# Wnt5b promotes the cell motility essential for metastasis of oral squamous cell carcinoma through active Cdc42 and RhoA

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**Abstract.** The activation of Wnt signaling has been reported in many types of squamous cell carcinoma. In this study, using human oral squamous cell carcinoma (OSCC) cells with different metastatic potential, we investigated the involvement of Wnt signaling in metastasis. Further, we aimed to elucidate the characteristic biological features related to high metastatic potential and to identify new target molecules for the suppression of OSCC lymph node metastasis. We compared SAS-Venus (SAS OSCC cells expressing green fluorescent protein) and SAS-LM8, which is a highly metastatic cell line derived from SAS-Venus by *in vivo* selection. The SAS-LM8 cell line had greater ability of migration and invasion compared to SAS-Venus. Furthermore, a higher number of filopodia-like protrusive structures were produced in SAS-LM8 cells compared to SAS-Venus cells, and the levels of active Cdc42 and active RhoA protein were higher in SAS-LM8 cells compared to SAS-Venus cells. We did not observe any differences in the expression of Wnt/β-catenin target genes between the two cell lines; however, the mRNA levels of *Wnt5b* were higher in SAS-LM8 cells compared to SAS-Venus cells. To confirm the involvement of Wnt5b in migration in OSCC cells, we examined the effects of the siRNA-mediated knockdown of Wnt5b in SAS-Venus cells and SAS-LM8 cells. The siRNA treatment significantly inhibited migration and the formation of filopodia-like protrusive structures. Conversely, when stimulated with Wnt5b, the migration and formation of filopodia-like protrusions were significantly enhanced and the levels of active Cdc42 and active RhoA proteins were also increased. These results indicate that Wnt5b is involved in the migration ability of OSCC cells through active Cdc42 and RhoA.

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## Introduction

Squamous cell carcinoma (SCC) is the most frequent malignant tumor of the head and neck region. Head and neck squamous cell carcinoma (HNSCC) is the sixth leading cancer by incidence worldwide, with approximately 500,000 new cases per year (1).

The most life-threatening aspects of cancer are invasion and metastasis. Cervical lymph node metastasis is frequently detected in HNSCC, including oral squamous cell carcinoma (OSCC). The presence or absence of cervical lymph node metastasis has a great impact on the prognosis of the patient, therefore, it is important to control cervical lymph node metastasis. However, little is known about the molecular mechanisms underlying lymph node metastasis in HNSCC.

The metastatic spread of tumor cells is a sequential multistep process beginning with the detachment of individual tumor cells from the primary tumor. The progression to metastasis requires that the individual tumor cells adhere to and invade through the basement membrane, migrate through the extracellular matrix, and then intravasate into blood or lymphatic vessels, throught which they can disseminate to distant sites. Tumor cells must then extravasate out of the vessel and invade the target organ before forming a metastatic tumor, which most commonly occurs in the cervical lymph nodes in HNSCC (2,3).

Many signal transduction systems are associated with the invasion and metastasis of cancer. Among them, the Wnt signaling pathway, which is conserved in various species from worms to mammals, and involved in various differentiation events during embryonic development. Aberrant Wnt activation can lead to tumor formation and malignant transformation. Wnt ligands bind to their cognate receptors on the cell surface and transduce signals through at least 3 distinct pathways: the canonical β-catenin pathway, the non-canonical planar cell polarity (PCP) pathway, and the $Ca^{2+}$ pathway (4-6).

Wnt proteins are cysteine-rich secreted glycoproteins that play critical roles in both carcinogenesis and embryonic development. At least 19 Wnt members have been shown to be present in humans and mice (7).

Wnt family members can be divided into 2 distinct types based on their ability to induce transformation of the mouse mammary epithelial cell line C57MG (8). The highly transforming members include Wnt1, Wnt3, Wnt3A, and Wnt7A,

while the intermediately transforming or non-transforming members include Wnt2, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7b, and Wnt11. It is thought that the Wnts that show high transforming activity in C57MG cells activate the canonical Wnt signals, and that those that show intermediately transforming or non-transforming activity in C57MG cells activate the non-canonical Wnt signals (8).

In addition to cancer, abnormal Wnt signaling has been reported to cause various kinds of disease, including neuronal disease, bone and cartilage disease, diabetes, and renal disease (9).

In cancer, mutations in genes involved in canonical Wnt signaling have been found in several cancer types, often resulting in the intracellular accumulation of β-catenin (9-11). The non-canonical Wnt signals were traditionally thought not to be involved in tumorigenesis because of their failure to induce transformation of C57MG cells. However, recent evidence has shown that some Wnts involved in non-canonical Wnt signaling are upregulated in human cancers; it is thought that abnormal activation of non-canonical Wnt signaling pathways might play a role in malignant transformation (8).

In this study, we used human OSCC cell lines with different metastatic potential to investigate the involvement of the canonical and non-canonical Wnt signals in the metastatic potential of cancer cells.

## Materials and methods

*Cell lines and culture*. The SAS cell line was derived from a poorly differentiated SCC that was originally isolated from the surgical specimens of a Japanese woman with a primary tongue lesion (12). Human SCC cell lines, SAS-Venus and SAS-LM8, were kindly provided by Professor T. Yoneda, Department of Biochemistry, Osaka University Graduate School of Dentistry, Japan. Morita *et al* (13) stably overexpressed Venus protein into SAS cells (SAS-Venus) and established highly metastatic SAS cells (named SAS-LM3) after three rounds of *in vivo* selection. SAS-LM8 used here was also derived from SAS-Venus but after 8 rounds of *in vivo* selection. SAS-LM8 cells have metastatic ability and motility almost equivalent to SAS-LM3 cells *in vivo*.

The SAS-Venus and, SAS-LM8 cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) at 37˚C in a humidified atmosphere with 5% $CO_2$.

*Proliferation assay*. Cells ($2x10^4$) were plated on 6-well plates and allowed to grow and expand. The cells were then trypsinized and counted after 8 days and evaluated using a growth curve.

