Desmoglein 3 – Influence on oral carcinoma cell migration and invasion

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A B S T R A C T

Desmoglein 3 (Dsg3) is an adhesion receptor in desmosomes, but its role in carcinoma cell migration and invasion is mostly unknown. Our aim was to quantitatively analyse the motion of Dsg3-modified carcinoma cells in 2D settings and in 3D within tumour microenvironment mimicking (TMEM) matrices. We tested mutant constructs of C-terminally truncated Dsg3 (Δ238 and Δ560), overexpressed full-length (FL) Dsg3, and empty vector control (Ct) of buccal mucosa squamous cell carcinoma (SqCC/Y1) cells. We captured live cell images and analysed migration velocities and accumulated and Euclidean distances. We compared rodent collagen and Matrigel® with human Myogel TMEM matrices for these parameters in 3D sandwich, in which we also tested the effects of monoclonal antibody AK23, which targets the EC1 domain of Dsg3. In monolayer culture, FL and both truncated constructs migrated faster relative to Ct cells. Of the mutants, the shorter form (Δ238) exhibited faster migration and invasion than Δ560 cells. In the Transwell, all of the cells invaded faster through Myogel than Matrigel®-coated wells. In 3D sandwich, AK23 antibody inhibited only the invasion of FL cells. We conclude that different experimental 2D and 3D settings can markedly influence the movement of oral carcinoma cells with various Dsg3 modifications.

1. Introduction

Desmosomes (DSMs) are specialized intercellular junctions in which the transmembrane adhesion proteins desmogleins and desmocollins belong to the cadherin superfamily [1]. Desmoglein 3 (Dsg3) is a member of the desmoglein subfamily and is also known as pemphigus vulgaris antigen (PVA) [2,3]. Dsg3 is upregulated in oral carcinoma cells, but its explicit role in cancer progression remains obscure [4]. A correlation between Dsg3 expression level and cancer progression has been reported, suggesting that Dsg3 may contribute to the spread of cancer [1]. Supporting this view, overexpression of Dsg3 in an oral buccal mucosa squamous cell carcinoma cell line (SqCC/Y1) has been shown to reduce E-cadherin expression with concomitant accelerated cell migration and invasion [5,6]. Although a plethora of studies have demonstrated Dsg3 as crucial in cell cohesion, relatively little is known about its function in cancer metastasis [1,6]. Monoclonal antibody (AK23) induces PV blisters by targeting the first extracellular domain (EC1) of Dsg3, but its function in cancer is unclear [7,8].

The tumour microenvironment (TME) plays a leading role in cancer invasion and metastasis. It is composed of vessels, mesenchymal cells including carcinoma-associated fibroblasts (CAFs), inflammatory cells, extracellular matrix (ECM) molecules, and various soluble factors [9–11]. Migrating cell front is an active regulator of ECM modulation in cancer spreading, and abnormal ECM structure and increased tissue stiffness influence cell movement [12–15]. Conventional cancer studies have focused primarily on two-dimensional (2D) models, and while
these facilitate understanding of the mechanical details of cell-cell interactions, they do not offer knowledge about cell-matrix interaction [16–18]. In vitro three-dimensional (3D) tumour microenvironment mimicking (TMEM) models partially cover the gap between 2D and in vivo models [19,20] since they resemble the matrix structure and rheology surrounding the cancer cells in vivo [21,22]. One major rheological parameter is stiffness, which modulates cell migration, invasion, and cell-cell adhesion [23,24]. Quantitative analysis of different parameters, such as cell speed and nuclear size, within 3D matrices can be obtained by time-lapse microscopy with fluorescent labelled cells [21,22].

Organotypic models with rat tail type I collagen together with mouse Engelbreth-Holm-Swarm (EHS) sarcoma-derived basement membrane matrix, Matrigel® are commonly used to investigate 3D cancer cell invasion [25]. However, these matrices do not simulate the human TME [26,27]. Matrigel®, mouse sarcoma derived matrix contains basement membrane (BM) molecules, such as laminin, type IV collagen, entactin and proteoglycans [28]. These molecules act as thin scaffolds to separate cell and tissue compartments, and support tissue structures during organogenesis [29]. Therefore, our group has introduced human uterine leiomyoma tissue-based myoma discs and Myogel models for 3D in vitro invasion assays [30–32]. Myogel, contains in addition to type I and III collagens, tenasin and laminin, also several small molecular weight proteins, such as cytokines, growth factors and growth factor receptors, which induce cancer cells invasion [31]. Myogel is therefore an ideal matrix for human cancer cell invasion assays [31,32].

The aim of this study was to portray the role of Dsg3 in migration and invasion of an oral mucosa carcinoma cell line SqCC/Y1 with stable overexpression of full-length (FL) Dsg3 and its C-terminally truncated Dsg3ΔC mutants [6] in various reconstructed TMEM models. Here we analysed, for the first time, the effects of truncated Dsg3 mutants and FL constructs in oral bucal mucosa carcinoma cell line, SqCC/Y1. We found that AK23 antibody, against the EC1 of Dsg3 inhibited only FL carcinoma cells invasion. Our preliminary study suggests that the EC domain of Dsg3 might potentially serve as a novel target for anticancer drugs. However, further functional and in vivo animal studies are still required to show if AK23 would be beneficial in oral cancers.

