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Disruption of CADM1-dependent cell-cell adhesion in human oral squamous cell carcinoma cells results in tumor progression, possibly through an increase of MMP-2 and MMP-9 expression

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ABSTRACT

Objectives: This study aimed to clarify the molecular mechanism underlying the higher invasion and metastasis abilities of LMF4 cells than those of HSC-3 cells by comparing the expression levels of the tumor suppressor factor, cell adhesion molecule 1 (CADM1).
Methods: We explored 1) whether CADM1 expression level was downregulated in LMF4 cells compared with HSC-3 cells, 2) whether CADM1 expression knockdown increased the expression levels of matrix metalloproteinases (MMPs), 3) the exact cellular signaling pathways responsible for increased MMP expression after knockdown of CADM1 expression, and 4) whether disruption of CADM1-dependent HSC-3 cell adhesion increased the migratory and invasive activities of HSC-3 cells.
Results: CADM1 expression was lower in the LMF4 than in the HSC-3 cells. The knockdown of CADM1 increased the expression of MMP-2 and MMP-9 in HSC-3 cells. In addition, the upregulation of MMP-2 expression after CADM1 knockdown was abrogated by the mitogen-activated protein (MAP)/extracellular signal-regulated kinase kinase (MEK) inhibitor U0126 and the phosphoinositide 3-kinase (PI3K) inhibitor LY294002. The upregulation of MMP-9 expression after the knockdown of CADM1 was abrogated by the c-Jun N-terminal kinase (JNK) inhibitor SP600125 and the p38 MAP kinase (MAPK) inhibitor SB203580 and LY294002. Anti-CADM1 neutralizing antibody evoked migratory and invasive abilities of HSC-3 cells.
Conclusion: The disruption of CADM1-dependent cell-cell adhesion in human oral squamous cell carcinoma cells resulted in tumor progression, possibly through an increase in MMP-2 expression in a MEK/PI3K-dependent manner and an increase in MMP-9 expression in a JNK/p38 MAPK/PI3K-dependent manner.

1. Introduction

Squamous cell carcinoma (SCC) is the most common oral cavity cancer [1]. Epithelial-mesenchymal transition (EMT) plays an important role in SCC cell metastasis [2]. In our previous study, we clarified the signal transduction mechanism underlying the transforming growth factor beta 1 (TGF-β1)-induced EMT that promoted invasive and metastatic abilities in the human oral SCC (hOSCC) cell line HSC-4 [3,4]. We also demonstrated that the migration ability of HSC-4 cells was upregulated by integrin α3β1/FAK activation [3]. In addition, we found that TGF-β1 induced elevation of the Slug expression level, increasing the migration ability in HSC-4 cells. TGF-β1 also induced the invasive ability in HSC-4 cells through the Slug/Wnt-5b/matrix metalloproteinase (MMP)-10 signaling axis [4]. In contrast, we showed that Sox9 was involved in upregulating N-cadherin expression in

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Abbreviations

CADM1	cell adhesion molecule 1
DMEM	Dulbecco's modified Eagle's medium
EMT	epithelial-mesenchymal transition
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
hOSCC	human oral squamous cell carcinoma
IKK-2	NF-κB kinase-2
JNK	c-Jun N-terminal kinase
MAP	mitogen-activated protein
MAPK	MAP kinase
MEK	MAP-ERK kinase
MITF	microphthalmia-associated transcription factor
MMP	matrix metalloproteinase
NSCLC	non-small-cell lung cancer
SCC	squamous cell carcinoma
PI3K	phosphoinositide 3-kinase
RT-qPCR	quantitative real-time reverse transcriptase polymerase chain reaction
siRNA	small interfering RNA
TGF-β1	transforming growth factor beta 1

TGF-β1-stimulated HSC-4 cells [5]. These results suggested that Slug and Sox9 are important EMT-related transcription factors that promote the invasion and metastasis of hOSCC cells.

Momose *et al.* established that LMF4 cells retained higher metastatic activity than their original HSC-3 cell line [6]. However, little is known about the molecular mechanisms underlying the differential metastatic abilities of HSC-3 and LMF4 cells. We previously showed that activation of the TGF-β/Smad signaling pathway did not drive EMT in HSC-3 cells [3].

In this study, we investigated whether the invasive and metastatic activities of LMF4 cells increase EMT-independently. In addition, we tried to clarify the molecular mechanism underlying the higher invasion and metastasis abilities in LMF4 cells than in HSC-3 cells by comparing the expression level of cell adhesion molecules in LMF4 cells with that in HSC-3 cells because adhesion molecules are key players in changing cell shape and migratory activity [7]. Cell adhesion molecule 1 (CADM1), also known as a tumor suppressor in lung cancer 1, immunoglobulin superfamily 4, nectin-like protein 2, spermatogenic Ig superfamily, and synaptic cell adhesion molecule are calcium-independent cell-to-cell adhesion molecules [8–11]. CADM1 binds to another CADM1 molecule in an adjacent cell, forming a homodimer in a Ca^{2+}/Mg^{2+}-independent manner [12]. *CADM1* is a tumor suppressor gene in lung cancer, laryngeal SCC [13], and other cancers [14]. CADM1 suppressed the invasion and migration abilities of cancer cells. Upregulation of CADM1 expression suppresses tumor cell proliferation and promotes apoptosis [13]. In addition, CADM1 inhibits the invasive ability of melanoma cells by downregulating MMPs [15]. Moreover, the EMT-related transcription factor Twist1 suppresses CADM1 expression in cancer cells, resulting in the upregulation of their invasive ability and suppression of apoptosis [16].

First, we compared the expression of EMT markers and transcription factors in HSC-3 and LMF4 cells. Then, we explored 1) whether CADM1 expression level was downregulated in LMF4 cells compared with HSC-3 cells, 2) whether downregulation of CADM1 expression increased MMPs expression level, 3) what type of cellular signaling caused an increase in MMPs expression after downregulation of CADM1 expression, and 4) whether disruption of CADM1-dependent HSC-3 cell-cell adhesion increased migratory and invasive activities in HSC-3 cells, resulting in the promotion of invasion ability in HSC-3 cells.

