).

Attachment Assays—Attachment assays were performed as follows. Briefly, FCS or 10 μg/ml of FN and LN-1 solution in phosphate-buffered saline (PBS) was spread evenly on the surface of 96-well plates. The protein was allowed to adsorb for either 1 or 12 h at 37 °C. Nonspecific interactions were blocked with 0.2% bovine serum albumin (BSA) in PBS. Cell suspensions were prepared in serum-free DMEM, and 2.5×10^4 cells were seeded on 96-well plates. After 30- to 40-min incubation, non-adherent cells were removed and adherent cells were fixed with 70% ethanol. Following staining with a 0.1% solution of crystal violet or methylene blue for 30 min, the cells were rinsed and the crystal violet or methylene blue adsorbed onto the adherent cells was dissolved with 0.5% Triton X-100. The optical density was then measured at 595 nm (25). The number of the cells that adhered to the plate was calculated by using a cell-number standard curve (cell number *versus* absorbance values) and presented. For peptide inhibition assays the peptides GRGDNP and GRGESP, at a final concentration of 1 mM, were incubated with the cell suspension for 10 min before seeding of the cells to the wells.

Detachment Assays—Suspended cells (1.2×10^4) were plated on 24-well plates and incubated for 24 h. Cells were then dissociated from the culture plate by incubation with 0.125% trypsin/0.1 mM EDTA or 0.025% trypsin/0.02 mM EDTA at 37 °C for the indicated times. DMEM containing 10% FCS or trypsin inhibitor was then added to the cells to inhibit trypsin. Following removal of the detached cells, the number of remaining cells was determined using crystal violet as described above. The data are also presented as a percentage of remained adherent cells to untreated cells. Detachment assays were performed in Transwell plates with a 0.4-μm pore size (Corning, Corning, NY). Cells (1×10^5) were seeded in the upper chamber of the Transwell plates, incubated for 24 h, and then treated with 0.125% trypsin/0.1 mM EDTA that was added to the lower chamber. At the indicated time trypsin was inactivated with 0.2% trypsin inhibitor, the cells in the upper chamber were rinsed, and the remained cells were determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (26).

RT-PCR Analysis—Total RNA were extracted from the cells with the Isogen kit (Wako, Osaka Japan) and was reverse transcribed using ReverTra Ace (Toyobo, Osaka, Japan). The resulting cDNAs were employed as templates for specific PCR reactions using GoTaq DNA polymerase (Promega, Madison, WI). The sequences for PCR primers were listed in Table 1 and 2. RT-PCR of glyceraldehyde-3-phosphate dehydrogenase was used as an internal control.

siRNA-mediated Protein Knockdown—MDCK/neo cells (2×10^5) were seeded per well in 6-well plates in antibiotic-free DMEM with FCS. After 24-h incubation, MDCK/neo cells were transfected with an siRNA duplex directed against LN-$\alpha 3$ (Sigma Genosys, Ishikari, Japan) using Lipofectamine 2000 (Invitrogen). The sequence of the siRNA target was 5′-AAATGACTACGAAGCCAAACT-3′ (27). After incubation for 5 h, FCS was then added and the cells were incubated for an addi-

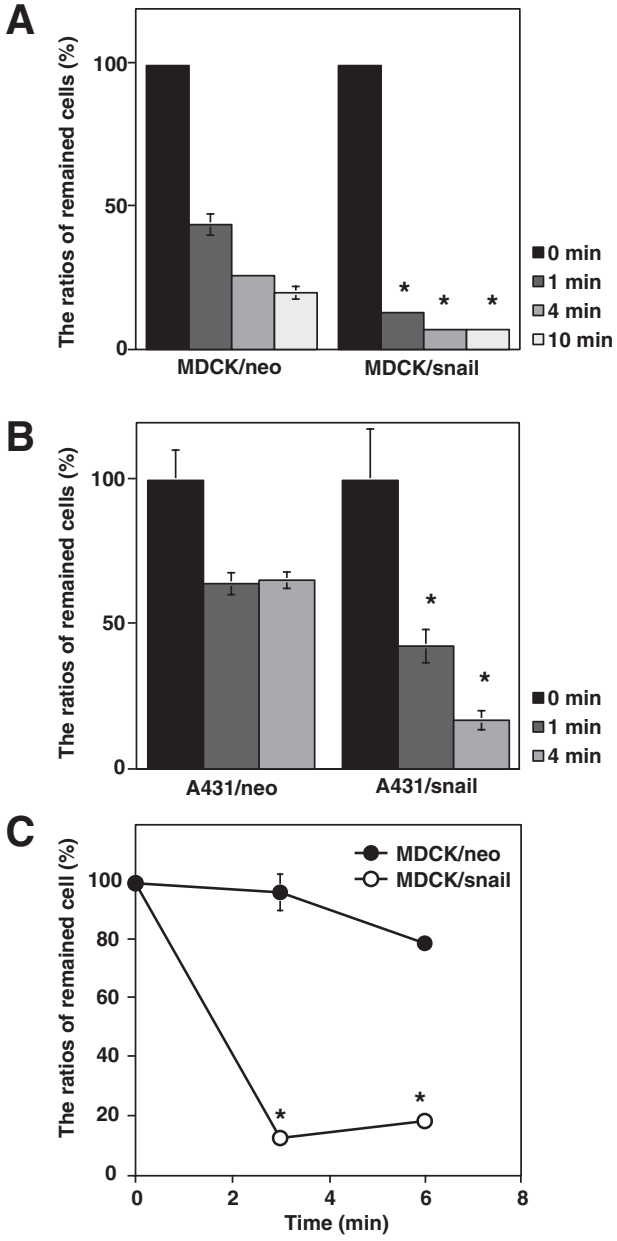

FIGURE 1. **Cell detachment is enhanced in snail-expressing cells.** *A*, detachment assays of MDCK/neo and MDCK/snail cells in 24-well plates. Cells were sparsely seeded (1.2×10^4 cells/well) on 24-well plates to and then incubated for 24 h at 37 °C. Cell monolayers were then treated with a solution of 0.125% trypsin/0.1 mM EDTA for the indicated times. The remaining cells adhered to the plates were counted by staining with crystal violet and the data are presented as the percentage of the adhered cells to total (untreated) cells. *B*, detachment assays of A431/neo and A431/snail cells in 96-well plates. Cells were seeded (2×10^4 cells/well) on 96-well plates and then incubated for 24 h at 37 °C in DMEM without FCS. Detachment assay was performed as described above. *C*, detachment assays of MDCK/neo and MDCK/snail cells in Transwell plates. Cells were seeded (1×10^5 cells/well) in the upper chamber of the Transwell plates, incubated for 24 h at 37 °C, and then treated with 0.125% trypsin/0.1 mM EDTA that was added to the lower chamber. At the indicated times, the remaining cells in the upper chamber were counted by a MTT assay as previously described (26). Data are the mean ± the S.E. for triplicate determinations. *, $p < 0.05$ *versus* MDCK/neo (*A* and *C*) or A431/neo (*B*) cells.

tional 24 h. The cells were then harvested and seeded for further experiments.