*Cell migration and invasion assay*. To measure the cell migration activity, transwell chamber assays were performed using BD BioCoat cell culture inserts (Becton-Dickinson, MA, USA). Cells were resuspended in serum-free DMEM and then added to the upper chamber at a density of $5x10^4$ cells/insert. DMEM containing 10% FBS was added to the lower chamber. After incubation at 37˚C for 48 h, the number of cells that invaded into the lower chamber was counted. To measure the cell invasion activity, transwell chamber assays were performed using BD BioCoat Matrigel invasion chambers (Becton-Dickinson) in a similar manner as that described for the cell migration assay. After incubation at 37˚C, the cells that penetrated the membrane onto the lower side were fixed with formalin. The invasiveness of the cells was determined by counting the area occupied by the cells on the lower side of the filter through the pores under a fluorescence microscope at magnification x100. Seven fields were randomly selected for each assay.

*Immunocytochemical staining*. Cells grown on cover slips were fixed with PBS containing 4% paraformaldehyde for 15 min and then rendered permeable with PBS containing 0.1% Triton X-100 for 3 min at 4˚C. For staining of β-catenin, after blocking with 2% bovine serum albumin in PBS for 30 min, the cells were incubated with anti-β-catenin mouse monoclonal antibody (Transduction Laboratories, KY, USA; diluted at 1:500) in the blocking solution at 4˚C overnight. After washing, the cells were incubated with a rhodamin-conjugated goat anti-mouse secondary antibody (Leinco Technologies, MO, USA; diluted at 1:150) in PBS for 30 min at room tempurature.

For staining of actin filaments, Alexa Fluor 546-conjugated phalloidin (Invitrogen, CA, USA) was used. After blocking, the cells were incubated with the Alexa Fluor 546 phalloidin (diluted at 1:40) in the blocking solution at room temperature for 20 min. Fluorescent images were obtained using a confocal laser scanning microscope (Carl Zeiss, Germany).

*GTPase activity assays*. The activity of the Cdc42, Rac1, and RhoA GTPases was determined using a RhoA/Rac1/Cdc42, Activation Assay Combo Biochem kit (Cytoskeleton, CO, USA) according to the manufacturer's protocol. Briefly, cell lysates were incubated with a GST fusion protein corresponding to either the p21-binding domain (amino acid residues 67-150) of PAK1 or the Rho-binding domain (amino acid residues 7-89) of Rhotekin, and the precipitates were bound to glutathione-coupled sepharose beads. The bound Cdc42, Rac1, and RhoA were separated by SDS-PAGE and detected by immunoblotting with antibodies against Cdc42, Rac1 or RhoA (Cytoskeleton). The bands were scanned and their intensities were quantified using the public domain ImageJ program.

*RNA extraction and reverse transcriptase PCR*. A 0.5-μg aliquot of total RNA was reverse-transcribed into single-stranded cDNA and subsequent PCR was performed using a Takara RNA PCR kit (AMV) Version 3.0 (Takara, Shiga, Japan) by monitoring GAPDH levels as a quantitative control. The primer sequences used for PCR amplification were as follows: 5'-CAACTACATGGTTTACATGTTC-3' and 5'-GCCAGTGGACTCCACGAC-3' for *GAPDH*; 5'-CCAGCGTGGACAATGGCTAC-3' and 5'-TGAGCTCGAGTCATTGCATAC-3' for *β-catenin*; 5'-AGACTCCAGCGCCTTCTCTCCG-3' and 5'-CTGTGAGGAGGTTTGCTGTGGCC-3' for *c-myc*; 5'-TCTAAGATGAAGGAGACCATC-3' and 5'-GCGGTAGTAGGACAGGAAGTTGTT-3' for *cyclin D1*; 5'-AACTCCCGCGTCATAGAAATAATG-3' and 5'-ACCCAAAGAATGGCCAAGTTCATG-3' for *MMP7*; 5'-CTGGAGCTTGAAAATCTGCCG-3' and 5'-GGTTTTTCGGTTCGTGAGTGC-3' for *uPAR*; 5'-AATGGGAAGTCCAGGCAGTGTATC-3' and 5'-ACAGCGTTCTCCAGTAACAGCTG-3' for

