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# The interplay of matrix metalloproteinase-8, transforming growth factor- $\beta$ 1 and vascular endothelial growth factor-C cooperatively contributes to the aggressiveness of oral tongue squamous cell carcinoma

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**Background:** Matrix metalloproteinase-8 (MMP-8) has oncosuppressive properties in various cancers. We attempted to assess MMP-8 function in oral tongue squamous cell carcinoma (OTSCC).

**Methods:** MMP-8 overexpressing OTSCC cells were used to study the effect of MMP-8 on proliferation, apoptosis, migration, invasion and gene and protein expression. Moreover, MMP-8 functions were assessed in the orthotopic mouse tongue cancer model and by immunohistochemistry in patient samples.

**Results:** MMP-8 reduced the invasion and migration of OTSCC cells and decreased the expression of MMP-1, cathepsin-K and vascular endothelial growth factor-C (VEGF-C). VEGF-C was induced by transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) in control cells, but not in MMP-8 overexpressing cells. In human OTSCC samples, low MMP-8 in combination with high VEGF-C was an independent predictor of poor cancer-specific survival. TGF- $\beta$ 1 treatment also restored the migration of MMP-8 overexpressing cells to the level of control cells. In mouse tongue cancer, MMP-8 did not inhibit metastasis, possibly because it was eliminated in the peripheral carcinoma cells.

**Conclusions:** The suppressive effects of MMP-8 in OTSCC may be mediated through interference of TGF- $\beta$ 1 and VEGF-C function and altered proteinase expression. Together, low MMP-8 and high VEGF-C expression have strong independent prognostic value in OTSCC.

Matrix metalloproteinases (MMPs) are one of the key players in cancer progression. MMPs degrade tissue barriers and enable tumour invasion and metastasis (Egeblad and Werb, 2002). However, it has become evident that the contribution of MMPs is far more diverse (Overall and Kleinfeld, 2006). Matrix

metalloproteinase-8 was among the first MMPs reported to have tumour-suppressive features (Balbin *et al*, 2003). Originally identified in neutrophils, MMP-8 is produced by various cells (Van Lint and Libert, 2006), including oral tongue squamous cell carcinoma (OTSCC) cells (Moilanen *et al*, 2002).

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The oncosuppressive effects of MMP-8 have been reported in malignancies such as melanoma (Gutiérrez-Fernández *et al*, 2008; Palavalli *et al*, 2009), skin (Balbin *et al*, 2003), breast (Agarwal *et al*, 2003; Montel *et al*, 2004; Decock *et al*, 2008; Soria-Valles *et al*, 2014), lung (Gutiérrez-Fernández *et al*, 2008) and tongue carcinomas (Korpi *et al*, 2008), but contrary evidence also exists (Stadlmann *et al*, 2003; Väyrynen *et al*, 2012).

The role of MMP-8 in head and neck squamous cell carcinomas is contradictory (Korpi *et al*, 2008; Pradhan-Palikhe *et al*, 2010; Mäkinen *et al*, 2012; Nurmenniemi *et al*, 2012). Head and neck squamous cell carcinomas are heterogeneous regarding their epidemiology, aetiology and survival rates (Rethman *et al*, 2010). Our previous study showed that MMP-8 expression by tumour cells improves patient survival, and MMP-8 protects mice from carcinogen-induced OTSCC (Korpi *et al*, 2008). There are few studies demonstrating the effects of MMP-8 in cancers at the cellular and molecular levels (Gutiérrez-Fernández *et al*, 2008; Pellinen *et al*, 2012; Thirkettle *et al*, 2013; Soria-Valles *et al*, 2014; Decock *et al*, 2015). We thus examined the action of MMP-8, a tumour-suppressive proteinase with promising diagnostic value in OTSCC.

## MATERIALS AND METHODS

**Cells.** Human OTSCC cell lines HSC-3 (Japan Health Sciences Foundation, Tokyo, Japan), SCC-15 and SCC-25 (American Type Culture Collection, Manassas, VA, USA) were cultured as described (Bitu *et al*, 2013). The cells were transfected with lentiviral particles containing *MMP8* (GenBank: NM\_002424.2) (MMP-8+ cells) or a non-coding control sequence (Amsbio, Abingdon, UK) (control cells) and selected by puromycin to obtain stable, polyclonal cell lines. In case of HSC-3 cells, two transfection clones were done. Cathepsin K-silenced (shCat-K) HSC-3 cells were previously described (Bitu *et al*, 2013). Short tandem repeat profiling of the cells was done by IdentiCell (Aarhus, Denmark). Mycoplasma infection was excluded by regular testing with MycoTrace PCR Detection Kit (PAA Laboratories GmbH, Cölbe, Germany). To collect conditioned media, the cells were washed with PBS prior to adding Opti-MEM (Life Technologies, Carlsbad, CA, USA). Recombinant human TGF- $\beta$ 1 (10 ng ml<sup>-1</sup>) (R&D System, Minneapolis, MN, USA) was included where indicated. After 24 h, small amounts of media were taken for zymography before adding cOmplete Tablets proteinase inhibitors (Sigma-Aldrich, St Louis, MO, USA). The media were clarified by centrifugation. The cells were harvested in elution buffer (50 mM Tris-HCl, 10 mM CaCl<sub>2</sub>, 150 mM NaCl, 0.05% (v/v) Brij-35 (Sigma-Aldrich) with cOmplete Tablets proteinase inhibitors (Sigma-Aldrich) at 4 °C overnight and clarified by centrifugation.

**Polymerase chain reaction.** Total RNA was extracted from subconfluent cultures with TRI Reagent (Sigma-Aldrich) according to the product protocol and transcribed into cDNA by SuperScript III First-Strand Synthesis System (Life Technologies). Semi-quantitative PCR reactions were conducted with AmpliTaq Gold DNA Polymerase (Life Technologies). Primers are described in the Supplementary Methods. The annealing temperature was 64 °C (*MMP8*) and 54 °C (*ACTB*).