Anoikis Assay—Poly-2-hydroxyethyl methacrylate (poly-HEMA) (Sigma, St. Louis, MO) was applied to 6-well plates (0.5 ml/well of a 12 mg/ml stock solution in ethanol) and allowed to

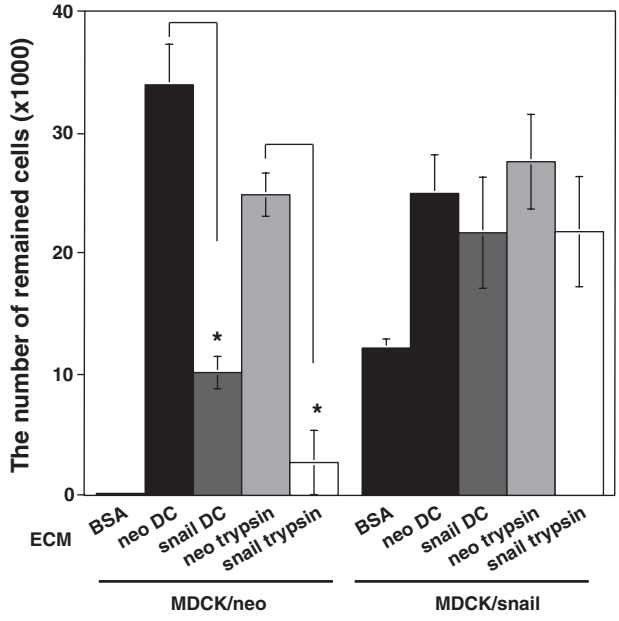

FIGURE 2. **The ECM produced by MDCK/snail cells is less adhesive for MDCK/neo cells than that produced by MDCK/neo cells.** To obtain the deposited ECM for this assay, MDCK/neo and MDCK/snail cells were seeded (5×10^4 cells/well) on 24-well plates and incubated for 48 h. The cells were then removed from the matrix protein by treatment with 0.1% deoxycholic acid (DC) or 0.125% trypsin/0.1 mM EDTA (trypsin). The ECM deposited by MDCK/neo (neo DC and neo trypsin) or by MDCK/snail (snail DC and snail trypsin) were washed with DMEM with 10% FCS and PBS. Blocking of nonspecific interactions was achieved by incubation with 0.2% BSA. MDCK/neo and MDCK/snail cells were then seeded (5×10^4 cells/well) onto the ECM-covered plates and incubated for 40 min at 37 °C in DMEM without FCS. After removing the non-adherent cells with PBS, adherent cells were quantified by staining with crystal violet. Data are the mean ± the S.E. for triplicate determinations. *, $p < 0.05$ *versus* ECM deposited by MDCK/neo cells.

air dry. This procedure was repeated twice. MDCK/neo and MDCK/snail cells (5×10^5/well) were cultured either on poly-HEMA-coated or non-coated plates for 24 h. After incubation, detached and suspended cells from poly-HEMA-coated plates were harvested in DMEM and centrifuged at $500 \times g$ for 5 min. Cells from non-coated plates were trypsinized, collected, and centrifuged in a similar manner. The pellets were stained with 100 μl of propidium iodide (PI) solution (10 μg/ml in PBS) at 25 °C for 15 min. The number of viable cells was then analyzed by using flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA).

Lactate Dehydrogenase (LDH) Assay—MDCK/neo and MDCK/snail cells (1×10^5) were cultured either on poly-HEMA coated, or non-coated 24-well plates for 24 h at 37 °C. The medium was recovered from cell culture and was assayed for LDH, released from dying cells, using a previously described method (28). Data for released LDH were normalized relative to total LDH and expressed as a percentage. All measurements were performed in triplicate.

Promoter Assay—The mouse integrin αV promoter, with the indicated deletions of the 5′-flanking region, was cloned upstream from the luciferase reporter gene (pGL3-Basic vector, Promega). The luciferase expression vectors, including the fragments deleted up to positions −3200, −1500, −933, −617, −310, −108, +22, to +97 were designated pIαV(−3.2k)-Luc, pIαV(−1.5k)-Luc, pIαV(−933)-Luc, pIαV(−617)-Luc, pIαV(−310)-Luc, pIαV(−108)-Luc, pIαV(+22)-Luc, and pIαV(+97)-Luc, respectively (29). Cells (1×10^5) were

