

# Slit-2 facilitates interaction of P-cadherin with Robo-3 and inhibits cell migration in an oral squamous cell carcinoma cell line

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**Slits are a group of secreted glycoproteins that act as molecular guidance cues in cellular migration. Recently, several studies demonstrated that Slit-2 can operate as candidate tumour suppressor protein in various tissues. In this study, we show Slit-2 expression in basal cell layers of normal oral mucosa colocalized with P-cadherin expression. In contrast, there is a loss of Slit-2 and P-cadherin expression in mucosa of oral squamous cell carcinoma (OSCC). Our *in vitro* investigations reveal a correlation of P-cadherin and Slit-2 expression: OSCC cells with induced P-cadherin expression (PCI52\_PC) display an increased Slit-2 expression. However, abrogating P-cadherin function with a function-blocking antibody decreases Slit-2 secretion confirming a direct link between P-cadherin and Slit-2. Moreover, experiments with OSCC cells show that Slit-2 interferes with a Wnt related signalling pathway, which in turn affects Slit-2 expression in a feedback loop. Functionally, transwell migration assays demonstrate a Slit-2 dose-dependent decrease of PCI52\_PC cell migration. However, there is no influence on migration in mock control cells. Responsible for this migration block might be an interaction of P-cadherin with Roundabout (Robo)-3, a high affinity receptor of Slit-2. Indeed, proximity ligation assays exhibit P-cadherin/Robo-3 interactions on PCI52\_PC cells. Additionally, we detect a modulation of this interaction by addition of recombinant Slit-2. Down-regulation of Robo-3 expression via small interfering RNA neutralizes Slit-2 induced migration block in PCI52\_PC cells. In summary, our experiments show antitumorigenic effects of Slit-2 on P-cadherin expressing OSCC cells supposedly via modulation of Robo-3 interaction.**

## Introduction

Oral squamous cell carcinoma (OSCC) is the most frequent malignant tumour of the oral cavity. Despite improved therapeutic intervention the 5 years survival rate is still only 50% (1). This underlines the importance to explore novel molecular markers, which could help to improve diagnosis and treatment of OSCC.

Cadherins belong to an important family of glycosylated Ca<sup>2+</sup>-dependent adhesion molecules (2). They are comprised of a large extracellular domain responsible for homophilic cell–cell interactions, a transmembrane domain and a highly conserved cytoplasmic tail that is bridged to the actin cytoskeleton by binding directly and indirectly to various cytoplasmatic proteins (3). Cadherins are localized to adherens junctions, participate in the maintenance of cell–cell contacts and control diverse morphogenetic events. In pathological processes, they play a dominant role in tumour metastasis and cell migration (4). The best characterized and most widely distributed members of the cadherin superfamily are the classical cadherins.

**Abbreviations:** cDNA, complementary DNA; DMEM, Dulbecco's modified Eagle's medium; GSK, glycogen synthase kinase; mRNA, messenger RNA; OSCC, oral squamous cell carcinoma; PLA, proximity ligation assay; rm, recombinant mouse; qRT-PCR, quantitative reverse transcription–polymerase chain reaction; Robo, Roundabout.

P-cadherin, an important epithelial molecule, is one of the classical cadherins. It is exclusively expressed in the regenerative basal and suprabasal cell layers of the epithelium (5), but the functional role of P-cadherin in tissue regeneration and homeostasis is largely unknown. Our main goal is to determine the role of P-cadherin and its association with other molecules in malignant oral epithelial cells.

In a previous study, we demonstrated that P-cadherin is an indispensable component in reconfiguring mesenchymal OSCC cells with epithelial features by affecting the glycogen synthase kinase (GSK) 3- $\beta$ -signalling pathway. Induced expression of P-cadherin leads to an increased GSK3- $\beta$  activation, a nuclear decrease of the E-cadherin repressor Snail via phosphorylation by GSK3- $\beta$  and subsequent cytoplasmatic translocation of Snail in OSCC cells (6). GSK3- $\beta$  is a serine/threonine kinase that phosphorylates many proteins including proteins of the Wnt-signalling pathway (7). A further key component of the canonical and non-canonical Wnt signalling is  $\beta$ -catenin which binds and activates members of the Lef-1/Tcf family of DNA-binding proteins and is also affected by GSK3- $\beta$ . Furthermore,  $\beta$ -catenin functions as a part of the cadherin complex, which regulates in this way cell–cell adhesion and cell migration (8). In normal cells, most  $\beta$ -catenin is retained at the cell membrane or it undergoes rapid turnover by the multiprotein destruction complex containing GSK3- $\beta$ , hence there is less free  $\beta$ -catenin available for nuclear shuttling (9).

Initially, the Slit/Roundabout (Robo)-signalling pathway was described in the nervous system where it regulates axon guidance, branching and neural migration (10,11). In non-neuronal cells, Slit has been found to inhibit chemotaxis of leukocytes, Langerhans cells and vascular smooth muscle cells (12–14), whereas endothelial cells and breast cancer cells are attracted by Slit (15,16). Slits are large multidomain leucine-rich repeat-containing proteins with three known members in mammals: Slit-1, Slit-2 and Slit-3, which are secreted (17). Slit ligands bind to Robo receptors, a membrane-protein family that all share a single transmembrane domain. The Robo family constitutes four Robos (Robo-1, Robo-2, Robo-3 and Robo-4) (18).

Recently, several studies revealed that Slit-2 affecting cell migration especially exhibits tumour-suppressive effects in various human cancers (19–22). A recent article reported that Slit-2 inhibits growth and metastasis of squamous cell carcinoma (23). However, little is known about the antitumorigenic effects of Slit-2 in OSCC.

In this study, we show for the first time a connection of Slit-2 and P-cadherin expression. We demonstrate that Slit-2 expression and secretion depends on P-cadherin expression and function. Furthermore, our results suggest a feedback loop of signalling events where Slit-2 affects GSK3- $\beta$  signalling resulting in Slit-2 expression. Functionally, addition of Slit-2 to OSCC cells expressing P-cadherin can slow down cell migration. *In vitro*, we show an interaction of P-cadherin with Robo-3, a high affinity receptor of Slit-2 (24), on aberrant oral epithelial keratinocytes, which can be modulated by addition of Slit-2. Altogether, our results indicate that Robo-3 receptor may act as a modulator of P-cadherin function meaning Slit-2-induced P-cadherin/Robo-3 complex formation on the one hand decreases cell migration, on the other hand helps to maintain appropriate amounts of Slit-2 expression levels in oral epithelium.

## Material and methods

### Cell lines and culture conditions

The OSCC cell line PCI 52, derived from a larynx carcinoma (pT2N0M0G2), was stably transfected with a full length complementary DNA (cDNA) clone of P-cadherin as described by Bauer *et al.* (6). Clones were designated as PCI52\_PC1-3. PCI52\_PC and PCI52\_mock cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Pan-Biotech, Aidenbach, Germany)

with 10% fetal calf serum (Gibco, Karlsruhe, Germany), 1% penicillin/streptomycin (Gibco) and 1% L-glutamine (Gibco). All cells were grown under selection with G418 (Gibco).