**Quantitative RT-PCR.** Total RNA was isolated using TRIzol Reagent (Life Technologies) according to the manufacturer's protocol. First-strand cDNAs were synthesised from 2  $\mu$ g of DNase-treated RNA using the SuperScript II Reverse Transcription Kit (Life Technologies). Diluted cDNA (1 : 3) was used to perform qRT-PCR using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in a 7900 RT-PCR System (Applied Biosystems). Primers are described in the Supplementary Methods. The PCR cycles were 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each reaction was performed in

triplicate and individually analysed. The results were calculated by using the 2<sup>- $\Delta\Delta$ Ct</sup> relative quantification method.

**Western blot.** Media and cell proteins were analysed by western blot as described (Korpi *et al*, 2008) in reducing (vascular endothelial growth factor-C (VEGF-C), MMP-14, N-cadherin and vimentin antibodies) or non-reducing (all other antibodies) conditions. Where indicated, the conditioned medium (20  $\mu$ g protein) was incubated for 2 h at 37 °C with or without 1 mM 4-aminophenylmercuric acetate (APMA) before analysis. The primary antibodies were rabbit anti-MMP-8 #ABT38 (Millipore, Billerica, MA, USA), 1  $\mu$ g ml<sup>-1</sup>, mouse anti-TGF- $\beta$ 1 MAB240 (R&D systems), 2  $\mu$ g ml<sup>-1</sup>, mouse anti-E-cadherin #13-1700 (Invitrogen, Carlsbad, CA, USA), 1 : 1000, rabbit anti-Slug C19G7 (Cell Signaling Technologies Inc., Danvers, MA, USA), 1 : 1000, mouse anti-N-cadherin 3B9 (Invitrogen), 1  $\mu$ g ml<sup>-1</sup>, mouse anti-vimentin V9 (DAKO A/S, Glostrup, Denmark), 1 : 750, goat anti-VEGF-C AF752 (R&D Systems), 0.2  $\mu$ g ml<sup>-1</sup>, rabbit anti-MMP-1 AB8105 (Chemicon International, Temecula, CA, USA), 1  $\mu$ g ml<sup>-1</sup>, and mouse anti-MT1-MMP LEM-2/15.8 (Millipore), 1  $\mu$ g ml<sup>-1</sup>. Equal sample loading was controlled by Coomassie Blue staining of the gel before blotting (medium samples) or mouse  $\beta$ -actin ab8226 (Abcam, Cambridge, UK), 0.5  $\mu$ g ml<sup>-1</sup> immunoblotting (cell extracts). The secondary antibodies were rabbit anti-mouse antibody E0354, swine anti-rabbit antibody E0353 (DAKO A/S) and horse anti-goat antibody BA9500 (Vector Laboratories Inc., Burlingame, CA, USA), 1 : 1000. Pierce ECL Western Blotting Substrate (Thermo Scientific, Waltham, MA, USA) was used for visualisation. Band intensities were quantified by Fiji software (Schindelin *et al*, 2012).

**Radioimmunoassay.** 1.5  $\times$  10<sup>6</sup> control and MMP-8 + HSC-3 cells (three different cell passages) were seeded onto 55 cm<sup>2</sup> plates. The cells were washed with PBS and 6 ml of Opti-MEM (Life Technologies) was added and collected after 5 days. Equal volumes of the three media were pooled and concentrated (4 $\times$ ). Carboxyterminal telopeptide of type I collagen (ICTP) (Risteli *et al*, 1993) was measured using ICTP-RIA kit (Orion Diagnostica Oy, Espoo, Finland) according to the manufacturer's instructions.

**Organotypic 3D leiomyoma cell invasion assay.** Human leiomyoma tissue samples were obtained during routine surgeries after obtaining written consent of the donors. The study was approved by the Ethics Committee of the Oulu University Hospital (#35/2014, 04.01.2016). The invasion assays were performed as described (Nurmenniemi *et al*, 2009) using 7  $\times$  10<sup>5</sup> control and MMP-8 + HSC-3, SCC-25 and SCC-15 cells. After 14 days, the tissues were processed into histological samples (Teppo *et al*, 2013).

**Apoptosis and proliferation.** *In Situ* Cell Death Detection Kit, POD (Roche Diagnostics, Basel, Switzerland) was used according to the manufacturer's instructions. The cells were analysed by light microscope. Six samples of control and MMP-8 + HSC-3, SCC-25 and SCC-15 cells were analysed. Cell Proliferation ELISA, BrdU (colorimetric) kit (Roche Diagnostics) was used with 1  $\times$  10<sup>4</sup> control and MMP-8 + HSC-3 cells according to the manufacturer's instructions using eight technical replicates.

**Cell migration and movement analyses.** 4  $\times$  10<sup>4</sup> control and MMP-8 + HSC-3 cells were seeded into 24-well plates with ibidi inserts (ibidi GmbH, Martinsried, Germany). The next day the inserts were removed, the cells were washed with PBS and medium (1% FBS) was added. Phase-contrast time-lapse images were collected on an Olympus IX81 inverted microscope equipped with a 10 $\times$  /0.3 objective and a grey-scale camera (Olympus XM10, Germany). The cells were maintained at 37 °C and 5% CO<sub>2</sub> by a controlled microscope stage incubator (Okolab, Pozzuoli NA, Italy) mounted on the microscope. Images were acquired every 10 min for

12 h at multiple stage positions using a motorised stage (Prior) and Cell<sup>^</sup>P software (Soft imaging system GmbH, Münster, Germany). Fiji software (Schindelin *et al*, 2012) was used to measure wound areas. For analyses of cell movement, cells were segmented from the time-lapse images using the maximally stable extremal regions approach (Matas *et al*, 2004). The Kalman filter-based multi-object tracking approach (Huttunen and Heikkilä, 2008) was used for tracking of the segmented cells. New tracks were initiated in each frame throughout the sequence to allow quantification of dynamic events taking place between successive frames. The analyses were performed with MATLAB (MathWorks, Natick, MA, USA). The application of the combination of the segmentation and tracking approaches for cell segmentation from phase-contrast images has been described in detail previously (Kaakinen *et al*, 2014). Six samples were studied for each cell group.