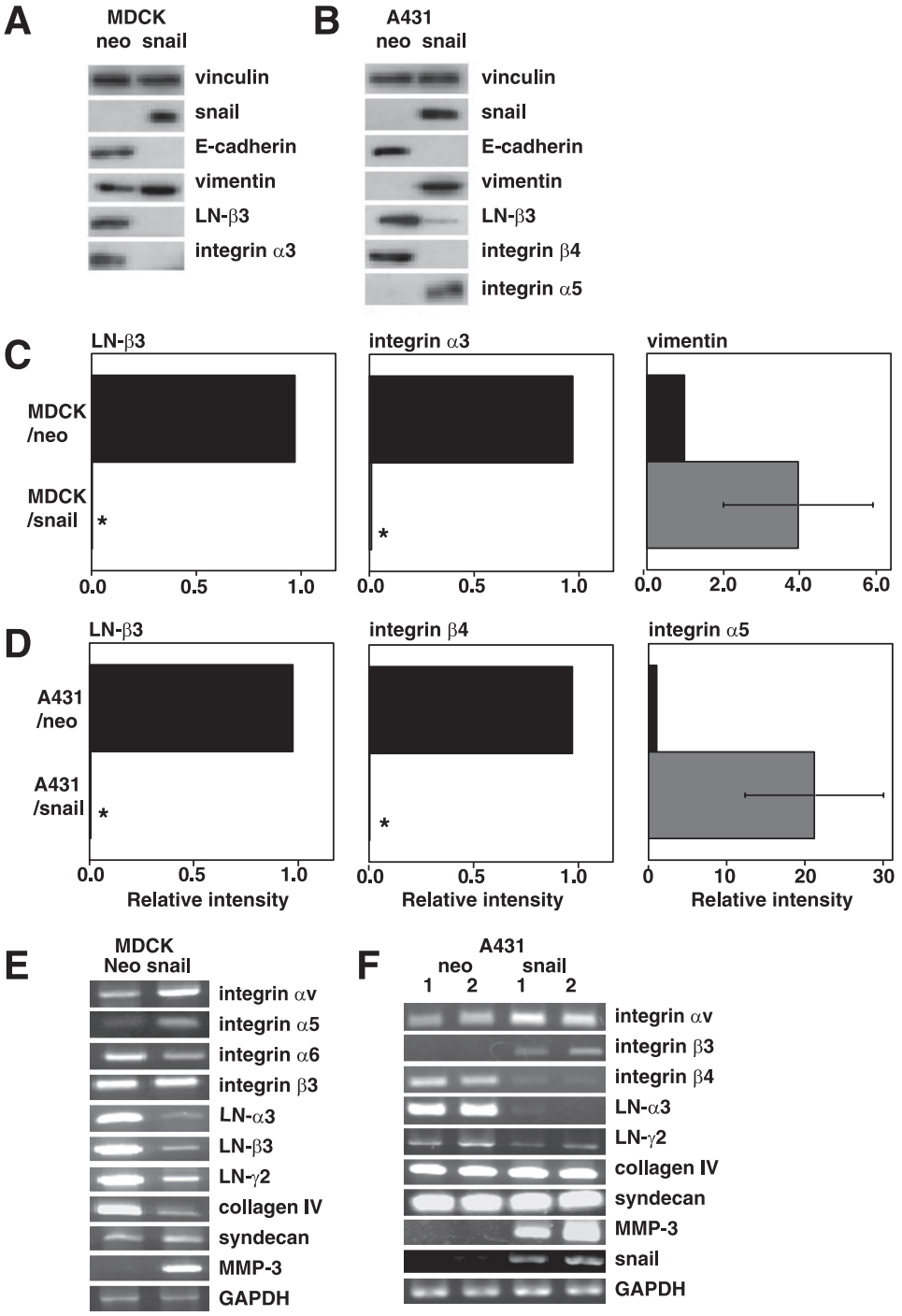

FIGURE 3. **Snail regulates the expression of integrins and ECM proteins.** *A* and *B*, effect of snail on the protein expression of integrins and ECM proteins. MDCK/neo and MDCK/snail cells (*A*) and A431/neo and A431/snail cells (*B*) were lysed in SDS sample buffer and subjected to Western blot analysis with the indicated antibodies. Vinculin was served as an internal control for protein loading. Measurement of protein expression level was achieved by using ImageJ (National Institutes of Health). The data are presented as the relative intensity of the bands of from MDCK/snail cells to that in MDCK/neo cells (*C*) and the bands from A431/snail to that in A431/neo cells (*D*). *, $p < 0.05$ *versus* MDCK/neo (*C*) or A431/neo (*D*) cells. Effect of snail on mRNA expression of integrins and ECM proteins in MDCK cells (*E*) and in A431 cells (*F*). RT-PCR of glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. The sequences for PCR primers are listed in Tables 1 and 2.

seeded into 24-well plates 24 h prior to transfection. One microgram of reporter vector and 20 ng of pRL-CMV vector were transfected per well using Lipofectamine Plus. After 48 h, both firefly (FL) and *Renilla* luciferase (RL) activities were measured using the Dual luciferase reporter assay kit (Promega). FL activities were normalized to the RL activities. The experiments were performed in triplicate.

Migration Assay—Migration assays were performed in Transwell plates with an 8-μm pore size (Corning) as described previously (26) with minor modifications. Briefly, 0.1 ml of cells (1×10^6 cells/ml) in DMEM supplemented with 0.1% BSA was seeded into the upper wells. FN or osteopontin was added to 0.6 ml of DMEM supplemented with 0.1% BSA in the lower wells at a final concentration of 10 μg/ml. Conditioned medium of MC3T3-E1 cells or fresh medium containing 10% FCS (0.15 ml) was added to 0.45 ml of DMEM supplemented with 0.1% BSA in lower wells. After 4 h of incubation, the cells remaining in the upper wells were completely removed. The cells that had migrated to the lower surface of the filter were subjected to the MTT assay.

Statistical Analysis—Statistical analysis was performed by Student's *t* test. Differences were considered to be significant at $p < 0.05$.

RESULTS

Snail Enhances Cell Detachment from Tissue Culture Dishes—We observed that the transfection of snail into MDCK cells enhanced cell detachment from tissue culture dishes. We therefore quantified detachment of MDCK/snail cells and compared it to that of MDCK/neo cells. Cells were sparsely seeded to inhibit cell-cell contacts. Following incubation for 24 h at 37 °C, the cells were treated with 0.125% trypsin/0.1 mM EDTA for up to 10 min. The remaining adherent cells were then stained with crystal violet, and the percentage of remaining cells to total cells was assessed. Approximately 88% of MDCK/snail cells detached within 1 min by trypsin/EDTA treatment (Fig. 1*A*). In contrast, only ~55% of MDCK/neo cells were detached at this time point. We also found that ~83% of A431/snail cells whereas only 35% of A431/neo cells detached within 4 min by 0.025% trypsin/0.02 mM EDTA treatment (Fig. 1*B*). It has been

TABLE 1
Dog primer sequences for RT-PCR

Gene	Forward primer	Reverse primer
Integrin αV	TCTGTTGCTGCCACTGACAT	AACTGGTCTGGCTCTGTATA
Integrin α5	GGCAGCCACGGCGTCCCGCTGTG	GGCATCAGAGGTGGCAGGTGGCTT
Integrin α6	GAACTGCTTTTATCGGTCTC	AGAATGTCCAAGTAGTTCAG
Integrin β3	GATGAGGCCCTGCCTCTGGGCTC	CTTGCCGAACTTGGATGGAGAA
LN-α3	ACAGATGGGGAAGGAAACAAC	AGTGGCCCGCTTTGCTTGG
LN-β3	CTGTCGAGAAGGGTTTGGTG	TCTGCTCCATCTTGCTCCTG
LN-γ2	AATGGGAAGTCCAGGCAGTGTGTC	ACAGCGCTCGCCGGTGACAGC
Collagen IV	ATGTCCATGGCGCCCATCGC	CTTCAAGGTGGATGGCGTGG
Syndecan	AGGACGAGGGGAGCTATGACC	GTGGGGGCCTTCTGATAAG
MMP-3	TGGGTCTCTTTCACTCGGCT	GGATAACCGGCTTGTACCTC
GAPDH	TGAAGGTCGGTGTGAACGGATTTGGC	CATGTAGGCCATGAGGTCCACCAC

TABLE 2
Human primer sequences for RT-PCR

Gene	Forward primer	Reverse primer
Integrin αV	AGAATCATTCCTATTCTCTG	TTCTTCTTGAGGTGGCCGGA
Integrin β3	CCTACATGACCGAAAATACCT	AATCCCTCCCCACAAATACTG
Integrin β4	GGTCCAGGAAGATCCATTTCAA	TAGCAGACCTCGTAGGCTGTGA
LN-α3	ACAGATGGAGAGGGAAACAAC	ATTTGCCTGCTTGGCTTGG
LN-γ2	TCAGCCAGAAGGTTTCAGATGCC	GGCCAGCTTCACTGTTGCTCAAGCAG
Collagen IV	ATGTCAATGGCACCCATCAC	CTTCAAGGTGGACGGCGTAG
Syndecan	AGGACGAAGGCAGCTACTCCT	TTTGGTGGGCTTCTGGTAGG
MMP-3	GAACAATGGACAAAGGATACAAC	AAATGAAAACGAGGTCCTTGCTAG
Snail	ACTACAGCGAGCTGCAGG	GTGTGGCTTCGGATGTGC
GAPDH	GCATCCTGGGCTACACTG	GTGAGGAGGGGAGATTCAG

proposed that cell-cell junctional complexes can inhibit the accessibility of trypsin to cell surface proteins that mediate cell attachment to the substratum. E-cadherin is considered to be the main component of junctional complexes that mediates this inhibition. To remove the effect of adhesion proteins on the accessibility of trypsin, we performed the detachment assay in Transwell chambers. MDCK/snail or MDCK/neo cells (1×10^5 cells/well) were seeded in the upper part of the chambers and, 24 h later, 0.1% trypsin/0.02% EDTA was added to the lower part of the chambers. Cells remaining adhered to the upper chamber were then counted by MTT assay. Over 80% of MDCK/snail cells were detached after 3 min of trypsin treatment compared with only 3% of MDCK/neo cells (Fig. 1*C*). Therefore, altered accessibility of trypsin to the proteins involved in cell-substrate attachment is not the reason why MDCK/snail cells show accelerated cell detachment compared with control cells.