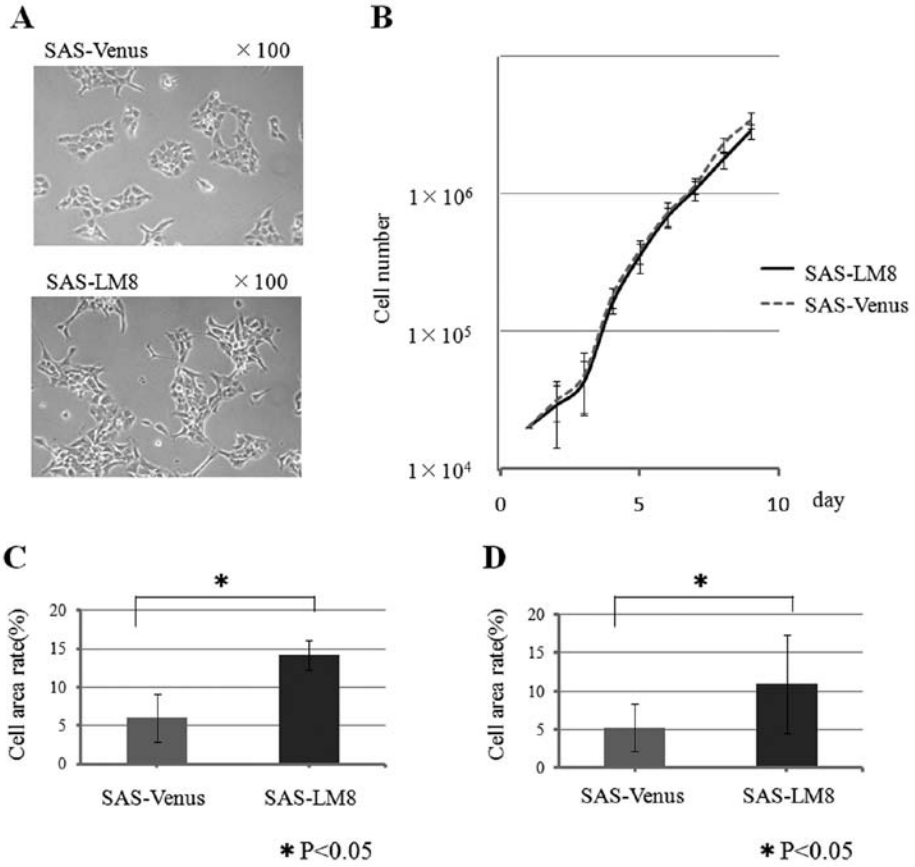


Figure 1. (A) The morphology of the SAS-Venus and SAS-LM8 cells was observed with a phase contrast microscope (magnification x100). (B) Cells ($2x10^4$) were plated on 6-well plates and allowed to grow and expand. The cells were then trypsinized and counted after 8 days and evaluated with a growth curve. (C) Enhancement of migration activity in SAS-LM8. Migration activity was measured using a transwell chamber assay. Each column indicates the mean ± SD of 3 separate experiments. (D) Enhancement of invasion activity in SAS-LM8. Invasion activity was measured using a Matrigel invasion chamber assay system (Beckton-Dickinson). Each column indicates the mean ± SD of 3 separate experiments.

*laminin-5γ2* chain; 5'-GGGTTTTTCGGTTCGTGAGT GC-3' and 5'-CCATTGGGCATCCAGAAGAGAGC-3' for *MT1-MMP*; 5'-CAAGTACTCGGGCAAAGAGG-3' and 5'-CTTCCTCTGCTTATCTG-3' for *S100A4*; 5'-CAGTTC AAGACCGTGCAGAC-3' and 5'-TGGAACCTACCC ATCCCATA-3' for *Wnt5a*; 5'-CGGGAGCGAGAGAAGA ACT-3' and 5'-TACACCTGACGAAG CAGCAC-3' for *Wnt5b*; 5'-TGACCTCAAGACCCGATACC-3' and 5'-CAAGTG AAGGCAAAGCACAA-3' for *Wnt11*.

*Silencing by siRNA*. The *Wnt-5b* siRNA (Qiagen, Valencia, CA, USA) used in this study is a 21-bp duplex oligoribonucleotide corresponding to the human *Wnt-5b* mRNA sequence and has the sense sequence 5'-CUCCUGGUGGUCAUUAGC UUU-3'.

Logarithmically growing cells were seeded at a density of $1x10^6$ cells per 10-cm dish and transfected with 5 nM *Wnt-5b* siRNA using HiPerFect HTS reagent (Qiagen) according to the manufacturer's instructions. Seventy-two hours after transfection, the cells were used for an *in vitro* migration assay and immunocytochemical staining of actin filaments as described above. AllStars Negative Control siRNA (Qiagen) was used as a negative control. The efficiency of the siRNA was checked by RT-PCR.

*Cell stimulation with recombinant Wnt5b*. For stimulation with Wnt5b, cells were cultured with recombinant human Wnt5b (500 ng/ml) (R&D Systems, Minneapolis, MN, USA) for 48 h and then used for shape change observations, the *in vitro* migration assay, immunocytochemical staining of actin filaments and the GTPase activity assays as described above.

*Statistical analysis*. The Student's t-test was used to compare data between 2 groups. p-values of <0.05 were considered to be statistically significant.

## Results

*Morphology, migration, and invasion of SAS-Venus and SAS-LM8 cells*. SAS-Venus cells showed a polygonal shape with few pseudopod-like protrusive structures around the colonies, while SAS-LM8 cells showed a slightly spindle-shaped morphology with many pseudopod-like protrusive structures. However, both cell type maintained cell-cell contact in the colonies (Fig. 1A), and no difference was observed between their proliferative capacities (Fig. 1B).

The motility of the SAS-LM8 and SAS-Venus cells was assessed by a transwell chamber migration assay. In the migration assay, the area occupied by the cells on the lower side of