#### Tissue samples

For immunohistochemical studies, archival formalin-fixed and paraffin-embedded OSCC tissues and healthy control samples from the same OSCC patients were obtained from the Department of Pathology of the University Hospital Regensburg.

#### Immunohistochemistry

Paraffin-embedded tissue sections were deparaffined and rehydrated. The slides were incubated with 0.05% protease XXIV (Roth, Karlsruhe, Germany), with 0.1% hyaluronidase (Roth), with 3% peroxidase (Roth) and blocked with 3% bovine serum albumin (Sigma–Aldrich, Steinheim, Germany) in phosphate buffered saline (Roth) containing 0.1% Tween 20 (Sigma, Steinheim, Germany) (phosphate buffered saline with Tween-20) for 1 h at 37°C. Goat polyclonal antibody anti-Slit-2 (1:5, Santa Cruz Biotechnology, Heidelberg, Germany) was used as primary antibody for 16 h at 4°C. Afterwards, the paraffin sections were incubated with the secondary antibody donkey anti-goat IgG-horseradish peroxidase (Santa Cruz Biotechnology) (1:400) for 1 h at 37°C and diaminobenzidine solution (DakoCytomation, Hamburg, Germany). The cell nuclei were counterstained with hematoxylin (Roth). All images were taken using the microscope Olympus BX 61. Modifications for P-cadherin immunohistochemistry: the slides were incubated with mouse monoclonal anti-P-cadherin antibody (BD Transduction Laboratories, Philadelphia, NJ) (1:100) for 1 h.

#### Immunocytochemistry

PCI52\_PC and PCI52\_mock cells were plated on glass coverslips and fixed. After blocking with 3% peroxidase and 3% bovine serum albumin in phosphate buffered saline with Tween-20 goat polyclonal anti-Slit-2 antibody (1:5) was used for 16 h at 4°C. Afterwards, the cells were incubated with the secondary antibody donkey anti-goat IgG-horseradish peroxidase (1:400) for 1 h at 37°C and diaminobenzidine.

#### Isolation of RNA from snap frozen tissues

Snap frozen tissues of normal and OSCC mucosa (250 mg) were cut with a scalpel, placed in 2.5 ml, –80°C pre-chilled RNAlater-ICE (Ambion, Austin, TX) and incubated for 16 h at –20°C. Afterwards, RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and was used for real-time polymerase chain reaction analyses.

#### Real-time polymerase chain reaction analyses

For real-time polymerase chain reaction, total cellular RNA was isolated from PCI52\_PC and PCI52\_mock cells using RNeasy Mini Kit according to the manufacturer's instructions. Reverse transcription of RNA to cDNA was performed using transcriptor high fidelity cDNA synthesis kit (Roche, Penzberg, Germany) according to the manufacturer's protocol. Quantitative gene expression was done using Brilliant II Fast quantitative polymerase chain reaction Master Mix from Stratagene (Agilent Technologies, Santa Clara, CA) with Taqman UPL probes (Roche) (Beta-actin: 5'-ATTGGCAATGAGCGGTTTC-3' and 5'-TGAAGGTAGTTTCGTGGATGC-3'; Robo-3: 5'-ACATCCCTCAGGAGATCTGG-3' and 5'-TCACCTTGCCTCCCTTGG-3'; Slit-1: 5'-GGCCAGTGTATGGATGAAG-3' and 5'-GGATCTCACA-GAGCTGTCCAC-3'; Slit-2: 5'-CATGGAGGAACCTGCCACTTA-3' and 5'-TCCATCAGCACAAATACACCA-3'; Slit-3: 5'-ACCGCTTCCAGTGCAAAG-3' and 5'-GCATGTCCCGTTATCTTGC-3' and P-cadherin: 5'-GCTGGGGAAAGTATTCATGG-3' and 5'-CCTCAGTGACCTTCTTCTTG-3'). Beta-actin messenger RNA (mRNA) was used for normalization.

#### Beta-catenin/Tcf inhibitor FH535 treatment

PCI52\_mock cells were incubated with 20, 40 and 100 µM or without FH535 beta-catenin/Tcf inhibitor (Calbiochem, Nottingham, UK) for 24 h (25). For control, PCI52\_mock cells were incubated with dimethyl sulfoxide. After FH535 beta-catenin/Tcf inhibitor treatment total cellular RNA was isolated.

#### Function blocking P-cadherin assay

PCI52\_PC cells were seeded in 96-well plates and incubated with function blocking mouse monoclonal antibody Cadherin P (1:10; Thermo Scientific, Bonn, Germany), which binds to the N-terminal domain of P-cadherin, or with IgG<sub>1</sub> control antibody (Abcam, Cambridge, UK) for 16 h. Supernatant human Slit-2 levels were detected with an enzyme-linked immunosorbent assay for human Slit-2 according to the manufacturer's instructions (USCN Life Science, Wuhan, China). This experiment was repeated eight times. To visualise the impact of P-cadherin function blocking antibody, a proximity ligation assay (PLA) was performed with PCI52\_PC cells after function blocking P-cadherin antibody treatment.

#### Proximity ligation assay

For detection of protein interactions, the PLA (Olink Bioscience, Uppsala, Sweden) was performed. PCI52\_PC cells were seeded with or without 100 ng recombinant mouse (rm) Slit-2 (R&D Systems, Wiesbaden, Germany) on glass coverslips. After reaching 80% confluence, the assay was performed according to the manufacturer's instructions. Briefly, after fixation and blocking, the cells were incubated with the primary antibodies rabbit polyclonal anti-P-cadherin C-terminal (Abnova, Heidelberg, Germany) and goat polyclonal anti-Robo-3 C-terminal (antibodies-online GmbH, Aachen, Germany) (1:50). Subsequently, the cells were incubated with secondary antibodies conjugated with oligonucleotides. After hybridization and ligation of the oligonucleotides, the DNA was amplified by addition of an amplification mixture. A detection mixture detected the amplicons resulting in red fluorescence signals. In the final step, the nuclei were counterstained with 4',6-diamidino-2-phenylindole and the cells were mounted with mounting medium (Vectashield; Vectorlabs, Peterborough, UK). As control, the cells were incubated without any antibody and with an antibody directed against the N-terminus of P-cadherin, respectively. Additionally, PLAs were performed with human oral keratinocytes (ScienCell, San Diego, CA). Paraffin-embedded tissue sections of normal oral mucosa were also used for PLAs. Paraffin sections were deparaffined, rehydrated and demasked with 10 mM sodium citrate buffer (Roth). Therefore, PLA proceeds as described above. For evaluation, the microscope Olympus BX 61 was used. To quantify the number of P-cadherin/Robo-3 interactions 42 and 160 cells, respectively, were counted. Quantification: counts/nucleus.