2. Materials and methods

2.1. Materials

The LMF4 cell line, a hOSCC cell line HSC-3 subline, retained higher metastatic activity than the HSC-3 cell line [6]. The LMF4 and HSC-3 cell lines were established and stored at Tokyo Medical and Dental University. The NF-κB kinase-2 (IKK-2) inhibitor TPCA-1 was purchased from R&D Systems Inc. (Minneapolis, MN, USA). The mitogen-activated protein kinase (MAPK) kinase (MEK) inhibitor U0126, stress-activated protein kinase/c-Jun N-terminal kinase (JNK) inhibitor SP600125, and phosphatidylinositol-3 kinase (PI3K) inhibitor LY294002 were

Table 1
Sequence of primers for RT-qPCR.

Target mRNA	Oligonucleotide sequence (5′–3′)	Predicted size (bp)
CADM1	(F) GACGCGCTTGAGTTAACATGTGA	116
	(R) AACAGGTTGGGCCCAGACAG	
CDH1	(F) AGGATGACACCCGGGACAAC	85
(E-cadherin)	(R) TGCAGCTGGCTCAAGTCAAAG	
CDH2	(F) CGAATGGATGAAAGACCCATCC	171
(N-cadherin)	(R) GCCACTGCCTTCATAGTCAAACACT	
KRT18	(F) AGGAGTATGAGGCCCTGCTGAA	128
(cytokeratin18)	(R) TTGCATGGAGTTGCTGCTGTC	
MITF	(F) AGGCATGAACACACATTCACGAG	113
	(R) CAGGATCCATCAAGCCCAAGA	
MMP2	(F) CTCATCGCAGATGCCTGGAA	104
(MMP-2)	(R) TTCAGGTAATAGGCACCCTTGAAGA	
MMP9	(F) ACGCACGACGTCTTCCAGTA	94
(MMP-9)	(R) CCACCTGGTTCAACTCACTCC	
SNAIL2	(F) GAAGCATTTCAACGCCTCCAA	110
(Slug)	(R) GTTGTGGTATGACAGGCATGGAGTA	
SNAIL1	(F) GACCACTATGCCGCGCTCTT	69
(Snail)	(R) TCGCTGTAGTTAGGCTTCCGATT	
Sox9	(F) GGAGATGAAATCTGTTCTGGGAATG	149
	(R) TTGAAGGTTAACTGCTGGTGTTCTG	
Twist1	(F) CCGGAGACCTAGATGTCATTG	181
	(R) GCCTGTCTCGCTTTCTCTTTT	
VIM	(F) GGTGGACCAGCTAACCAACGA	183
(vimentin)	(R) TCAAGGTCAAGACGTGCCAGA	
GAPDH	(F) GCACCGTCAAGGCTGAGAAC	89
	(R) TGGTGAAGACGCCAGTGGA	

purchased from Merck Millipore (Frankfurt, Germany). The p38 MAPK inhibitor, SB203580 (adezmapimod), was purchased from Selleck Chemicals (Houston, TX, USA). All other reagents were of analytical grade.

2.2. Cell culture

HSC-3 and LMF4 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Rockville, MD, USA). The cells were grown at 37 °C and 5% CO_2.

2.3. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR of the investigated genes using human gene-specific primers (Table 1) was performed according to the methods described in our previous paper [17]. The target gene expression was normalized to an internal GAPDH reference and expressed in terms of fold change relative to the control sample using the 2-ΔΔCt method [18].

2.4. Downregulation of gene expression with small interfering RNA (siRNA)

Gene knockdown analysis in HSC-3 cells was conducted with human siRNA specific to CADM1 (siCADM1) (PDSIRNA2D, SASI_Hs_02_00319227, Sigma-Aldrich, Merck), according to methods described in a previous paper [17]. si-Control (Control siRNA-A sc-37007, Santa Cruz Biotechnology , Dallas, TX, USA) was used as a negative control. Suppression of gene expression by siRNA was evaluated using RT-qPCR for the target genes.

2.5. Western blot analysis

Sample preparation and western blotting experiments were performed using previously described procedures [5]. The target proteins were analyzed using chicken anti-SynCAM mAb primary antibody (at a 1:200 dilution, clone 3E1; CM004-3, Medical and Biological Laboratories (MBL), Tokyo, Japan), while a mouse anti-β-actin antibody (at a 1:1000 dilution, clone C4; Santa Cruz Biotechnology) was used as the loading control on the polyvinylidene fluoride membranes (Merck Millipore). The membrane signals were detected using an alkaline phosphatase substrate kit (BCIP/NBT Substrate Kit; Vector Laboratories Inc., Burlingame, CA, USA). The densities of the protein bands were calculated using ImageJ software (version 1.53k) (NIH, Bethesda, MD, USA).

2.6. Contact inhibition analysis using neutralizing antibody

Briefly, 1×10^5 cells of HSC-3 cells in 12-well dishes were treated with 10 μg/mL anti-human CADM1 chicken antibody (Clone 9D2, CM005-3, MBL) or normal chicken IgY (PM084, MBL) for 48 h under serum-free conditions, according to the method described by Furuno *et al.* [19]. The mRNA expression levels of MMP-2 and MMP-9 were examined using RT-qPCR as described above.

2.7. Immunofluorescence analysis of HSC-3 and LMF4 cells

The cells were plated on 8-well chamber slides and incubated at 37 °C for 48 h. Chamber slides were fixed with 4% paraformaldehyde at room temperature for 30 min. Cells were then incubated with specific antibodies with 1:1000 dilutions of chicken anti-SynCAM mAb (CM004-3; MBL) antibodies for 16 h at 4 °C. After washing with phosphate-buffered saline, the cells were stained with secondary antibodies Alexa Fluor® 488 goat anti-mouse or anti-rabbit antibodies (1:400; Life Technologies) and tetramethylrhodamine B isothiocyanate-conjugated phalloidin (1:500; Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature and then stained with DAPI (Wako Pure Chem. Ind., Osaka, Japan). Slides were washed and examined under a fluorescence microscope (IX70; Olympus, Tokyo, Japan).

2.8. Identification of cell signaling pathways for MMP-2/MMP-9 expression

First, 4×10^5 cells/well of HSC-3 cells were transfected with si-CADM1 or si-control RNA, according to the method described in Section 2.4. After incubation for 24 h, some cells were further treated with various inhibitors (10 μM) against the targeted signal transduction molecules for 24 h. Then, the cells were used for RT-qPCR analysis of gene expressions, as described in Section 2.3.

