Guardians of the oral cavity

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CHAPTER 3

HUMAN SALIVARY PEPTIDE HISTATIN 1 STIMULATES EPITHELIAL AND ENDOTHELIAL CELL ADHESION AND BARRIER FUNCTION

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Histatins are multifunctional histidine-rich peptides secreted by the salivary glands and exclusively present in the saliva of higher primates, where they play a fundamental role in the protection of the oral cavity. Our previously published results demonstrated that histatin-1 (Hst1) promotes cell-substrate adhesion in various cell types and hinted that it could also be involved in cell-cell adhesion, a process of fundamental importance to epithelial and endothelial barriers. Here we explore the effects of Hst1 on cellular barrier function. We show that Hst1 improved endothelial barrier integrity, decreased its permeability for large molecules, and prevented translocation of bacteria across epithelial cell layers. These effects are mediated by the adherens junction protein E-cadherin (E-cad) and by the tight junction protein zonula occludens 1, as Hst1 increases the levels of zonula occludens 1 and of active E-cad. Hst1 may also promote epithelial differentiation as Hst1 induced transcription of the epithelial cell differentiation marker apolipoprotein A-IV (a downstream E-cad target). In addition, Hst1 counteracted the effects of epithelial-mesenchymal transition inducers on the outgrowth of oral cancer cell spheroids, suggesting that Hst1 affects processes that are implicated in cancer progression.
INTRODUCTION

The oral cavity is among the most heavily colonized sites of the body and is an important point of entry for toxins and harmful microorganisms causing systemic infections. An intact epithelial barrier is thus crucial for preventing penetration of such agents (1). Adding to the barrier functions of the epithelium, saliva contains a myriad of proteins and peptides that are involved in the protection of oral tissues. Among these are the histatins, histidine-rich peptides that are exclusively present in the saliva of humans and higher primates. Histatins perform a wide variety of functions, including detoxification of harmful food components (2), inhibition of enamel demineralization (3), and killing of yeasts and bacteria (4, 5). Furthermore, some histatins play a role in oral wound closure and healing in vitro (6, 7). Recently we showed that one member of this family, histatin-1 (Hst1), significantly improves cell-substrate and cell-cell adhesion, which could enhance the epithelial barrier function (8).

The epithelial barrier arises as a result of cell-cell adhesion, which depends on structures called desmosomes, adherens junctions, and tight junctions that together form connections between neighboring cells (9). Adherens junction formation is mediated by cadherins, calcium-dependent cell adhesion proteins. The transmembrane E-cadherin (E-cad) molecules on adjacent cells interact homophilically with each other (on the outside of the cell membrane) and are associated with the actin cytoskeleton (on the inside) via several proteins (10). E-cad is essential in mesenchymal-epithelial transition (MET) – the process that transforms mesenchymal cells into epithelial cells (11) – which is important in the maintenance of tissue morphology, cell differentiation, and cell polarity (12).

In this study, we explored the involvement of Hst1 in mechanisms regulating cell adhesion, barrier function, and MET-like processes in epithelial and endothelial cells. Our results show that Hst1 promotes epithelial and endothelial cell adhesion, improves epithelial and endothelial barrier function, and affects expression and activity of junctional proteins and a differentiation marker. Furthermore, Hst1 seems to promote MET or inhibit epithelial-mesenchymal transition (EMT) as it counteracts the EMT effects of cytokines in a spheroid model. Our results imply that Hst1 is an important contributor to the maintenance of the oral epithelium.

MATERIALS AND METHODS

Solid-phase peptide synthesis

Hst1 (DS$^p$HEKRHHGYRRKFHEKHHSHREFPFYGDYGSNYLYDN; $S^p =$ phosphoserine) and Hst5 (DSHAKRHHGYRRKFHEKHHSHRGY) were manufactured by solid-phase peptide synthesis using 9-fluorenlymethoxycarbonyl (Fmoc) chemistry with a Syro II synthesizer (Biotage, Uppsala, Sweden) essentially as described previously (8, 13). Solvents were obtained from Actu-All Chemicals (Oss, The Netherlands), preloaded NovaSyn TGA resins from NovaBiochem (Merck GMBH, Darmstadt, Germany) and the N-α-Fmoc-amino acids from Orpegen Pharma (Heidelberg, Germany) and Iris Biotech (Marktredwitz, Germany). Peptides were purified by preparative reverse-phase HPLC (Thermo Fisher Scientific, Waltham, MA, USA) on a Grace Spring column (Grace, Columbia, MD, USA) containing Vydc C18 TP
beads, 10μm (Vydac Hesperia, CA, USA). Elution was performed with a linear gradient from 15% to 45% acetonitrile containing 0.1% trifluoroacetic acid, 214 nm absorbance monitored, and peak fractions pooled and lyophilized. Reverse-phase HPLC showed a purity of at least 95% and authenticity was confirmed by mass spectrometry as previously described (13). For experiments, lyophilized peptides were first dissolved in a minimal amount of DMSO and subsequently, to a final peptide concentration of 1 mM, in water. The final concentration of DMSO during experiments never exceeded 0.08%, and the same amount of DMSO (vehicle) was added to the controls.