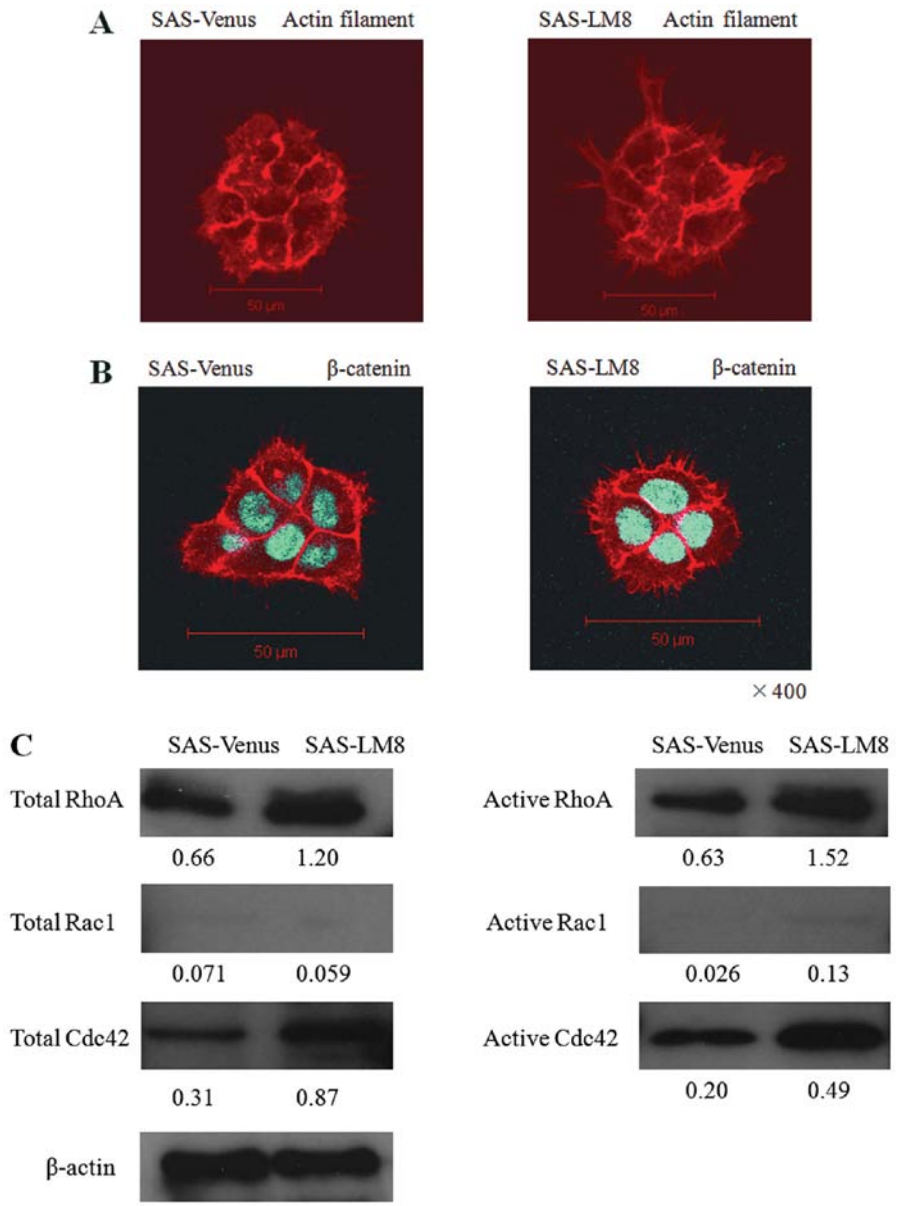


Figure 2. (A) The expression of actin filaments were examined by immunofluorescence staining. Actin filaments were stained using Alexa Fluor 546-Phalloidin. (B) The expression of β-catenin was examined by immunofluorescence staining. β-catenin was stained using an anti-β-catenin mouse monoclonal antibody and rhodamine-conjugated secondary antibody. Confocal microscopy (x400). (C) Activation of Rho family members in SAS-Venus and SAS-LM8 cells. Cell lysates were incubated with a GST fusion protein corresponding to either the p21-binding domain (amino acid residues 67-150) of PAK1 or the Rho-binding domain (amino acid residues 7-89) of Rhotekin, and the precipitates were collected. The lysates were separated by SDS-PAGE and detected by immunoblotting with antibodies against Cdc42, Rac1, or RhoA. The bands in 3 independent experiments were scanned and their intensities were quantified using the public domain ImageJ program. We used 10 $\mu$g of protein (total cell lysate) to detect total RhoA, Rac1, and Cdc42 (left panel). We used 1,000 $\mu$g of pull-down protein to detect the active form of each protein (right panel).

the filter after migration through the pores was measured. The cell area rate of the SAS-LM8 cells (14.2%) was significantly higher than that of the parental cell line SAS-Venus (6.0%: $p<0.05$) (Fig. 1C). Similarly, in the invasion assay, the cell area rate of the SAS-LM8 cell line (11.0%) was significantly higher than that of the parental cell line SAS-Venus (5.3%: $p<0.05$) (Fig. 1D).

*Immunofluorescence analysis of actin filaments and β-catenin.* Having observed that the cell lines exhibit significant differences in cell motility, we next assessed the rearrangement of the actin cytoskeleton and filopodia-like protrusive structures in SAS-Venus and SAS-LM8 cells via immunofluorescent staining of actin filaments using Alexa Fluor 546-conjugated phalloidin. Actin rearrangements and the formation of filopodia-like protrusions were more evident in SAS-LM8 than in SAS-Venus (Fig. 2A). Immunofluorescence analysis of β-catenin revealed that it was located at the cell membranes and in the cytoplasm in SAS-LM8 and SAS-Venus cells; no difference in β-catenin was observed (Fig. 2B).

*Activation of Rho family members.* Rho family members including the Cdc42, Rac1 and RhoA GTPases have been reported to contribute to the rearrangement of actin fila-

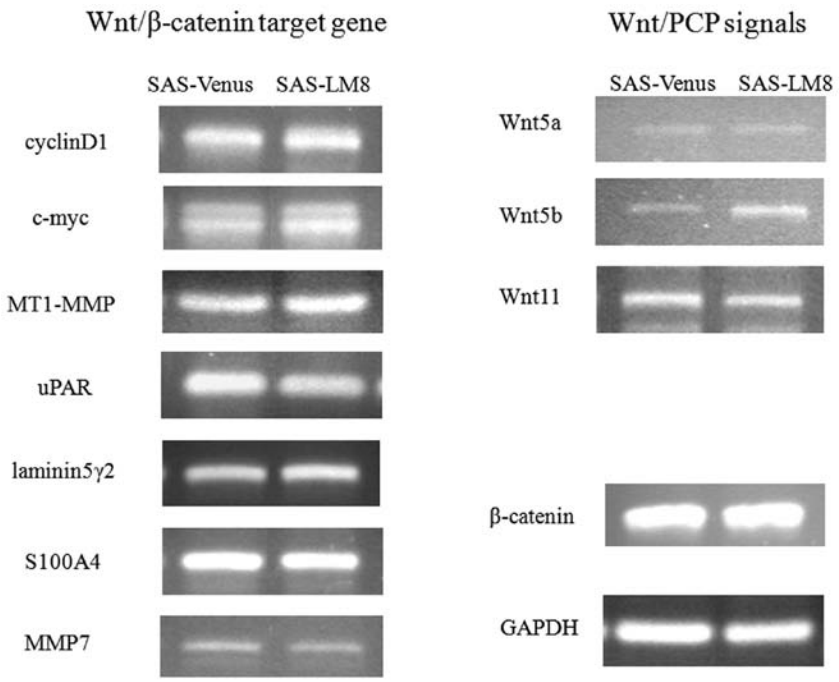


Figure 3. Expression of Wnt signaling related genes. The expression of target genes of the Wnt/β-catenin pathway (cyclin D1, *c-myc, MT1-MMP, uPAR*, laminin-5γ2, *S100A4, MMP-7*) (left panel) and representative non-canonical Wnt genes that transduce Wnt/PCP signals (Wnt5a, Wnt5b, Wnt11), were measured by semi-quantitative RT-PCR. GAPDH was used as an internal control (right panel).

ments. Therefore, the levels of active Cdc42, Rac1, and RhoA GTPases in SAS-LM8 and SAS-Venus cells were measured by GTPase activity assays. The level of active Cdc42 and active RhoA was higher in SAS-LM8 than in SAS-Venus (Fig. 2C).