**Transwell migration.** Transwell inserts (Corning Inc., Corning, NY, USA) were stabilised with 600  $\mu$ l medium for 1 h and  $5 \times 10^4$  control and MMP-8 + HSC-3 cells (two different clones) were seeded into the chambers in (0.5% lactalbumin) medium with and without 10 ng ml<sup>-1</sup> TGF- $\beta$ 1 (R&D Systems). Similarly, the effect of 50 ng ml<sup>-1</sup> VEGF-C (ProspecBio, Rehovot, Israel) was tested (one clone). After 24, 48 and 72 h, the inserts were fixed with 4% formaldehyde, washed with PBS and stained with 1% Toluidine Blue + 1% sodium tetraborate in ddH<sub>2</sub>O. After removing the excess dye with a cotton swab, the inserts were dipped in 1% SDS and the absorbances of three technical replicates were measured at 650 nm.

**Microarray.**  $9 \times 10^4$  control and MMP-8 + HSC-3 cells were seeded into six-well plates. The next day, three cultures per cell line were scratched throughout with a 1 ml pipette tip at ~2.5 mm intervals horizontally and vertically (migratory phenotype); three wells were left unwounded (stationary phenotype). Medium (1% FBS) was added and after 10 h RNA was extracted by Qiagen RNA kit (Qiagen, Düsseldorf, Germany). Equal amounts of each RNA from the three cultures were pooled. Affymetrix GeneChip Human Genome U133 Plus 2.0 Arrays were used and the experimental procedures were performed according to the Affymetrix GeneChip Expression Analysis Technical Manual using 1  $\mu$ g of total RNA as template (see Supplementary Methods for more details). Finally, the arrays were scanned on a GeneChip Scanner 3000. dChip software (Li and Wong, 2001) was used for expression analyses (GEO, GSE85435) and DAVID 6.7 for Gene Ontology analyses (Dennis *et al*, 2003).

**Zymography.** Gelatin zymography was performed as described (Pirilä *et al*, 2007) with three different passages of control and MMP-8 + HSC-3 and SCC-25 cells.

**TGF- $\beta$ 1 ELISA.** Human TGF-beta-1 Quantikine ELISA Kit (R&D Systems) was used according to the kit protocol to measure TGF- $\beta$ 1 levels in the conditioned media of control and MMP-8 + HSC-3 cells ( $\pm$  acidic activation).

**Patient samples.** Paraffin-embedded archival resection specimens of OTSCC ( $n=57$ ) were obtained from the Department of Pathology, Oulu University Hospital. The patients had been diagnosed between the years 1984–2001. The survival data were acquired from Statistics Finland and other relevant data from the patient records. The baseline characteristics of the patients are presented in Supplementary Table S1. We were unable to retrieve treatment data of three patients and follow-up data of one patient. The use of the patient samples and the data inquiry were approved by the Ethics Committee of the Oulu University Hospital (#49/2010, 16.08.2010) and by the National Supervisory Authority for Welfare and Health, Valvira (6865/05.01.00.06/2010, 05.10.2010).

**Orthotopic mouse model of OTSCC.** Cultured parental, control and MMP-8 + HSC-3 cells were detached and washed with medium. The cell suspensions in cold serum-free DMEM (Sigma-

Aldrich) were mixed with cold Matrigel 1:1 to a final concentration of  $8 \times 10^6$  cells ml<sup>-1</sup>. Seven-week-old BALB/c nude male mice (Charles River, Germany) were anaesthetised with a mixture of 0.5 mg ketamine and 0.1 mg xylazine hydrochloride subcutaneously before 25  $\mu$ l of cell suspension ( $2 \times 10^5$  cells) was injected into the lateral part of the tongue ( $n=12$  for each of the cell clones). The mice were weighed daily and killed with CO<sub>2</sub> if they lost more than 10% of their body weight or 13 days after the injection. Tongues and draining lymph nodes were fixed in 4% formalin immediately after removal. For positron emission tomography (PET) scanning, eight ( $\pm 1$ ) days after injections, three mice from parental and five mice from control and MMP-8 + groups (18.5 g  $\pm$  1.8 g) were used. While kept under constant anaesthesia, the mice were positioned with the head in the centre of the field of view inside the Triumph LabPET-8<sup>TM</sup> small animal PET/SPECT/CT scanner (Trifoil Imaging Inc., Chatsworth, CA, USA). More details of PET imaging are described in Supplementary Methods. All experiments complied with the ARRIVE guidelines and were conducted in accordance with the European Convention for the Protection of Vertebrate Animals for Experimental and Other Scientific Purposes' guidelines on accommodation and care of animals. The experiments were also approved by the Animal Welfare Committee at UiT-The Arctic University of Norway.

**Histology and immunohistochemistry (IHC).** Leiomyoma (6  $\mu$ m) and mouse tissue (4  $\mu$ m) samples were stained with Mayer's H&E. The lymph nodes were investigated for metastatic foci at 100  $\mu$ m intervals. Immunohistochemistry with mouse anti-pancytokeratin AE1/AE3 antibody (Dako A/S), 1:150, was performed on leiomyoma tissue to identify carcinoma cells. Invasion was quantified as described (Nurmeniemäki *et al*, 2009) using Fiji (Schindelin *et al*, 2012). The IHC staining of mouse anti-Ki-67, NCL-L-Ki67-MM1 (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK), 1:100, mouse anti-E-cadherin #13-1700 (Invitrogen), 1:300, mouse anti-N-cadherin 3B9, (Invitrogen), 1:200, and mouse anti-vimentin V9 (DAKO A/S, 1:1500, were performed similarly to pancytokeratin staining. IHC staining was done with a Dako Autostainer except for Ki-67 samples, which were incubated with primary antibody at 37 °C for 30 min followed by incubation at 4 °C overnight. Vectastain Elite ABC kit (Vector Laboratories Inc.) was used with the biotinylated secondary antibodies. DAB was used as a chromogen and the samples were counterstained with Mayer's haematoxylin. For staining of the mouse tumours, a heat-induced antigen retrieval (10 mM citrate buffer pH 6.0) was performed. After blocking endogenous peroxidase activity (Peroxidase block, Dako, EnVision + system) and non-specific protein binding with normal goat serum, sections were incubated 1 h at room temperature with rabbit anti-MMP-8 monoclonal antibody EP1252Y (Abcam), 1:100. HRP-labelled secondary antibody and DAB were used for visualisation (EnVision + system-HRP for rabbit primary antibodies) before counterstaining with Harris Haematoxylin. Neutrophils were used as an internal positive control. The patient samples were selected on the basis of H&E staining to be representative for the tumour mass in the resected specimen. Matrix metalloproteinase-8 (Hanemaaijer *et al*, 1997) immunostaining was performed as described (Korpi *et al*, 2008). VEGF-C staining was performed using goat anti-VEGF-C antibody AF752 (R&D Systems), 1:50. High-temperature antigen retrieval was performed in Tris-EDTA buffer for 15 min followed by Goat on Rodent HRP-Polymer (Biocare Medical, Concord, CA, USA) and with DAB as a chromogen. The samples were mounted in Glycergel (Dako A/S) or Aquamount (BDH Laboratory Supplies). Negative controls were omission of the primary antibody or replacement with an antibody isotype control.