**Cell lines**

Caco-2 human intestinal epithelial cells, kindly provided by K. Wolthers (Academic Medical Center, University of Amsterdam), were cultured at passages 25 to 35 in DMEM without pyruvate (Thermo Fisher Scientific) at 5% CO₂. TR146 human oral epithelium cells, the gift of Cancer Research UK (London, United Kingdom), were cultured in DMEM (Thermo Fisher Scientific) at 10% CO₂. Human umbilical vein endothelial cells (HUVECs; passage 3) were isolated as previously described (14) and cultured in 2% gelatin-coated plates in M199 basal medium (Thermo Fisher Scientific) supplemented with 10% human serum at 5% CO₂. All media were supplemented with 10% fetal calf serum (FCS; GE Healthcare, Waukesha, WI, USA), penicillin, and streptomycin (10 U/mL and 10 μg/mL, respectively; Thermo Fisher Scientific). Cells from exponentially growing cultures were cultured at 37°C in a moist atmosphere and regularly tested for mycoplasma.

**Bacterial strains**

*Streptococcus suis* strain BM407 (serotype 2, clonal complex 1, isolated from a Vietnamese patient with meningitis) (15, 16) and unencapsulated mutant strain 10Δcps2 (originated from strain 10 belonging to serotype 2, clonal complex 1, isolated from a Dutch pig with meningitis) (17) were grown in Todd-Hewitt broth with 5% yeast (Difco Laboratories, Detroit, MI, USA) or on Columbia blood agar (Difco Laboratories) at 37°C and 5% CO₂.

**Real-time impedance-based cell adhesion assay**

Cell-substrate adhesion was monitored using the xCelligence system (18) (ACEA Biosciences, San Diego, CA, USA). Caco-2 cells were plated in 16-well E-plates (ACEA Biosciences) at 1.5 X 10⁴ cells per well in DMEM containing 1% FCS and incubated in the presence of vehicle or 10 µM Hst1. Impedance was measured every 10 min for 70 h. Cell attachment is expressed as the cell index (CI), representing the change in electrical impedance.

**Trypsin cell adhesion assay**

Caco-2 cells were plated in 12-well plates at 10⁴ cells per well and incubated in the presence of vehicle or 10 µM Hst1 for 10 d. Cells were washed with PBS, and 0.01% trypsin-EDTA (Thermo Fisher Scientific) was added. Phase-contrast images were acquired continuously at
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37°C in an atmosphere containing 5% CO₂ with an inverted DM IRBE time-lapse microscope with a Plan Apo X40 objective (Leica, Wetzlar, Germany). Images were analyzed using custom-made software called CiMicro (Amsterdam, The Netherlands; http://software.cellularimaging.nl). Cells that round up and detach from the surface appear as white spheres in phase contrast. The area covered by attached cells divided by the total image area gives the percentage of cell attachment.

S. suis translocation through polarized Caco-2 cells

Caco-2 cells were plated at 10⁶ cells per well on 12 Transwell polycarbonate filters (1.12 cm², pore size 0.4 µm; Corning, Corning, NY, USA) and cultured for 8 d, washed with PBS to remove antibiotics, and incubated in medium without antibiotics containing vehicle or 10 µM Hst1 for 18 h. S. suis BM407 or 10Δcps2 were added to the apical side of the cell cultures at a multiplicity of infection of 100 and incubated for 4 h. The amount of bacterial translocation to the lower compartment was quantified by colony-forming units (CFUs) per milliliter (CFU/ml) counting, essentially as previously described (16). To eliminate between-session variation in the experiments, factor correction was applied using Factor Correction 2015.2.0.0 software (19). The effect of Hst1 on S. suis viability was tested by incubating bacteria for 6 h in the presence or absence of 10 µM Hst1, and survival was measured by CFU/ml counting.

Cell spreading assays

HUVEC cells from 2 donors were seeded at 3 X 10⁴ cells per well on coverslips (Thermo Fisher Scientific) in 25-well replica dishes (4 cm²/well; Greiner Bio-One, Monroe, NC, USA) in endothelial serum-free medium (Thermo Fisher Scientific) containing vehicle or 10 µM Hst1. Cell spreading was scored using a DM RA HC microscope (Leica) as previously described (8). Cells displaying a spreading morphology divided by the total cell number gave the percentage of cell spreading.

