Migration and invasion of oral squamous carcinoma cells is promoted by WNT5A, a regulator of cancer progression

Zdenka Prgomet[1,2], Lena Axelsson[2], Pia Lindberg[1], Tommy Andersson[2]

[1]*Oral Pathology, Faculty of Odontology, Malmö University, Malmö, Sweden;* [2]*Cell and Experimental Pathology, Department of Laboratory Medicine, Lund University, Clinical Research Centre, Skåne University Hospital, Malmö, Sweden*

BACKGROUND: Oral squamous cell carcinoma (OSCC) constitutes 90% of all cancers in the oral cavity, and the prognosis for patients diagnosed with OSCC is still poor. The identification of novel therapeutic targets and prognostic markers for OSCC is therefore essential. Previous studies of OSCC revealed an increased expression of WNT5A in the tumor tissue. However, no functional studies of WNT5A-induced effects in OSCC have been performed.
METHODS: Two different OSCC cell lines were used for analysis of WNT5A expression by Western blot, whereas WNT5A-induced responses were analyzed by measuring calcium (Ca^{2+}) signaling, PKC activation, migration and invasion.
RESULTS: Despite the lack of WNT5A expression, both cell lines responded to recombinant WNT5A (rWNT5A) with activation of the non-canonical WNT/Ca^{2+}/PKC pathway. This effect was ascertained to be mediated by WNT5A by use of the WNT5A antagonist, Box5. To investigate how WNT5A affects tumor progression, rWNT5A-induced alterations in BrdU absorbance (reflecting the number of tumor cells) were analyzed. rWNT5A had no effect on BrdU absorbance but instead promoted tumor cell migration and invasion. These results were confirmed by the use of the WNT5A-mimicking peptide Foxy5, while the rWNT5A-induced migration was blocked by secreted Frizzled-related protein I (SFRP1), protein kinase C inhibitors or the intracellular Ca^{2+} chelator, MAPT.
CONCLUSIONS: These novel data clearly show that WNT5A activates the non-canonical WNT/Ca^{2+}/PKC pathway and increases migration and invasion of OSCC cells. This may indicate how an increased WNT5A expression in the tumor tissue is likely to promote progression of OSCC.

Introduction

Oral squamous cell carcinoma (OSCC) accounts for approximately 90% of all malignancies in the oral cavity, and the most common location is the tongue (1, 2). Despite advances in therapy, OSCC has a poor prognosis; the 5-year survival is only 50% (3) due to local invasion, spread to lymph nodes, and distal metastasis (4, 5). Normal oral squamous epithelial cells are limited in their growth and localization by interactions with the basement membrane and neighboring stromal cells (6, 7). Following cancer transformation, these cells increase their growth potential (decreased apoptosis and increased proliferation) and become more motile and invasive (3, 8, 9).

The WNT5A protein is an important regulator of cancer cell behavior and progression in different types of cancer (10, 11). WNT5A is a member of the WNT protein family, which in humans includes 19 hydrophobic cysteine-rich glycoproteins that share 20–85% amino acid identity (12, 13). WNT proteins act as ligands and bind to specific receptors/coreceptors on the cell surface. The predominant receptors/coreceptors mediating the effects of WNT ligands are Frizzled receptors (seven-pass transmembrane proteins), ROR1, ROR2, Ryk and the low-density lipoprotein receptor-related proteins, LRP-5 and LRP-6 (11–14). WNT proteins activate multiple intracellular signaling cascades and affect cellular functions including apoptosis, survival, proliferation, differentiation, adhesion, polarity, and migration (14, 15).

WNTs activate two principal signaling pathways in mammalian cells, the canonical WNT/β-catenin pathway and the non-canonical WNT pathway. The non-canonical WNT pathways, WNT/planar cell polarity (PCP) pathway and WNT/Ca^{2+} pathway, differ from the canonical pathway in that they do not involve β-catenin signaling (12, 14, 15). Apart from the direct signaling effects, WNT5A can also counteract the activity of the canonical signaling pathway

(15). The WNT/PCP pathway signals through activation of Jun N-terminal kinase (JNK) (14), whereas the WNT/Ca^{2+} pathway involve intracellular Ca^{2+} signaling but also activation of protein kinase C (PKC) (10, 12, 16, 17). It has been reported that PKC and JNK activities play important roles in regulation of OSCC cell migration (18, 19).

Several endogenous-secreted inhibitory proteins control WNT signaling, for example, the Dickkopf (Dkk) family of proteins that inhibit the canonical pathway, and secreted Frizzled-related proteins (SFRPs) that are able to inhibit both types of WNT signaling pathways (20, 21).

Previously, studies have shown an increased WNT5A expression in OSCC tissue (19, 22, 23). However, the functional role of WNT5A/Ca2+ signaling has not yet been examined in OSCC. The aim of this study was to determine whether an increased WNT5A expression either promotes or suppresses cellular functions that directly affect OSCC progression.