*Expression of Wnt signaling related gene*. To investigate whether canonical Wnt/β-catenin signaling might contribute to the cell migration differences between the cell lines, the expression of several target genes of the Wnt/β-catenin pathway was examined using semi-quantitative RT-PCR. The selected Wnt-related genes included *β-catenin, cyclin D1* (14,15), *c-myc* (16), *MT1-MMP* (17), *MMP-7* (18), *laminin-5γ2* (19), *uPAR* (20,21) and *S100A4* (22). However, no significant changes were observed in the expression of the tested target genes of the Wnt/β-catenin pathway (Fig. 3).

We also assessed the expression of Wnt5a, Wnt5b, and Wnt11, which are representative non-canonical Wnts that transduce Wnt/PCP signals (23). The mRNA level of *Wnt5b* was higher in SAS-LM8 than in SAS-Venus, whereas no significant changes were observed in the expression of *Wnt5a* and *Wnt11* (Fig. 3).

*Changes in cell motility result in siRNA-mediated knockdown of Wnt5b*. Having found that *Wnt5b* was differentially expressed in the OSCC cell lines used in this study, we next examined the role of *Wnt5b* in their migration ability via siRNA-mediated knockdown of *Wnt5b*. We first confirmed via RT-PCR that treatment with the Wnt5b siRNA reduced the expression of Wnt5b mRNA in the cell lines by comparison with treatment with a control siRNA (si-control) (Fig. 4A). In the migration assay, the cell area rate of SAS-Venus si-Wnt5b (2.5%) was significantly lower than that of SAS-Venus si-control (6.1%: $p<0.05$), and the cell area rate of SAS-LM8 si-Wnt5b (5.9%), was significantly lower than that of SAS-LA8 si-control (10.2%; $p<0.05$), indicating that knockdown of *Wnt5b* significantly inhibited cell migration in both cell lines (Fig. 4A).

*Changes in the ability of migration and invasion of cells stimulated with recombinant Wnt5b*. To confirm the siRNA result indicating that *Wnt5b* was involved in migration and invasion, we carried out assays of cells stimulated with recombinant Wnt5b protein.

In the migration assay, the cell area rates of SAS-Venus and SAS-LM8 cells stimulated with Wnt5b (51.3 and 90.3%, respectively) were significantly higher than those of the SAS-Venus and SAS-LM8 not stimulated with Wnt5b (7.0 and 33.1%, respectively; $p<0.05$) (Fig. 4B). These values correspond to 7.3- and 2.7-fold increase in migration upon Wnt5b stimulation of SAS-Venus and SAS-LM8 cells, respectively.

Similarly, in the invasion assay, SAS-Venus and SAS-LM8 cells stimulated with Wnt5b showed significantly higher cell area rates (26.3 and 25.0%, recpectively) than SAS-Venus and SAS-LM8 cells not stimulated with Wnt5b (3.3 and 7.4%, respectively; $p<0.05$) (Fig. 4C). These results correspond to 8.0-and 3.4-fold increase in invasion by SAS-Venus and SAS-LM8 cells, respectively, and indicate that stimulation with Wnt5b promoted cell invasion in both cell lines.

*Morphological changes upon stimulation of cells with recombinant Wnt5b*. SAS-Venus and SAS-LM8 cells stimulated with recombinant Wnt5b, showed an increase in the formation of the pseudopod-like protrusive structures around the colonies,