**Evaluation of IHC of OTSCC patient sample.** Two independent researchers (JHK, TS), blinded from clinical data, evaluated the

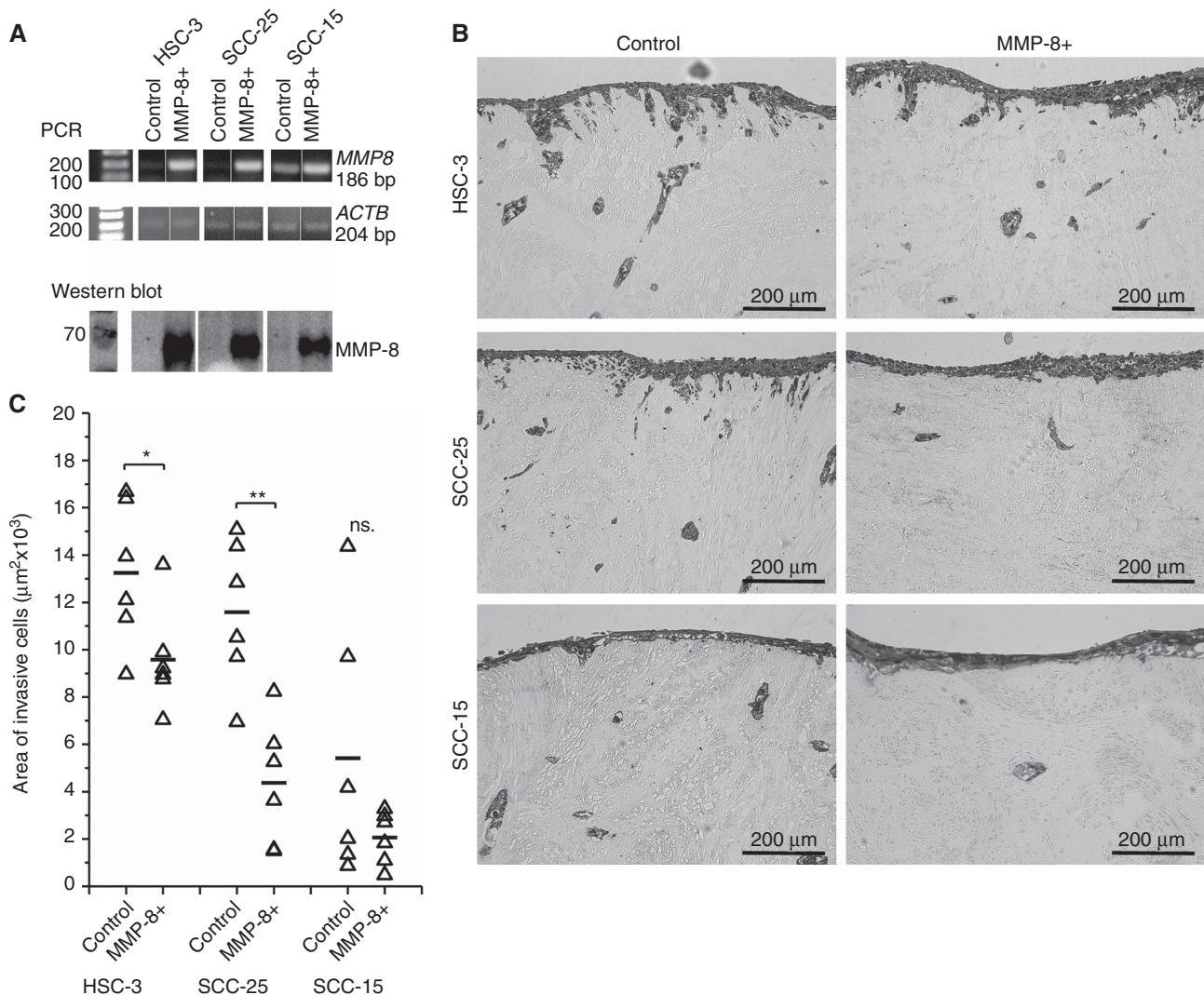
immunostained sections. The full evaluation of MMP-8 staining was described earlier (Korpi *et al*, 2008). The evaluation of VEGF-C staining was performed using a two-point scale similar to that previously described (Shih *et al*, 2000; Shintani *et al*, 2004). Briefly, the percentage of positively stained cancer cells was counted and the cases were considered VEGF-C positive if more than 80% of the cells had positive cytoplasmic staining.

**Statistical analysis.** SPSS 18.0 software for Windows (SPSS Inc., Chicago, IL, USA) was used and a  $P$ -value  $< 0.05$  was considered statistically significant (indicated in the diagrams as  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ ). Student's  $t$ -test and Mann-Whitney  $U$  test were used for comparisons of two independent groups and repeated measures ANOVA for dependent values at various time points. Pearson Chi-square test was used to calculate statistically significant differences between prognostic and clinicopathological variables. A correlation coefficient between MMP-8 and VEGF-C expression was calculated with Pearson's correlation coefficient. Life tables were calculated according to the Kaplan-Meier method. Survival curves were compared with the log-rank test. Uni- and

multivariate survival analyses were done with the Cox proportional hazards model using the following covariates: VEGF-C and MMP-8 co-expression (MMP-8 - / VEGF-C + or other), gender, age at the time of diagnosis ( $< 55$ ,  $55-70$  and  $> 70$  years), tumour stages (1-2 and 3-4), tumour histologic grades (1, 2 and 3) and adjuvant therapy (no adjuvant or radiotherapy). Multivariate analysis was done using backward stepwise selection of variables. A  $P$ -value of 0.05 was adopted as the limit for inclusion of a covariate. The backward stepwise algorithm was used to choose the best combination of prognostic factors to explain the mortality or recurrences in the study population. The hazard ratios (HRs) and 95% CIs are provided for each covariate.