Materials and methods

Cell lines, chemicals, and peptides

The oral squamous tongue carcinoma cell lines, SCC9 (CRL-1629, lot 4372272, received June 2010) and SCC25 (CRL-1628, lot 58075871, received December 2011), were purchased from ATCC (USA). ATCC also provided molecular characterization of these two cell lines, performed in 2008 (SCC9) and 2009 (SCC25). The cells were routinely maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (Gibco, Life Technologies Europe BV, Netherlands) supplemented with 10% fetal bovine serum, 400 ng/ml hydrocortisone, 5 U/ml penicillin, 0.5 U/ml streptomycin, and 2 mM L-glutamine (Sigma-Aldrich Chemie, Germany) at 37°C in an atmosphere of 5% CO$_2$. Cells were detached for subculture with trypsin-EDTA (Sigma-Aldrich Chemie, Germany) for 1 min followed by incubation in Versene (Gibco, Invitrogen, Scotland) for 10 min. The cell lines were routinely analyzed for the absence of mycoplasma using an EZ-PCR kit from Biological Industries (Israel). Recombinant WNT5A (645-WN), recombinant SFRP1 (5396-SF-025), and the WNT5A antibody (AF645) were purchased from R&D Systems (Minneapolis, MN, USA) and the Foxy5 and Box5 peptides from Storkbio (Estonia). Rabbit anti-goat/HRP (P0449), goat anti-rabbit/HRP (P0448), and goat anti-mouse/HRP (P0447) were purchased from DAKO (Denmark). Mouse anti-β-actin (clone AC-15), phorbol 12-myristate 13-acetate (PMA; P8139), and Fura-2-AM were purchased from Sigma-Aldrich Chemie (Germany). MAPT-AM (454867) was purchased from Calbiochem, and GF109203X (inhibitor of PKC α, β1, β2, and γ) and Go 6983 (inhibitor of PKC α, β, γ, δ, and ζ) were purchased from Selleck Chemicals (Houston, TX, USA). Rabbit anti-phospho-MARCKS (2741) was purchased from Cell Signaling (Danvers, MA, USA).

Western blot analysis

Cells were lysed in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 30 mM sodium pyrophosphate, 1 mM EDTA, 1.5 mM MgCl$_2$, 0.1 mM sodium orthovanadate, 10% glycerol, and 1% Triton X-100) supplemented with protease inhibitors (1 tablet Complete mini EDTA-free and 1 tablet PhosSTOP, both from Roche, Germany) for 30 min on ice and centrifuged at 14 000 rpm for 30 min at 4°C. Protein content was determined with a Pierce BCA Protein Assay kit (Thermo Scientific, Waltham, MA, USA). Samples containing equal amounts of protein were suspended in 4X Laemmli sample buffer, boiled, and loaded on a 10% sodium dodecyl sulfate polyacrylamide electrophoresis gel. Protein separation by electrophoresis was followed by semi-dry blotting onto a PVDF membrane for 1 h. The membrane was blocked in 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature (RT) and incubated with WNT5A primary antibody (1/200) in 5% skim milk in TBST overnight at 4°C. After washing with TBST, the membrane was incubated for 1 h at RT with the secondary antibody in 5% skim milk in TBST. The membrane was developed using enhanced chemiluminescence with a Chemiluminescence HRP substrate (Millipore, Billerica, MA, USA). β-actin was used as a loading control.

Measurements of calcium signaling

The cells were cultured on glass coverslips in culture medium overnight at 37°C in an atmosphere of 5% CO$_2$. The cytosolic free calcium levels were measured by loading the cells with the fluorescent calcium indicator, fura-2-AM (4 μM), for 30 min at 37°C and 5% CO$_2$. The glass coverslips with attached cells were washed once with calcium buffer (136 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO$_4$, 1.1 mM CaCl$_2$, 1.2 mM KH$_2$PO$_4$, 5 mM NaHCO$_3$, 5.5 mM glucose, and 20 mM HEPES) and mounted in a cover-glass holder containing 0.5 ml calcium buffer (37°C). The cells were excited at 340 and 380 nm, and the fluorescent fura-2 signal was recorded at 510 nm and expressed as the ratio of the 340 to the 380 nm signal. Normally, the camera was focused on 5–7 OSCC cells to detect the cytosolic free Ca^{2+} response to rWNT5A. A heterogeneous calcium response was observed in both cell lines meaning that not all cells responded.

Measurements of BrdU-positive cells

To analyze possible effects on cell number, the Cell Proliferation ELISA, BrdU kit (cat no. 11647229001, Roche, Germany) was used. Briefly, after the first 24 h of the wound-healing assay (see next section), 10 μM BrdU was added to and incubated with the cells for another 24 h. After 48 h, the cells were fixed with FixDenat for 30 min and incubated with anti-BrdU-POD for 90 min. Antibody binding was detected by addition of a specific substrate and measurement of absorbance according to manufacturer's instructions.

Analysis of cell migration

Cell migration was analyzed using a wound-healing assay. Cells were cultured in 48-well plates at 37°C and 5% CO$_2$ until they reached 100% confluence. Experiments were initiated by scratching the cell layers with a pipette tip. The wells were rinsed with PBS to remove detached cells. The remaining cells were incubated with serum-free medium

supplemented with or without rWNT5A, Foxy5, Box5, SFRP1, MAPT, GF109203X, or Go 6983, as indicated in the figure legends, for 48 h at 37°C and 5% CO$_2$. Images of the scratches were taken at 0 and 48 h, and the effect on cell migration was calculated as percentage of the scratched area that was closed using the Image-J software (NIH, Bethesda, MD, USA). For the experiments with MAPT, GF109203X, or Go 6983, the cells were pre-incubated for 30 min before scratching.

Analysis of cell invasion
A cell invasion assay was performed in BD BioCoat Matrigel Invasion Chambers (354480, BD Biosciences, MA, USA) with a pore size of 8 μm. After detachment with Trypsin-EDTA and Versene, the cells were resuspended in serum-free media and mixed with either 0.4 μg/ml of rWNT5A or carrier alone (0.1 mM EDTA, 0.5% CHAPS in PBS pH 6.8) and finally the single cells were added to the upper chamber. To the lower chamber, 10% serum containing medium was added. Cells were allowed to invade for 48 h, before fixation in 4% paraformaldehyde and staining with crystal violet. After removal of the Matrigel, the membranes were mounted on glass slides and all intact cells that had invaded the membrane were counted.