2.9. Cell migration assay with Boyden chamber

Boyden chamber-based cell migration assays were performed as follows: HSC-3 or LMF4 cells were cultured with DMEM (500 μL/well) without FBS for 48 h. Then, the cells were further treated with normal chicken IgY or neutralizing antibody of CADM1 (Clone 9D2, CM005-3, MBL, 10 μg/mL) for 48 h. The cells treated with the antibody were plated into the upper chamber of the Boyden chamber apparatus (8.0 μm pore size) in serum-free media at a density of 0.25×10^5 cells/chamber. The cells were then allowed to migrate into the lower chamber that contained medium with 10% FBS for 48 h at 37 °C. After 48 h, the cells on the membrane were fixed in 4% paraformaldehyde for 30 min and stained with DAPI for 10 min. The migrated cells to the underside of the membrane were counted in nine random fields under a DM IL LED/DFC700T fluorescence microscope (Leica Microsystems, Deerfield, IL, USA). The data presented here represent the average of quadruplicate experiments.

2.10. Cell invasion assay with Boyden chamber coated with Matrigel

A Matrigel-coated membrane with pores was used as an insert in the Boyden chamber for invasion assays (Corning Inc., Corning, NY, USA). Cells were treated with neutralizing antibody against CADM1 (clone 9D2, CM005-3, MBL) as described in Section 2.6. The cells were treated with 10 μg/mL anti-CADM1 antibody or normal chicken IgY as a control under serum-free conditions for 48 h. Subsequently, the cells were inoculated into the upper compartment of a Boyden chamber (8.0 μm pore size) at a density of 2×10^5 cells/chamber in a serum-free medium. Cells were allowed to migrate into medium containing 10% FBS in the lower chamber for 48 h at 37 °C. After 48 h incubation, the filter was fixed in 4% paraformaldehyde for 30 min and stained with DAPI for 10 min. Cells that migrated to the lower side of the membrane were counted. Values represent the averages of experiments conducted in triplicate.

2.11. Statistical analysis

All experiments were performed at least in triplicates. Results are expressed as mean ± standard deviation. Differences in gene expression between HSC-3 and LMF4 cells were analyzed using an unpaired two-tailed Student's *t*-test. Statistical comparison between every pair of two samples among the multiple samples in hOSCC cells was conducted using Tukey's multiple comparison test with SPSS Statistics 24 software (IBM, Armonk, NY, USA). Differences were considered statistically significant at $^*P < 0.05$ and $^{**}P < 0.01$.

3. Results

3.1. Investigation of the expression levels of EMT-related molecules in hOSCC HSC-3 and LMF4 cells

The expression level of the epithelial marker E-cadherin mRNA in

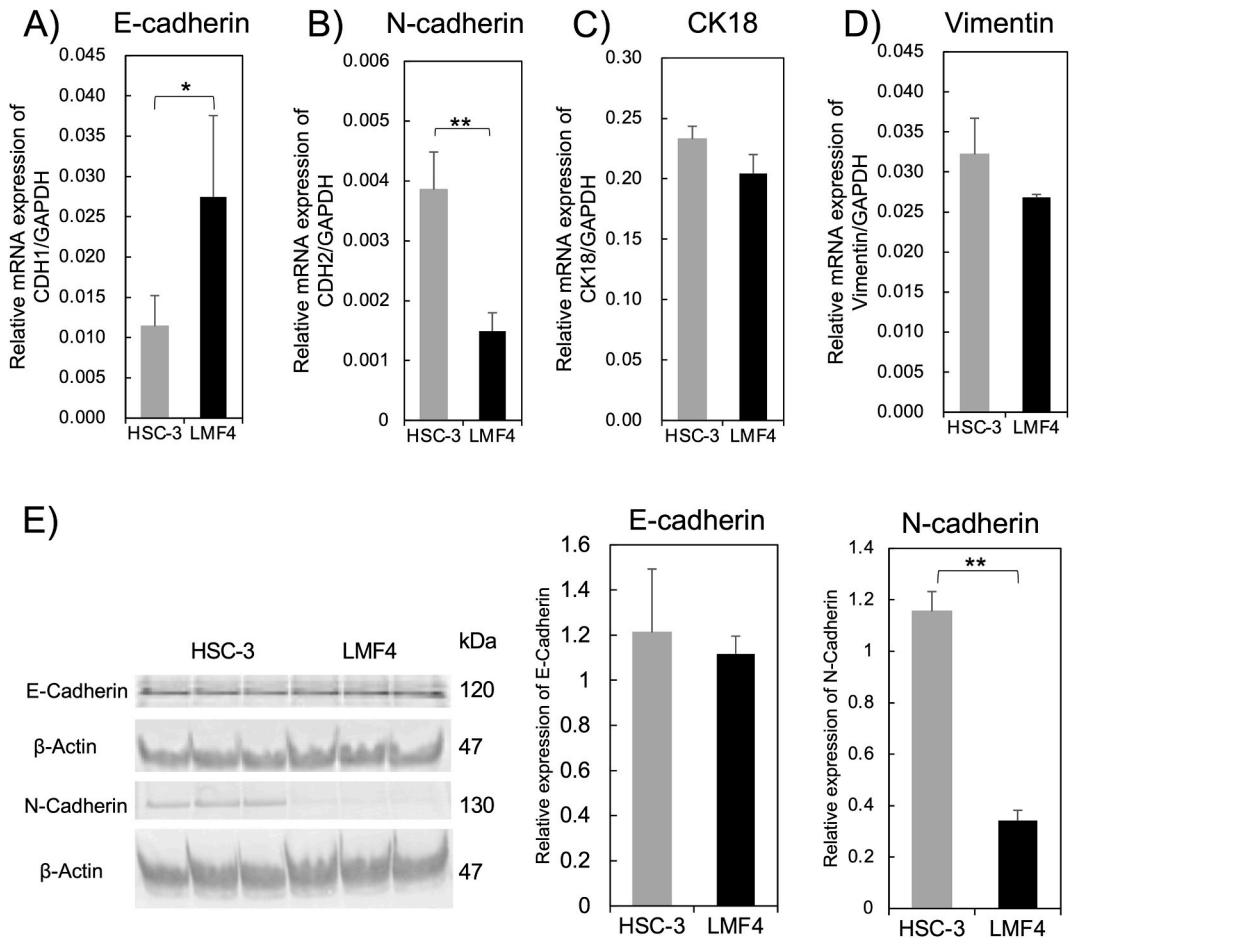

Fig. 1. *Investigation of the expression levels of EMT-related molecules in hOSCC HSC-3 and LMF4 cells.*
A)~D) The mRNA expression levels of epithelial markers, A) E-cadherin and C) cytokeratin 18, and mesenchymal markers, B) N-cadherin and D) vimentin in HSC-3 cells (gray bar) and LMF4 cells (black bar) were analyzed using RT-qPCR. Values were normalized to GAPDH mRNA levels. Data are presented as the mean ± SD of quadruplicate experiments. Differences in values between HSC-3 and LMF4 cells were statistically analyzed using Student's *t*-test (**$P < 0.01$ and *$P < 0.05$). E) The protein expression levels of E-cadherin and N-cadherin were analyzed using western blot analysis. Western blot analysis was repeated three times, and the representative data were indicated. For the statistical evaluation of the obtained band intensity, β-actin was used as the loading standard, and the values obtained from the concentration of each band were normalized to β-actin protein levels. Data are presented as the mean ± SD of triplicate experiments. Differences in values between HSC-3 and LMF4 cells were statistically analyzed using Student's *t*-test (**$P < 0.01$ and *$P < 0.05$).