#### Immunoprecipitation experiment

PCI52\_PC cells were incubated with 100 ng rm Slit-2 for 16 h. For control, PCI52\_mock and PCI52\_PC cells without Slit-2 treatment were used. After Slit-2 incubation, cell lysates were prepared using radioimmunoprecipitation assay buffer (Sigma). For immunoprecipitation experiments, cell lysates were mixed with 4 µg goat polyclonal anti-Robo-3 antibody and 100 µl magnetic protein A/G microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and purified using µMACS columns (Miltenyi Biotec) according to the manufacturer's instructions. The samples eluted from µMACS columns with sodium dodecyl sulfate sample buffer (Roth) were analysed by western blotting.

#### Western blot analyses

For western blot analysis, immunoprecipitates were separated with sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Blot membranes were blocked, subsequently incubated with primary mouse monoclonal anti-P-cadherin antibody (1:1000) for 16 h at 4°C. As loading control, goat polyclonal anti-Robo-3 antibody (1:1000) was used to confirm the P-cadherin/Robo-3 interaction. All appropriate secondary antibodies conjugated with horseradish peroxidase were purchased from Pierce (Rockford, IL). Modifications for P-cadherin and Slit-2, respectively, western blot analysis: blots were incubated with primary mouse monoclonal anti-P-cadherin (1:1000) and goat polyclonal anti-Slit-2 antibody (1:500), respectively, for 16 h at 4°C. Equal loading was verified with rabbit monoclonal antibody to beta-actin (1:10 000; Abcam). Modifications for beta-catenin western blot analysis: PCI52\_mock cells were incubated with 100 ng rm Slit-2 for 15, 30, 60 and 120 min or without rm Slit-2 and subsequently, cell lysates were prepared. Blots were incubated with primary rabbit monoclonal anti-phospho-beta-catenin antibody (This antibody detects endogenous levels of beta-catenin only when phosphorylated at serines 33, 37 or threonine 41.) (1:1000; Cell Signaling Technology, Danvers, MA), mouse anti-beta-catenin (1:1000; BD Transduction Laboratories) or rabbit monoclonal anti-beta-actin for 16 h at 4°C.

#### Transient transfection of small interfering RNA-Robo-3

PCI52\_PC and PCI52\_mock cells were transfected with small interfering RNA (siRNA) specific to human Robo-3 (Thermo Scientific). Cells were seeded the day before transfection in six-well plates in DMEM without antibiotics. Cells were transfected with 20 µM siRNA or 20 µM of control siRNA (non-targeting, Thermo Scientific) using Dharmafect 1 (Thermo Scientific). After 48 h, the extent of mRNA knockdown was determined by quantitative reverse transcription–polymerase chain reaction (qRT–PCR). After 3 days, siRNA transfected cells were used for migration assays.

#### Migration assay

Migration assays were performed using 24-well dishes and ThinCert cell culture inserts (Greiner Bio-One, Frickenhausen, Germany). Inserts were placed in wells of a 24-well plate forming a migration chamber. The migration chamber consisted of an upper and lower compartment separated by a membrane with 8 µm pore diameter. The lower 24-well compartment was filled with DMEM containing 10% fetal calf serum. In the upper compartment, PCI52\_PC or PCI52\_mock cells were seeded with 10 and 100 ng or without rm Slit-2 and siRNA-transfected cells, respectively, in DMEM containing 0.2% bovine

serum albumin. After incubation for 4.5 h at 37°C cells which migrated to the reverse side of the membrane were stained with calcein-AM (Calbiochem), released into the lower compartment by trypsinization for 20 min. Quantification of cells was performed measuring the relative fluorescent units with a microplate reader (Tecan, Crailsheim, Germany).

#### Statistical analyses and evaluation

All statistical analyses were done using the Mann–Whitney-*U*-test. For evaluation of the quantitative polymerase chain reaction and migrations experiments, raw data were set to 100% and standard deviation was calculated in subsequent experiments.

## Results

Our area of interest is to investigate the role of P-cadherin and its association with other molecules in OSCC. Novel studies report on a connection of cadherins with the Slit/Robo system (26–30). For this reason, we examined if P-cadherin acts in concert with this receptor system.

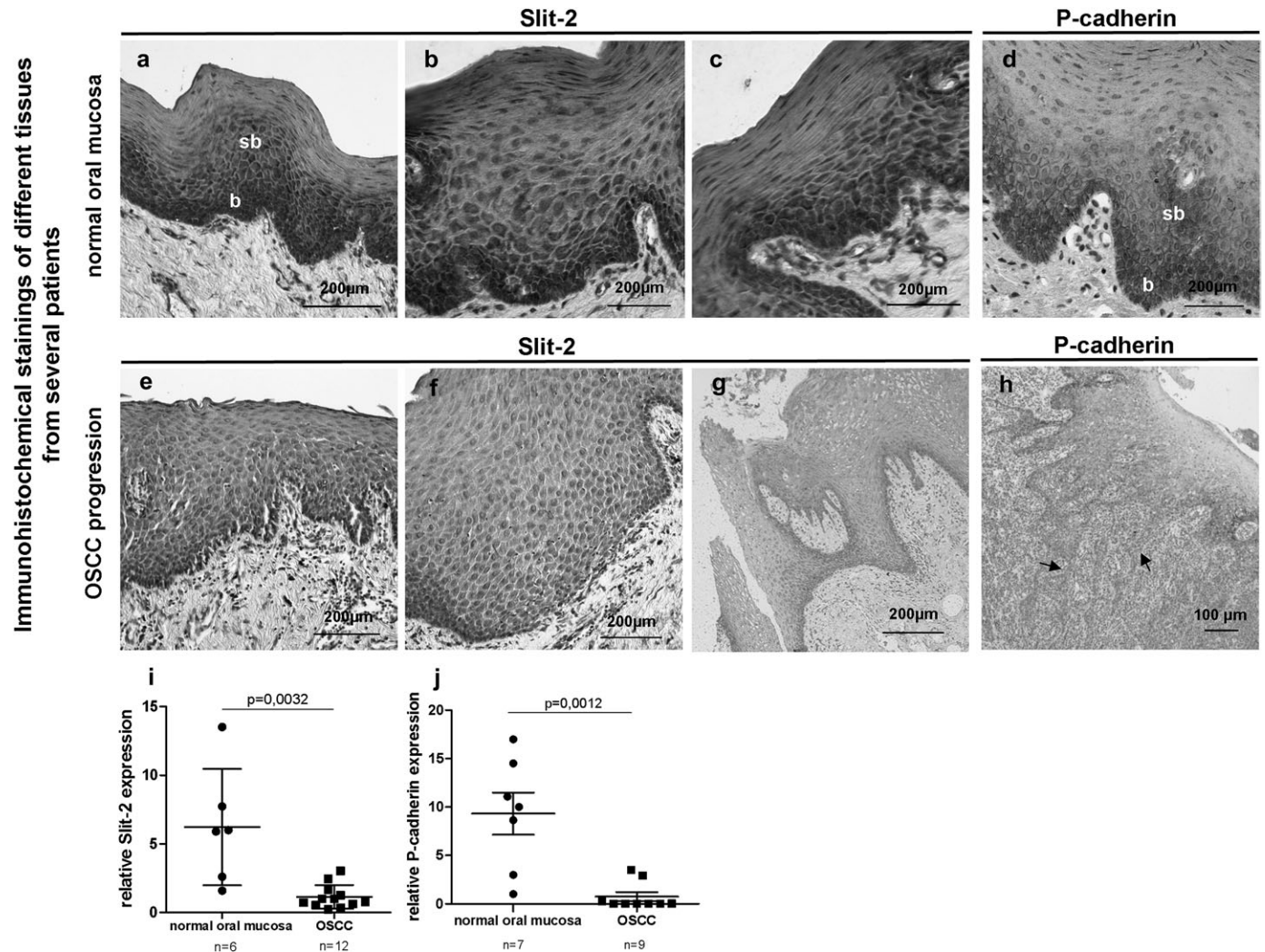
#### Expression of Slit-2 was decreased in OSCC