2. Materials and methods

2.1. Cell lines and their characteristics

SqCC/Y1 cells derived from human buccal mucosa squamous cell carcinoma (SCC) were cultured in Epilife medium with 60 mM calcium concentration (Gibco) supplemented with EDGS (Epilife Defined Growth Supplement, Gibco) as described elsewhere [6]. To investigate the role of Dsg3 in SCC cell movement, two different constructs encoding the C-terminally truncated Dsg3 (ΔC) mutants Δ238 (EC1-2) and

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**Fig. 1. Full-length, mutant forms of Desmoglein 3 (Dsg3) and 2D migration pattern analysis of SqCC/Y1 cell lines. (A)** Schematic diagram of full-length (FL) and two C-terminally truncated mutants ΔC (Δ238 and Δ560) of Dsg3 tagged with myc epitopes (red) [6]. Abbreviations: EC: extracellular domain; EA: extracellular anchorage domain; TM: transmembrane domain; ICD: intracellular domain; C: C-terminal end; N: N-terminal end. (B) Trajectory plots show migration patterns of 30 cells from four SqCC/Y1 cell lines. (C) Rose diagrams indicate directionalities of 30 cells from each cell line. Velocity (D) and accumulated (E) and Euclidean (F) distances of vector control, FL, and truncated mutants of Dsg3. A total of 144 cells were analysed from each cell line. **p < 0.01 and > 0.001, ***p < 0.001.
Δ560 (ECI-EA) were created along with full-length h. Dsg3,myc (FL) and empty vector control (Ct) in SqCC/Y1 cells [Fig. 1A] [6]. Two antibodies (Abs) against Dsg3, mAb 5H10 directed to N-terminus and rabbit anti-Dsg3 directed to C-terminus, were used for the protein expression analysis. Although little or no reduction in total endogenous Dsg3 expression was detected in cell lines with transduction of mutants by Western blot analysis, disruption of cell-cell junctions with concomitantly compromised adhesion strength was shown in these cell lines [6].

Carcinoma-associated fibroblasts (CAFs) and normal oral fibroblasts (NOFs) were established from oral squamous cell carcinoma tissue (OSCC) and healthy oral mucosa, respectively, and cultured in DMEM medium (Sigma-Aldrich) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml ascorbic acid, 250 μg/ml fungizone, 1 mmol/L sodium pyruvate (Sigma-Aldrich), and 10% heat-inactivated foetal bovine serum (FBS) (Perbio Science, Erembodegem, Belgium) as described earlier [33].

2.2. Lentiviral transduction for fluorescent labelling

SqCC/Y1 cell lines were transduced with nuclear histone-2B (H2B) -coupled mCherry expression plasmid Six.2 V5/DEST (a kind gift from Dr. Cindy E. Dieteren, Radboud UMC, the Netherlands) [34]. CAFs with stable transduction of GFP were generated with non-silencing GIPZ lentiviral shRNAmir control particles (pGIPZ vector contains GFP in order to track shRNAmir expression; Thermo Fischer Open Biosystems) according to the manufacturer’s instructions. These cells were cultured in the medium in the same manner as SqCC/Y1 and CAFs.

2.3. Horizontal 2D migration assay, imaging, cell tracking, and migration analysis

We used the IncuCyte® Live Cell Imaging System (Essen Bioscience, Hertfordshire, UK) for 2D migration imaging. Ct, FL, Δ238, and Δ560 cell lines were seeded in EpiLife medium and separately plated on uncoated 6-well plates at a density of 1.5 × 10⁵ cells/well. The plates were placed in an IncuCyte® and nine regions in each well were snap-shotted at 10-min intervals in the first 3 h and thereafter at 15-min intervals to 48 h. For each selected region, 198 images were acquired to generate video files. For each cell line, nine video files were obtained and 100 frames in each video were selected for analysis; images with rounded cell shape and overgrown cultures were excluded. A total of 900 frames for each of the four cell lines were included in the cell tracking analysis. MetaMorph software (Molecular Devices Corp., Downingtown, PA, USA) was used for cell tracking. X and Y coordinate values for 16 cells/video/region at every frame (n = 100) were recorded to represent random cell migration, and therefore, 144 cells in each cell line were analysed. The generated 57,600 coordinates/cell line were used for later retrieval and analysis using ImageJ Fiji software (NIH, Bethesda, MD, USA).

Chemotaxis and Migration Tool in ImageJ Fiji (NIH, Bethesda, MD, USA) plugin was used for the migration analyses. ‘Plot graph’ was used to plot cell trajectories/paths, and ‘diagram feature’ tool was selected to display rose diagram to observe directionality between different cell lines. Velocity and accumulated and Euclidean distances were measured to quantify differences. ‘Accumulated distance’ for each track/cell is defined as the total distance covered or migrated within a trajectory (Supplementary Fig. S1). The ‘Euclidean distance’ is the mean distance between the end point and origin of each track/cell (Supplementary Fig. S1).

2.4. Myogel rheology

Use of human uterus leiomyoma tissue was approved by the Regional Ethics Committee of the Northern Ostrobothnia Hospital District (license number 35/2014). The gel from rat tail type I collagen (Corning Inc) was prepared according to the manufacturer’s protocol. Myogel (2.4 mg/ml), Matrigel® (2.4 mg/ml; BD Biosciences), Matrigel®-collagen (2.4 mg/ml, BD Biosciences; 1 mg/ml, Corning Inc.), and Myogel-collagen (2.4 mg/ml; 1 mg/ml, Corning Inc.) were diluted with serum free 1:1 DMEM/F-12 medium (Life Technologies) at 4 °C; their rheology was tested following a previously published protocol [35]. Storage modulus/stiffness (G’), loss modulus (G”), and complex viscosity (η *) were measured with two different methods using a rheometer (Discovery HR-1, TA Instruments, Elstree, Herts, UK). Logarithmic strain sweep from 0.1% to 300% using angular frequency of 10 rad/s was applied to evaluate linear viscoelastic regions of gels. Linear frequency sweep from 0.1 to 0.55 Hz using 1% strain was performed at the linear viscoelastic region to observe gel behaviour under different angular frequencies.