LMF4 cells was significantly higher than that in HSC-3 cells (Fig. 1A). Additionally, E-cadherin protein expression in LMF4 cells was comparable to that in HSC-3 cells (Fig. 1E). mRNA expression of the epithelial marker cytokeratin 18 (CK18) was comparable to that in HSC-3 cells (Fig. 1C). In contrast, the mRNA and protein expression levels of the mesenchymal marker N-cadherin in LMF4 cells were significantly lower than that in HSC-3 cells (Fig. 1B and E, respectively). In addition, the mRNA expression of the mesenchymal marker vimentin in LMF4 cells was comparable to that in HSC-3 cells (Fig. 1D).

3.2. Investigation of the mRNA expression levels of EMT-related transcription factors in HSC-3 and LMF4 cells

The mRNA expression levels of EMT-related transcription factors Slug and Snail were significantly lower in LMF4 cells than those in HSC-3 cells (Fig. 2A and B, respectively). In addition, the mRNA expression levels of the EMT-related transcription factors Sox9 and Twist1 in LMF4 cells were comparable to those in HSC-3 cells (Fig. 2C and D, respectively).

3.3. Expression level of tumor-related cell adhesion molecule CADM1 in LMF4 cells was lower than that in HSC-3 cells

We focused on the differential expression levels of cell adhesion molecules between HSC-3 and LMF4 cells because LMF4 cells showed loose cell-to-cell adhesion similar to the mesenchymal shape [7]. The mRNA expression of CADM1, a known tumor suppressor [20], was markedly downregulated in LMF4 cells compared with that in HSC-3 cells (Fig. 3A). In addition, CADM1 protein level in LMF4 cells was significantly lower than that in HSC-3 cells (Fig. 3B). We also investigated the localization of CADM1 by immunofluorescence staining. CADM1 was intensely stained in HSC-3 cells but not in LMF4 cells (Fig. 3C). CADM1 was mainly localized to the cell membrane in HSC-3 and LMF4 cells (Fig. 3C).

3.4. Downregulation of CADM1 expression level with si-CADM1 increased the expression of MMP-2 and MMP-9 in HSC-3 cells

First, we confirmed whether a siRNA specific to CADM1 (si-CADM1) suppressed the expression of CADM1 in HSC-3 cells. si-CADM1 significantly decreased CADM1 expression in HSC-3 cells at both the mRNA

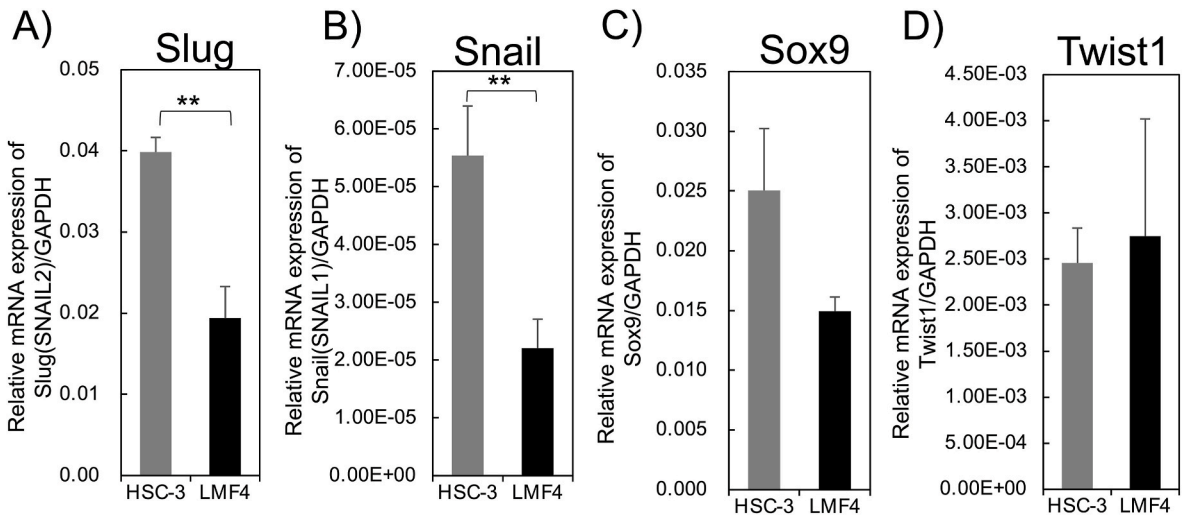

Fig. 2. *Investigation of the mRNA expression levels of EMT-related transcription factors in HSC-3 and LMF4 cells.*
The mRNA expression levels of EMT-related transcription factors, A) Slug, B) Snail, C) Sox9, and D) Twist1 in HSC-3 cells (gray bar) and LMF4 cells (black bar) were analyzed using RT-qPCR. Values were normalized to GAPDH mRNA levels. Data are presented as the mean ± SD of quadruplicate experiments. Differences in values between HSC-3 and LMF4 cells were statistically analyzed using Student's *t*-test (**$P < 0.01$).