Analysis of PKC activity
PKC activity was analyzed using Western blotting. Cells were cultured in 6-well plates at 37°C and 5% CO$_2$ until they reached 80% confluence. The cells were rinsed with PBS and incubated with serum-free medium supplemented with or without rWNT5A and Box5, as indicated in the figure legends, for 2 h at 37°C and 5% CO$_2$. The cells treated with rWNT5A together with Box5 were pre-incubated with Box5 in serum-free medium overnight. PMA was added to the cells 90 sec prior the end of the 2 h incubation. The experiment was carried out by Western blotting. The membrane was blocked with 5% BSA in TBST for 1 h prior the incubation with the P-MARCKS primary antibody (1/500) in 5% BSA in TBST overnight at 4°C.

Statistical analysis
The data are presented as means ± standard error of the means (SEM), and statistical analysis was performed using either a two-tailed Student's t-test or analysis of variance (ANOVA; with Dunnett's multiple comparison test for post-analysis) using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). All experiments were repeated at least three times.

Results
Expression of WNT5A in OSCC cell lines
WNT5A protein expression was examined in two OSCC cell lines, SCC9 and SCC25, by Western blotting. In accordance with previous experience of the amount of protein needed to detect endogenous expression levels of WNT5A, 10 μg of total protein was first loaded from lysates of five cell lines, the malignant melanoma cell line HTB63 (positive control), the OSCC cell lines SCC9 and SCC25, and the breast cancer cell line MDA-MB468 supplemented with 4 ng/μl rWNT5A (positive control) and without such a supplementation (negative control) (Fig. 1A). Selection of

positive and negative controls was based on previous studies showing that HTB63 cells express and secrete high levels of endogenous WNT5A (10), whereas MDA-MB468 cells do not express endogenous WNT5A (24). The MDA-MB468 cell lysate supplemented with rWNT5A served as a second positive control. WNT5A was detected in HTB63 cell lysate as well as in MDA-MB468 cell lysate supplemented with rWNT5A, but not in lysates of unsupplemented MDA-MB468 cells, SCC9 cells or SCC25 cells (Fig. 1A). To further control whether SCC9 and SCC25 cells lack expression of WNT5A, we performed experiments to exclude the possibility that the absence of WNT5A was not due to a failure of the WNT5A antibody to detect the WNT5A protein in these cell lysates. We loaded 65 μg of protein on the gel and now we detected a distinct but weak WNT5A protein band in the OSCC cell lines as well as in WNT5A-negative cell line MDA-MB468 (Fig. 1B).

Measurement of calcium signaling
WNT5A is known to trigger the non-canonical WNT/Ca^{2+} signaling pathway in several types of cancer cells (10, 25, 26). To examine the activation of WNT/Ca^{2+} signaling pathway in OSCC cell lines SCC9 and SCC25, cytosolic free Ca^{2+} levels were measured in single, adherent OSCC cells loaded with Fura-2, before and after stimulation with 0.4 μg/ml rWNT5A. This concentration was chosen based on studies performed in breast cancer cells (27). In both cell lines, stimulation with rWNT5A induced a prompt increase in the cytosolic free Ca^{2+} level (Fig. 1C,D). The specificity of the rWNT5A-induced effect was examined by pre-incubating the cells by an inhibitor of WNT5A. The t-boc-Met-Asp-Gly-Cys-Glu-Leu hexapeptide, called Box5, was identified as an antagonist of WNT5A signaling (10). Fura-2-AM was added during the last 30 min of the pre-incubation with Box5. Pre-treatment of SCC9 and SCC25 cells for 40 min with 100 μM Box5, followed by stimulation with 0.4 μg/ml rWNT5A in the presence of 100 μM Box5, significantly reduced the rWNT5A-induced Ca^{2+} response (Fig. 1E,F). To examine whether the WNT5A-induced Ca^{2+} signal could be blocked, the intracellular Ca^{2+} chelator, MAPT, was added. Pre-treatment of SCC9 and SCC25 cells for 30 min with 5 μM MAPT-AM together with Fura-2-AM followed by stimulation with 0.4 μg/ml rWNT5A in the presence of 5 μM MAPT-AM significantly inhibited the rWNT5A-induced Ca^{2+} response (Fig. 1G,H).