To address this issue, immunohistochemical stainings of P-cadherin and Slit-2 were performed on paraffin sections of healthy oral mucosa and patients suffering from OSCC. Immunohistochemical

stainings of tissue sections from different patients showed that in normal oral mucosa, Slit-2 was localized in basal and suprabasal cell layers (Figure 1a–c). It is known that P-cadherin is also expressed in these epithelial cell layers (Figure 1d). However, during neoplastic transformation of OSCC, Slit-2 revealed a rather low and dyslocalized expression pattern in the oral mucosa (Figure 1e–g). It is suggested that there is a strong P-cadherin expression in dysplasia, whereas its membrane bound expression is lost during OSCC invasion (Figure 1h, arrows) (6). To confirm the immunohistochemical results, we investigated Slit-2 and P-cadherin mRNA expression of OSCC patients and healthy controls. qRT–PCR of cDNA obtained from snap frozen tissues demonstrated a significant decrease of Slit-2 expression ( $P = 0.0032$ ) in patients suffering from invasive OSCC compared with control patients (Figure 1i). Interestingly, qRT–PCR experiments of cDNA from snap frozen tissues of patients with progressed OSCC revealed a significant decrease of P-cadherin expression ( $P = 0.0012$ ) in contrast to healthy controls as well (Figure 1j).

#### P-cadherin overexpression induced Slit-2 expression in the OSCC cell line PCI 52

To analyse the role of Slit-2 and P-cadherin in detail, we used the highly undifferentiated OSCC cell line PCI 52 deficient of the



**Fig. 1.** Representative immunohistochemical stainings of different tissues from several patients show that in normal oral mucosa, Slit-2 expression (a–c) is localized in basal and suprabasal epithelial cell layers like P-cadherin (d). During neoplastic transformation of OSCC, Slit-2 reveals a rather low and dyslocalized expression pattern in the oral mucosa (e–g). Tissue sections of OSCC patients display a loss of membrane-bound P-cadherin at the invasion front of OSCC (h). qRT–PCR of cDNA obtained from snap frozen tissues confirms a significant decrease of Slit-2 (i) and P-cadherin (j) expression in OSCC patients compared with healthy controls (b, basal; sb, suprabasal cell layers of normal oral mucosa; arrows, invasion front).

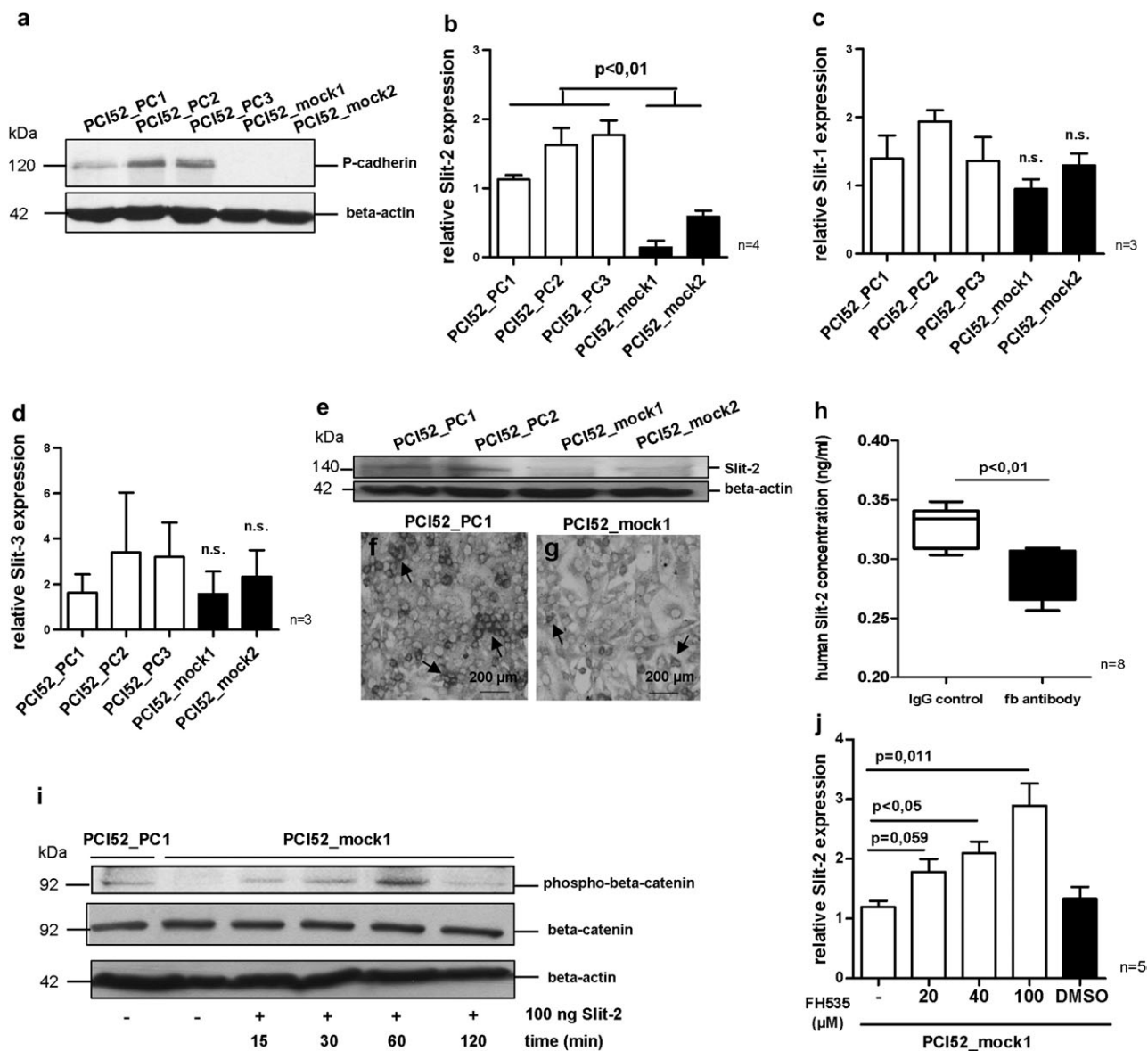
classical cadherins as an *in vitro* model system. This cell line was stably transfected with full length P-cadherin. The chosen cell clones were designated as PCI52\_PC1-3. Figure 2a shows the P-cadherin expression of the selected PCI52\_PC cell clones and PCI52\_mock control cells in a western blot analysis. Immunohistochemical stainings of PCI52\_PC cells revealed that P-cadherin was localized at the cell surface membrane, whereas in PCI52\_mock control cells, there was no visible P-cadherin expression (6).