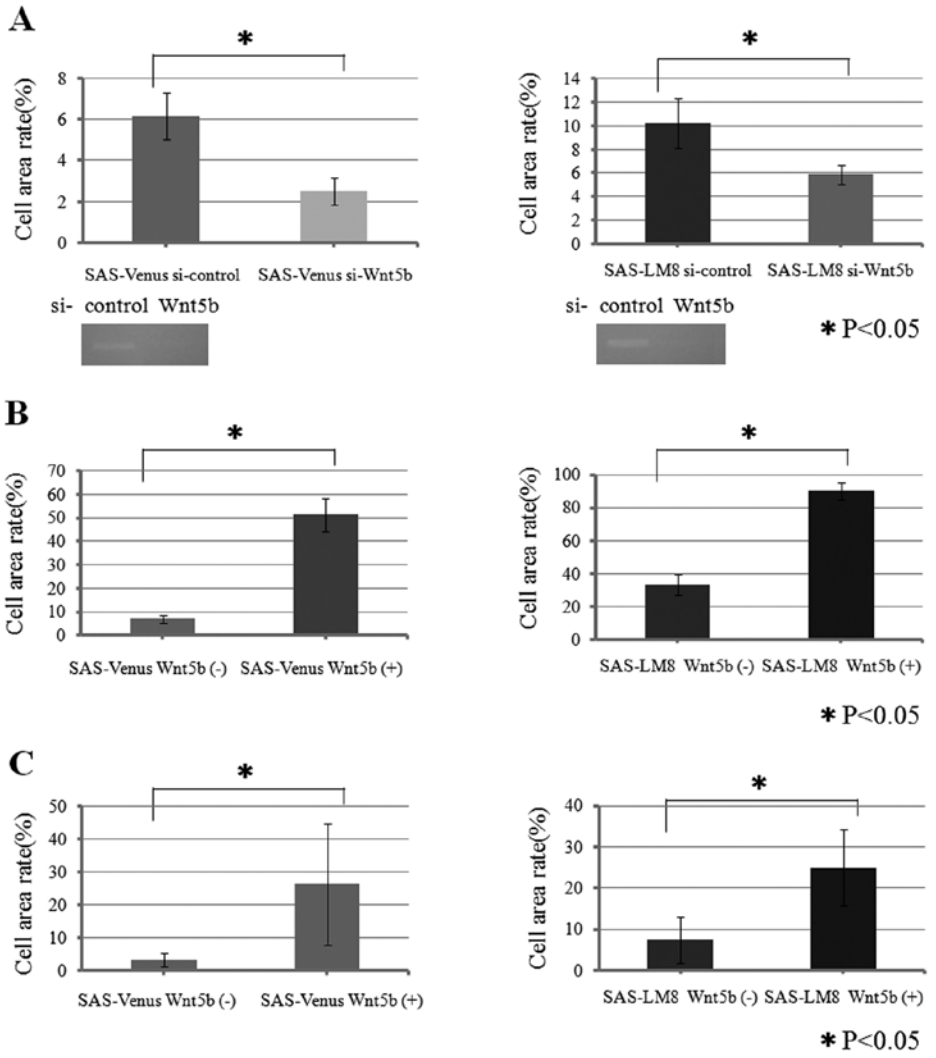


Figure 4. (A) Migration assay for cells treated with Wnt5b siRNA. The reduction of Wnt5b expression in SAS-Venus and SAS-LM8 cells by siRNA (si-control; gel images are shown below the microscopy images for each cell line). Migration activity was measured using a transwell chamber assay. Each column indicates the mean ± SD of 3 separate experiments. (B) Enhancement of migration activities by treatment with recombinant Wnt5b (500 ng/ml, 48 h). Migration activity was measured using a transwell chamber assay. (C) Enhancement of invasion activities by treatment with recombinant Wnt5b (500 ng/ml, 48 h). Invasion activity was measured using a Matrigel invasion chamber assay system (Becton-Dickinson). Each column indicates the mean ± SD of 3 separate experiments.

while cell-cell interaction was maintained in both cell lines (Fig. 5A).

*Changes in actin filaments upon Wnt5b knockdown or stimulation*. Immunofluorescent staining of actin filaments revealed that treatment with *Wnt5b* siRNA significantly inhibited the formation of filopodia-like protrusive structures in both SAS-Venus and SAS-LM8 cells. Conversely, stimulation with recombinant Wnt5b resulted in a clear increase in filopodia-like protrusions in both SAS-Venus and SAS-LM8 cells (Fig. 5B).

*Activation of Rho family members upon stimulation with recombinant Wnt5b*. Having found that stimulation with recombinant Wnt5b promoted the rearrangement of the actin cytoskeleton and the formation of filopodia-like protrusive structures, we next carried out GTPase activity assays on cells stimulated with recombinant Wnt5b. While the total protein levels of Cdc42, Rac1, and RhoA were similar in Wnt5b-stimulated and unstimulated cells, the levels of the active forms of these proteins were clearly higher in the Wnt5b-stimulated cells than in the unstimulated cells (Fig. 6).

## Discussion

It is important to control the invasion and metastasis of cancer cells. However, little is known about the molecular mechanisms underlying invasion and lymph node metastasis in HNSCC. We aimed to clarify the characteristic biological features related to high metastatic potential and identify new target molecules for the suppression of lymph node metastasis of OSCC. To this end, we used cell lines with different metastatic potential to investigate the involvement of canonical and non-canonical Wnt signaling in OSCC metastasis.

First, we compared the cell shape, proliferation, and motility of the cell lines. SAS-LM8 cells exhibited a slight spindle shape, while SAS-Venus cells showed a polygonal shape. However, in both cell types, colonies formed with