Influence of rWNT5A on cell proliferation and migration
Based on the finding that rWNT5A activates a non-canonical WNT/Ca^{2+} signal in OSCC cells, and because, in most cases, OSCC cells have been described to invade collectively (7), the effect of rWNT5A was investigated both on the number of BrdU-positive cells and migration in a wound-healing assay. BrdU was added for the last 24 h of a 48-h incubation with rWNT5A. rWNT5A had no influence on the number of BrdU-positive cells at any of the concentrations tested (0.1–0.6 μg/ml), neither in SCC9 (Fig. 2A) nor in SCC25 (Fig. 2B) cells. However, 0.4 μg/ml rWNT5A significantly increased migration of both SCC9 ($P < 0.01$; Fig. 2C) and SCC25 ($P < 0.05$; Fig. 2D) cells in a wound-healing assay. The fact that rWNT5A had no effect
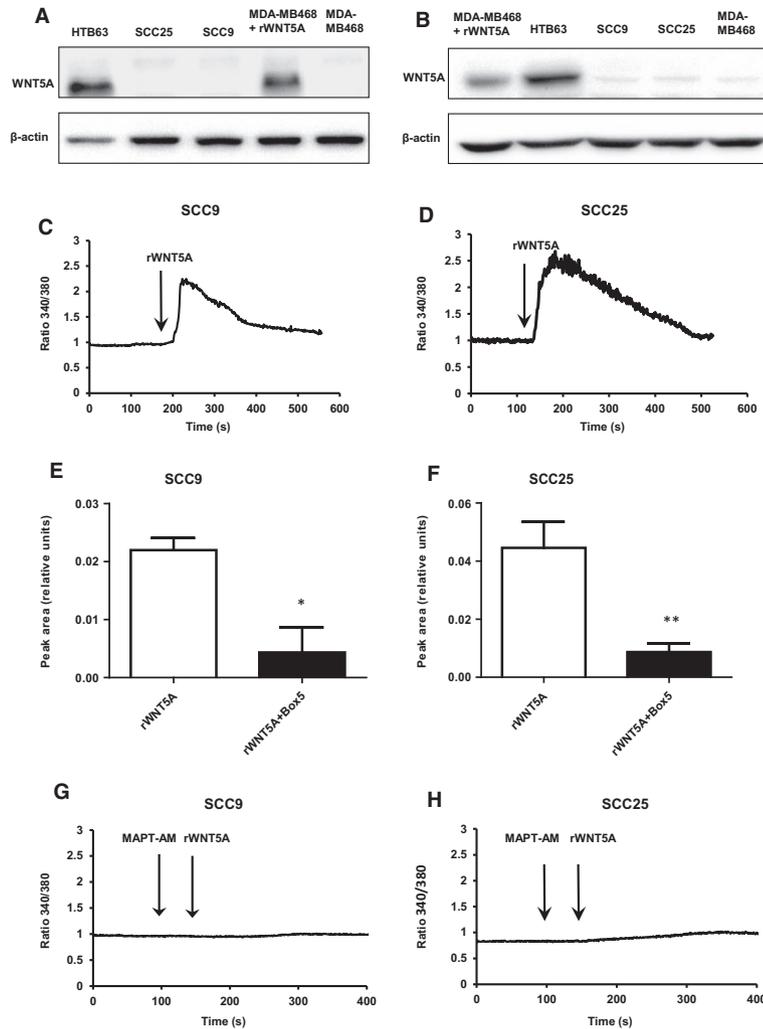
Figure 1 Expression of WNT5A protein and signaling effects of rWNT5A in OSCC cells. (A and B) The expression of endogenous WNT5A in two OSCC cell lines, SCC9 and SCC25, is very low compared to malignant melanoma cell line HTB63 (positive control). Lysate of the breast cancer cell line MDA-MB468 loaded with or without 4 ng/µl rWNT5A served as additional positive and negative control, respectively. Ten µg (A) or sixty-five µg (B) of protein was loaded in each lane. (C and D) Stimulation of SCC9 (C) and SCC25 (D) cells with 0.4 µg/ml rWNT5A resulted in an increase in cytosolic free Ca^{2+}. Cytosolic free Ca^{2+} levels are presented as the ratio of the Fura-2 signals generated by excitation at 340 and 380 nm. (E and F) Pre-incubation of SCC9 (E) and SCC25 (F) cells with 100 µM Box5 for 40 min prior to stimulation with 0.4 µg/ml rWNT5A led in both cases to a significant decrease in cytosolic free Ca^{2+} response. (G and H) Pre-incubation of SCC9 (G) and SCC25 (H) cells with 5 µM MAPT for 30 min and its presence during stimulation with 0.4 µg/ml rWNT5A significantly inhibited the rWNT5A-induced Ca^{2+} signal. Data are expressed as mean ± standard error of the means (SEM). Statistical analysis was performed with unpaired Student's t-test; *, $P < 0.05$; **, $P < 0.01$.

on the number of BrdU-positive cells provides a solid basis for studying cell migration in a wound-healing assay. A further increase in the concentration of rWNT5A to 0.6 µg/ml did not cause a statistically significant enhancement of either SCC9 or SCC25 cell migration (Fig. 2C,D). Reduction of the rWNT5A concentration still elicited a migratory response in SCC9 ($P < 0.01$ to 0.05) but not in SCC25 ($P > 0.05$) cells. For further studies, 0.4 µg/ml rWNT5A was used, that is, the concentration normally used for studying rWNT5A-induced effects in breast cancer cells (27).

To confirm the rWNT5A-induced effect on cell migration by an independent means, the cells were stimulated with the WNT5A-derived hexapeptide Foxy5. This peptide has previously been shown to mimic the effect of rWNT5A on breast cancer cells (28) and malignant melanoma cells (10). Foxy5 had in accordance with previous observations (Fig. 2) no effect on the number of BrdU-positive cells in either cell line, not even at 150 µM (Fig. 3A,B). However, it significantly increased cell migration at 25 µM in SCC9 cells (Fig. 3C) and at 50 µM in SCC25 cells (Fig. 3D). Consequently, Foxy5 mimics the effects of rWNT5A in two independent assays and in two different cell lines.