2.5. Adhesion assay in different coatings

The 96-well plate surfaces were coated 24 h with 10 μg/ml bovine serum albumin (BSA; Sigma), 10 μg/ml fibronectin (FN; Sigma), 0.62 mg/ml Matrigel® (BD Biosciences), and 0.62 mg/ml Myogel. Assays with four SqCC/Y1 cell lines were performed three times according to a published protocol [36].

2.6. Immunofluorescence (IMF) in coated coverslips

IMF was performed to examine the role of ECM in Dsg3 expression in Ct, FL, Δ238, and Δ560 cell lines. Coverslips were coated overnight with EpiLife medium, 0.62 mg/ml Matrigel® (BD Biosciences), or 0.62 mg/ml Myogel diluted in EpiLife medium before cell seeding. Stable expression of Dsg3 and its truncated mutants was visualized with the mouse mAb 5H10 (Santa Cruz) in conjunction with anti-mouse IgG conjugated with Alexa Fluor 568 (Santa Cruz), as described previously [6]. In addition, expression and distribution of E-cadherin and F-actin were also analysed by fluorescent staining with the monoclonal antibody against E-cadherin HEC1-1 and A488 conjugated phalloidin. Images were acquired via Leica DMi4000 B epifluorescent microscope. Further IMF experiments were carried to evaluate endogenous Dsg3 expression in FL and Dsg3ΔC mutants relative to the Ct; the technique is described in the Supplementary methods.

2.7. Transwell® vertical migration and invasion assay

Assays were performed as described previously [5,31]. Briefly, cells were suspended in serum free 1:1 DMEM/F-12 medium with 1% bovine serum albumin (BSA), and keratinocyte growth medium (KGM, Gibco) was used in the bottom well. For the invasion assay, Transwells (Corning Inc.) were coated with 50 μl of Matrigel® (2.4 mg/ml; BD Biosciences) or Myogel (2.4 mg/ml with 0.2% low melting agarose (LMA) in EpiLife medium).

2.8. 3D sandwich cultures

Nuclear histone-2B (H2B) -coupled mCherry expressing SqCC/Y1 cell lines were embedded between polymerized 3D TME gel matrix layers in a µ-Plate Angiogenesis 96-well plate (ibidi, Germany) and prepared as described elsewhere [37]. Rat tail type I collagen gel (2.4 mg/ml; Corning Inc.), Matrigel® (2.4 mg/ml; BD Biosciences), Matrigel®-collagen (2.4 mg/ml, BD Biosciences; 1 mg/ml, Corning Inc.), and Myogel-collagen (2.4 mg/ml; 1 mg/ml, Corning Inc.) were used as ECM matrices. KGM (Gibco) containing 2% FBS was used on top of polymerized gels for feeding the cultures.

Co-culture model was formed as explained previously for monolayers. A 2:1 ratio of SqCC/Y1-H2B cells to CAF-GFPs or NOFs was used for standard stromal interaction. 1000 SqCC/Y1-ΔH2B, two ΔC-H2B cells were gently mixed with 500 CAF-GFPs and NOFs, respectively, embedded in Myogel-collagen (2.4 mg/ml; 1 mg/ml, Corning Inc.).
2.9. AK23 antibody treatment against Dsg3 using 3D sandwich monocultures

Mouse monoclonal antibody AK23 (Medical & Biological Laboratories, Nagoya, Japan) was used for the study. AK23 is characterized to cross react also with human species of Dsg3: it binds to the aminoterminal region (N-terminus) at the first extracellular domain (EC1) of Dsg3 [8]. To find out the role of AK23 targeting EC1 domain in human cancer, we evaluated its ability to inhibit SqCC/Y1 cell invasion through Myogel mixed with rat tail type I collagen (Corning Inc.) gel to the published sandwich culture protocol [37]. AK23 or mouse IgG1 isotype as a control was mixed with Myogel-collagen (2.4 mg/ml; 1 mg/ml, Corning Inc.) to 1 µg/ml of the final concentrations before embedding the cells in the matrices. Cells were treated with either IgG1 isotype control or AK23 to 1 µg/ml concentration in KGM (Gibco) medium containing 2% FBS (Life Technologies).

2.10. 3D image acquisition and deconvolution

CellSens system (Olympus, Japan) with its top stage incubator enabled time-lapse imaging of sandwich cultures at 37 °C in 5% CO2 humidified atmosphere. Images were taken with CPlanFLN PhC 10x/0.30, UPlanFLN 20x/0.50 objectives (both from Olympus, Japan) for Texas Red (TxBRed) and GFP channels. Images consisting of 1376 × 1038 pixels were taken every 30 min with 7.9 µm Z spacing with Olympus XM10 CCD controlled by cellSens Dimension (Build-10236) for 18–24 h. 3D image stacks were further processed with Huygens Professional (Scientific Volume Imaging, Hilversum, the Netherlands) deconvolution wizard using original microscope parameters before analysis. Cell segmentation and tracking for the analysis are described in the Supplementary methods.