Snail Reduces the Expression of ECM Proteins—We next determined whether the rapid detachment of snail-expressing cells was due to changes in the ECM produced by these cells. We therefore compared the adhesion of MDCK/neo or MDCK/snail cells to the ECM deposited by either cell type. MDCK/snail cells adhered to the ECM deposited by both the MDCK/neo and MDCK/snail cells, with a comparable extent. However, MDCK/neo cells were much less adherent to the ECM laid down by MDCK/snail cells than to their own ECM (Fig. 2). This result suggests that the ECM components deposited by the MDCK/snail cells must be altered in some way. The expression of ECM proteins involved in binding to the receptors of MDCK/neo cells might be reduced in MDCK/snail cells. The ECM prepared with deoxycholic acid had more ability to support adhesion of MDCK/neo cells than that prepared with trypsin. Trypsin might degrade the proteins that involved in the adhesion of MDCK/neo cells. Furthermore, the expression of receptors for ECM proteins may be altered in MDCK/snail cells, thereby MDCK/snail cells adhered to the ECM from both cell types to a comparable level. The major adhesive components expressed and secreted by epithelial cells are LN, heparan sulfate proteoglycan, and collagens. We therefore determined protein and mRNA expressions of these molecules in MDCK/neo, MDCK/snail, A431/neo, and A431/snail cells. LN-β3 protein was undetectable in MDCK/snail nor A431/snail cells but was expressed in MDCK/neo and A431/neo cells, as measured with an antibody against LN-β3 (Fig. 3, *A–D*). The expressions of the LN-α3, -β3, and -γ2 subunits were also reduced in MDCK/snail cells, and the expressions of LN-α3 and γ2 were reduced in A431/snail cells when assayed by RT-PCR (Fig. 3, *E* and *F*). In agreement with a previous report, the expression of vimentin, as measured by Western blot, was enhanced in MDCK/snail and in A431/snail cells compare with MDCK/neo cells and A431/neo cells (Fig. 3, *A–C*). In contrast, the level of mRNA expression of syndecan was similar in MDCK/neo and MDCK/snail cells and A431/neo or A431/snail cells when assayed by RT-PCR (Fig. 3, *E* and *F*). We also found not only LN-5 protein expression but also the expressions of receptor proteins for LN-5 such as integrin α3 and α6 were strongly suppressed in MDCK/snail cells as assessed by Western blot (Fig. 3, *A* and *C*) or by RT-PCR (Fig. 3*E*), respectively. The expression of integrin β4 was also suppressed in A431/snail cells as assessed by both Western blot (Fig. 3, *B* and *D*) and by RT-PCR (Fig. 3*F*). These data indicate that snail regulates the expression of specific ECM proteins and their receptors. We also determined the expressions of decorin, collagen I, integrin α2, integrin β1, and MMP-2, and found they were unaltered in snail-expressing cells (data not shown).

Inhibition of LN-α3 Synthesis with siRNA Does Not Enhance Cell Detachment—To investigate whether the reduction in LN-5 protein expression might be responsible for the changes in cell detachment from the ECM observed in snail-expressing cells, LN-5 synthesis was inhibited by introduction of siRNA

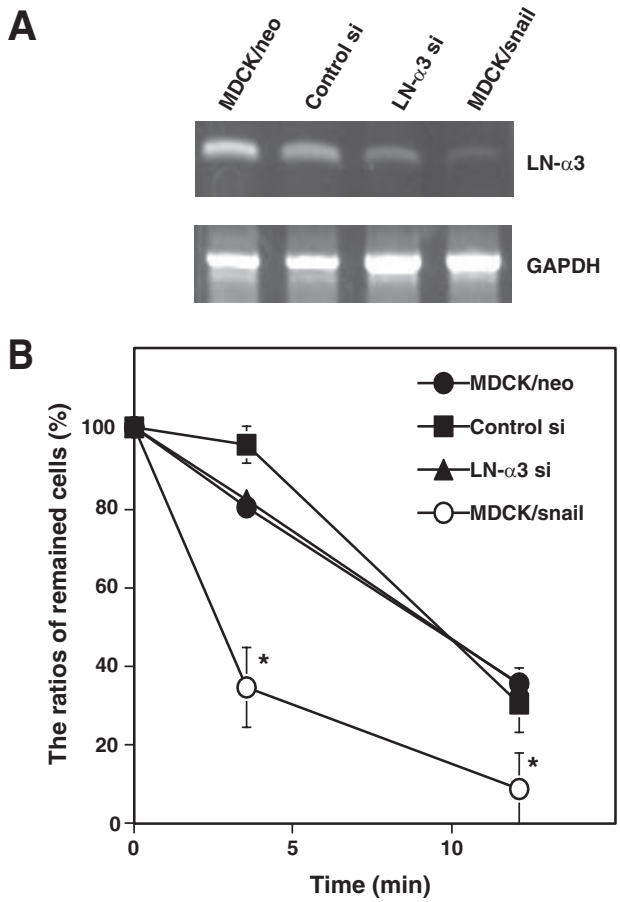

FIGURE 4. **Suppression of LN-α3 expression by siRNA does not affect cell detachment.** *A*, RT-PCR analysis of LN-α3 transcripts. MDCK/neo cells were transfected with control siRNA (*Con si*) or with siRNA targeted against LN-α3 (*LN-α3 si*). The expression of LN-α3 mRNA or of control glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA was assayed by RT-PCR using specific primers listed in Table 1. *B*, effect of LN-α3 siRNA on detachment of MDCK/neo cells. MDCK/neo cells, MDCK/neo cells that were transfected with control siRNA (*Con si*) or with LN-α3 siRNA (*LN-α3 si*) and MDCK/snail cells were seeded (1×10^5 cells/well) on 24-well plates and incubated for 24 h at 37 °C. Detachment assays were performed as described in the legend to Fig. 1*A*. The data are presented as the percentage of the cells that remained adhered to the plate. Data are the mean ± the S.E. for triplicate determinations. *, $p < 0.05$ *versus* MDCK/neo cells.