HUVEC transepithelial/endothelial resistance and permeability analysis

HUVECs from 3 donors were seeded at 6 X 10⁴ cells per insert in 24-well Transwell inserts (0.33 cm², pore size 0.4 µm, Greiner Bio-One) coated with 2% gelatin. Cells were grown on filters for 3 d, after which medium from the apical side of the insert was replaced with medium containing 5, 10, or 20 µM Hst1 or vehicle as a negative control. As a positive control, vehicle with 312.5 µM 8-(4-chlorophenylthio(CPT))-cAMP (Sigma-Aldrich, St. Louis, MO, USA) and 17.5 µM RO-20-1724 (cAMP/RO) (Merck) was used, which enhances barrier function (20). After 16 h, transepithelial/endothelial resistance (TER) was measured using a Millicell ERS-2 voltohmmeter (Merck) as previously described (21). Subsequently, fluorescent tracers of different sizes [766-Da Cy3, 50 µg/ml (GE Healthcare) and 70-kDa FITC-dextran, 250 µg/ml (Thermo Fisher Scientific)] were added to the apical side of the Transwell insert, and samples from the upper and lower compartments were collected after 4 h. Tracer concentrations were measured with a ClarioStar fluorescence plate reader (BMG Labtech, Cary, NC, USA),
and the percentage of tracer passage to the lower compartment was calculated on the basis of the initial concentration in the upper compartment. Factor correction was applied (19).

**Confocal microscopy**

Caco-2 cells were plated at 10^5 cells per well on coverslips (Thermo Fisher Scientific) in 25-well plates (Greiner Bio-One) and incubated in the presence of vehicle or 10 µM Hst1 for up to 21 d. Cultures were fixed with 2% paraformaldehyde for 30 min, and permeabilized and blocked with TNBS (PBS supplemented with 1% fetal bovine serum and 0.1% Triton X-100) for 30 min. Cells were incubated with primary antibodies (Table 1) for 2 h, then with FITC (green)- or Cy3 (red)-conjugated secondary antibodies (Table 1) for 1 h, followed by 15 min incubation with 1:1000 dilution Hoechst 33342 (Thermo Fisher Scientific) to stain DNA. To visualize F-actin, cultures were incubated with Texas Red conjugated phalloidin (Thermo Fisher Scientific) at 1:100 dilution for 1 h. All antibodies were diluted in TNBS. Samples were mounted with Vectashield antifade mounting medium (Vector Laboratories, Burlingame, CA, USA) and stored at 4°C. Confocal stacks were acquired with a Leica TSC XSP-8 or TSC XSP-8 SMD mounted on a Leica DMI6000 inverted microscope using a HC Plan Apochromat X63 objective. Fluorescence intensity and colocalization were quantified using Huygens software (Scientific Volume Imaging, Hilversum, The Netherlands). Fluorescence intensity, in this case, is the sum (the sum of all pixel values in an image) in Huygens software (under Statistics), divided by the nuclear staining intensity to correct for the number of cells. Since immunostainings were performed on different days, the values were only comparable at each time point and are represented as fold change relative to the vector control.

**Real-time quantitative PCR**

Caco-2 cells were plated at 8.5 X 10^4 cells per well in 24-well plates and incubated in the presence of vehicle or 10 µM Hst1 for 5, 10, 15 or 20 d. Cells were collected using 500 µl Trizol reagent (Thermo Fisher Scientific). Total RNA was isolated according to the manufacturer’s protocol and dissolved in RNAse-free water. RNA yield was measured using NanoDrop (Thermo Fisher Scientific) and 1 µg of RNA was treated with DNase-I (Thermo Fisher Scientific) and reverse transcribed into first strand cDNA using a QuantiTect Reverse Transcription Kit (Qiagen, Germantown, MD, USA). Quantitative PCR (qPCR) was performed on 20X diluted cDNA samples with primers listed in Table 2 using a CFX96 system (Bio-Rad, Hercules, CA, USA) as previously described (22). Data were normalized to the geometric mean of 4 housekeeping genes. Factor correction was applied (19).

**Western blot analysis**

Caco-2 cells were plated at 3 X 10^5 cells per well in 6-well plates and incubated in the presence of vehicle or 10 µM Hst1 for 5, 10, or 15 d. Cells were scraped and collected with ice-cold lysis buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 20 mM N-ethylmaleimide) supplemented with protease inhibitor mixture (Sigma-Aldrich). Lysates
were boiled for 10 min with Laemmli loading buffer, fractionated by SDS-PAGE and transferred to a PVDF membrane (Thermo Fisher Scientific), blocked with 5% milk, and incubated with the primary antibodies listed in Table 1 overnight at 4°C and for 1 h with secondary antibodies IRDye 680 (red) and IRDye 800 (green) (Table 1). Infrared signal was detected using the Odyssey imaging system (Li-Cor Biosciences, Lincoln, NE, USA). Immunoblot images were analyzed by manually specifying band boundaries and measuring total intensity using Odyssey 3.0 software, generating background-subtracted values. Finally, intensities of proteins of interest were divided by loading control tubulin.