2.11. Myoma organotypic 3D invasion assay

Organotypic myoma disc invasion experiments were done according to a published protocol with a few modifications [38,39]. Briefly, myomas were cut to 5 mm slices and into discs with an 8-mm biopsy punch (Kai Industries Co., Japan). The discs were placed with caution in Transwell ® inserts (Corning Inc.) and 7 × 105 cells from each SqCC/Y1 cell line were suspended in 50 µl of serum free 1:1 DMEM/F-12 medium supplemented with 1% BSA, and was placed on top of each disc. After that, 0.5 ml of KGM (Gibco) with 10% heat-inactivated fetal bovine serum (FBS, Life Technologies) was added to the wells and cells were allowed to attach. On the following day, discs with cells attached were placed on top of nylon discs on curved steel grids in 12 well plates containing 1 ml of KGM with 10% FBS. After two weeks of culturing, myoma discs were fixed with 4% formalin. Invasion area and depth of four SqCC/Y1 cell lines were quantified after immunohistochemical pancytokeratin AE1/AE3 (Dako) staining. Sections were digitally recorded at ×100 magnification with a Leica DMRB photomicroscope using QWin V3 software. The areas of stained non-invading and invading cells were measured with ImageJ, excluding the nonuniform invasion in the edges of the myomas.

2.12. Statistical analysis

Statistical evaluation was performed using ANOVA of IBM SPSS 23.0 software for Windows (SPSS Inc., Chicago, IL, USA). Statistical tests applied for data analysis are indicated in the figure legends. We considered a P-value of < 0.05 to be statistically significant and are indicated with asterisks. Graphic indications of calculated p-values are *p < 0.05 and > 0.01, **p < 0.01 and > 0.001; ***p < 0.001.

3. Results

3.1. Enhanced migration of both Dsg3 FL and Dsg3ΔC mutant cell lines

Since structural variations in Dsg3 have effects on cell-cell adhesion, we tested whether Dsg3 overexpression or knockdown has a role in horizontal migration by utilizing 2D live cell imaging technique. Our previous study has demonstrated the dominant negative action of Dsg3 truncated mutants on cell-cell adhesions [6], and we decided to use this model system to address the specific role of Dsg3 in the context of cell migration. We quantitated the mean velocity and accumulated Euclidean distances. Relative to Ct cells, which showed similar migration paths, almost half of the FL cells exhibited longer distance migration and turned around in random directions. However, the Δ238 mutant showed a more irregular migration pattern and the Δ560 mutant exhibited a more restricted pattern with a shorter Euclidean distance comparable to the Ct cells (Fig. 1B). The directionality of cell migration presented by ‘rose diagram’ indicated that Δ238 was more unique than other three cell lines with more restricted directionality (Fig. 1C). Mean migration velocities of FL and two mutants were around 24% higher than in Ct cells (Fig. 1D). Similarly, all cell lines with Dsg3 variation had about 24% greater accumulated distances than Ct (Fig. 1E). Euclidean distances in FL and Δ238 cells were about 34% longer than in Ct cells (Fig. 1F). However, in Δ560 there was no difference compared with the respective Ct cells. Between the two mutants, there was no difference in velocity (Fig. 1D) and accumulated distances (Fig. 1E). However, Δ238 cells exhibited enhanced (p = 0.002) Euclidean distances compared to Δ560 cells (Fig. 1F).

3.2. Myogel rheology

To evaluate stiffness/storage modulus and viscosity of four TME matrices, we analysed their rheology using a rheometer. Matrigel ®, Matrigel ®-collagen and Myogel-collagen were similar in firmness. Myogel was softer and less viscous than Matrigel ® (Supplementary Fig. S2A-D). Myogel combined with collagen gel was stiffer and more viscous, but still softer and less viscous than Matrigel ® and Matrigel ®-collagen (Supplementary Fig. S2C-D). Matrigel ®-collagen was the stiffest and most viscous of all the matrices analysed (Supplementary Fig. S2C-D).

3.3. Exogenous expression of Dsg3 FL and Dsg3ΔC mutants affected cell adhesion on different matrices mimicking the tumour microenvironment

To address whether the alteration of Dsg3 levels and action has any influence on cell adhesion, we performed the adhesion assays on various coated substrates. We observed that the adhesion of both Ct and FL lines on Myogel was poorer than on Matrigel ® (p < 0.001 and p = 0.04, respectively, Supplementary Fig. S3). By contrast, Δ560 mutants seemed to favour Myogel over Matrigel ® (p = 0.04), with better attachment on Myogel (Supplementary Fig. S3). Δ238 mutants adhered almost similarly on all the substrates. Regarding Matrigel ®, both mutants, Δ238 (p < 0.01) and Δ560 (p < 0.001) showed poor attachment, compared with Ct. Even compared with FL, the mutants attached poorly on Matrigel ® (p < 0.001). In terms of Myogel, there was no significant differences in attachments of FL and both mutants, compared to controls (Supplementary Fig. S3). Both on Matrigel ® and Myogel, there was no differences observed between both mutants. These results suggest that the alterations in Dsg3 expression and function had an impact on the cell adhesion property of different substrates.