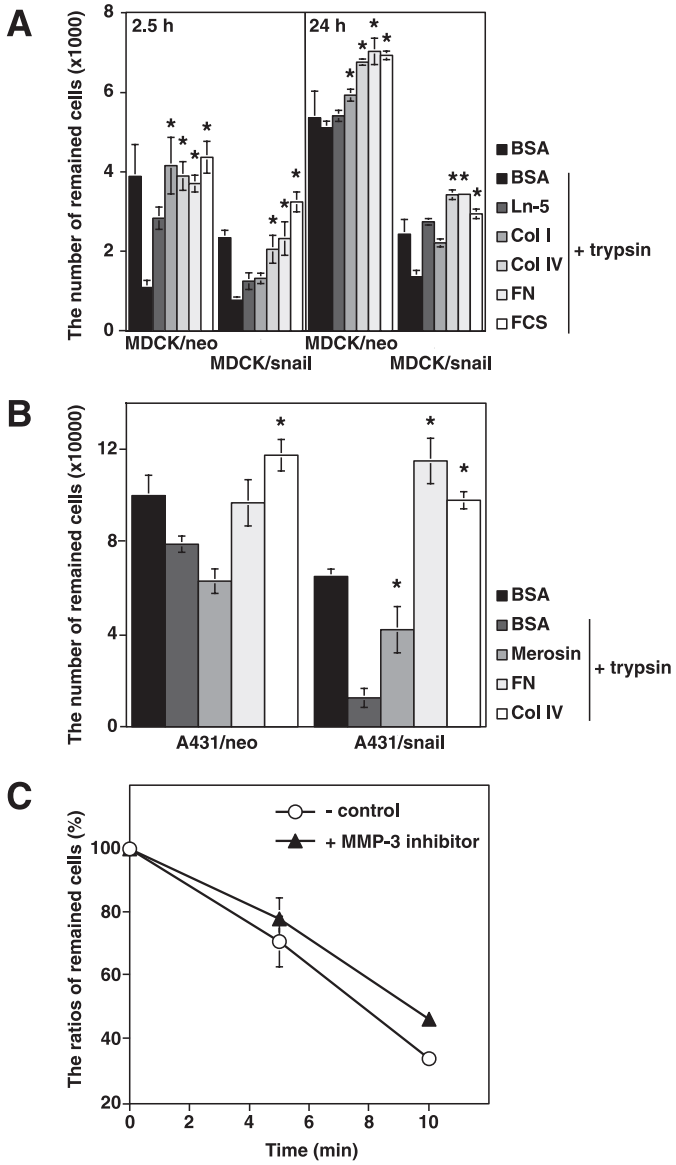

FIGURE 5. **Collagen IV suppresses the detachment of MDCK/snail and A431/snail cells.** *A*, effects of various ECM proteins on cell detachment of MDCK cells. The wells of 24-well plates were coated with 0.25 ml of 1 μg/ml purified LN-5, 10 μg/ml collagen I, collagen IV, FN, or 10% FCS. Twenty-four hours later, MDCK/neo and MDCK/snail cells were seeded (3.5×10^4 cells/well) onto these wells and incubated for 2.5 or 24 h in DMEM without FCS. These cells were then treated with 0.025% trypsin/0.02 mM EDTA for 90 s. The detached cells were removed, and the remaining cells on the plate were fixed with 70% ethanol and stained with crystal violet and quantified. *, $p < 0.05$ *versus* trypsin-treated cells on BSA-coated wells. *B*, effects of various ECM proteins on cell detachment of A431 cells. A431/neo and A431/snail cells were seeded (8×10^4 cell/well). Detachment assay was performed as described above. *, $p < 0.05$ *versus* trypsin-treated cells on BSA-coated wells. *C*, effect of MMP-3 inhibitor on detachment of MDCK/snail cells. Cells were incubated without or with MMP-3 inhibitor at 1 μM for 24 h and subjected to detachment assay using enzyme free cell dissociation buffer.

targeted to the LN-α3 subunit. The expression of LN-α3 was reduced in MDCK/neo cells that had been transfected of siRNA targeted to LN-α3 (LN-α3 si) compared with that in cells transfected with control siRNA (control si) (Fig. 4*A*). The morphology of the LN-α3 si cells remained unchanged compared with control si cells (data not shown). We then assayed the effect of knockdown of LN-α3 on cell detachment. There was no difference in detachment between LN-α3 si and control si cells (Fig. 4*B*). These data suggest that the observed reduction of LN-5 in snail-expressing cells is not solely responsible for the increased cell detachment in these cells. Thus, we tested the effect of other matrix proteins on cell detachment. MDCK/neo, MDCK/snail, A431/neo, and A431/snail cells were seeded on the wells precoated with various ECM proteins, and detachment assay was performed after incubation for 2.5 and 24 h (Fig. 5*A*) or 24 h (Fig. 5*B*). The detachment of MDCK/neo and MDCK/snail cells by trypsin/EDTA treatment was significantly suppressed in the wells precoated with collagen IV, FN, and 10% FCS. We also found the detachment of A431/snail cells was significantly suppressed in the wells precoated with collagen IV and FN. Because the expression of collagen IV was reduced in MDCK/snail cells (Fig. 3*E*), this reduction might be responsible for increased detachment of MDCK/snail cells at least in part. Although the detachment of MDCK/neo cells by 0.025% trypsin/0.02 mM EDTA treatment was much reduced after 24-h incubation, the levels of detachment of MDCK/snail cells were unaltered

between 2.5- and 24-h incubation. This suggests that the degradation of ECM proteins might be enhanced in MDCK/snail cells. Snail has been reported to induce the expression of MMP-9, a member of MMPs known to be involved in the degradation of BM proteins. Although we observed remarkable expression of MMP-3 in snail-expressing cells (Fig. 3*E* and 3*F*), MMP-3 inhibitor (1 μM) showed no significant effect on detachment of MDCK/snail cells (Fig. 5*C*).

Snail Does Not Confer Resistance to Anoikis—Recently, snail has been reported to act as a survival factor. Snail gene expression protects against cell death of the neural crest (30) and against cell death induced by the withdrawal of survival factors or pro-apoptotic signals (31). Loss of contact to substrate induces anoikis. Because snail enhances the detachment of cells, we investigated if snail expression might confer resistance to anoikis. To analyze the cellular response to anoikis, cells were cultured under non-adherent conditions by seeding on poly-HEMA-coated dishes. We determined the extent of cell death by flow cytometry as a failure of the cells to exclude the membrane-impermeable dye, propidium iodide (PI). After 24 h of culture, MDCK/snail cells showed a similar, or higher, level of anoikis than that of MDCK/neo cells (Fig. 6*A*). These data suggested that snail is unable to protect cells against anoikis. Similar conclusions were reached when cell death was determined by LDH assay (Fig. 6*B*).

Snail Enhances Cell Attachment to FN—The fact that snail enhances cell detachment from the ECM but does not suppress anoikis suggests that, in order for the detached, snail-expressing tumor cells to survive and proliferate in the body, they must re-attach to the substratum at a different site. Indeed, MDCK/snail cells attached to culture dishes much faster than control cells (Fig. 7*A*). Furthermore, attachment to FCS- or FN-coated plates was significantly enhanced in MDCK/snail and A431/snail cells compared with MDCK/neo and A431/neo cells when assayed over a short period (30 min) (Fig. 7, *B* and *D*). The addition of RGD peptides (1 mM), which inhibit the binding of FN to its cell surface receptor, completely abrogated the attachment of MDCK/snail cells (Fig. 7*C*).