### TABLE 1. Study antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host species</th>
<th>Vendor</th>
<th>Catalog no.</th>
<th>Western blot</th>
<th>Immunofluorescence</th>
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<tr>
<td>E-cad</td>
<td>Rabbit</td>
<td>Cell Signaling Technology, Danvers, MA, USA</td>
<td>3195</td>
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<td>1:150</td>
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<tr>
<td>Occludin</td>
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<td>Zymed Laboratories, San Francisco, CA, USA</td>
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<td>1:1000</td>
<td>1:300</td>
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<tr>
<td>Tubulin</td>
<td>Rabbit</td>
<td>Cell Signaling Technology</td>
<td>2148</td>
<td>1:2000</td>
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</tr>
<tr>
<td>ZO-1</td>
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<td>Mouse IRDye 680</td>
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<td>Jackson ImmunoResearch Laboratories</td>
<td>-</td>
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</table>

### TABLE 2. Study primers

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<tr>
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<td>GCGCTGGAGCTATGGTCG</td>
<td>ATTACGGAGGTCTAGATGG</td>
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<tr>
<td>ocln</td>
<td>CTCCCGTTTGGATAAAGAATGG</td>
<td>TGCAATCCCTTCAGATCTTC</td>
</tr>
<tr>
<td>tjp1 (zo-1)</td>
<td>ATTTCTCACAAGGGAAGCCCTCTGA</td>
<td>GCATCTGCGAGGGCAATGGA</td>
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<tr>
<td>actb*</td>
<td>CACCTCTACATGAGCTTGCTTG</td>
<td>ATAGCAGAAGCTAGAACGTAC</td>
</tr>
<tr>
<td>gapdh*</td>
<td>TCACCACATGAGAAGGCG</td>
<td>GCTAAGCAGTTGTTGTGCA</td>
</tr>
<tr>
<td>hprt1*</td>
<td>TGACACTGGCAGAAGCAATGCA</td>
<td>GGTCTTTTTTCACGCAAGCT</td>
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<tr>
<td>ubc*</td>
<td>ATTTGGGTCGCGGTTTCTTG</td>
<td>TGCCCTGACATTCTCGATGG</td>
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</table>

*Housekeeping gene.
Spheroid outgrowth assay

Spheroids were made by plating TR146 cells at 3 X 10^5 cells per well in uncoated 6-cm dishes in the presence of vehicle or 10 µM Hst1 and shaking at 50 rpm for 24 h at 37°C in a moist atmosphere containing 10% CO₂. Spheroids were plated in 12-well plates at 1 spheroid per well in DMEM containing 10% FCS or 50 ng/ml epidermal growth factor (EGF) and 5 ng/ml TGF-β and allowed to attach for 5 h. Subsequently, phase-contrast images were acquired every 15 min for 70 h at 37°C in an atmosphere containing 10% CO₂ with an inverted DM IRBE microscope (Leica). The surface areas occupied by the spheroids were quantified by CiMicro software.

Statistical analysis

Data obtained from at least 3 independent experiments are expressed as means ± SD, calculated and plotted by GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Statistical significance was determined by Student's t test or ANOVA. A value of P < 0.05 was considered significant.

RESULTS

Hst1 promotes epithelial cell adhesion

We first investigated whether cell-substrate adhesion is affected by Hst1 in human intestinal epithelial cells (Caco-2), an established model system for epithelial barrier studies (23), using two different methods (Fig. 1). With the xCelligence system (18) we directly measured the effects of Hst1 on cell-substrate adhesion. In this system, cells are seeded on an electrode, and the number of connections between the cells and the electrode is represented as CI, which is proportional to the extent of adhesion. We found that the presence of 10 µM Hst1 in the medium resulted in a higher CI (P < 0.05; Fig. 1A), indicating enhanced cell-substrate adhesion. Additionally, confluent Caco-2 cell cultures grown on culture plastics in the presence or absence of Hst1 were trypsinized, and cell detachment was monitored by time-lapse microscopy. Because trypsin detaches cells both from their substrate and from each other, this showed the combined effect of Hst1 on both modes of cell adhesion. First the tight cell-cell barrier is broken for detachment from the substrate to occur. We found that Hst1 inhibited cell detachment, as the area of cells attached to the substrate was notably larger for up to 40 min after trypsinization in cultures grown in the presence of Hst1 (P < 0.01; Fig. 1B). During trypsinization, the cells become round and detach from the substrate, which can be seen as cells appearing as white spheres under a phase-contrast microscope (Fig. 1C).