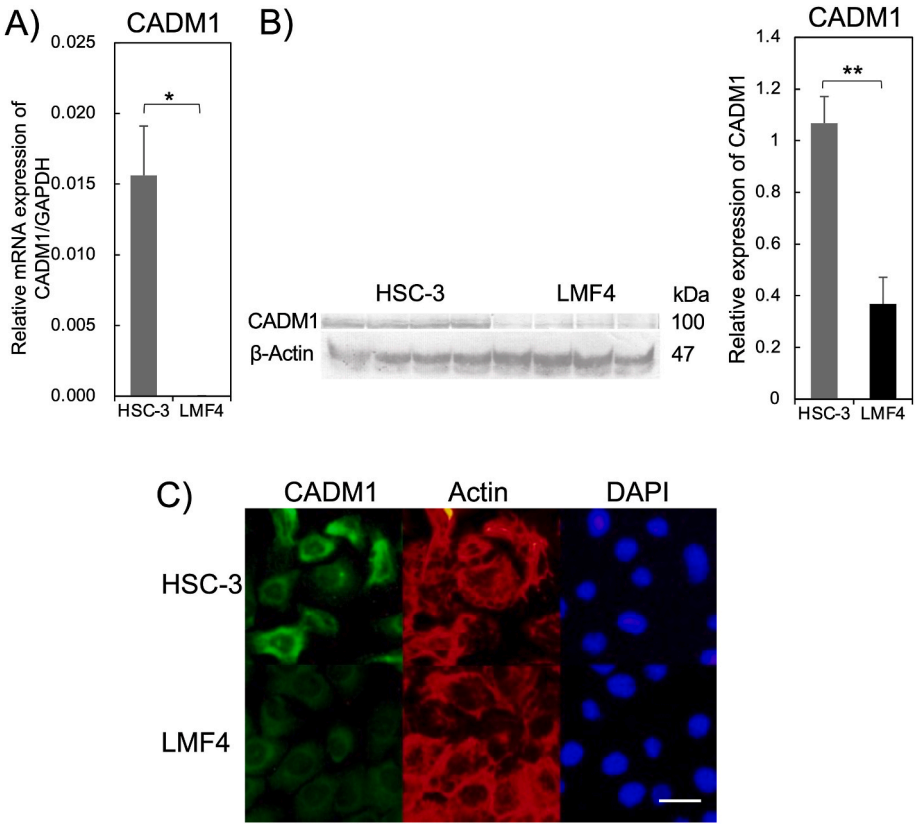

Fig. 3. *Investigation of the expression level of tumor-related cell adhesion molecule CADM1 in HSC-3 and LMF4 cells.*
A) The level of CADM1 mRNA in HSC-3 cells (gray bar) and LMF4 cells (black bar) was examined using RT-qPCR. Data are presented as the mean ± SD of quadruplicate experiments. B) The protein expression levels of CADM1 were analyzed using Western blot analysis. Western blot analysis was repeated three times, and the representative data were indicated. For the statistical evaluation of the obtained band intensity, β-actin was used as the loading standard, and the values obtained from the concentration of each band were normalized to β-actin protein levels. Differences in values between HSC-3 and LMF4 cells were statistically analyzed using Student's *t*-test (**$P < 0.01$ and *$P < 0.05$). C) The localization and intensity of CADM1 in HSC-3 and LMF4 cells were examined by fluorescence immunostaining. Both cells were immunostained with anti-CADM1 antibody (green), and with phalloidin (red) to detect F-actin and DAPI (blue) to detect nuclei. Scale bars represent 25 μm.