## RESULTS

**Overexpression of MMP-8 decreases invasion.** Overexpression of *MMP8* was confirmed by PCR and western blot (Figure 1A) and routinely checked. In case of HSC-3 cells, clone #1 showed higher



**Figure 1.** MMP-8 reduced the invasion of OTSCC cells. *MMP8* gene expression and  $\beta$ -actin (*ACTB*) were measured from the same RT reaction samples. Overexpression of pro-MMP-8 was routinely confirmed by western blot from 20  $\mu$ g of conditioned media (representative result shown) (A). Organotypic 3D leiomyoma tissues were used to analyse cell invasion (B). The areas of invasive cytokeratin positive cells were quantified with Fiji software (C).  $*P < 0.05$ ,  $**P < 0.01$ , n.s. = not significant. PCR was performed using samples from three different individual experiments (representative results shown). The myoma experiment was performed with duplicate leiomyoma disks per culture condition; three sections per disk were used in analyses.

MMP-8 expression (Supplementary Figure S1A) and was used in experiments unless otherwise mentioned. Endogenous MMP8 expression was detected only in SCC-15 (Figure 1A). Based on protein size ( $\sim 70$  kDa), the detected band represents pro-MMP-8. The active form of MMP-8 was detected in conditioned medium after treatment with APMA (Supplementary Figure S1B). The activation in cell culture conditions presumably occurs only temporally and locally. The overexpressed MMP-8 was functionally active, as demonstrated by radioimmunoassay with a higher concentration of the type I collagen degradation product ICTP ( $0.63 \mu\text{g l}^{-1}$ ) in ( $4 \times$  concentrated) MMP-8 + conditioned medium compared to the control ( $< 0.25 \mu\text{g l}^{-1}$ ). The invasion of MMP-8 + HSC-3 ( $P = 0.037$ ) and SCC-25 ( $P = 0.001$ ) was reduced compared to controls (Figure 1B and C).

**MMP-8 impairs migration.** MMP-8 did not promote apoptosis (Supplementary Figure S2A). Among the epithelial to mesenchymal markers, only vimentin expression was slightly decreased in MMP-8 + HSC-3 cells (Supplementary Figure S2B). The change was also observed in the invasive areas of MMP-8 + cells in leiomyoma invasion assays (Supplementary Figure S2C). In further analyses, the two aggressive cell lines (HSC-3 and SCC-25) were used. Matrix metalloproteinase-8 significantly suppressed the migration of HSC-3 cells ( $P = 0.015$ ; Figure 2A and B) by reducing both the number of moving cells ( $P = 0.015$ ) and the magnitude of cell movement towards the empty area ( $P = 0.001$ ; Figure 2C). The proliferation of MMP-8 + HSC-3 cells showed no difference (Supplementary Figure S2D; Ki-67 staining not shown).

**MMP-8 affects the gene expression of various factors.** To examine the overall effects of MMP-8, we compared the gene expression of stationary and migrating control and MMP-8 + HSC-3 cells. The 20 most upregulated and downregulated genes and the genes that were changed only in stationary or migrating cells are shown in Supplementary Table S2 (fold change  $\geq 1.5$ ). Supplementary Table S3 lists the genes involved in various cancer-related events, including *VEGFC*, *MMP1* and *MMP9*.

**MMP-8 changes the proteinase web.** We confirmed the decreased expression of MMP-1 and increased expression of MMP-9 at the protein level in HSC-3 and SCC-25 cells (Figure 3A and B). MMP-8 + HSC-3 cells also expressed significantly ( $P = 0.002$ ) lower levels of *CTSK* than control cells. Consistently, shCat-K HSC-3 cells expressed significantly ( $P < 0.001$ ) increased levels of *MMP8* compared to controls (Figure 3C). The expression of MMP-14 (MT1-MMP) was not changed (Figure 3D).

**MMP-8 impairs VEGF-C expression and serves as prognostic tool together with VEGF-C.** TGF- $\beta$ 1 treatment increased VEGF-C levels approximately 2.5-fold in HSC-3 control cells. However, in only one of the three experiments with corresponding MMP-8 + HSC-3 cells was a slight increase observed (Figure 4A). This difference between control and MMP-8 + cells was not observed in HSC-3 clone #2 probably due to its low MMP-8 overexpression (not shown). VEGF-C treatment did not revert the impaired migration of MMP-8 + HSC-3 cells (Transwell migration, not shown). The impaired (or even diminished) VEGF-C expression in TGF- $\beta$ 1-treated MMP-8 + cells compared to control cells prompted us to examine the association of the two proteins in human OTSCC samples. We found that low expression of MMP-8 strongly correlated with high expression of VEGF-C ( $\rho = 0.556$ ,  $P < 0.001$ ). Both high VEGF-C and low MMP-8 expression levels associated with shortened cancer-specific survival ( $P = 0.001$  and  $P = 0.01$ , respectively). However, the combined MMP-8 - /VEGF-C + -status was an even stronger predictor for poor cancer-specific survival ( $P < 0.001$ ; Figure 4B). In multivariate analysis, the combined MMP-8 - /VEGF-C + status was an independent predictor of cancer-specific deaths, with an HR of 7.157 (95% confidence intervals (CIs) CI 2.219–23.082], along with high