Next, the effect of rWNT5A was examined on cell invasion. Despite the fact that SCC9 is a poorly invasive cell line (29–31), the effects of rWNT5A on cell invasion were tested in both SCC9 and SCC25 cells. rWNT5A at a concentration of 0.4 µg/ml had only a limited influence on invasion of SCC9 cells (Fig. 4A) but on the other hand significantly increased invasion of SCC25 cells (Fig. 4B).
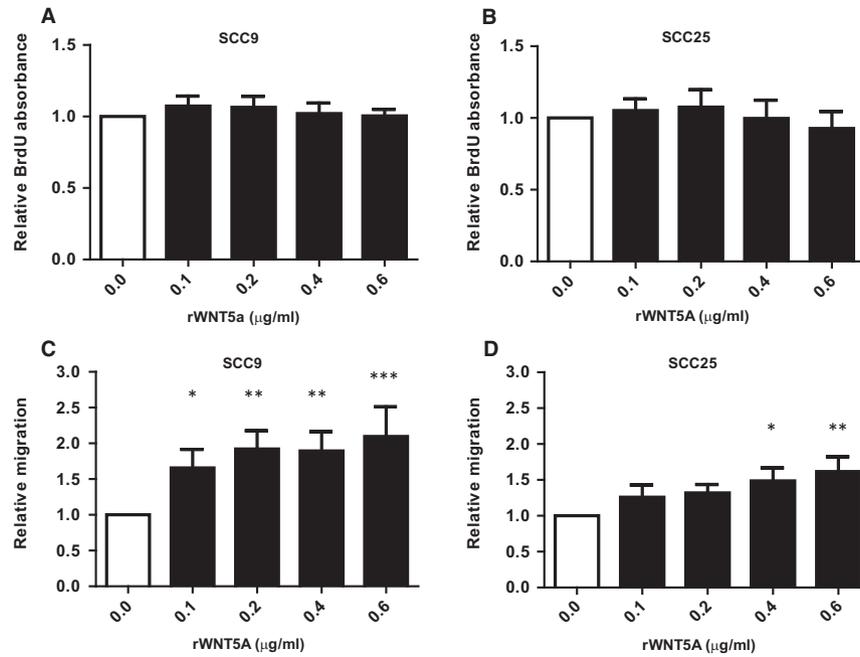
Figure 2 Functional effects of rWNT5A on OSCC cells. (A and B) The presence of rWNT5A (0.1–0.6 µg/ml) for 48 h had no effects on the number of BrdU-positive cells when compared to vehicle alone in either SCC9 (A) or SCC25 (B) cells. (C and D) In contrast, addition of rWN5A for 48 h increased the migration of SCC9 (C) and SCC25 (D) cells in a wound-healing assay. Data are expressed as mean ± SEM. Statistical analysis was performed with one-way analysis of variance (ANOVA; with Dunnett's multiple comparison test for post-analysis); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
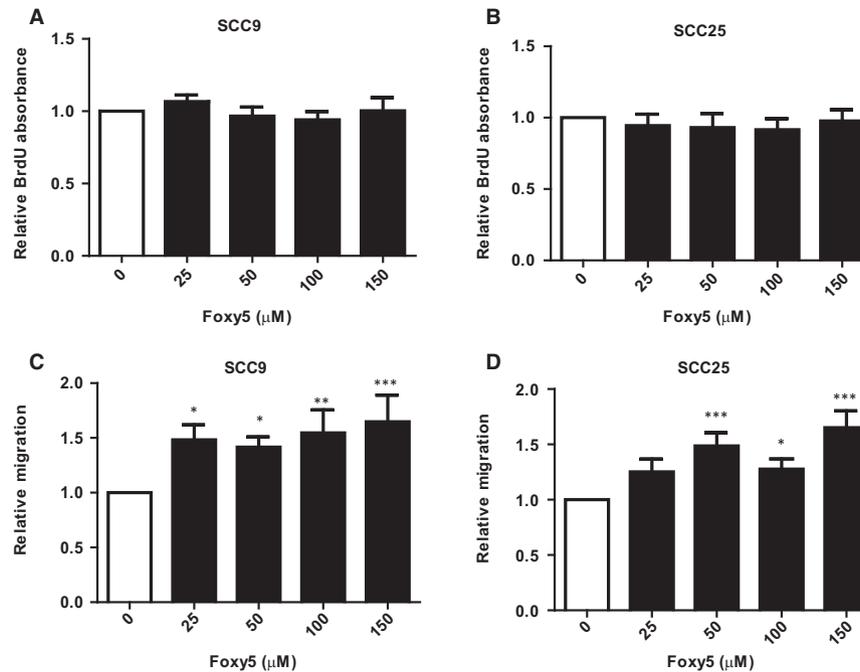

Figure 3 Functional effects of Foxy5 on OSCC cells. (A and B) The presence of Foxy5 (25–150 µM) for 48 h had no effects on the number of BrdU-positive cells when compared to vehicle alone in either SCC9 (A) or SCC25 (B) cells. (C and D) In contrast, addition of Foxy5 for 48 h increased the migration of SCC9 (C) and SCC25 (D) cells in a wound-healing assay. Data are expressed as mean ± SEM. Statistical analysis was performed with one-way analysis of variance (ANOVA; with Dunnett's multiple comparison test for post-analysis); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Furthermore, as Box5 has been documented to selectively inhibit the effects of WNT5A (10), including the calcium response in OSCC cells (Fig. 1), it was used as an additional control for the rWNT5A-induced cell migration. The effect of rWNT5A (0.4 µg/ml) on OSCC cell migration was abolished