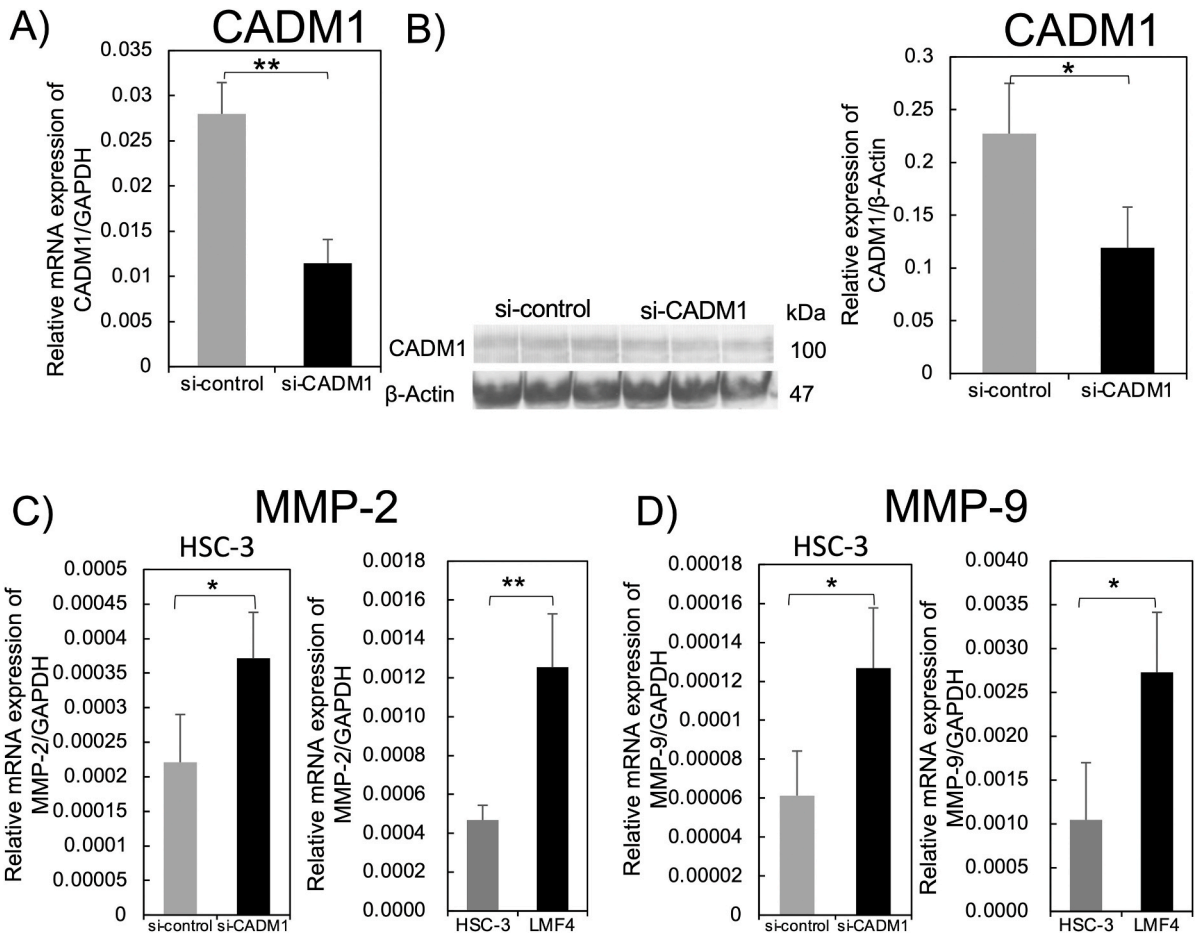

Fig. 4. *Downregulation of CADM1 expression level with si-CADM1 increased expression of MMP-2 and MMP-9 in HSC-3 cells.*
A) The mRNA expression levels of CADM1 in HSC-3 cells treated with si-control or si-CADM1 were analyzed using RT-qPCR. B) The expression levels of CADM1 in HSC-3 cells treated with si-control or si-CADM1 were analyzed using western blotting. Western blot analysis was repeated three times, and the representative data were indicated. For the statistical evaluation of the obtained band intensity, β-actin was used as the loading standard, and the values obtained from the intensity of each band were normalized to β-actin protein levels. Differences in values between si-control and si-CADM1 in HSC-3 cells were statistically analyzed using Student's *t*-test (*$P < 0.05$). C) The comparison of expression levels of MMP-2 between si-control-treated-, and si-CADM1-treated-HSC-3 cells (left graph) and that between HSC-3 and LMF4 cells (right graph) were analyzed using RT-qPCR. D) The comparison of expression levels of MMP-9 between si-control-treated- and si-CADM1-treated-HSC-3 cells (left graph) and that between HSC-3 and LMF4 cells (right graph) were analyzed using RT-qPCR. Values were normalized to GAPDH mRNA levels. Data are presented as the mean ± SD of quadruplicate experiments. Differences in the values between si-control-treated- and si-CADM1-treated-HSC-3 cells or that between HSC-3 and LMF4 cells were statistically analyzed using Student's *t*-test (**$P < 0.01$ and *$P < 0.05$).

and protein levels (Fig. 4A and B, respectively). Then, we evaluated the effects of si-CADM1 on MMP-2 and MMP-9 expressions in HSC-3 cells (Fig. 4C and D). We found that si-CADM1 significantly increased MMP-2 and MMP-9 mRNA expressions in HSC-3 cells (Fig. 4C, left graph; 4D, left graph). The expression levels of MMP-2 and MMP-9 in LMF4 cells were significantly higher than those in HSC-3 cells (Fig. 4C, right graph; 4D, right graph).

3.5. Identification of cell signaling pathways that caused an increase of MMP-2/MMP-9 expression levels after the suppression of CADM1 expression in HSC-3 cells

We investigated the types of intracellular signal transduction in HSC-3 cells that increased the expression levels of MMP-2 and MMP-9 after the knockdown of CADM1 expression using si-CADM1. We found that MEK inhibitor U0126 (10 μM) and PI3K inhibitor LY294002 (10 μM) abrogated the si-CADM1-promoted upregulation of MMP-2 mRNA expression in HSC-3 cells (Fig. 5A). In addition, JNK inhibitor SP600125 (10 μM), p38 MAPK inhibitor SB203580 (10 μM), and the LY294002 (10 μM) abrogated the si-CADM1-promoted upregulation of MMP-9 mRNA expression (Fig. 5B). On the other hand, the treatment of NF-κB kinase-2 (IKK-2) inhibitor TCPA-1 tended to suppress the CADM-1 siRNA-induced upregulation of both MMP-2 and MMP-9. However, the statistical significance between the CADM1 siRNA-treated HSC-3 cells without TCPA-1 administration and those with TCPA-1 administration was not observed (Fig. 5A and B).

3.6. Disruption of CADM1-dependent HSC-3 cell-cell adhesion promoted the migratory and invasive activities in HSC-3 cells

First, we compared the migratory activities of HSC-3 and LMF4 cells. The migratory activity of HSC-3 cells was comparable to that of LMF4 cells (Fig. 6A). Treatment with neutralizing antibodies against CADM1 (anti-CADM1) significantly enhanced the migratory activity of HSC-3 cells (Fig. 6B). However, the statistically significant enhancement was mot observed in the migratory activity of LMF4 cells treated with anti-CADM1 (Fig. 6C). In contrast, the invasive activity of HSC-3 cells was significantly lower than that of LMF4 cells (Fig. 6D). Treatment with anti-CADM1 neutralizing antibody significantly enhanced the invasive activity of HSC-3 cells (Fig. 6E).

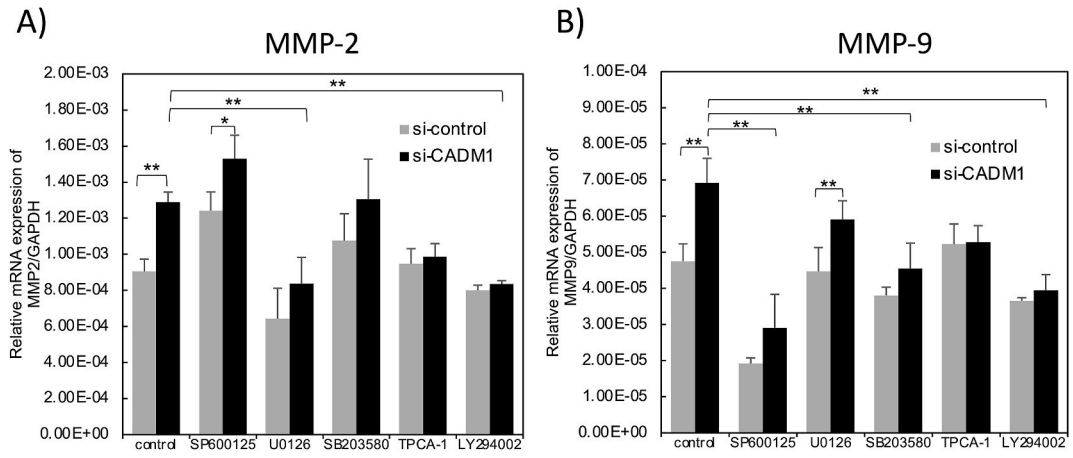

Fig. 5. *Identification of the cell signaling pathway that caused an increase of MMP-2/MMP-9 expression after the suppression of CADM1 expression in HSC-3 cells.* The expression levels of A) MMP-2 and B) MMP-9 in HSC-3 cells transfected with si-control (gray bar) or si-CADM1 (black bar) were evaluated using RT-qPCR. Cells were treated with several inhibitors against intracellular signaling molecules as described in the Materials and methods section. Values were normalized to GAPDH mRNA levels. Data are presented as the mean ± SD of quadruplicate experiments. Statistical comparison between every pair of two samples among the multiple samples in HSC-3 cells was conducted using Tukey's multiple comparison test (**$P < 0.01$ and *$P < 0.05$).