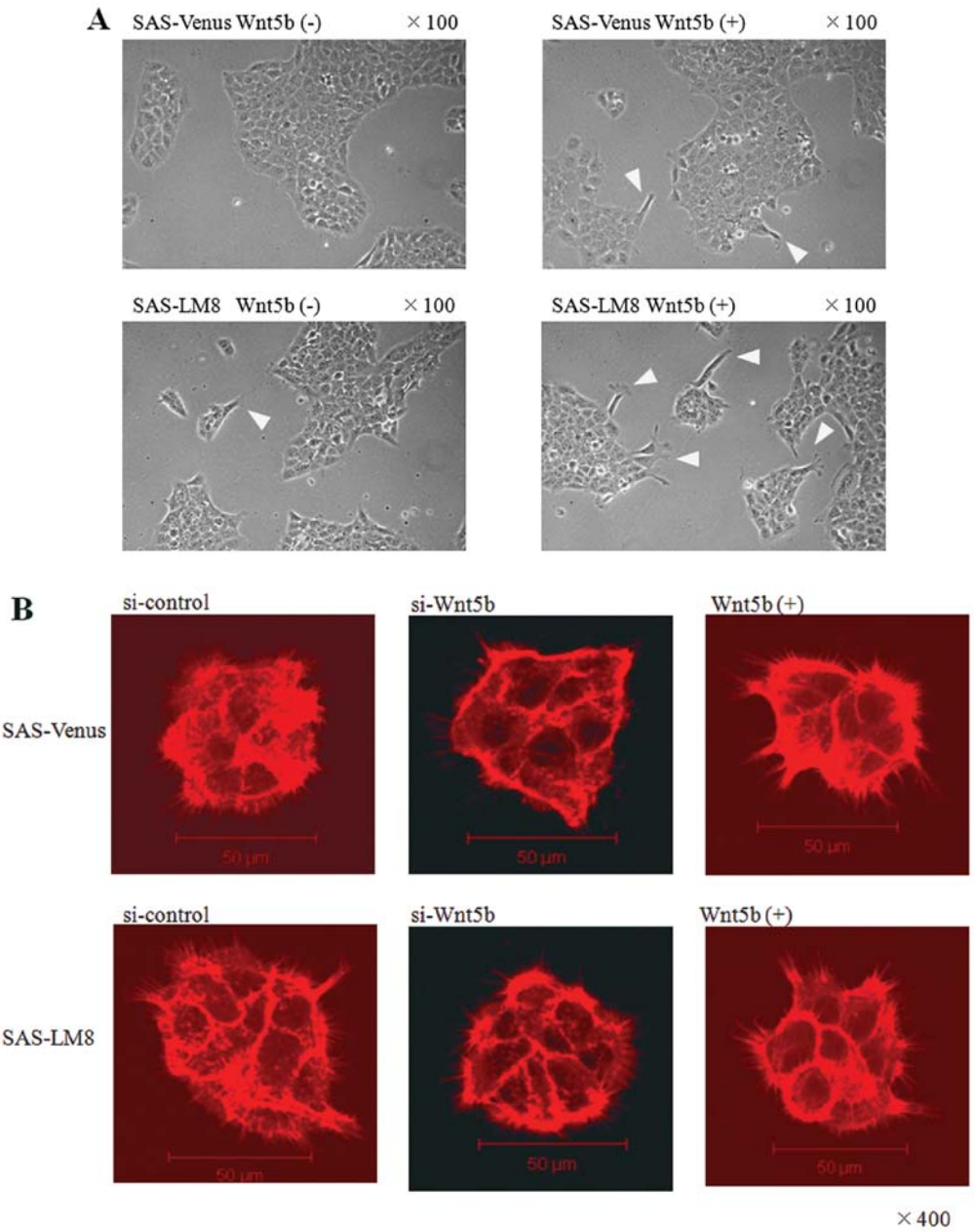


Figure 5. (A) Morphological changes of SAS-Venus and SAS-LM8 cells when stimulated with recombinant Wnt5b (500 ng/ml,48 h) observed with a phase contrast microscope (x100). Filopodia-like protrusive structures were more clearly produced in SAS-Venus and SAS-LM8 cells treated with recombinant Wnt5b (arrowheads). (B) Changes in the actin filaments of SAS-Venus and SAS-LM8 cells treated with *Wnt5b* siRNA and stimulated with recombinant Wnt5b (500 ng/ml, 48 h) were observed with confocal microscopy (x400). Actin filaments were stained using Alexa Fluor 546-Phalloidin.

intimate intercellular interaction, and the cell-cell interactions were maintained (Fig. 1A). Moreover, there was no difference in proliferative capacity of the cell lines (Fig. 1B). Distinct migration and invasion differences were observed between the cell lines; SAS-LM8 showed significantly higher migration and invasion ability than SAS-Venus (Fig. 1C and D).

Migration of the cancer cell is essential to the metastatic process, particularly for the detachment from the primary tumor, invasion into surrounding tissue and intravasation steps. Increased motility of cancer cells is one of the malignant transformation steps of cancer, and is a direct cause of cancer invasion and metastasis.

Therefore, the differences in migration ability of the SAS-Venus and SAS-LM8 cells are likely to be largely involved in the difference in metastatic potential of these cell lines. It is known that when cells migrate, reconstitution of the actin cytoskeleton occurs. In our study, immunofluorescent staining of actin filaments revealed that the rearrangement of the actin cytoskeleton and formation of filopodia-like protrusive structures were more pronounced in SAS-LM8 cells than in SAS-Venus cells (Fig. 2A). Rho family GTPases, including Cdc42, Rac1, and RhoA, have been reported to contribute to the rearrangement of actin filaments (24-26). Rho proteins cycle between an inactive GDP-bound form and an active GTP-bound form. In their active form, they can interact with a variety of effector proteins (24-26). The levels of active Cdc42, and active RhoA in SAS-LM8 were higher than in SAS-Venus (Fig. 2C), whereas Rac1 was present at a very low

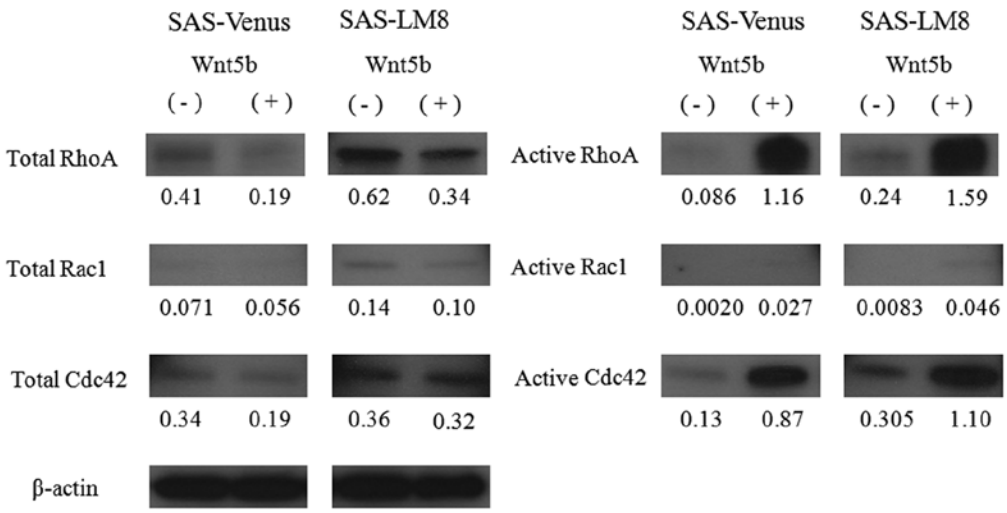


Figure 6. The activity of Rho family members in cells stimulated with recombinant Wnt5b (500 ng/ml, 48 h) was determined. After stimulation with recombinant Wnt5b, cell lysates were incubated with a GST fusion protein corresponding to either the p21-binding domain (amino acid residues 67-150) of PAK1 or the Rho-binding domain (amino acid residues 7-89) of Rhotekin, and the precipitates were collected. Proteins were detected by immunoblotting with antibodies against Cdc42, Rac1, or Rho. The mean band intensities from 3 independent experiments is shown. Ten $\mu$g of protein (total cell lysate) was used to detect total RhoA, Rac1 and Cdc42 (left panel). We used 1,000 $\mu$g of pull-down protein to detect the active form of the protein (right panel).

level in both of the cell lines. These results suggest that the activation of Cdc42 and RhoA was associated with the high migration ability of the SAS-LM8 cells.