in the presence of 100 µM Box5 in both SCC9 ($P < 0.05$; Fig. 4C) and SCC25 ($P < 0.05$; Fig. 4D) cells. It should be noted that Box5 alone had no effect on the basal migration of either SCC9 or SCC25 cells. To ascertain that the rWNT5A-induced effect on cell migration was indeed due to WNT5A
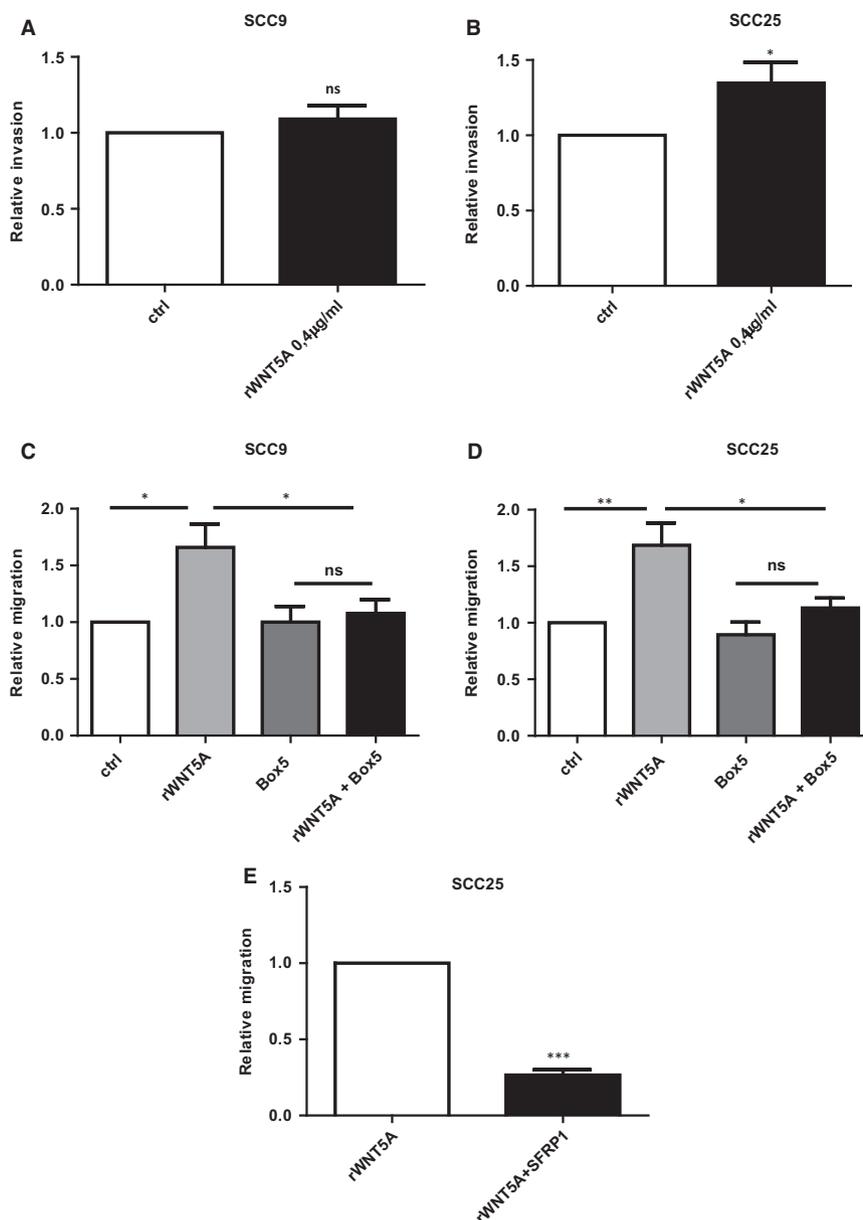
Figure 4 Effects of rWNT5A, Box5, and SFRP1 on migration and invasion of OSCC cells. (A and B) Invasion of SCC9 (A) and SCC25 (B) cells in the absence or presence of rWNT5A (0.4 µg/ml) was studied for 48 h in BD Invasion chambers. (C and D) Box5 (100 µM) significantly decreased rWNT5A-induced migration of SCC9 (C) and SCC25 (D) cells. (E) The effect of SFRP1 on rWNT5A-induced SCC25 cell migration was investigated by adding SFRP1 (3.5 µg/ml) together with rWNT5A (0.4 µg/ml) for 48 h. Data are expressed as mean ± SEM. Statistical analysis was performed with paired or unpaired Student's t-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

and not to any possible contaminant, the SCC25 cells were stimulated with rWNT5A in the presence of the natural WNT5A inhibitor rSFRP1 (molar ratio 1:10) as previously described (27). The effect of rWNT5A (0.4 µg/ml) on migration was significantly inhibited in the presence of 3.5 µg/ml rSFRP1 in SCC25 ($P < 0.001$; Fig. 4E) cells.

Influence of rWNT5A on PKC activation
In this study, we evaluated WNT5A-induced PKC activation through phosphorylation of the endogenous PKC substrate myristoylated alanine-rich protein kinase C substrate (MARCKS) in the presence or absence of Box5.

rWNT5A (0.4 µg/ml) increased the phosphorylation of MARCKS, and this phosphorylation was abolished in the presence of 100 µM Box5 both in SCC9 (Fig. 5A) and SCC25 (Fig. 5B) cells.