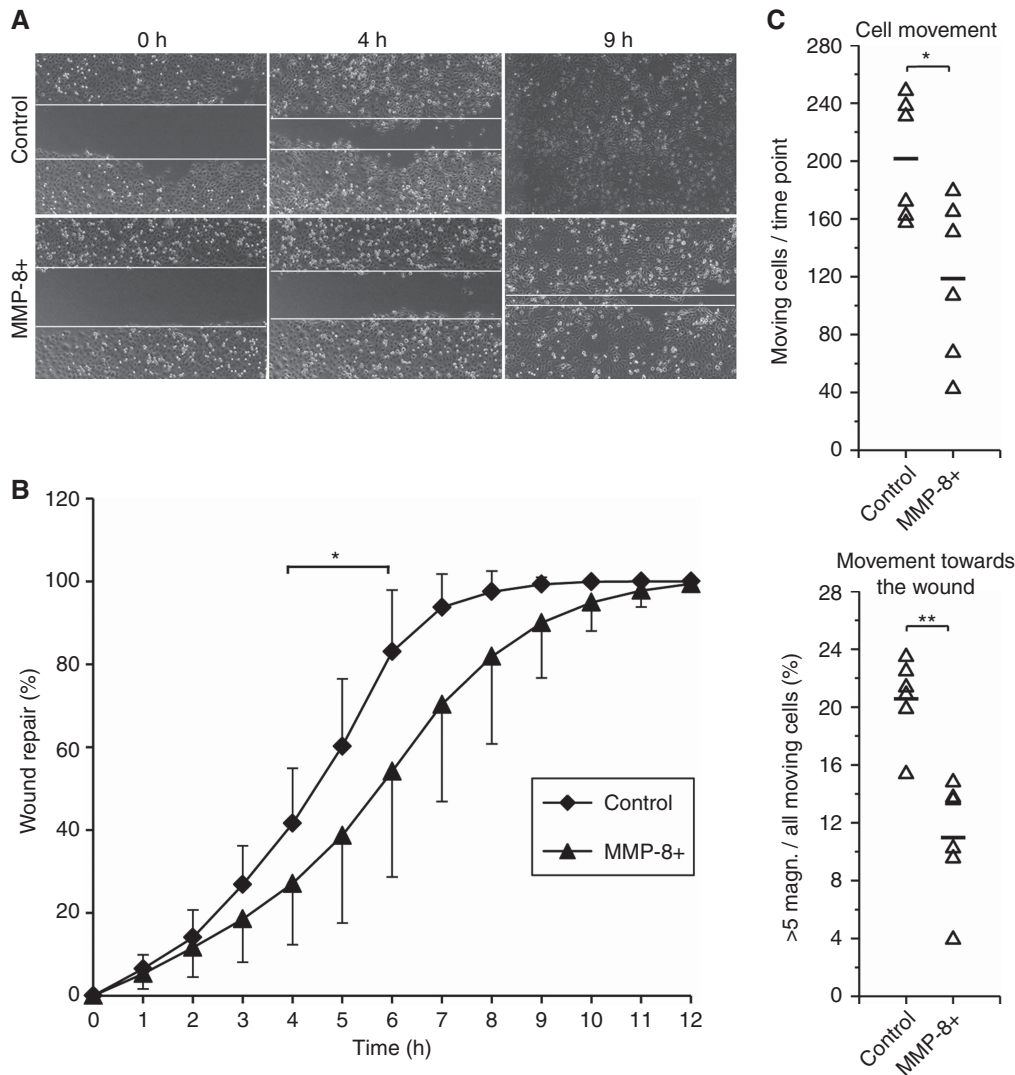
cancer stage and age over 70 at the time of the diagnosis (Figure 4C). Matrix metalloproteinase-8 and VEGF-C did not correlate with clinicopathological variables, except high VEGF-C expression was associated with lymph node metastasis (Supplementary Tables S1 and S4).

**MMP-8 impairs migration via TGF- $\beta$ 1 interference.** Since TGF- $\beta$ 1 did not induce VEGF-C expression in MMP-8 + HSC-3 cells as observed in control HSC-3 cells, we next examined its function in MMP-8 + cells. The relative level of the active TGF- $\beta$ 1 was slightly reduced (n.s.) in MMP-8 + cells (Figure 5A). A transwell assay confirmed the impaired migration of MMP-8 + HSC-3 cells ( $P = 0.001$  after 48 h and  $P = 0.017$  after 72 h, Figure 5B), which was also verified in clone #2 (Supplementary Figure S3). TGF- $\beta$ 1 treatment increased the migration of MMP-8 + HSC-3 cells, restoring the migration to the level of control cells after 72 h (Figure 5B). After 24 h, both cell lines migrated more in the presence of TGF- $\beta$ 1, but the difference in response to TGF- $\beta$ 1 between the cell lines was not definite at that time point (data not shown). Differential effect of TGF- $\beta$ 1 between control and MMP-8 + cells was observed in similar experiment performed with clone #2 (Supplementary Figure S3). However, in this clone the effect was not as clear as in clone #1 possibly due to substantially lower expression of MMP-8. The statistical analysis combining the average results of the different clones in transwell experiments or for other assays with two clones were not performed, because the resulting sample number would have been only two.

**MMP-8 vanishes in tumour-stroma interface in mouse OTSCC.** Lastly, we investigated whether overexpression of MMP-8 in HSC-3 cells would reduce metastases in mouse OTSCC. Tumour take was 100% for all cell clones. The cell suspension was injected into the left side of the mouse tongue, confirmed by PET-imaging (Supplementary Figure S4A upper panel, more details in Supplementary Results, Supplementary Figure S4B and C). Cancer cells in tumours from the parental (Supplementary Figure S5A and B) and the control cell line (Supplementary Figure S5C and D) did not show any cytoplasmic MMP-8 staining. In the tumour islands with MMP-8 + cells, cytoplasmic staining of MMP-8 was detected mainly in the most keratinised cells located in the centre of the tumour. In the tumour-stroma interface, the cancer cells showed weak or absent cytoplasmic MMP-8 staining (Supplementary Figure S5E and F). From the 13 mice killed at time point 8–9 days, nine mice had lymph node metastases (included in the comparisons of the number of mice with and without metastases) (Supplementary Figure S6A and B). No significant difference in metastases was found between the groups (Supplementary Figure S6A–S6C).