Hst1 improves epithelial and endothelial barrier function

The intestine as well as the oral cavity are heavily colonized by bacteria, and an intact epithelial cell barrier is essential to prevent invasion of these microorganisms. Recently S. suis, an emerging zoonotic pathogen that can use an oral route to infect humans and pigs (24), was shown to translocate across a Caco-2 monolayer by disrupting tight junctions (16). We previously showed that Hst1 improves the epithelial barrier in Caco-2 cells because it causes an increase
of TER (8). To examine whether Hst1 can enhance the impermeability of an epithelial barrier to pathogens, we challenged the Caco-2 monolayers for 4 h with live bacteria, namely S. suis strains BM407 and unencapsulated mutant strain 10Δcps2. These 2 strains were used because BM407 crosses the epithelium via a paracellular route (in between cells), while 10Δcps2 is also able to translocate through cells (16). Using the Transwell translocation technique developed by Ferrando et al. (16), we found that an 18 h pretreatment of Caco-2 cells with Hst1 significantly inhibited the translocation of S. suis BM407 across the epithelial layer, as measured by CFU/ml counting (P < 0.05; Fig. 2A, left). This was not due to a bactericidal effect because Hst1 did not affect S. suis viability (Fig. 2B). Furthermore, Ferrando et al. (16) showed previously that a 6 h incubation with S. suis does not affect Caco-2 cell viability. In contrast to wild-type S. suis, Hst1 had no significant effect on the translocation of the unencapsulated mutant (Fig. 2A, right).

To further investigate the effect of Hst1 on barrier function, we measured cell layer permeability to tracers of different sizes. We could not detect tracer permeability in Caco-2
Histatin 1 stimulates barrier function cells (data not shown), which form a very tight barrier. Therefore, we used HUVEC monolayers, which are relatively permeable under control conditions. To establish whether HUVECs respond to Hst1, we first performed a cell spreading assay. We found that in the presence of Hst1, cell attachment and spreading were significantly improved at 1 h, 1.5 h ($P < 0.001$) and 2 h ($P < 0.05$) after seeding (Fig. 2C). Next we assessed the effect of Hst1 on the HUVEC barrier function by measuring changes in the TER. The TER values were increased in HUVEC monolayers treated...
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with 20 µM Hst1 or cAMP/RO (used as a positive control because it enhances barrier function (20)) (Fig 2D), indicating an improved endothelial barrier function. Furthermore, monolayers treated with 20 µM Hst1 or cAMP/RO were less permeable to a 70-kDa FITC-dextran tracer than mock-treated cultures (Fig. 2E, right), while there was no effect of Hst1 on the permeability of HUVECs to a 0.766-kDa Cy3 tracer (Fig. 2E, left). Together, these results indicate that Hst1 decreases permeability of the epithelial and endothelial barriers to bacteria and large molecules.

**Hst1 affects expression and activity of junction proteins**

To investigate the mechanism behind the adhesion-promoting effect of Hst1, we assessed the abundance and localization of several junctional proteins by immunostaining and confocal microscopy. We found that 3 and 6 d after seeding, Hst1-treatment caused a 2-fold increase in E-cad signal in Caco-2 cells (Fig. 3A,B). Because functional E-cad is associated with the cytoskeleton (25-28), colocalization of E-cad and F-actin is an indication of E-cad activity. We found that co-localization of F-actin and E-cad was increased in the presence of Hst1 at d 3 ($P < 0.05$) and 6 after seeding (Fig. 3C,D). Additionally, we found a 1.5-fold ($P < 0.05$) increase in intensity of zonula occludens 1 (ZO-1) staining in Hst1-treated cultures at 2 wk after seeding (Fig. 3E,F).

These observations prompted us to test whether Hst1 affected the expression of proteins involved in junction formation. We found no effect of Hst1 on mRNA levels of E-cad, occludin, claudin 1, or catenin α1 and catenin δ1 (Fig. 4A-E), or on protein levels of E-cad and occludin (Fig. 4F-H), and we were not able to detect ZO-1 protein levels. However, we observed that Hst1 increased mRNA levels of ZO-1 (by ~30% at d 10 and by ~10% at d 20 after seeding, $P < 0.001$, Fig. 4I). Furthermore, mRNA expression of apolipoprotein A-IV (ApoA-IV) – an E-cad-regulated differentiation marker (29) – was increased by 1.5-fold in the presence of Hst1 at d 5 after seeding ($P < 0.001$; Fig. 4J).