We previously reported that the aberrant cytoplasmic accumulation of β-catenin can induce Tcf/Lef-mediated transcriptional activity, and the Rho family member-mediated rearrangement of the actin cytoskeleton in OSSC (27).

Canonical Wnt signals are transduced through the Wnt/GSK3β signaling pathway, which determines cell fate through the activation of the β-catenin/Tcf and Snail/EMT signaling cascades. Non-canonical Wnt signals are transduced through the Wnt/PCP signaling pathway, which regulates tissue polarity and cell movement and includes RhoA, Rac1, Cdc42 as downstream effectors (23). Therefore, we investigated whether canonical and/or non-canonical Wnt signaling contributed to the difference between the migration ability of SAS-Venus and SAS-LM8 cells.

Our examination of canonical Wnt signals, revealed that there was no difference between the cell lines in the localization of of β-catenin (Fig. 2B) or the expression of Wnt/β-catenin target genes (Fig. 3).

When we examined Wnts known to be involved in non-canonical Wnt/PCP signaling, namely, Wnt5a, Wnt5b, and Wnt11, we found that the mRNA level of *Wnt5b* was increased in SAS-LM8 compared to SAS-Venus (Fig. 3). Wnt5b has an 80.5% total-amino-acid identity with Wnt5a; however, there are many remaining questions about the function of Wnt5b. Whether the function of Wnt5b is the same as that of Wnt5a. It has been reported that Wnt5a inhibits cell proliferation, migration, and invasion in thyroid cancer (28), and colon cancer (29), but promotes cell migration and invasion in malignant melanoma (30) and gastric cancer (31). These latter findings suggest that Wnt5a might enhance the migration activity of cancer cells and promote invasion and metastasis. The *Wnt5b* gene has been linked to diseases other than cancer. For example, *Wnt5b* may contribute to the susceptibility to type 2 diabetes and may be involved in the pathogenesis of this disease through the regulation of adipocyte function (32,33). However, there are only a few reports on the relationship between *Wnt5b* and malignant tumors, and to the best of our knowledge, there is only one report indicating that *Wnt5b* promotes the invasion of HNSCC (34).

In our study, no difference was observed in the expression of *Wnt5a* between the cell lines; however, compared to SAS-Venus, SAS-LM8 cells exhibited higher migration ability and higher expression of Wnt5b, which shows high sequence identity with Wnt5a. In order to clarify the role of *Wnt5b* in the migration capability of OSCC cell lines, we examined the effects of *Wnt5b* knockdown by using siRNA in SAS-Venus and SAS-LM8 cells. We found that *Wnt5b* knockdown significantly inhibited the migration of both SAS-Venus and SAS-LM8 cells (Fig. 4A). Consistent with these results, the stimulation of SAS-Venus and SAS-LM8 cells with recombinant Wnt5b resulted in a significant increase in migration and invasion (Fig. 4B and C). Taken together, these results indicate that Wnt5b was involved in the migration ability of the OSSC cells tested in this study.

The siRNA-mediated knockdown of *Wnt5b* also caused a decrease in the formation of filopodia-like protrusive structures in SAS-Venus and SAS-LM8 (Fig. 5B). Conversely, when stimulated with recombinant Wnt5b, SAS-Venus and SAS-LM8 cells formed more filopodia-like protrusions (Fig. 5), and the levels of active Cdc42 and active RhoA increased (Fig. 6). These results suggest that the activation of Cdc42 and RhoA is particularly important for the increase in the cell migration ability induced by Wnt5b.

In the process of cancer invasion, invadopodia are formed locally and penetrate the basement membrane (35). Once they are through the basement membrane and interstitial tissue, the tumor cells form filopodia-like protrusive structures at the invading front, in addition to local stress fibers. Therefore, it is thought that the invasion of the cancer is promoted by the activation of Cdc42 and RhoA, which leads to subsequent rearrangement of the actin cytoskeleton.

When a cancer cell moves, we can classify its migration modes into amoeboid, mesenchymal and collective migration (36,37). Cdc42, RhoA, and Rac1 have been found to be involved in each of the migration styles (26). The amoeboid and mesenchymal modes are observed when single cells migrate, while collective migration is a mode of movement in which cell-cell adhesion of a plurality of cells is maintained. It is becoming clear that collective migration is involved in the dissemination of tumor cells, particularly for tumors such as SCCs. In the classical view of metastasis, it is thought that tumor cells must undergo EMT to migrate as single cells. However, imaging of tumor cell behavior in a 3D culture revealed that epithelial-type tumor cells can spread as groups or sprouts, and full EMT appears not to be essential for tumors to spread into the surrounding tissue (38).

In our study, SAS-Venus and SAS-LM8 cells formed colonies with intimate intercellular interactions. Furthermore, cell-cell interactions were maintained and no significant changes were observed in the expression of E-cadherin (data not shown). Cdc42 has been reported to be involved in the collective migration of cancer cells via myosin (39). We observed an increase in the levels of active Cdc42 and active RhoA in SAS-LM8; thus, in addition to single-cell migration, SAS-LM8 cells might have a high potential for formation of invadopodia and collective cell migration, which likely contributes to the high metastasis potential of SAS-LM8.

In conclusion, we demonstrated that Wnt5b is involved in cell motility through the activation of Cdc42 and RhoA via the non-canonical Wnt signaling pathway. Therefore, the elevated expression of Wnt5b may become an important index for the evaluation of OSCC invasion and metastasis. Furthermore, Wnt5b shows promising as a therapeutic target for the prevention of OSCC metastasis.

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