When we analysed the Slit expression in PCI52\_PC and PCI52\_mock cells by qRT-PCR, we discovered a correlation between P-cadherin and Slit-2 expression. Figure 2b depicts a significantly increased Slit-2 expression ( $P < 0.01$ ) in stable P-cadherin transfected OSCC cell clones (PCI52\_PC1-3) compared with mock control cells (PCI52\_mock1-2). In contrast, no correlation could be seen between P-cadherin and Slit-1 and Slit-3 expression, respectively

(Figure 2c and d). Indeed, western blot analysis demonstrated a higher Slit-2 protein level in PCI52\_PC cells than in PCI52\_mock cells (Figure 2e). Additionally, immunocytochemical stainings confirm a stronger Slit-2 expression in PCI52\_PC cells than in PCI52\_mock cells (Figure 2f and g). In a confluent monolayer, Slit-2 is located pericellular in cell patches of PCI52\_PC cells (arrows in Figure 2f), whereas in PCI52\_mock cells, only a few single cells show Slit-2 expression (arrows in Figure 2g). Hence, the qRT-PCR data as well as the western blot results and immunocytochemical studies suggest a supposable link between P-cadherin and Slit-2 expression.

#### *Slit-2 secretion was enhanced in the OSCC cell line PCI 52 by P-cadherin overexpression*

To show the direct connection between Slit-2 and P-cadherin, we blocked P-cadherin with a function blocking P-cadherin antibody in



**Fig. 2.** Western blot analysis displays a strong P-cadherin expression in the stably transfected OSCC cell line PCI 52 (PCI52\_PC1-3), whereas no P-cadherin expression can be seen in mock transfected OSCC cells (PCI52\_mock1-2) (a). After P-cadherin overexpression Slit-2 expression increases significantly in PCI52\_PC cells compared with PCI52\_mock cells (b), whereas both Slit-1 and Slit-3 expression reveal no correlation with P-cadherin expression (c and d). Western blot analysis shows a higher Slit-2 protein level in PCI52\_PC cells than in PCI52\_mock cells (e). Immunocytochemical stainings affirm a stronger Slit-2 expression in PCI52\_PC cells contact each other (f, see arrows) in contrast to sparse Slit-2 expression in a few PCI52\_mock single cells (g, see arrows). Blocking P-cadherin function with function blocking (fb) P-cadherin antibody results in a significant decrease of Slit-2 secretion in PCI52\_PC cells compared with cells with IgG control antibody treatment (h). Within an hour after Slit-2 incubation phosphorylation of beta-catenin appears in PCI52\_mock cells, which have only trace amounts of phospho-beta-catenin (i). Inhibition of the beta-catenin/Lef/Tcf complex with FH535 inhibitor in PCI52\_mock cells results in a dose-dependent increase of Slit-2 expression (j).

confluently grown PCI52\_PC cells. Subsequently, the Slit-2 concentration in the supernatant was measured by enzyme-linked immunosorbent assay. The function-blocking antibody binds to the N-terminal domain of P-cadherin and prevents P-cadherin function but does not detach the cells from the culture plate. In our experiment, we observed that blocking P-cadherin function resulted in a significant decrease of Slit-2 secretion ( $P < 0.01$ ) compared with cells with IgG control antibody treatment (Figure 2h).

#### Slit-2 induced phosphorylation of beta-catenin in the OSCC cell line PCI 52

Screening for signalling pathways that could be involved in the Slit-2 expression, we identified the Wnt related GSK3-beta-signalling pathway. In a previous study (6), we showed that GSK3-beta is activated in PCI52\_PC cells and, consequently, the transcription factor Snail is translocated from the nucleus into the cytoplasm in contrast to PCI52\_mock cells. Additionally, beta-catenin, which is another important part in this pathway is abundant in the cytoplasm in our PCI52\_mock cells (6). Interestingly, western blot analysis demonstrated that in PCI52\_mock cells, beta-catenin is temporarily phosphorylated within an hour after Slit-2 incubation indicating that Slit-2 influenced in some way beta-catenin phosphorylation (Figure

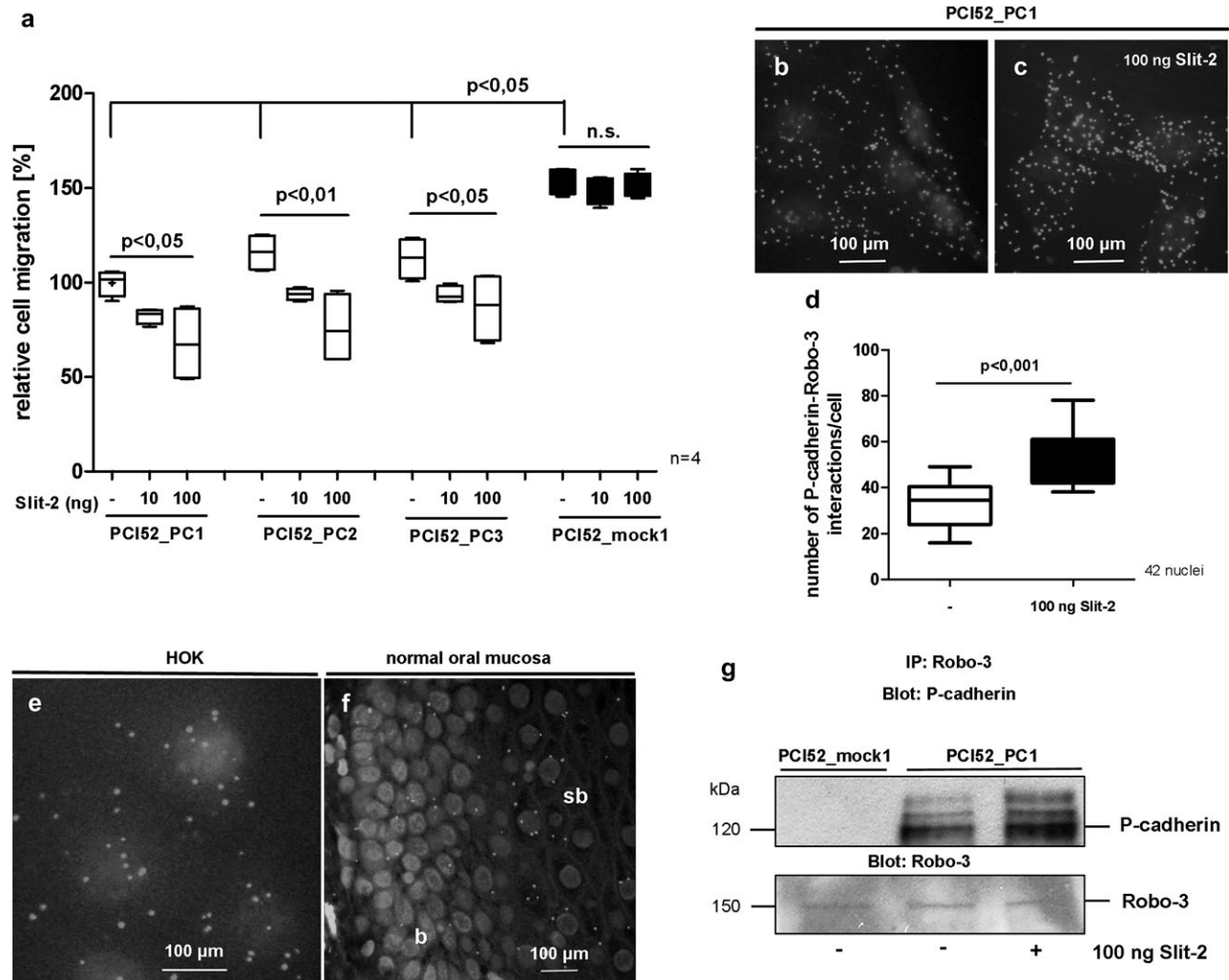
2i). However, after prolonged incubation of Slit-2 for  $>1$  h led to a decrease of beta-catenin phosphorylation (Figure 2i).