Figure 1E,F show that MAPT significantly inhibited the WNT5A-induced Ca^{2+} signal. In addition, the WNT5A-induced migration of SCC25 cells was eliminated by MAPT (5 µM; Fig. 5C). As WNT5A activates protein kinase C (PKC) in OSCC cells (Fig 5A,B), two conventional PKC pathway inhibitors previously used to inhibit PKC activity in OSCC cells (18, 32–34) were used to study the role of PKC in WNT5A-induced SCC25 cell migration. Both PKC
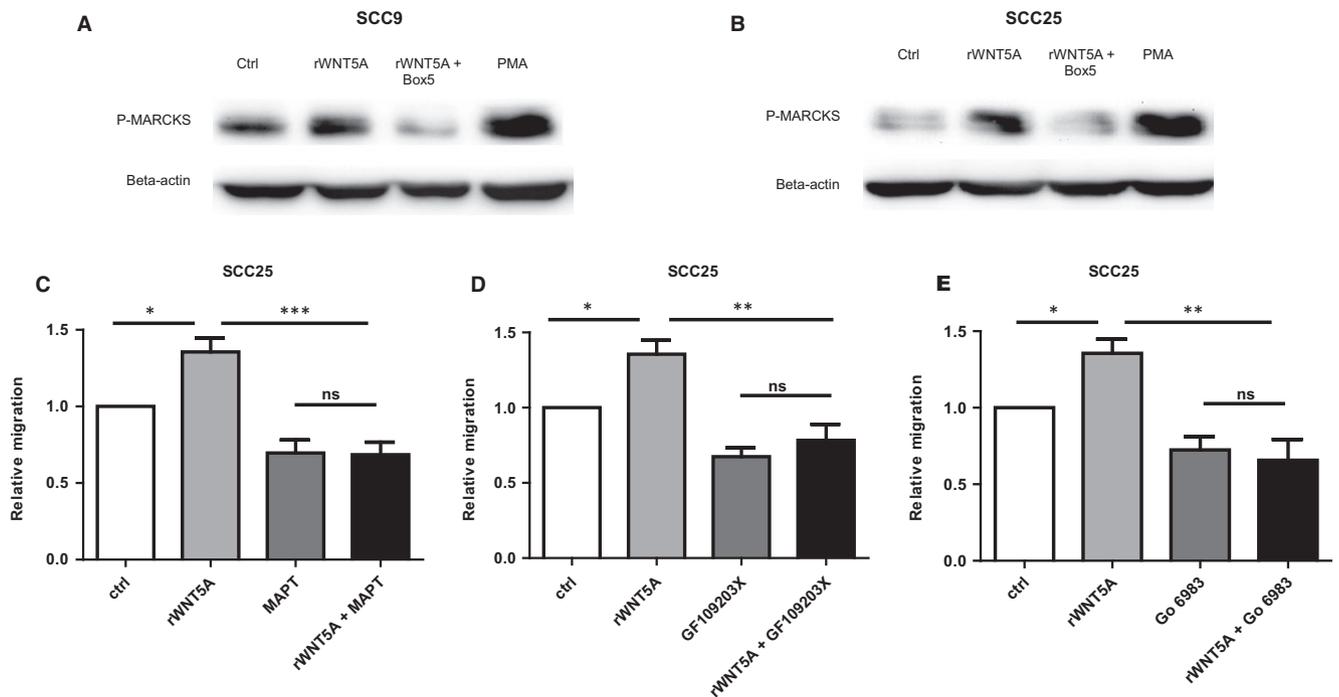
Figure 5 rWNT5A-induced PKC activation and effects of MAPT and two different PKC inhibitors on rWNT5A-induced OSCC cell migration. (A and B) Stimulation of SCC9 (A) and SCC25 (B) cells with rWNT5A (0.4 µg/ml) for 2 h, increased the activation of PKC (phosphorylation of MARCKS). Cells pre-treated with Box5 (100 µM overnight) and then stimulated with rWNT5A (0.4 µg/ml) for 2 h in the presence of Box5 (100 µM) did not respond with an activation of PKC (phosphorylation of MARCKS). PMA (10–20 nM) was used as a positive control for activation of PKC (phosphorylation of MARCKS). The blots shown are representative of 4 separate experiments. (C–E) SCC25 cells were pre-incubated (30 min) with MAPT-AM (5 µM; C), GF109203X (1 µM; D) and Go 6983 (1 µM; E) before the cells were incubated with each of these inhibitors together with rWNT5A (0.4 µg/ml) during the 48-h migration assay. Data are expressed as mean ± SEM. Statistical analysis was performed with paired or unpaired Student's *t*-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

inhibitors (1 µM) abolished the migration of SCC25 cells mediated by WNT5A (Fig. 5D,E).

Discussion

The functional role of WNT5A/Ca2+ signaling has so far not been examined in OSCC, despite the fact that an increased WNT5A expression has been documented in a study of primary OSCC cancer tissues (19, 22) as well as in an experimental rat model of tongue carcinoma (23).

In this study, we used two different OSCC cell lines. The two cell lines, SCC9 and SCC25, were both derived from tongue carcinomas, the most common location for OSCC. We found very low expression of WNT5A protein in both SCC9 and SCC25 cells, in good agreement with the previously published data on WNT5A mRNA levels in SCC25 cells (35). The fact that both SCC9 and SCC25 lack endogenous expression of WNT5A make them good cell models to study the functional role of WNT5A by reinstalling WNT5A signaling through addition of recombinant WNT5A. Similar models have previously been employed in *in vitro* studies of WNT5A in malignant melanoma and breast cancer cells (10, 27). Analysis of cytosolic free Ca^{2+} levels in OSCC cell lines demonstrated that rWNT5A induced non-canonical WNT/Ca^{2+} signaling in these cells. The rWNT5A-induced Ca^{2+} responses in the OSCC cells were similar to that previously reported for the

malignant melanoma cell line A2058, which also expresses WNT5A at low level (10).

It is well established that non-canonical WNT5A signaling regulates tumor cell migration differently depending on the type of cancer (10, 25, 27, 36). Therefore, we investigated whether OSCC cells became more or less motile after stimulation with rWNT5A. OSCC cells are known to predominantly migrate collectively, meaning that they remain connected through cell–cell junctions throughout their migratory response (7). Consequently, we used a wound-healing assay to monitor WNT5A-induced effects on migration. We found that rWNT5A significantly increased migration without affecting cell proliferation of both SCC9 and SCC25, a situation similar to that previously documented for malignant melanoma cells (17).