3.4. Expression of Dsg3ΔC mutants caused reduced junctional protein expression

Since variations in cell adhesion were observed among cell lines, we then compared the expression of several junctional proteins, such as...
Dsg3, E-cadherin, and F-actin, by immunofluorescence, in cells seeded on different matrices, i.e. coverslips alone or coated with Matrigel® and Myogel, respectively. The monoclonal antibody SH10 that binds to EC1 domain of Dsg3 was used to examine the Dsg3 expression. Though Dsg3 immunostaining showed positive signals at the junctions in all cell lines the protein expression and distribution at the cell borders were much less in both mutant lines compared to Ct and FL cells. Many cells exhibited gaps or showed lack of staining at the borders as indicated by yellow arrows (Supplementary Fig. S4). For E-cadherin staining, the monoclonal antibody HECD-1 that binds to the N-terminus of E-cadherin was used (Supplementary Fig. S5), and the control cells showed different staining patterns with respect to different substrates. While strong diffuse staining was detected in cells grown on Matrigel®, disrupted junctional staining was shown in cells incubated on Myogel. Such a disruption in junctional staining seemed to be compromised in FL, but more severe in mutants grown on both Matrigel® and Myogel (arrows in Supplementary Fig. S5) than on coverslips. The formation of cortical F-actin was apparent in control cells grown on all substrates, with slightly stronger staining in cells on Myogel (Supplementary Fig. S6). The strong cortical F-actin was also noticeable in FL cells cultured on Matrigel® compared with coverslip. However, the cells grown on Myogel exhibited some degree of reduction in cortical F-actin formation. For mutant cell lines, cortical F-actin was stronger on Matrigel® than on coverslips or Myogel. Again, more diffuse actin staining was observed in cells grown on Myogel than on coverslips, and some cells even showed a lack of cortical actin staining. In general, the reduction of cortical F-actin staining appeared to be more severe in Δ238 than Δ560 grown on coverslips (Supplementary Fig. S6). The strong cortical F-actin was also noticeable in FL cells cultured on Matrigel® compared with coverslip. However, the cells grown on Myogel exhibited some degree of reduction in cortical F-actin formation. For mutant cell lines, cortical F-actin was stronger on Matrigel® than on coverslips or Myogel. Again, more diffuse actin staining was observed in cells grown on Myogel than on coverslips, and some cells even showed a lack of cortical actin staining. In general, the reduction of cortical F-actin staining appeared to be more severe in Δ238 than Δ560 grown on coverslips (Supplementary Fig. S6). Compared with Ct, we also studied endogenous Dsg3 expression of the overexpressed and two mutant cell lines by using monoclonal antibody 5H10 (Supplementary Fig. S7). All mutant expression caused disruption of junctions and accumulation of Dsg3 in the cytoplasm after treating cells to inhibit protein degradation. Mutant expression caused disruption in the endogenous protein, but not in the overexpressed FL compared with control (Supplementary Fig. S7). Taken together, these results suggest strongly that the expression of Dsg3 mutants in cells caused disruption of cell-cell junctions, including the E-cadherin-mediated adherens junctions, although some variation was seen on different substrates.

### 3.5. Enhanced vertical migration and invasion in cells expressing Dsg3ΔC mutants

Since the overall horizontal migration of the overexpressed and mutant cell lines was higher than that of the control, we next evaluated the cell vertical migration and invasion through the Transwell inserts. After incubation of 72 h, the differences in cell migration speed began to emerge, with only the Δ238 cells showing faster migration than all three cells (Fig. 2A). Compared to Ct, mutant Δ560 cells migrated almost in the same speed. At 96 h, more FL and mutant cells than controls had passed the uncoated membrane of inserts. Δ238 cells migrated faster than the other Δ560 mutants (Fig. 2A). Next, the Transwell invasion assay was performed and it showed that all cells, except the FL passed through Myogel-LMA faster than Matrigel® coated wells. Through Myogel-LMA, the FL and Δ238, in particular, invaded significantly faster than Ct and Δ560 cells (Fig. 2B). Whereas through Matrigel® coated wells, only the FL cells invaded faster than the Ct. In general, these data support the observation made in the 2D cell migration assay that changes in Dsg3 expression at the junctions produce different cell migration and invasion behaviours.

### 3.6. Dsg3ΔC mutants invaded faster in 3D matrices

We also analysed the 3D invasion speed and distances (accumulated and Euclidean) of SqCC/Y cell lines by a sandwich assay and monitored the cell movement using an advanced live cell imaging technique. In this case, cells with transduction of H2B were embedded within four different types of matrices. We observed that within collagen, only the Δ238-H2B mutants moved faster than other three cell lines (Fig. 3A). However, in Matrigel® as well as in Matrigel®-collagen, both mutants moved quicker than FL-H2B and Ct cell lines. In Myogel-collagen, both mutants moved quicker than Ct and FL-H2B cell lines, also the FL-H2B moved faster than Ct cells (Fig. 3A). Again, a similar trend was observed in collagen and also in Matrigel®-collagen for the accumulated distances (Fig. 3B). However in Matrigel®, both mutants had increased accumulated distances relative to Ct and FL-H2B cell lines, and among the mutants, Δ238-H2B cells had the highest. Both mutants had increased accumulated distances relative to Ct in Myogel®-collagen, and only the Δ560-H2B mutant had higher accumulated distances than FL-H2B. Finally, as for the Euclidean distances, again both mutants showed enhanced migration ability in collagen and Matrigel®-collagen compared with Ct cells, whereas in Matrigel® only Δ560-H2B mutant had increased Euclidean distances compared with FL-H2B. Both mutants had
increased Euclidean distances compared to FL-H2B in Matrigel®-collagen and in collagen Δ238-H2B mutant had the highest Euclidean distances compared to other three cell lines. In addition, both FL-H2B and Δ560-H2B mutant had increased Euclidean distances in Myogel-collagen compared with Ct and Δ560-H2B mutant (Fig. 3C).

3.7. Δ238 mutants invaded faster in CAF co-culture

Next, we analysed the effects of CAFs and NOFs on the invasion of these oral carcinoma cell lines. For this purpose, we used 3D sandwich assay in which Ct and the two mutants were embedded with CAFs or NOFs within Myogel-collagen matrices. In sandwich monocultures, both mutants had higher speed than Ct (Fig. 4A), whereas in co-cultures with CAF-GFPs, only Δ238-H2B cells invaded faster than Ct-H2B or Δ560-H2B (Fig. 4B). In contrast, when co-cultured with NOFs, both mutants moved slower than controls (Fig. 4C).
cytokeratin antibody AE1/AE3 staining and microscopy (Fig. 6C). The parameters of cell invasion area (Fig. 6A) and depth (Fig. 6B) in each cell line were measured. The resulting data indicated that only the Δ238 mutant invaded more and deeper than other three cell lines. However, Δ560 mutant invaded with large cell clusters (Fig. 6A-C).