#### Slit-2 expression was influenced in the OSCC cell line PCI 52 by blocking of beta-catenin

On the other hand, we found that beta-catenin also modifies the Slit-2 expression. Inhibition of beta-catenin/Lef/Tcf complex with the FH535 inhibitor resulted in a dose-dependent increase of Slit-2 mRNA expression ( $P = 0.011$ ) in PCI52\_mock cells pointing to an involvement of beta-catenin together with further transcription factors in Slit-2 expression (Figure 2j).

#### Slit-2 decreased migration of the P-cadherin overexpressing OSCC cell line PCI 52

In our next experiments, we investigated the functional role of Slit-2. In a previous study (6), we showed that the migration of P-cadherin overexpressing cell clones is decreased compared with mock control cells. Here, we demonstrated a further dose-dependent decrease ( $\sim 50\%$ ,  $P < 0.05$  and  $P < 0.01$ ) in cell migration after addition of extra Slit-2 to P-cadherin overexpressing OSCC cells (Figure 3a). However, addition of Slit-2 to PCI52\_mock cells did not show any



**Fig. 3.** After addition of Slit-2 PCI52\_PC cells display a dose-dependent decrease in cell migration, whereas PCI52\_mock cells do not show any reaction on cell migration after Slit-2 addition (a). PLAs demonstrate an interaction of P-cadherin with Robo-3 receptor *in vitro* on PCI52\_PC cells (b). After addition of Slit-2 to PCI52\_PC cells, an increased number of P-cadherin/Robo-3 interactions can be observed (c and d). P-cadherin/Robo-3 interactions can also be seen in human oral keratinocytes (HOK) (e) and in basal cell layers of normal oral mucosa (f). Coimmunoprecipitation experiments confirm the occurrence of increased P-cadherin/Robo-3 interactions following Slit-2 treatment (g) (b = basal, sb = suprabasal cell layers of normal oral mucosa).

effect on cell migration (Figure 3a). Furthermore, no Slit-2 influence on chemoinvasion could be observed (data not shown).

*Slit-2 amplified P-cadherin/Robo-3 interactions in the P-cadherin overexpressing OSCC cell line PCI 52*

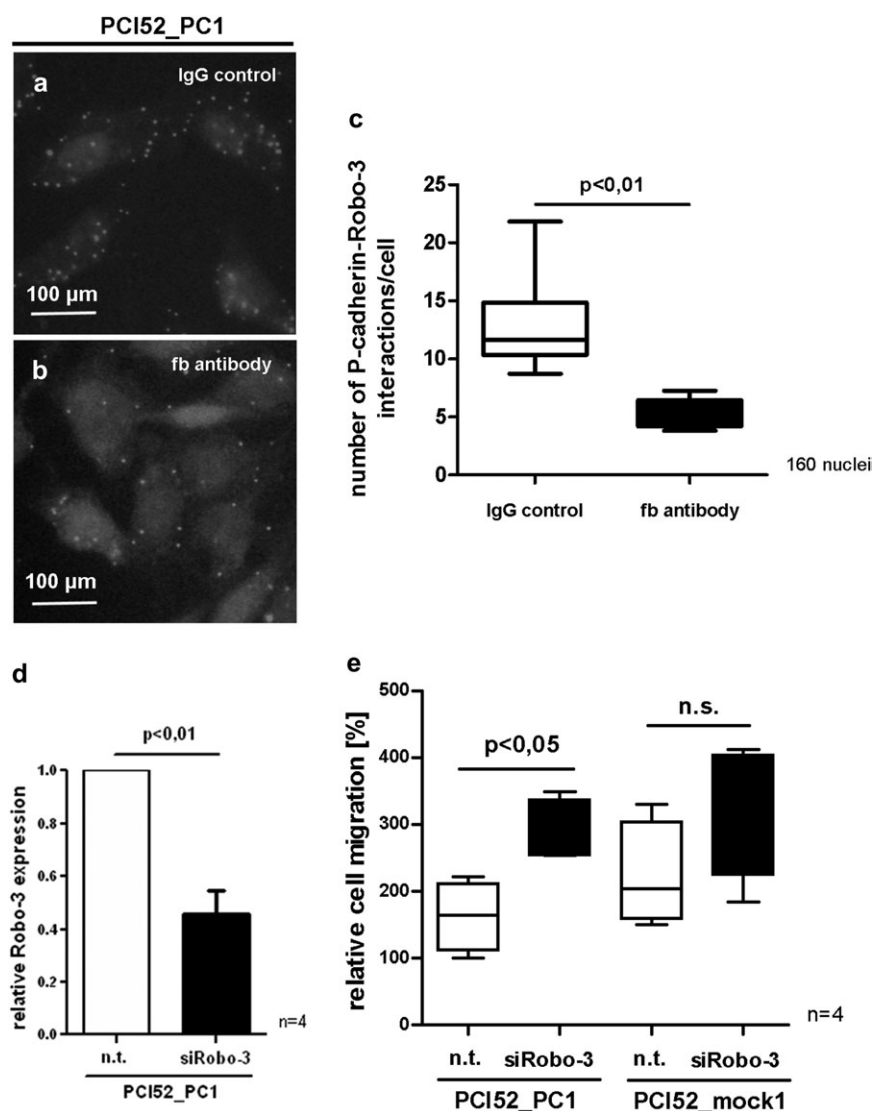
To explain the effect of Slit-2 exerted on P-cadherin overexpressing OSCC cells, we were curious as to whether there was a link between P-cadherin and Robo receptors. It is known that Slit proteins are ligands for these receptors. During our experiments, we observed that Robo-3, a high affinity receptor of Slit-2, is expressed in the same basal and suprabasal cell layers of oral epithelium (data not shown). Therefore, we concentrated on Robo-3 and its interaction with Slit-2 and P-cadherin.