4. Discussion

Since LMF4 cells retained higher metastatic activity than HSC-3 cells [6], we investigated the molecular mechanism underlying the differential metastatic activities of HSC-3 and LMF4 cells. We examined the expression of EMT-related markers in HSC-3 and LMF4 cells. mRNA expression of the epithelial marker E-cadherin in LMF4 cells was significantly higher than that in HSC-3 cells (Fig. 1A). Moreover, expression of the mesenchymal marker N-cadherin in LMF4 cells was significantly lower than that in HSC-3 cells (Fig. 1B and E). In addition, the mRNA expression levels of the epithelial marker CK18 and the mesenchymal marker vimentin in LMF4 cells were comparable to those in HSC-3 cells (Fig. 1C and D). We also compared the mRNA expression levels of EMT transcription factors in HSC-3 and LMF4 cells. We found that the mRNA expression levels of Slug and Snail in LMF4 cells were significantly lower than those in HSC-3 cells (Fig. 2). In addition, the mRNA levels of Sox9 and Twist in LMF4 cells were comparable to those in HSC-3 cells (Fig. 2). Moreover, we examined whether EMT was induced by TGF-β treatment (10 ng/mL, 24 h incubation) in LMF4 cells. The mRNA expression levels of epithelial markers E-cadherin and CK18 in LMF4 cells were not suppressed by TGF-β1 treatment (data not shown). We also showed that TGF-β1 treatment did not decrease mRNA expressions of E-cadherin and CK18 but decreased vimentin mRNA expression in HSC-3 cells [3]. These results suggested that the acquisition of highly invasive and metastatic activity by LMF4 cells was not caused by EMT-related mechanisms.

Subsequently, we focused on cell adhesion molecule/tumor suppressor CADM1: the downregulation of CADM1 expression was frequently observed in non-small cell lung cancer (NSCLC) and possibly correlated with the enhancement of cell migratory and invasive activities in the tumor cells [20]. Herein, we found that the expression of CADM1 in LMF4 cells was significantly lower than that in HSC-3 cells at both the mRNA and protein levels (Fig. 3), suggesting that the downregulated expression of cell adhesion molecules CADM1 participates in the acquisition of highly invasive and metastatic activities in LMF4 cells. Downregulation of CADM1 in HSC-3 cells transfected with si-CADM1 significantly promoted the upregulation of mRNA expression of the tumor invasive factors MMP-2 and MMP-9 (Fig. 4C and D, left graphs, respectively). Moreover, LMF4 cells showed higher mRNA expression levels of MMP-2 and MMP-9 than HSC-3 cells (Fig. 4C and D, right graphs, respectively). You *et al.* demonstrated that the upregulation of CADM1 suppresses the invasive activity of melanoma cell line by downregulating the expressions of MMP-2 and MMP-9 [15]. We also found that downregulation of CADM1 promoted MMP-2 expression in HSC-3 cells via MEK/extracellular signal-regulated kinase (ERK)- and PI3K/AKT-mediated signal transduction (Fig. 5A). In addition, CADM1 downregulation promoted MMP-9 expression in HSC-3 cells via JNK-, p38 MAPK-, and PI3K/AKT-mediated signal transduction (Fig. 5B). MMP-2 expression was mediated by the MEK/ERK signaling pathway in hOSCC cells [21] and AKT signaling pathway in vascular smooth muscle cells [22]. In contrast, the upregulation of MMP-9 expression is mediated by the JNK signaling pathway in human pancreatic ductal adenocarcinoma cells [23], whereas the p38 MAPK signaling pathway is also involved in MMP-9 expression in chondrocytes [24]. Wang *et al.* reported that focal adhesion kinase-activated PI3K/AKT signaling increased MMP-9 expression in NSCLC [25]. Therefore, these results did not conflict with the experimental data.

The motility of HSC-3 cells was lower than that of LMF4 cells but higher than that of HSC-2 and HSC-4 cells in the gold colloidal phagokinetic track assay [7]. In our experimental system with a Boden chamber assay, the migratory activity of LMF4 cells was comparable to that of HSC-3 cells (Fig. 6A). In addition, disruption of CADM1-dependent HSC-3 cell-cell adhesion by an anti-CADM1 neutralizing antibody significantly enhanced the migratory activity of HSC-3 cells (Fig. 6B). Moreover, the administration of an anti-CADM1 neutralizing antibody into LMF4 cell culture did not affect the migratory activity of LMF4 cells (Fig. 6C), possibly because CADM1 expression level in LMF4 cells was much lower than that in HSC-3 cells (Fig. 3A, B, and C). These results suggest that suppressing CADM1 expression promotes tumor progression by inducing migratory activity in CADM1-rich hOSCC cells. On the other hand, we did not examine the effect of an anti-CADM1 neutralizing antibody against expressions of MMP-2, or -9 in LMF4 cells. In contrast, the invasive activity of LMF4 cells was significantly higher than that of HSC-3 cells (Fig. 6D). Moreover, disruption of CADM1-dependent HSC-3 cell adhesion by an anti-CADM1 neutralizing antibody significantly enhanced the invasive activity of HSC-3 cells (Fig. 6E). These results strongly suggest that decreased cell-cell adhesive activity of the tumor suppressor CADM1 positively regulates the invasive and metastatic activities of hOSCC cells.

The negative upstream effector of CADM1 expression in LMF4 cells has not yet been identified. The EMT transcription factor Twist1 directly interacts with the CADM1 promoter and represses CADM1 expression in