In contrast, cell invasion experiments revealed that rWNT5A is less effective in inducing cell invasion of SCC9 compared to SCC25 cells. These data confirm previous reports showing that SCC9 cells are poorly invasive (29–31). These data are somewhat intriguing as in our study, SCC9 cells were shown to be more migratory in response to rWNT5A as compared to SCC25 cells. The most reasonable explanation for why SCC9 cells are less invasive compared to SCC25 cells is that cell migration and cell invasion are two different functional responses. The fact that SCC25 cells responded more to stimulation with rWNT5A in the cell invasion assay compared to SCC9

cells suggests that SCC25 cells are more capable of degrading extracellular matrix (MMP's, uPA etc.) than SCC9 cells. However, the precise mechanism behind such a cellular difference in extracellular matrix degradation requires further investigations.

The importance of WNT5A-induced non-canonical signaling for OSCC cell migration was investigated by blocking the Ca^{2+} signal with MAPT and by incubating the cells with two different PKC inhibitors. Inhibition of the rWNT5A-induced Ca^{2+} signal by MAPT, an intracellular Ca^{2+} chelator, resulted in repression of WNT5A-induced OSCC cell migration. Activation of the WNT5A/Ca^{2+} signaling pathway triggered activation of PKC in both SCC9 and SCC25 cells, as demonstrated by WNT5A-induced phosphorylation of the endogenous PKC substrate MARCKS. This ability of WNT5A to activate PKC has previously been demonstrated by its phosphorylation of the PKC isoforms α, β, and γ in melanoma cells (37). To study the functional effects of WNT5A-induced PKC activation we have in the present study used two conventional PKC inhibitors, GF109203X (inhibitor of PKC α, β1, β2, and γ) and Go 6983 (inhibitor of PKC α, β, γ, δ, and ζ). These inhibitors have previously been used in OSCC cells (32–34). In the present study, both PKC inhibitors significantly repressed rWNT5A-induced OSCC cell migration. These data indicate that WNT5A promotes migration of OSCC cells by a non-canonical WNT5A/Ca^{2+}/PKC signaling pathway. Notably, these PKC inhibitors also inhibited the basal migration of OSCC cells. This observation is most likely explained by the fact that non-stimulated OSCC cells express large amounts of PKCα and PKCδ as well as increased activity of PKCα (18). Furthermore, it was shown that PKC inhibition reduced the basal migration in a wound-healing assay (32). Taken together with our present results, these previous findings indicate an elevated expression and activity of PKCα in non-stimulated OSCC cells explaining our finding of inhibitor-repressed basal cell migration.

In a recently published study by Liu et al., WNT5A has been shown to be recovered in complex with CTHRC1 and that OSCC cell migration is mediated through activation of RhoA, Rac1, and JNK (19). However, they did not directly stimulate the cells with WNT5A. Their approach is different from ours, as we have used recombinant WNT5A to induce cell migration of OSCC cells, an effect that we show is mediated via activation of a WNT5A/Ca^{2+}/PKC pathway. We strongly believe that our study complement the study conducted by Liu et al. Thus, both studies indicate that WNT5A plays a role in the regulation of OSCC cell migration through the WNT/PCP pathway and/or the WNT/Ca^{2+} pathway.

In a recent study published by Pourreyron et al., it was shown that WNT5A is upregulated at the leading edge in non-melanoma skin cancer, as well as in tumor-surrounding stroma including tumor-associated fibroblasts and endothelial cells, forming active WNT5A-gradients (acting as chemo-attractant) (21). They suggest that chemotactic migration of keratinocytes is enhanced only in the presence of a WNT5A-gradient. In contrast, our study shows that recombinant WNT5A present in homogenous concentration can induce cell invasion of OSCC cells, in the absence of a WNT5A-gradient. However, one cannot exclude that WNT5A could act as a chemo-attractant in OSCC *in vivo*, since in OSCC the surrounding stroma is very inflamed, infiltrated by numerous inflammatory cells expressing and releasing WNT5A, potentially giving rise to a WNT5A-gradient. It cannot be excluded that such a WNT5A-gradient might increase the invasive capability of the peripheral cancer cells.

Already in early stages of OSCC development, when cancer cells have just invaded through the basement membrane they can easily reach blood or lymph vessels and metastasize. This underlines the importance of a novel therapeutic approach that targets tumor cell migration and that can be combined with conventional chemotherapy and/or localized radiation. The WNT5A antagonist Box5 has previously been shown to impair WNT5A signaling and cell migration (10). In the present study, Box5 was also shown to inhibit WNT5A-induced Ca^{2+} signaling and migration of OSCC cells. These data not only confirm that we are studying WNT5A-induced effects, but might also indicate a potential novel therapeutic strategy to treat OSCC progression by inhibition of WNT5A-induced migration.

In summary, the current experimental study demonstrates that WNT5A/Ca^{2+}/PKC signaling affects migration and invasion of OSCC cells in a cellular context-dependent manner. These findings also indicate a need for a novel treatment strategy for OSCC that targets WNT5A signaling and thus OSCC invasion and metastasis.

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Acknowledgements

Conflict of interest

L.A. and T.A. are shareholders of WntResearch and T.A. is part-time Chief Scientific Officer of WntResearch. This does not alter the author's adherence to all the policies on sharing data and materials as stated for the *Journal of Oral Pathology and Medicine*.