**Hst1 inhibits the effects of EMT-inducing agents**

Cell-cell and cell-substrate adhesion are of key importance in EMT and MET, the processes underpinning cancer metastasis. E-cad levels inversely correlate with metastasis probability in primary tumors (30-32), and this protein is involved in metastasis of oral squamous carcinoma (33, 34). To assess whether Hst1 can affect these processes, we used spheroids of oral cancer cell line TR146 as a crude *in vitro* model of EMT during cancer invasion (35, 36). EMT was induced by plating the spheroids in medium containing serum or in serum-free medium supplemented with TGF-β (a major inducer of EMT during embryogenesis and fibrosis (37)) and EGF (27). These EMT factors cause expansion of spheroids, and indeed the surface area occupied by the spheroids plated in medium supplemented with serum or EGF + TGF-β was much larger than that of spheroids plated in medium without these factors (Fig. 5A). Expansion of the spheroids was then used as a rough proxy for EMT and metastasis. In medium with EMT-inducing factors, control spheroids rapidly lost their compact morphology because cells collectively adhered more to the surface and less to each other. In contrast, Hst1-treated
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Figure 3. Hst1 activates E-cad and promotes ZO-1 expression. Caco-2 cells were seeded at 2.5 X 10^4 cells/cm^2 and cultured in presence of vehicle or 10 µM Hst1 for up to 21 d. At each time point, cultures were immunolabeled and analyzed by confocal microscopy under strictly controlled conditions preventing photobleaching and always using same settings. Fluorescence intensity and colocalization were quantified by Huygens software. A) Graph shows total E-cad pixel intensity divided by nuclear signal to correct for cell number, and depicts fold change between Hst1-treated cultures and vehicle treatment. B) Sample images at d 6 after seeding. E-cad, green; nuclei, blue. C) Colocalization between F-actin and E-cad was quantified using Huygens software and is shown as fold change between Hst1-treated cultures and vehicle treatment. D) Sample images at d 6 after seeding; E-cad, green; F-actin, red; nuclei, blue. E) Graph shows total ZO-1 pixel intensity divided by the nuclear signal to correct for cell number, and depicts fold change between Hst1-treated cultures and vehicle treatment. F) Sample images at d 14 after seeding. ZO-1, green; nuclei, blue. Scale bars, 20 µM. * P < 0.05.
Histatin 1 stimulates barrier function

Figure 4. Effect of Hst1 on junctional proteins. A-E) Caco-2 cells were seeded at 8.5 X 10^4 cells per well in 24-well plates and cultured for 5, 10, 15 or 20 d in presence of vehicle or 10 µM Hst1. mRNA expression of E-cad (A), occludin (B), claudin-1 (C), catenin α1 (D), and catenin δ1 (E) were quantified by qPCR and normalized to housekeeping genes. F,G) Caco-2 cells were seeded at 3 X 10^5 cells per well in 6-well plates and cultured for 5, 10 or 15 d in the presence of vehicle or 10 µM Hst1. Protein expression of E-cad (F) and occludin (G) were quantified by Western blot analysis and normalized to housekeeping protein tubulin as a loading control. H) Typical Western blot with E-cad, occludin and tubulin bands. I,J) As described in A-E; mRNA expression of ZO-1 (I) and APOA-IV (J) are shown. *** P < 0.001.
Figure 5. Hst1 inhibits effects of EMT-inducing agents. A) Spheroids were formed by incubating TR146 cells for 24 h while shaking at 50 rpm. Spheroids were plated in presence of serum (left) or EGF + TGF-β (right) and monitored by time-lapse microscopy. Images were analyzed by measuring surface area occupied by spheroids using CiMicro software. B, C) Spheroids were formed as described above, but in presence of vehicle or 10 µM Hst1. Spheroids were plated in presence of serum (B, left) or EGF + TGF-β (B, right) and monitored by time-lapse microscopy. Surface area occupied by spheroids was quantified by CiMicro software. Graphs depict increase in surface area during each 10-h period. * P < 0.05, ** P < 0.01. C) Sample images of spheroid expansion over time.
spheroids remained more compact (Fig. 5B,C). In the first 15 h after plating, the surface increase was significantly lower for spheroids cultured in the presence of Hst1 (Fig. 5B), with a 1.8-fold difference ($P < 0.05$) when spheroids were incubated in medium additionally supplemented with serum (Fig. 5B, left) and 1.5-fold ($P < 0.01$) in medium supplemented with EGF + TGF-β (Fig. 5B, right). After this initial period, the surface increase was no longer inhibited by Hst1, but the total surface area of the Hst1-treated spheroids remained smaller than that of control spheroids throughout the experiment. These results indicate that Hst1 can counteract the effects of EMT-driving factors on the outgrowth of oral cancer spheroids.

**DISCUSSION**

Cell-cell adhesion is an essential part of barrier formation, and the integrity of epithelial and endothelial barrier is fundamental in maintaining low and selective permeability to fluids, metabolites and pathogens under physiological conditions. A dysfunction of the physiological barrier can lead to the onset of various diseases and bacterial infections (38).