To detect protein interactions between P-cadherin and Robo-3, we performed PLAs. This assay is a new method to depict and to quantify protein interactions in an immunohistological way. At the end of this assay, red-fluorescent signals display protein interactions. With this experiment, we detected interactions of P-cadherin with Robo-3 on PCI52\_PC cells distributed over the whole cell (Figure 3b). Interestingly, after addition of Slit-2 to PCI52\_PC cells, we observed an increase of these P-cadherin/Robo-3 interactions (Figure 3c). We

counted the fluorescent signals of 42 cells and noticed a significant rise of P-cadherin/Robo-3 interactions ( $P < 0.001$ ) in cells incubated with Slit-2 (Figure 3d). As additional cells for PLAs, we used human oral keratinocytes. In these normal cells, we also detected some P-cadherin/Robo-3 interactions spread across the whole cell (Figure 3e). Indeed, in basal cell layers of normal oral mucosa, P-cadherin interacted with Robo-3 (Figure 3f). Coimmunoprecipitation experiments confirmed this enhanced P-cadherin/Robo-3 association in PCI52\_PC cells following Slit-2 treatment. Subsequent western blot analyses of P-cadherin after Robo-3 precipitation revealed an increase of P-cadherin/Robo-3 signals indicating increased interactions after Slit-2 incubation (Figure 3g).

*Inhibition of P-cadherin function resulted in decreased P-cadherin/Robo-3 interactions*

Quantifying P-cadherin/Robo-3 interactions in a PLA experiment after treatment of PCI52\_PC cells with function blocking P-cadherin antibody, we observed 40% decrease of P-cadherin/Robo-3 interactions (Figure 4a–c,  $P < 0.01$ ). In the function blocking experiment, the N-terminal P-cadherin antibody prevents cell adhesion, which results in a reduced Slit-2 secretion as shown above in Figure 2h.



**Fig. 4.** After blocking P-cadherin function with function blocking (fb) P-cadherin antibody, PCI52\_PC cells reveal a significant decrease of P-cadherin/Robo-3 interactions by PLAs (a–c). Knockdown of Robo-3 with siRNA (d) in PCI52\_PC and PCI52\_mock cells results in a significant increase in cell migration in Slit-2 producing PCI52\_PC cells. However, in PCI52\_mock cells, there is no significant migration effect after Robo-3 knockdown (e) (n.t. = non-targeting).

As a result of this, the decreased Slit-2 concentration caused diminished P-cadherin/Robo-3 interactions as demonstrated with PLA assays (Figure 4a–c).

#### Knockdown of Robo-3 induced migration in the P-cadherin overexpressing OSCC cell line PCI 52

To analyse the role of the Robo-3 receptor during migration of the OSCC cells, we performed a knockdown of the Robo-3 receptor via transfection of siRNA-Robo-3. Transfection with siRNA against Robo-3 resulted in a ~60% knockdown of Robo-3-mRNA (Figure 4d,  $P < 0.01$ ). Subsequently, cell migration assays were performed. Interestingly, after Robo-3 knockdown in Slit-2 producing PCI52\_PC cells, we observed a significant increase in cell migration (Figure 4e,  $P < 0.05$ ). However, in PCI52\_mock cells, there was no significant migration effect after Robo-3 knockdown (Figure 4e). These data indicate that knockdown of Robo-3 receptor neutralized Slit-2 induced migration block in PCI52\_PC cells (as shown above in Figure 3a).

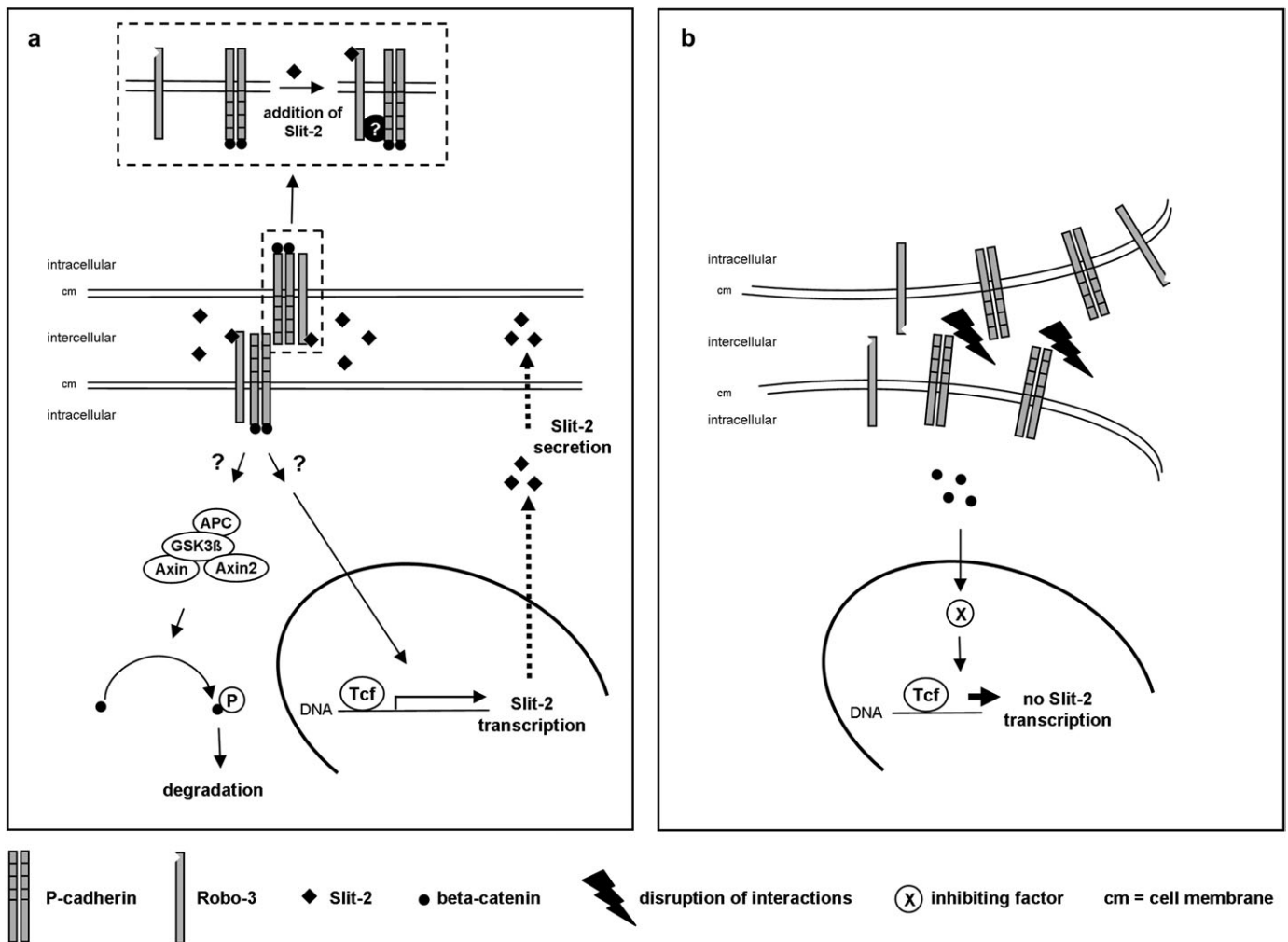
#### Discussion

In the present study, we show antitumorigenic effects of the secreted glycoprotein Slit-2 on P-cadherin expressing OSCC cells mediated by

the Robo-3 receptor. Our experiments demonstrate how P-cadherin might act in concert with the cell membrane receptor Robo-3 to induce a stationary phenotype by means of Slit-2 induced P-cadherin/Robo-3 complex formation in OSCC cells. In several reports, interactions of N-cadherin with Robo receptors have already been discussed (26,27,29).