Snail Regulates the Expression of Various Integrins—Enhancement of cell adhesion to FN, as well as inhibition of cell adhesion by RGD peptides, suggested that snail might modulate the expression of FN-binding integrins. We therefore compared the expression of various integrins in MDCK/snail and A431/snail cells to that in MDCK/neo and A431/neo cells. The expression of integrin α5 was up-regulated in A431/snail cells at protein level (Fig. 3, *B* and *D*) and in MDCK/snail cells at RNA level (Fig. 3*E*). At the RNA level, the expression of the vitronectin receptors integrin αV was also up-regulated in MDCK/snail and A431/snail cells (Fig. 3, *E* and *F*). The combined data therefore suggest that snail regulates the expression of specific components of the ECM, as well as integrins.

Snail Regulates the Promoter Activity of Integrin αV—Snail is known to regulate the transcription of E-cadherin. We therefore investigated whether the effect of snail on the expression of integrin αV was mediated via modulation of its promoter activity. The promoter activity of mouse integrin αV was significantly increased in the MDCK/snail cells compared with the MDCK/neo cells (Fig. 8). To better define the region of the promoter that is responsible for the effect of induction by snail, we transfected MDCK/snail cells with luciferase expression vectors encoding various deletions in the promoter. Successive 5′- deletions from position −3.2k to −108 in plαV-Luc resulted in a gradual decrease in luciferase activity. However, elimination of the region (−108 to +22) from plαV (−108)-Luc resulted in a remarkable decrease in luciferase activity (Fig. 8). This region contains the consensus binding site for the transcription factors AP-2, Ets, and Sp1.

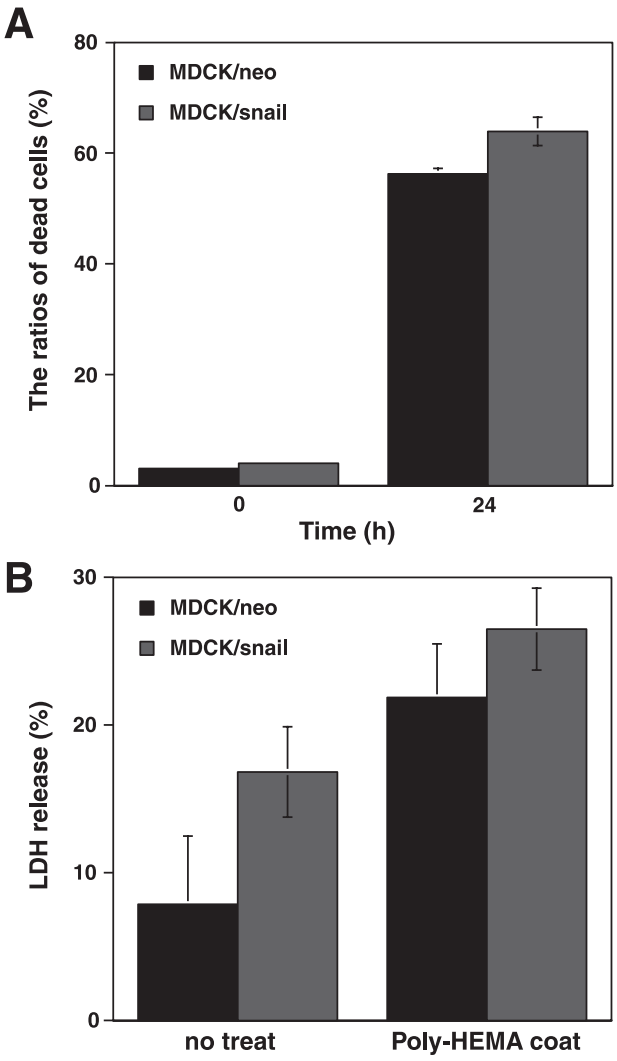

FIGURE 6. **Snail does not confer resistance to anoikis.** *A*, analysis of anoikis of MDCK/neo and MDCK/snail cells by PI staining and subsequent FACS analysis. MDCK/neo and MDCK/snail cells (5×10^5 cells/well) were cultured either on non-coated, or on poly-HEMA-coated 6-well plates for 24 h at 37 °C. After incubation, detached and suspended cells from poly-HEMA-coated plates were harvested (24 h). Cells from non-coated plastic tissue were trypsinized and harvested (0 h). Cells were stained with PI solution, and the percentage of dead cells was quantified by flow cytometry. *B*, analysis of anoikis with the LDH assay. MDCK/neo and MDCK/snail cells (1×10^5 cells/well) were cultured either on non-coated or poly-HEMA-coated 24-well tissue culture plates for 24 h at 37 °C. The medium was recovered, and LDH that released from dying cells was measured. Data for released LDH were normalized relative to total LDH and expressed as a percentage. All measurements were performed in triplicate.

Snail Promotes Migration of MDCK Cells toward FN, Osteopontin, and the Conditioned Medium of Osteoblasts—It has recently been reported that tumor-specific expression of $\alpha V\beta 3$ integrin enhances the spontaneous metastasis of breast