Our data show that Hst1 improves cell-cell adhesion, which is in line with our previous studies showing that Hst1 stimulates cell-substrate adhesion, and which suggested that Hst1 promotes epithelial barrier integrity (8, 39). In this study, we focused on Hst1 because we previously found that this was the only member of the histatin family that induced cell attachment and spreading (8). Throughout the study, we chose to use Hst1 at a concentration of 10 µM because the optimal concentration was between 5 and 10 µM in several different cell lines and cell types that we tested earlier (8, 39); 10 µM is also the approximate concentration of Hst1 in human saliva (40).

To explore cell-cell adhesion in a more physiologically relevant context, we quantified translocation of the pathogen *S. suis* through monolayers of Caco-2 cells and found that Hst1 inhibits the translocation of wild type (BM407), but not of the unencapsulated mutant strain (10Δcps2). The 2 strains use different strategies of translocation through the mucosal epithelium (16). As a result of the presence of the polysaccharide capsule that obstructs intracellular invasion, BM407 mostly passes across the epithelium *via* a paracellular route (in between cells) that is affected by Hst1, while the unencapsulated mutant 10Δcps2 is also able to translocate through cells (16) and is not significantly affected by Hst1.

It is thus feasible that Hst1 mostly blocks the paracellular translocation. However, in endothelial cells, our results showed that Hst1 mainly reduced barrier permeability to a 70-kDa tracer, which on the basis of its molecular weight is most likely to predominantly use the transcellular translocation route (41). These contrasting results could be accounted for if translocation of bacteria and dextrans are driven by distinct mechanisms that are differentially affected by Hst1. Alternatively, the effects of Hst1 on the presumably paracellular translocation of the 10Δcps2 strain could be undetectable because of the mutant's overall reduced translocation capabilities. In any case, our results demonstrate that Hst1 has a positive effect on physiologically relevant barrier functions in both epithelial and endothelial cells.

Strong links have been found between cell-substrate and cell-cell adhesion, termed adhesive cross talk (42). For instance, adhesion of cells to extracellular matrix promotes cell-cell adhesion...
Histatin 1 stimulates barrier function by stimulating accumulation of E-cad at the epithelial intercellular junctions (43). Also, cell-substrate adhesion exerts mechanical force on the cell, in turn affecting E-cad complexes, which are force sensitive (44). Furthermore, it was shown that cell-matrix adhesion can induce polarization of cells via E-cad-actin complexes (26, 45). Because junctional proteins are directly responsible for cell-cell adhesion and barrier function, we investigated how Hst1 affects these proteins. Formation of the cell-cell contacts through E-cad is associated with tight junction formation and relocation of ZO-1 to these structures (46). We found that Hst1 induced ZO1 gene expression and increased levels of ZO-1 and E-cad as detected by immunostaining. Moreover, we observed increased expression of ApoA-IV, an intestinal differentiation marker that is transcriptionally controlled by E-cad (26, 47). However, Western blot analysis did not reveal increased E-cad protein levels. This effect appears similar to previously published data that showed unchanged E-cad protein levels, while changes were clearly detected by immunofluorescence and immunoprecipitation (26, 45). The E-cad complex dynamically associates with the actin cytoskeleton, with α-catenin – one of the key E-cad partners – acting as regulator of actin dynamics (48, 49). In epithelial cells, a considerable fraction of E-cad in the plasma membrane is activated by association with the actin cytoskeleton and with the extracellular E-cad domains of the neighboring cells (25-28). The anti-E-cad antibody that we used recognizes the part of the E-cad cytoplasmic domain that interacts with β-catenin. It has been shown that this domain is unstructured in isolation but changes conformation when bound by β-catenin (50), possibly affecting antibody recognition. It is thus feasible that changes in E-cad conformation, activity, and localization, rather than increased protein levels, resulted in the elevated immunostaining efficiency that we observed. In support of this hypothesis, we also found that Hst1 promoted colocalization of E-cad with cytoskeletal F-actin, which is considered to represent active E-cad (25).

Junction components, including E-cad, constantly recirculate from and back to the plasma membrane, which is essential for dynamic maintenance of the epithelial barrier (10, 51). E-cad trafficking occurs in membrane vesicles whose formation, transport, and targeting are controlled by Rab GTPases (51). Other small GTPases, including RhoA and Rac1, are also involved in global regulation of cell adhesion (10, 51, 52). It is thus tempting to speculate that Hst1 could bind to an unidentified cellular receptor, activating Rab, Rac and/or Rho signaling. This in turn could stimulate recirculation, re-localization, and activation of ZO-1 and E-cad to promote barrier function. In support of such active and receptor-mediated mechanism of Hst1 internalization, intracellular localization of Hst1 is inhibited when cells are incubated in its presence at 4°C (6). Further upholding this hypothesis, the study showed that the effects of another member of the histatin family were blocked by an ERK inhibitor (6). Notably, we found that Hst1 induced ERK phosphorylation and that 2 different ERK inhibitors prevented stimulation of cell spreading by Hst1 in gingival fibroblasts (Supplemental Fig. 1).