4. Discussion

Desmogelin 3 (Dsg3) belongs to the desmosomal cadherin proteins, but its expression is not restricted to the desmosomes in keratinocytes. The function of non-desmosomal Dsg3 remains poorly understood. In this study, we evaluated the role of Dsg3 in oral carcinoma cell migration and invasion. We monitored in monolayers and in 3D models the cell migration and invasion of SqCC/Y1 cell lines with Dsg3 overexpression or the expression of truncated Dsg3AC mutants. For matrices, we applied both commercial rodent Matrigel® and collagen and locally manufactured human leiomyoma matrices. We showed that on plastic culture, FL cells were more mobile than Ct; this result is consistent with our previous findings [3,5]. In addition, we also measured the cell migration in two mutant lines expressing C-terminally truncated Dsg3 (Δ238 and Δ560) whose migration property had not been examined before, and we found the mutants also exhibited similar enhanced migration activity. In the Transwell assay, all cells invaded faster through Myogel-LMA than Matrigel® coated wells. In the Myogel-collagen sandwich assay, only the mutants invaded significantly faster than Ct, and CAFs elicited further the invasion of the shorter Δ238 mutant. Finally, we observed that monoclonal antibody AK23, which binds the EC1 domain of Dsg3, seemed to be able to inhibit cell invasion speed in FL cells compared with isotype IgG1 control. A summary of these findings is presented in Table 1.

Our previous report revealed that overexpression of Dsg3 enhances cell migration of epidermoid carcinoma and oral buccal mucosa SqCC/Y1 cell lines in the Transwell and scratch wound assays [3,5]. Moreover, RNAi and shRNA knockdown of Dsg3 inhibits migration of OSCC cell lines [40,41]. However, a recent study reported an opposite effect, with overexpression of Dsg3 in immortalized keratinocytes (HaCaT) slowing down and siRNA silencing of Dsg3 accelerating the cell migration in a 2D culture setting [42]. The reason for this discrepancy is unknown, but it could well be due to the different keratinocyte lines (oral versus skin cells) as well as the adhesion status with neighbouring cells in the population.

Recently, we have reported that the expression of Dsg3AC mutants in SqCC/Y1 resulted in the defect in desmosomes and cell-to-cell adhesion [6]. As observed in this study, those mutations lead to the changes in cell migratory behaviour as well. We also demonstrated the reduced expression and distribution of both Dsg3 and E-cadherin at cell periphery in these mutant cells. It is known that C-terminal domain of Dsg3 binds plakoglobin [43] and p120 catenin [44], and forms complexes with several signalling molecules, such as Src, Ezrin, caveolin-1 [2,3,5,45], and p38 MAPK [46]. Since Dsg3AC mutants showed a reduction of plakoglobin and p120 amounts [6], we speculated, and demonstrated here, that Dsg3 mutants have impacts on the cell signalling pathways that influence the migration and invasion as well. There are three Dsg3 mouse models, Dsg3tm1stan, Dsg3tm1pas, and Dsg3tm1bal with targeted disruption of the Dsg3 gene which caused recurrent blisters and erosion in the oral mucosa of a one-year old female baby [48]. Therefore, we believe it is valuable to learn more about the biological effects of Dsg3 mutants which might contribute to the pathogenesis of various human diseases.

Cancer cell movement pattern through the TME is determined by various cellular and matrix factors. Key cellular factors are cell adhesion, cytoskeletal dynamics, matrix degradation and cellular forces. Cellular factors depend on other surrounding ECM mechanical factors like stiffness, collagen types, fibre thickness, matrix pore size and

3.8. Antibody targeting to N-terminus of Dsg3 caused reduced invasion speed in FL cells

To explore the potential role of anti-Dsg3 antibody, which targets the adhesion site on the N-terminus of Dsg3, in cell migration and invasion, we treated SqCC/Y1 cell lines embedded in Myogel-collagen in 3D sandwich assay with monoclonal antibody AK23 targeting the EC1 domain of Dsg3 alongside the mouse IgG1 isotype control. Cells were treated with AK23 for 18 h, and only FL-H2B showed diminished invasion speed (Fig. 5B) compared with control IgG1-treated cells.

3.9. Dsg3AC mutants invaded deeper into the myoma discs

Finally, since the mutants and overexpressed cell lines were more invasive than Ct within different TME gelatinous matrices, we analysed the cell invasion pattern through solid 3D myoma discs. The cellular invasion depth was visualized by immunohistochemistry with Pan-
collagen-binding proteins for cross-linking [49]. Here, we hypothesized that human carcinoma cell lines will invade more naturally through leiomyoma tissue derived matrices compared to the rodent-based matrices. We investigated the role of Dsg3 in cell migration and invasion by utilizing our 3D TMEM matrix models, the soluble Myogel [31] and the solid myoma discs [30], both derived from human uterus.
leiomysarcoma tissue. Compared with the commercially available rodent matrices Matrigel® and rat tail type I collagen, we demonstrated once again that our locally made myoma matrices provided superior TME to mouse sarcoma basement membrane matrix by promoting cell locomotion in all cell lines tested [30–32]. Although matrix proteins, such as laminins, type I, III, and IV collagen, nidogen, epidermal growth factor, and heparan sulphate proteoglycans, are present in both Myogel and Matrigel® [31], myoma tissue contains also tenasin-C [26], active forms of MMP-2 [30], and LOX-1 [39], all of which are crucial for effective cancer invasion. Additionally, several soluble growth factors, their receptors, and other binding proteins are identified in myoma discs, and some of these factors, such as TGF-beta 1 and 2, HGF, and FGF2, participate in cancer cell movement [26,39]. Interestingly, the oral tongue carcinoma cells on top of Myogel, unlike on plastic, showed invasion inductive G13 signalling pathways [31]. We discovered that oral tongue carcinoma cells on top of Myogel, unlike on plastic, showed no significant difference; n.t., not tested; < , decrease; > , increase; significantly higher or lower (↑ or ↓, p < 0.05 and > 0.01; ↑↑ or ↓↓, p < 0.01 and > 0.001; ↑↑↑ or ↓↓↓, p < 0.001).