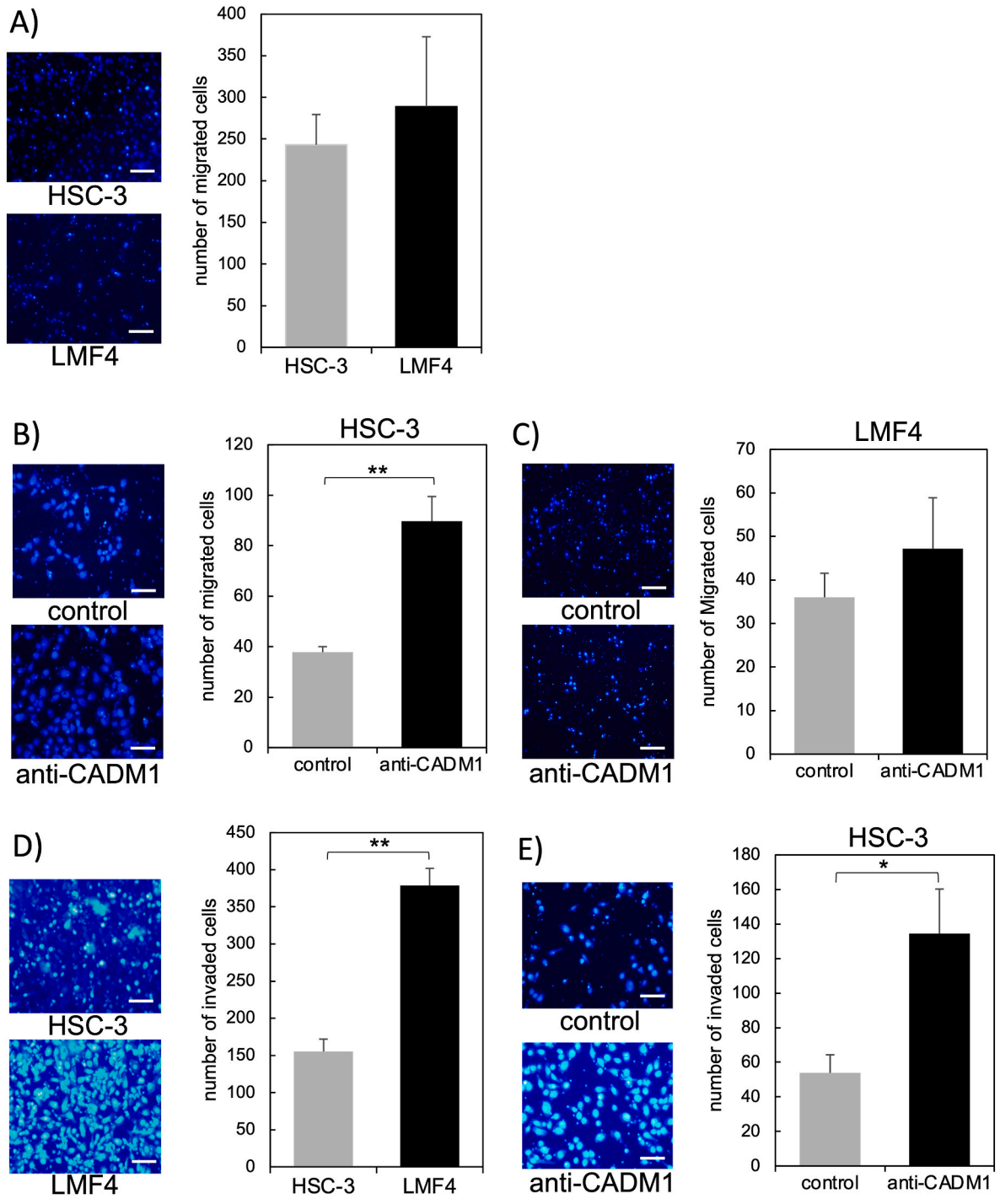

Fig. 6. *Disruption of CADM1-dependent HSC-3 cell-cell adhesion promoted the migratory and invasive activities in HSC-3 cells.*
A)~E) Cells that had migrated (A~C) or invaded (D and E) onto the lower side of the membrane were stained with DAPI and photographed under a phase-contrast microscopy (Scale bar, 50 μm). The cell number in each photograph was counted and represented by a bar graph. A) Cell migratory activity was evaluated in HSC-3 cells (gray bar) and LMF4 cells (black bar) by using a Boden chamber assay as described in the Materials and methods section. B) and C) Control antibody (gray bar) or anti-CADM1 neutralizing antibody (black bar) were added to the B) HSC-3 or C) LMF4 cell culture medium, respectively. Then, a comparison of migratory activities between control antibody-treated- and anti-CADM1 neutralizing antibody-treated-cells was performed by using a Boyden chamber assay as described in the Materials and methods section. D) Cell invasive activity was evaluated in HSC-3 cells (gray bar) and LMF4 cells (black bar) by using a Boden chamber coated with Matrigel as described in the Materials and methods section. E) Control antibody (gray bar) or anti-CADM1 neutralizing antibody (black bar) were added to the HSC-3 cell culture medium. Then, a comparison of invasive activities between control antibody-treated- and anti-CADM1 neutralizing antibody-treated-HSC-3 cells was performed by using a Boyden chamber coated with Matrigel as described in the Materials and methods section. Data from all experiments represent the mean ± SD from triplicate experiments. A) ~ E) Statistical analysis was performed using Student's *t*-test (**$P < 0.01$ and *$P < 0.05$).

melanoma cells [16]. However, the expression level of Twist1 mRNA in LMF4 cells was comparable to that in HSC-3 cells (Fig. 2D), suggesting that Twist1 is not a possible regulator that differentially downregulates CADM1 expressions in LMF4 and HSC-3 cells. Ito *et al.* demonstrated that microphthalmia-associated transcription factor (MITF) positively regulates CADM1 expression [26]; however, the expression level of MITF in LMF4 cells was comparable to that in HSC-3 cells (data not shown), suggesting that MITF does not appear to be a regulator that differentially upregulates CADM1 expression levels between LMF4 and HSC-3 cells. In contrast, CADM1 promoter methylation, such as epigenetic gene silencing, has been observed in NSCLC and cervical cancer [20,27,28]. Further studies are required to investigate the possibility of methylation of the CADM1 promoter in LMF4 cells.

5. Conclusion

Disruption of CADM1-dependent hOSCC cell-cell adhesion promoted the invasive activity of hOSCC cells, possibly through an increase in MMP-2 expression in a MEK/ERK- and PI3K/AKT-dependent manner and possibly through an increase in MMP-9 expression in a JNK-, p38 MAPK-, and PI3K/AKT-dependent manner.

Ethical approval

The authors declare that ethical approval is not asked for this article.

CRediT authorship contribution statement

Nanami Obara: data collection, data analysis and figures. **Seiko Kyakumoto:** data collection and data analysis. **Satoshi Yamaguchi:** source maintenance and data collection. **Hiroyuki Yamada:** study design. **Akira Ishisaki:** literature search, study design, data interpretation and writing. **Masaharu Kamo:** literature search, study design, data analysis, data interpretation, figures and writing.

Declaration of competing interest

The authors declare that they have no competing interests.

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