In our study, Slit-2 was immunolocalized in basal and suprabasal epithelial cell layers of normal oral mucosa. The same localization could be observed for the adhesion molecule P-cadherin. Slit-2 showed a low expression and diffuse localization in dysplastic stratified cell layers or at the onset of invasion. Kim *et al.* (23) detected Slit-2 mRNA in normal human esophageal mucosa by *in situ* hybridization, whereas Slit-2 expression was also reduced in esophageal squamous cell carcinoma.

Moreover, our results displayed a correlation of Slit-2 and P-cadherin expression in P-cadherin overexpressing OSCC cells and a dependency of Slit-2 secretion on P-cadherin function. Those findings suggest that the cell adhesion molecule P-cadherin stabilizes Slit-2 expression and facilitates Slit-2 secretion by a still unknown mechanism. A hypothetical model of the P-cadherin-Slit-2-mechanism derived from our data demonstrates Figure 5. It shows how Slit-2 might induce P-cadherin/Robo-3 complex formation and subsequent effects on Slit-2 transcription and secretion.



**Fig. 5.** Hypothetical model of the formation of Slit-2 induced P-cadherin/Robo-3 complex and subsequent effects on Slit-2 transcription and secretion: Activation of Robo-3 by Slit-2 results in the formation of a complex between Robo-3 and P-cadherin mediated by still unknown molecules (a, small picture). Extracellular Slit-2 binds to Robo-3 receptor with subsequent abundant cytosolic beta-catenin phosphorylation and degradation. Consequently, further inhibiting transcription factors cannot be expressed thus supporting Slit-2 transcription in a feedback loop. Additionally, P-cadherin–P-cadherin interactions affect Slit-2 expression and facilitate Slit-2 secretion (a). In contrast, disruption of P-cadherin–P-cadherin interactions causes a diminished Slit-2 secretion and a decrease of P-cadherin/Robo-3 interactions. Because of the disrupted cell adhesion and decreased Slit-2 secretion, beta-catenin is not phosphorylated possibly due to GSK3-beta inactivation. Thus, beta-catenin is translocated to the nucleus where genes are activated which in turn might contribute to the inhibition of Slit-2 transcription (b).

Additionally, our experiments displayed a re-expression of Slit-2 in OSCC cells after inhibition of binding beta-catenin to Lef/Tcf. One would expect a decrease of gene expression after blocking the beta-catenin/Lef/Tcf complex. However, in recent publications Blauwkamp *et al.* (31) and Hoverter *et al.* (32) have demonstrated that Lef/Tcf gene transcription heavily depends on the equipment of transcription factors which in combination can have transcription repressor function. Therefore, our observed effect of Slit-2 increase points to an involvement of beta-catenin in combination with further transcription factors effecting Slit-2 expression.

Besides, Slit-2 treatment affects phosphorylation of beta-catenin in OSCC cells which have more phosphorylated GSK3-beta meaning a more inactive form resulting in stabilized beta-catenin (6). Recently, Prasad *et al.* (33) and Tseng *et al.* (30) have confirmed that Slit-2 mediated its tumour-suppressive effect by regulating beta-catenin and phosphoinositide-3-kinase/AKT pathway in breast cancer and lung cancer cells. In line with these data, our studies suggest a feedback loop of signalling events whereby Slit-2 may affect its own transcription. This may lead to enhanced phosphorylation and degradation of cytoplasmic beta-catenin, limiting the amount of beta-catenin for nuclear shuttling and regulation of Slit-2 transcription in OSCC cells (Figure 5).

Furthermore, we could demonstrate an interaction of P-cadherin with Robo-3, a high affinity receptor of Slit-2, *in vitro* on aberrant oral epithelial keratinocytes by PLAs. The interaction was modulated by addition of Slit-2. In contrast, in mouse fibroblast L-cells, N-cadherin interacted with Robo receptor resulting in the inhibition of cadherin function (26). However, Shiau *et al.* (29) reported that N-cadherin acted in concert with Robo-2 in placode-derived cranial sensory neurons and strengthened placodal cell adhesion required for proper gangliogenesis.

Slit, which mediates its function by binding to Robo regulates migration of neurons, leukocytes and cancer cells (12,34,35). In our study, we also demonstrated a dose-dependent antimigratory effect of Slit-2 on OSCC cells overexpressing P-cadherin but not on P-cadherin-deficient OSCC cells. On the other hand, our experiments showed that knockdown of Robo-3 expression induced enhanced cell migration predominantly in P-cadherin overexpressing OSCC cells. Likewise, in experiments of Mertsch *et al.* (36) knockdown of Robo-1 neutralized the repulsive effect of Slit-2 in glioma cell lines. That implies that P-cadherin is one component in a complex with Slit-2 and Robo-3 for slowing down cell migration. Our results suggest that P-cadherin, Slit-2 and Robo-3 form a complex (Figure 5a, small picture) strengthening epithelial architecture *in vivo* by transmitting antimigratory signalling. In line with our data, other publications verified that Slit-2 inhibits migration of tumour cells *in vitro*. In medulloblastoma, Slit-2 inhibited the cell invasion in a variety of *in vitro* models (37). Furthermore, in CXCL12/CXCR4-induced breast cancer, Slit treatment inhibited cell chemotaxis, chemoinvasion and adhesion (38). Moreover, Mertsch *et al.* (36) showed that Slit-2 was chemorepulsive for glioma cell lines in a modified Boyden chamber assay, the same experimental setup we used. In a report of Yiin *et al.* (39), Slit-2 down-regulated Cdc42 activation through its receptor Robo-1 and attenuated migration of glioma cells *in vitro*. Likewise, Slit-2 has antimigratory effects on dendritic cells. By allergen, sensitization in the skin activated Slit-2 down-regulated the migration of Langerhans cells (13).

In summary, our study indicates that the Robo-3 receptor may act as a modulator of P-cadherin function. This implies that Slit-2 induced P-cadherin/Robo-3 complex formation on the one hand decreases cell migration, on the other hand helps to maintain appropriate amounts of Slit-2 expression levels in oral epithelium.

## Funding

Deutsche Forschungsgemeinschaft (BA3696/1-2) to R.B.

## Acknowledgements

We thank Anja Reck for the excellent technical assistance.

*Conflict of Interest Statement:* None declared.

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Received December 16, 2010; revised March 2, 2010;  
accepted March 25, 2011