## DISCUSSION

Our study provides evidence for the tumour-suppressive mechanisms of MMP-8 in OTSCC by interplay with TGF- $\beta$ 1 and VEGF-C. Previously identified protective actions of MMP-8 in cancers include the regulation of cell adhesion (Gutiérrez-Fernández *et al*, 2008), inactivation of  $\beta$ 1-integrins (Pellinen *et al*, 2012), induction of interleukins 6 and 8 (Thirkettle *et al*, 2013) and inhibition of TGF- $\beta$ 1 activity by decorin cleavage (Soria-Valles *et al*, 2014). The invasiveness of the cell lines used in this study corresponded to the previously reported differences in their aggressiveness (HSC-3 > SCC-25 > SCC-15) (Scott *et al*, 1988; Ramos *et al*, 1997). Consistent with the previous studies (Agarwal *et al*, 2003; Montel *et al*, 2004), we found that MMP-8 reduces the invasion and migration of carcinoma cells. In melanoma and lung carcinoma, MMP-8 also prevented metastases (Gutiérrez-Fernández *et al*, 2008). However, in the orthotopic mouse tongue



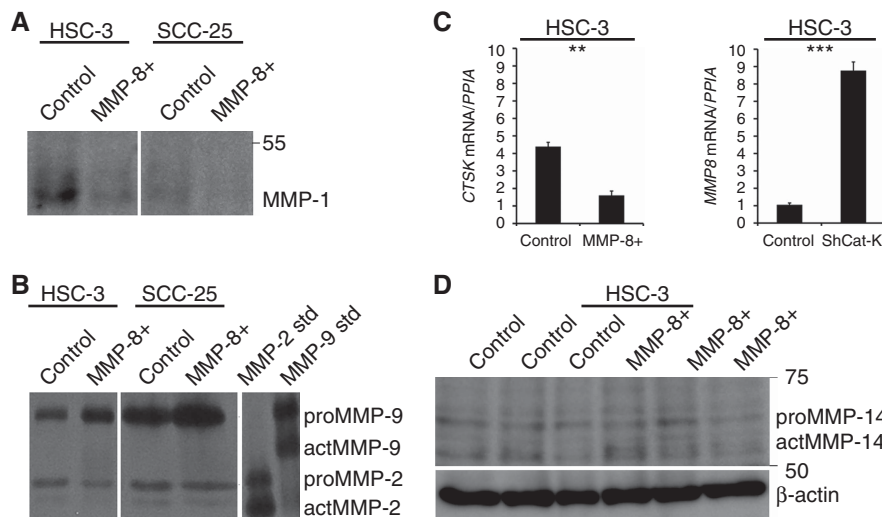
**Figure 2.** MMP-8 reduced the migration of HSC-3 cells. After removing the ibidi inserts, the migrating cells were photographed for 12 h (10 min intervals) (A). The open areas were measured using Fiji software and the results were calculated as a percentage of area closure (mean and standard deviation are shown) (B). Phase contrast time-lapse images were acquired at multiple stage positions using a motorised stage with Cell<sup>^</sup>P software and the results were analysed using a Kalman filter-based tracking approach with MATLAB (C). \* $P < 0.05$ , \*\* $P < 0.01$ . Six replicates were analysed for each cell group in all experiments.

carcinoma model, the expression of MMP-8 almost completely vanished in the tumour periphery. The cells with low MMP-8 expression may have invasion and growth advantages *in vivo*, causing a shift in MMP-8 expression. Moreover, the complex *in vivo* setting in the tumour microenvironment may modulate the effects of MMP-8 via the presence of regulatory factors, such as tissue inhibitors of metalloproteinases. Downregulation of MMP-8 in the tumour-stroma interface may have countered the possible oncosuppressive effects of MMP-8 *in vivo*, as the cells in the tumour periphery in particular contribute to invasion and metastasis.

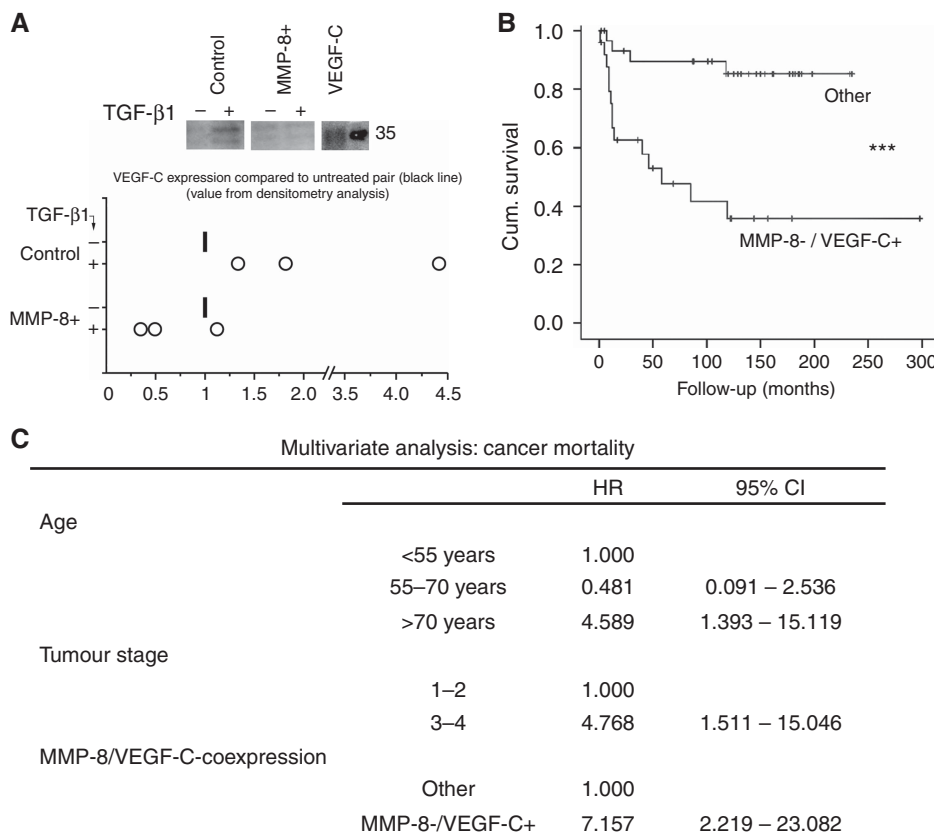
Matrix metalloproteinase-8 affects the level of MMP-3 in breast carcinoma (Decock *et al*, 2015). In OTSCC cells, MMP-8 overexpression decreased the expression of MMP-1, a tumour-promoting proteinase in OSCC (Sutinen *et al*, 1998; Kurahara *et al*, 1999; Jordan *et al*, 2004; Shimizu *et al*, 2008; George *et al*, 2010). Interestingly, MMP-9 expression was increased in MMP-8 + OTSCC cells. MMP-9 has both pro- and anti-tumourigenic roles in OSCC (Vilen *et al*, 2013). MMP-9 possesses antiangiogenic functions (Cornelius *et al*, 1998; Hamano *et al*, 2003; Bendrik *et al*, 2008) and is a negative prognostic factor when expressed by