### Table 1

**Differential comparison of FL and Dsg3ΔC mutants with Ct cells tested in migration and invasion assays using various matrices. Abbreviations:** 2D, two-dimensional; 3D, three-dimensional; Dsg3, Desmoglein 3; 1-UCP, IncuCyte assay with uncoated plates; TW, Transwell assay; SW, sandwich culture; AD-CP, adhesion assay with coated plates; NOF, normal oral fibroblasts; CAF, carcinoma-associated fibroblasts; AK23, monoclonal antibody AK23; SW + NOF, sandwich co-culture with normal oral fibroblasts; SW + CAF, sandwich co-culture with carcinoma-associated fibroblasts; SW + AK23, sandwich culture with AK23 treatment; MD, myoma discs; h, hours; →, no significant difference; n.t., not tested; < , decrease; > , increase; significantly higher or lower (↑ or ↓, p < 0.05 and > 0.01; ↑↑ or ↓↓, p < 0.01 and > 0.001; ↑↑↑ or ↓↓↓, p < 0.001).

<table>
<thead>
<tr>
<th>Matrices (Dimensionality)</th>
<th>In vitro assays</th>
<th>Compared parameters</th>
<th>Dsg3 FL</th>
<th>Dsg3Δ238</th>
<th>Dsg3Δ560</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plastic (2D)</td>
<td>1-UCP</td>
<td>Migration velocity</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>1-UCP</td>
<td>Accumulated distance</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>a</td>
</tr>
<tr>
<td>Collagen (3D)</td>
<td>SW</td>
<td>Invasion speed</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>b</td>
</tr>
<tr>
<td>Myogel (3D)</td>
<td>AD-CP</td>
<td>Adhesion</td>
<td>↓↓</td>
<td>↓↓</td>
<td>↓↓</td>
<td>c</td>
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<tr>
<td>Myogel-collagen (3D)</td>
<td>SW</td>
<td>Invasion speed</td>
<td>↑↑↑</td>
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<tr>
<td>Myogel-collagen (3D)</td>
<td>SW</td>
<td>Accumulated distance</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>b</td>
</tr>
<tr>
<td>Myoma disc model (3D)</td>
<td>MD</td>
<td>Invasion area</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑↑</td>
<td>b</td>
</tr>
</tbody>
</table>

a Migration parameters compared with Vect Ct cells in uncoated plastic plates.
b Results compared with Vect Ct cells.
c Adhesion on Matrigel coated plates compared with Vect Ct cells (Supplementary Fig. S3 online).
d Transwell invasion through Matrigel compared with Vect Ct cells.
e Adhesion on Myogel coated plates compared with Vect Ct cells (Supplementary Fig. S3 online).
f Transwell invasion through Myogel compared with Vect Ct cells.
g Results compared with Vect Ct cells embedded in sandwich co-cultures.
h AK23-treated Dsg3 FL and Dsg3ΔC mutants' results compared with IgG1 control treatment.

Cancer cell actions are additionally adjusted by the stiffness of the matrix. Cells on soft matrices are rounded, minimally adhesive and less proliferative than on stiff matrices, where they are proliferative and fibrogenic [24]. Matrix stiffness influences malignant epithelial cell growth and morphogenesis in vitro by affecting cell contractility and tensional homeostasis [50]. Additionally, increased collagen cross-linking, which enhances matrix stiffness, induced mammary epithelial cell invasive structures in 3D cultures and promoted focal adhesions and tumour progression in vivo [51]. In our experiments, although Matrigel® was stiffer than Myogel-collagen or Myogel, surprisingly, all carcinoma cell lines invaded faster in Myogel-collagen than in Matrigel. Similarly, also in a study analysing lung adenocarcinoma cell movement in collagen, collagen-Matrigel, or collagen with a double concentration of Matrigel, cells invaded fastest in collagen-Matrigel, although collagen itself was the stiffer matrix [52]. These results indicate that TME stiffness itself is not the only regulatory factor for invasion, but also the composition of TME modifies cell migratory behaviour.

Extracellular matrix (ECM) molecules, such as fibronectin (FN) and collagen, modulate OSCC invasion and cell adhesion [53]. Even though Dsg3 is important in cell-cell junctions, we showed that cell lines with different Dsg3 expression and function had an influence on cell attachment to TME matrices. Similar to oral tongue squamous cell carcinoma (HSC-3) cells [31], the buccal mucosa carcinoma cell lines Ct and FL adhered to Myogel coated substrates better than Matrigel®, indicating that substrates of human origin provide better support for cell

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adhesion. Unexpectedly, compared to Matrigel®, Δ560 mutant also showed enhanced adhesion on Myogel. We hypothesized that Dsg3 structural variations have an influence on cell adhesion and different matrices alter their behaviour. The defect of cell-cell adhesion could have an impact on cell-ECM adhesion, a crosstalk between cell-cell junctions and focal adhesions mediated by integrin family proteins [54]. In fact, our preliminary data suggest that Dsg3 mutant cells exhibit enhanced cell adhesion by phospho-myosin light chain (pMLC) staining (unpublished). Therefore, it is likely that these cells have changed their property in adhesion to different coated matrix as shown in the present study.