Several studies reported that Hst1 promotes wound healing (6, 7, 53-55). During the first phase of this process in vivo, EMT leads to loosening of cell-cell contacts and migration of the cells into the wound gap. The reverse process (MET) occurs to promote reestablishment of cell-cell junctions and wound reepithelialization (56). Our data shed some light on how
Hst1 may affect these processes. We observed that Hst1 counteracts the EMT-promoting and junction-disrupting (57) effects of TGF-β and EGF as well as of FCS – which contains growth factors (58) – in a spheroid model. Hence, by blocking EMT in the wound gap, Hst1 could stimulate MET and epithelium formation in the final stages of wound healing, when cells need to return to their differentiated state.

The involvement of EMT in cancer invasion and metastasis is still debated (59-61), but many studies have proposed that cancer cells require EMT, characterized by reduced levels of junction proteins, to disperse throughout the body (57, 62-66). After invading distant tissues, cancer cells likely rely on MET to establish secondary tumors (66, 67). EMT is thought to be the cause of drug-induced gingival overgrowth (68), and plays a role in the progression of oral cancer (69, 70). Because Hst1 counteracts EMT inducers, it could potentially inhibit the initial phases of metastasis. Interestingly, Hst1 has been patented as a prognostic marker in melanoma, with high expression of Hst1 in metastatic lesions correlating with worse overall survival (71). It is tempting to speculate that in this case, Hst1 could promote attachment of metastasizing cells to their target sites. The effects of Hst1 in the context of cancer as well as those related to epithelial tissues warrant future investigations.

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Supplementary figure 1. Hst1 activates ERK. A) ARPE-19 cells were incubated overnight in SFM supplemented with inhibitors against ERK (25 µM PD98059 and 10 µM U0126), p38 (10 µM SB202190) and JNK (20 µM SP600125). The following day cells were seeded at 7.5 X 10^4 cells per well in SFM and incubated for 2 h in the presence of vehicle, 10 µM Hst1 or 10 µM Hst5. Cell spreading is shown relative to vehicle treatment for each inhibitor. Both ERK inhibitors blocked the effect of Hst1.

B, C) Primary gingival fibroblasts were cultured for 2 d and incubated in 0.1% serum for 2 d. Indicated cultures were pretreated with ERK inhibitor PD98059 overnight. The following day, cells were treated with Hst1 or 10% FCS (positive control). The following day, cells were treated with Hst1 or 10% FCS (positive control). The amounts of phosphorylated ERK (p-ERK) and total ERK were quantified by Western blot. * P < 0.05.
MATERIALS AND METHODS

Cell lines
ARPE-19 human retinal pigment epithelial cells, kindly provided by the Department of Ophthalmology (AMC), were cultured in Medium 199 + F12 medium 1:1 (Thermo Fisher Scientific) at 5% CO₂. Primary gingival fibroblasts isolated from human gingiva, gifted by M. Boink (VU University Medical Center, Amsterdam, The Netherlands) were cultured in DMEM (Thermo Fisher Scientific) at 10% CO₂.

Cell spreading assays with signaling inhibitors
ARPE-19 cells were seeded at 5 X 10⁵ cells per well in 6 cm dishes, cultured until 60% confluent and incubated overnight in serum-free medium (SFM; Thermo Fisher Scientific) supplemented with 0.5% DMSO (control), 25 µM PD98059 (MEK1/2 inhibitor 1), 10 µM U0126 (MEK1/2 inhibitor 2), 10 µM SB202190 (p38 inhibitor) or 20 µM SP600125 (JNK1/2 inhibitor).
Subsequently, cells were seeded at 7.5 X 10⁴ cells per well in SFM (Thermo Fisher Scientific) on coverslips (Thermo Fisher Scientific) in 25-well replica dishes (Greiner Bio-One) and incubated in the presence of vehicle, 10 µM Hst1 or 10 µM Hst5 for 2 h. Cell spreading was scored using a DM RA HC microscope (Leica) as previously described (1).

Western blot analysis
Primary gingival fibroblasts were seeded at 1.5 X 10⁵ cells per well in 6-well plates and cultured until 60% confluent, incubated in medium with 0.1% FCS for 48 h and specified cultures were treated overnight with 25 µM PD98059 (MEK1/2 inhibitor 1). Cells were treated with 10 µM Hst1 or 10% FCS for 0, 5, 10, 15, 30 and 60 min. Treatments were added carefully at a 1:10 dilution without swirling in order not to activate phosphorylation by mechanical stimulation.
Cells were scraped and collected with ice-cold lysis buffer (2) and blotted as described in the main document, except that Odyssey blocking buffer (Li-Cor Biosciences) was used instead of milk, because milk contains phosphatases, and primary antibodies against ERK and phosphorylated ERK (Cell Signaling) at 1:1000 dilution overnight at 4°C.
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