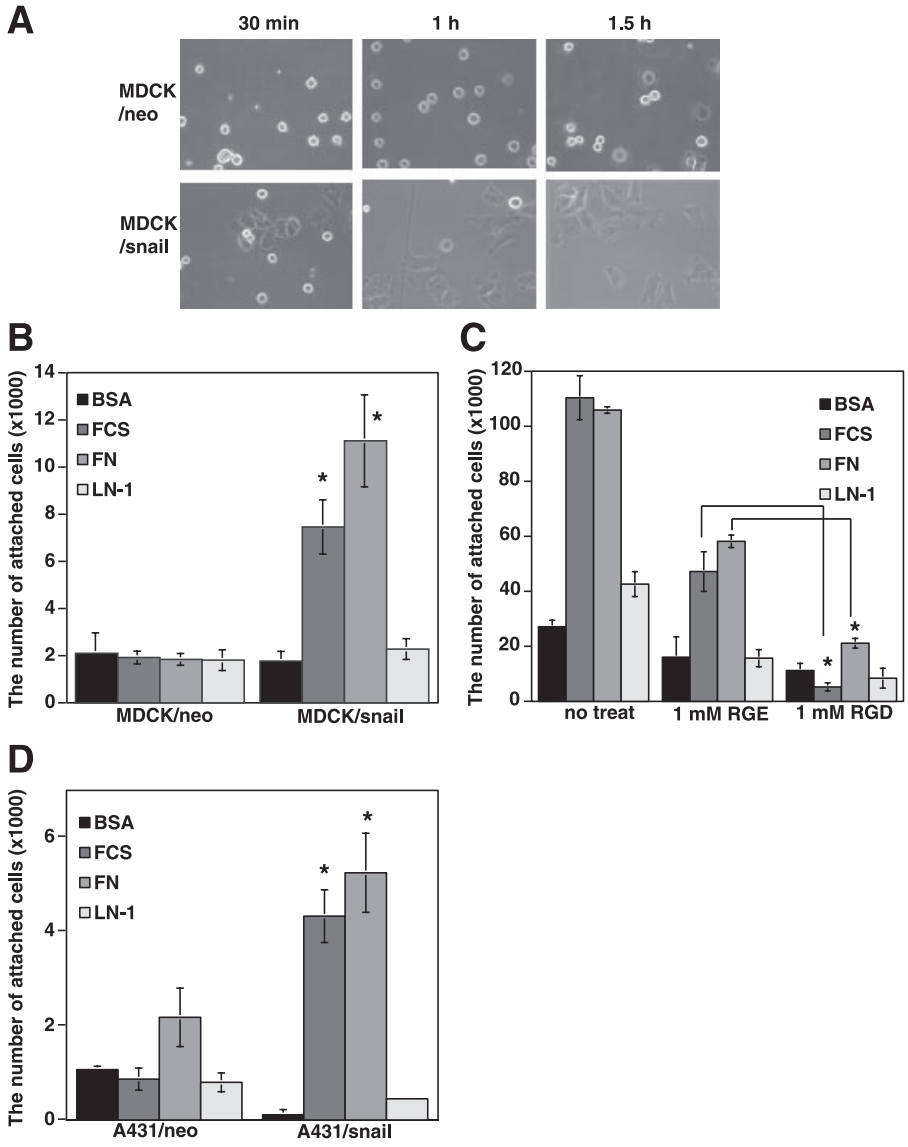

FIGURE 7. **Snail enhances cell adhesion to FCS- and FN-coated plates.** *A*, attachment of MDCK/neo and MDCK/snail cells to tissue culture plates. Representative micrographs of the cells, photographed at the indicated time after seeding, are shown. *B*, attachment assays of MDCK/neo and MDCK/snail cells to various ECM substrates. The wells of 96-well plates were coated with FCS or 10 μg/ml of FN or LN-1. Twenty-four hours later, cells were seeded (2.5×10^4 cells/well) onto these wells and incubated for 30 min at 37 °C in DMEM without FCS. After removing non-adherent cells, the adhered cells were quantified by staining with methylene blue. *, $p < 0.05$ *versus* MDCK/neo cells. *C*, inhibition of cell attachment by RGD peptides. MDCK/snail cells were incubated with or without 1 mM RGD or RGE peptides for 10 min before seeding on 96-well plates. An attachment assay was performed as described above. *, $p < 0.05$ *versus* RGE-treated cells. *D*, attachment assays of A431/neo and A431/snail cells to various ECM substrates. *, $p < 0.05$ *versus* A431/neo cells.

tumors to bone. The same study also showed the chemotactic migration of $\alpha V\beta 3$-overexpressing tumor cells toward a monolayer of bone stroma cells (32). Because we found an increase in the expression of integrin αV in snail-expressing cells, we tested if snail expression might also enhance the migratory activity of these cells. Migration of MDCK/snail cells toward osteopontin, an abundant ligand for integrin $\alpha V\beta 3$ in bone, was examined. Compared with MDCK/neo cells, the migration of MDCK/snail cells toward FN, osteopontin, or the conditioned medium of osteoblasts (CM MC3T3) was significantly enhanced (Fig. 9*A*). Furthermore, the migration of MDCK/snail cells to osteopontin and FCS was significantly suppressed by function blocking integrin $\alpha V\beta 3$ antibody (Fig. 9*B*). By contrast, the migration of MDCK/snail cells to FN was unaffected by this antibody. These findings suggest that snail may enhance cell migration to osteopontin and FCS, but not FN, by enhancement of the expression of integrin αV. Because the expression of integrin $\alpha 5$, one of FN receptors (33), was enhanced in MDCK/snail cell, this type of integrin might mediate the migration of these cells to FN.

DISCUSSION

Although snail has been reported to suppress the expression of adherence- or tight junctional-proteins, and act as an inducer of cell movement and tumor invasion, a role for snail in the regulation of cell-substratum association has not been defined. This is the first report showing that snail enhances not only cell detachment but also cell attachment. Our finding that snail enhances cell detachment suggests that the interaction between ECM proteins and their cell surface receptors may be weakened in snail-expressing cells. The fact, that the adhesion of MDCK/neo cells to ECM deposited by MDCK/snail cells is significantly lower than that to the ECM deposited by the MDCK/neo cells, also suggests a defect in the production of ECM in snail-expressing cells.

It has previously been reported that snail-transfected cells do not produce LN-5, the major protein in BM (17). We confirmed the reduction of LN-$\alpha 3$, -$\beta 3$, and -$\gamma 2$ in MDCK/snail and A431/snail cells. We propose that the deficiency in the expression of these proteins must therefore play a role in cell detachment in snail-expressing cells. However, we found that suppression of LN-$\alpha 3$ expression by transient transfection of siRNAs unaltered cell detachment. It is possible that a low level of LN-5 is sufficient for strong adhesion of MDCK/neo cells that have intact expressions of receptors for LN-5 such as integrin $\alpha 3$ and $\alpha 6$. Because MDCK/snail cells have reduced expressions of integrin $\alpha 3$ and $\alpha 6$ in addition to LN-5, they might show significant enhancement of detachment.

Detachment of cells from the ECM induces a programmed cell death termed anoikis. Snail overexpression has been associated with resistance to programmed cell death elicited by

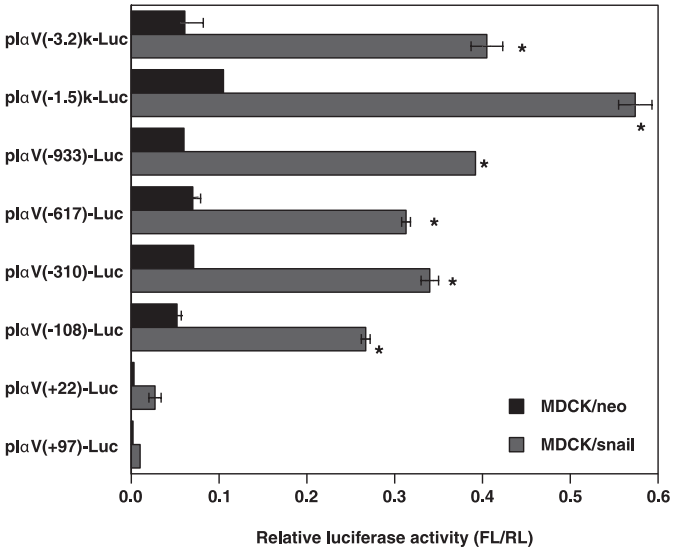

FIGURE 8. **The expression of snail enhances the promoter activities of integrin αV.** MDCK/neo and MDCK/snail cells were seeded (1×10^5 cells/well) onto 24-well and transfected with 1 μg of reporter vector and 20 ng of pRL-CMV vector. After 48-h incubation, both firefly (*FL*) and *Renilla* luciferase (*RL*) activities were measured using the Dual luciferase reporter assay kit (Promega). FL activities were normalized to the RL activities. Data are the mean ± the S.E. for triplicate determinations. *, $p < 0.05$ *versus* MDCK/neo cells.