Comparing Myogel-LMA with Matrigel®, we found that all four cell lines invaded faster and deeper through Myogel-LMA than Matrigel®, confirming the advantage of Myogel-LMA over Matrigel®. Interestingly, we observed that shorter mutants (Δ238) were more motile and moved fast through Myogel-LMA, and this result could arise from differing extent in disruption of cell junctions between these two Dsg3ΔC mutants.

Our previous study demonstrated that human epidermoid carcinoma and SqCC/Y1 cell lines with overexpression of Dsg3 invaded better than controls in Matrigel® and Matrigel®-collagen Transwell and 3D organotypic environment [5]. In support of our study, invasiveness through Matrigel® in Transwell was markedly reduced after Dsg3 silencing in several OSCC cell lines, a finding consistent with the notion that Dsg3 promotes cell migration and invasion [40,41]. A similar result was reproducibly demonstrated here, with Dsg3 overexpressing cells showing the fastest invasion through Matrigel®; nevertheless all four cell lines invaded faster and deeper through Myogel-LMA than Matrigel®.

Unlike Transwell, the 3D sandwich assay mimics the TME biomechanical factors [55] and offers quantification of cell movements in different matrices with a real-time live cell imaging technique [37]. Similar to the finding based on Transwell, the mutants invaded faster than controls, especially in Myogel-collagen compared with Matrigel®-collagen. This is in line with our previous finding, where in hanging drop cultures, HSC-3 cells invaded faster in Myogel-collagen than in Matrigel®-collagen [31].

We monitored the effect of normal and cancer-associated fibroblasts (CAFs) on carcinoma cell invasion in the sandwich assay and showed that, surprisingly, CAFs increased the speed of only the shorter mutant. CAFs have been demonstrated to form the predominant route to cancer and to serve as prognostic markers in OSCC [56]. In our earlier study based on myoma disc co-culture, OTSCC cell lines invaded more efficiently in the presence of CAFs derived from OSCC, bone marrow mesenchymal stem cells, and also together with tumour-associated macrophages [26,57,58]. In addition, the controls invaded faster together with NOFs than CAFs, which was contrary to our hypothesis. Although CAFs are known to be associated with cell invasion, they may also have anti-tumorigenic effects [59], as some CAFs secrete proteins, such as Decorin, Asporin, and Slit SLIT2, which inhibit EGFR, TGFBR, and ROBO1 carcinogenic signalling. These effects can diminish cancer invasion [60]. We also compared cell invasion of Dsg3ΔC mutants between gelatinous Myogel and solid myoma discs and found no difference between the two mutant lines.

Some monoclonal antibodies, such as Cetuximab and Panitumumab, which bind to epidermal growth factor receptors, are already in clinical use for head and neck SCC treatment, but the clinical trials have been disappointing [61,62]. Therefore, there is a need to identify other potential targets for monoclonal antibodies that could be effective against oral cancer. Masayuki Amagai’s group generated a panel of anti-mouse Dsg3 IgG monoclonal antibodies (mAbs) [7], and these mAbs mapped to different regions of extracellular domain of mouse Dsg3 (mDsg3). The mAb AK23 also is able to bind to human Dsg3, and thus, its ability to inhibit cell invasion was evaluated in this study. Although this study was preliminary, the data obtained indicate that AK23 may be able to inhibit invasion of FL cells but not the mutants. Dsg3ΔC mutants had a negative impact on the surface expression of endogenous Dsg3, which is less likely to be bound by AK23 sufficiently. Further studies with various dosages of AK23 as well as other antibodies targeting different regions of Dsg3 are necessary.

5. Conclusions

Taken together, our findings demonstrate that cell movement is not identical in 2D and 3D models. Alteration in Dsg3 expression and function had an impact on cell adhesion, migration, and invasion in both 2D and 3D TMEM matrices. In general, cells expressing Dsg3ΔC mutants exhibited enhanced migration and invasion, suggesting that loss of Dsg3 action promotes cell motility in the human TME matrix. The epithelial cell-cell junctions are compromised in Dsg3 mutants, whereas Dsg3 overexpression activates various signalling pathways that promote cell motility. In this study, we hypothesized that unbalanced Dsg3 molecule, both in case of overexpression and deficiency, promote migration and invasion of malignant SqCC/Y1 cell lines depending on the matrix. In vivo, Dsg3 imbalances might be present in mutated cancer cells. Thus, as we hypothesized, we showed here that migration in 2D was affected in both FL and truncated constructs. However, when the cells were facing tumour mimicking 3D matrix, only the Dsg3 mutants were quicker than FL. Additionally, our experiments demonstrated how the nature of the matrix, mouse derived Matrigel® versus human tumour derive Myogel could also affect on the invasion speed of human cancer cells. An antibody targeting Dsg3 on the cell surface might be able to inhibit cell invasion, however, further studies are required before drawing conclusions.

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Competing interests

The authors declare that they have no competing interests.

Author Contributions

TS conceived and designed the study. HW designed the horizontal 2D migration and immunofluorescence analysis. EHA planned and performed all experiments, analysed the results, and interpreted the data. SUA tracked and provided the quantitative data for the 3D sandwich assays. JR and EHA conducted the matrix rheology measurements. EHA wrote the first draft of the manuscript. Both EHA and TS wrote the main version of the manuscript. All authors reviewed/edited the manuscript. TS and HW share correspondence. TS takes full responsibility for the contents of the publication.