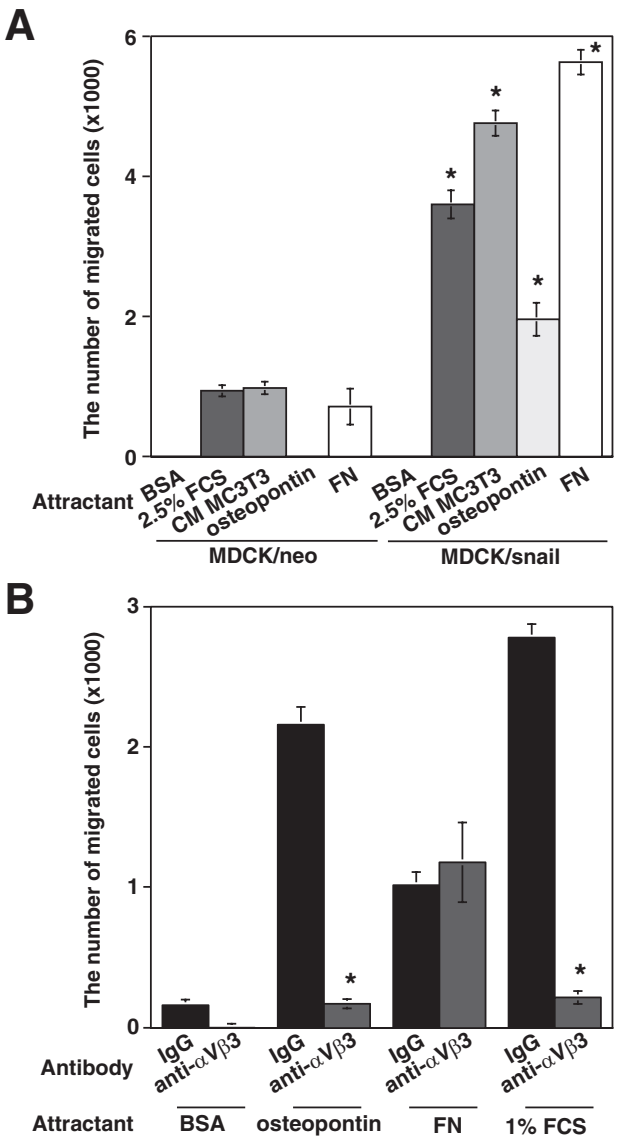

FIGURE 9. **Snail enhances cell migration toward FN, osteopontin, and the conditioned medium of osteoblasts.** *A*, migration assay of MDCK/neo and MDCK/snail cells to various attractant. Cells (1×10^6 cells/0.1 ml) were seeded into the upper wells. FN or osteopontin was added to the lower wells at a final concentration of 10 μg/ml. BSA was used at 0.1%. Conditioned medium of MC3T3 cells (CM MC3T3) or fresh medium containing 10% FCS was added to lower wells at 25% v/v. After 4 h of incubation at 37 °C, cells that had migrated to the lower surface of the filter were counted using the MTT assay. Data are the mean ± the S.E. for triplicate determinations. *, $p < 0.05$ *versus* MDCK/neo cells. *B*, inhibition of migration of MDCK/snail cell to osteopontin by function blocking integrin αVβ3 antibody. MDCK/snail cells were incubated with 25 μg/ml function blocking integrin αVβ3 antibody or control antibody in DMEM supplemented with 0.1% BSA for 20 min. Cells (8×10^4 cells/0.1 ml) were then seeded into the upper wells. Five μg/ml osteopontin, 1 μg/ml FN, and 1% FCS in DMEM supplemented with 0.1% BSA were added to the lower wells. Migration assay was performed as described above. *, $p < 0.05$ *versus* control antibody-treated cells.

DNA damage (34). In contrast to this, in our assays, the expression of snail did not confer resistance to anoikis. Recently, E-cadherin-mediated cell-cell adhesion in tumor cells was reported to suppress the anoikis (35). The reduction of E-cadherin in snail-expressing cells might be the reason why these cells are more sensitive to anoikis. The faster detachment of snail-expressing cells from the matrix, coupled with their sensitivity to anoikis, suggests that it may be more difficult for snail-expressing cells to metastasize *in vivo*. However, in our study, snail expression not only enhanced detachment from the ECM but also enhanced cell attachment to FN. This attachment to FN was completely abrogated in the presence of RGD peptides suggesting that it was mediated by cell surface integrin receptors. In agreement with this result, the expression of the FN receptors integrin αV and α5 were up-regulated in MDCK/snail and A431/snail cells. Thus increased snail expression may enhance the adhesion tumor cells to specific ECM substrates thereby conferring a selective advantage for tumor cell metastasis *in vivo*. It is not clear why MDCK/snail showed enhancement of detachment even though these cells have enhancement of expressions of FN and integrin αV and α5. We assume that enhancement of expression of FN and integrin αV or α5 might not be sufficient for surpassing the effect of reductions of other ECM proteins.

The promoter activity of mouse integrin αV was significantly higher in snail-expressing cells indicating that the effect of snail on integrin expression was mediated by its transcriptional activity. By elimination of various regions in the promoter region we identified the region within the integrin αV promoter (−108 to +22) that was transactivated by snail. Of note this region contains the binding sites for the transcription factors Ets and Sp1. This finding is consistent with a previous report that Sp1 and proximal Ets elements are crucial for snail modulation of the MMP-9 promoter (11). Several Sp1 elements are also present in the promoters of other genes such as vimentin, FN, as well as integrin α5, the expressions of which were up-regulated in MDCK/snail cells in this study. However, Sp1 sites also exist in the promoter and 5′-untranslated regions of the genes that showed reduced expression in snail-expressing cells such as integrin α6, α3, β4, LN-β3, and LN-γ2. The Ets element is present in the promoters of integrin α5 and MMP-3. Snail has also been shown to regulate the expression of E-cadherin by

binding to DNA elements that conform to the E-box consensus sequence. Several E-elements (CANNTG) are present in the promoter and 5′-untranslated region of the genes such as LN-β3, LN-γ2, integrin α3, α6, and β4, MMP-3, and integrin αV. Because snail regulates the expression of many genes, it might cooperate with other factors for the regulation of different promoters. These other factors may differ according to cell type.

The expression level of integrin $\alpha V\beta 3$ in various tumor tissues is strongly suggestive of a potential role for this receptor in tumor progression for invasive tumors that preferentially metastasize to bone (32, 36). Our study supports these findings as snail-expressing cells that showed enhanced expression of integrin αV also demonstrated enhanced cell migration to the conditioned medium of osteoblast MC3T3-E1 cells as well as toward osteopontin.

Osteopontin is an abundant ligand to the integrin $\alpha V\beta 3$ in bone. Although we cannot exclude the possibility that the migration-inducing factor in the media of osteoblast cells is a matrix protein derived from FCS, it is more likely that snail-expressing tumors have a greater propensity to metastasize to bone *in vivo*. Further analysis is required to determine the correlation between the level of snail expression and the level of bone metastasis.

The enhancement of integrin $\alpha V\beta 3$ expression in snail-expressing cells could provide a target for cancer treatment. Inhibition of binding between integrin $\alpha V\beta 3$ and its ligands by RGD peptides could potentially induce tumor cell death. Expression of snail might enhance migration and adhesion to specific substrates such as bone and protect cells from "anoikis" *in vivo*. The addition of an inhibitor specifically targeting integrin $\alpha V\beta 3$ may provide therapeutic benefits for the treatment of patients with snail-expressing tumors.

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