stromal cells but is a positive prognostic factor when expressed by cancer cells (Pellikainen *et al*, 2004; Mylona *et al*, 2007). The decreased expression of Cat-K in MMP-8 + HSC-3 cells is consistent with our previous study showing reduced invasion in shCat-K HSC-3 cells (Bitu *et al*, 2013). The mutual regulatory pathways between Cat-K and MMP-8 are unknown. Shared substrates, such as TGF- $\beta$ 1 (Zhang *et al*, 2011; Åström *et al*, 2014), may participate in regulating proteinases. TGF- $\beta$ 1 downregulates Cat-K (van den Brùle *et al*, 2005); both negative (Palosaari *et al*, 2000; Moilanen *et al*, 2002; Åström *et al*, 2014; Soria-Valles *et al*, 2014) and positive (Wen *et al*, 2015; Qin *et al*, 2016) regulatory pathways between MMP-8 and TGF- $\beta$ 1 have been reported. Our study provides further evidence for the mutual contribution of MMP-8 and TGF- $\beta$ 1 in cancer. Although a decreased level of active TGF- $\beta$ 1 in MMP-8 + HSC-3 cells was not statistically significant, it may be responsible for their reduced migration.

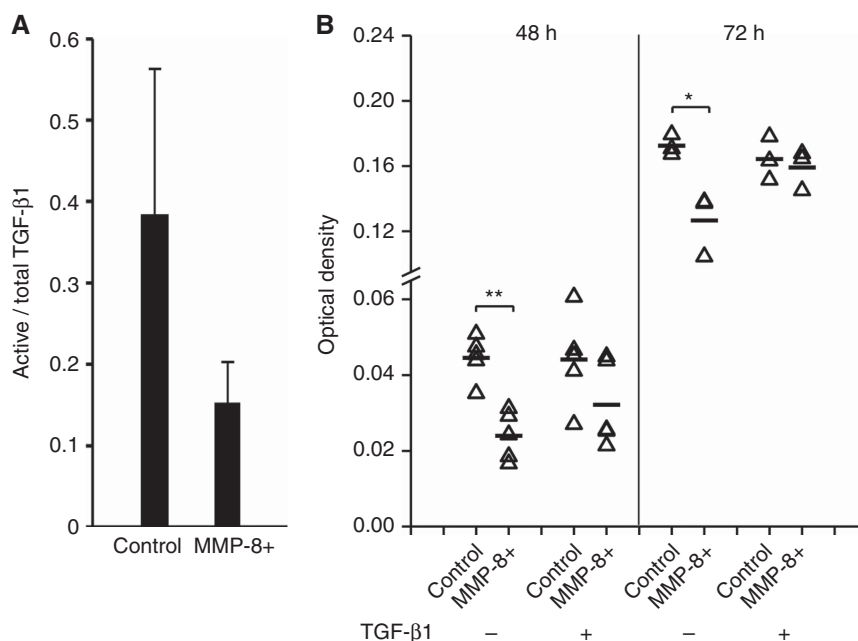
In macrophages, TGF- $\beta$ 1 induces the expression of VEGF-C (Cursiefen *et al*, 2011), a tumour-promoting molecule involved in lymphangiogenesis (Karaman and Detmar, 2014). Our finding that MMP-8 attenuates TGF- $\beta$ 1 effects and blocks subsequent



**Figure 3.** MMP-8 affected the expression levels of various proteinases. MMP-8 + HSC-3 and SCC-25 cells expressed less MMP-1 than controls cells analysed by western blot from 20 μg of conditioned media (A). The level of MMP-9 (but not MMP-2) was increased in conditioned media of MMP-8 + HSC-3 and SCC-25 cells analysed by zymography (B). Cathepsin-K (CTSK) mRNA levels were analysed from MMP-8 + and control HSC-3 cells, and *MMP8* mRNA levels from cathepsin K-silenced and control cells using real-time quantitative PCR (qRT-PCR). The Y axis shows the ratio of CTSK or MMP-8 mRNA levels to the peptidylprolyl isomerase (PPIA) mRNA level (with standard deviations) (C). The level of MMP-14 was not changed in MMP-8 + HSC-3 cells as analysed by western blot from 40 μg of cell protein extract (D). MMP-1 western blots and gelatin zymography were performed twice from two different individual experiments with HSC-3 and SCC-25 cells and qRT-PCRs in triplicates from two individual experiments. For MMP-14 western blot, three individual experiments of HSC-3 cells were analysed. \*\**P* < 0.01, \*\*\**P* < 0.001.



**Figure 4.** MMP-8 overexpression blocked TGF-β1-induced VEGF-C expression and low MMP-8/high VEGF-C associated with poor prognosis of OTSCC patients. VEGF-C was analysed by western blot from equal volumes of conditioned media (three individual experiments with different cell passages) with and without 10 ng ml<sup>-1</sup> TGF-β1. Representative samples are shown. The values from densitometer analyses (Fiji) were adjusted to the protein concentration of each media (A). VEGF-C and MMP-8 in OTSCC samples (*n* = 57) were detected by immunohistochemistry. The combined MMP-8 – /VEGF-C + -status was highly predictive for poor cancer-specific survival as analysed by Kaplan–Meier method and compared with the other combinations (MMP-8 + /VEGF-C +, MMP-8 + /VEGF-C – or MMP-8 – /VEGF-C –) using the log-rank test (*P* < 0.001) (B). Multivariate analysis was done using backward stepwise selection of variables, and a *P*-value of 0.05 was adopted as the limit for inclusion of a covariate. HRs, hazard ratios; CI, 95% confidence interval (C). \*\*\**P* < 0.001, log-rank test.



**Figure 5. TGF- $\beta$ 1 restored the reduced migration of MMP-8 + HSC-3 cells.** ELISA was used to examine TGF- $\beta$ 1 (active/total) in the conditioned media of MMP-8 + HSC-3 and control cells. TGF- $\beta$ 1 concentrations were calculated per sample protein concentration and the percentage of the active form was calculated. The bars represent standard deviation (A). Recombinant human TGF- $\beta$ 1 ( $10 \text{ ng ml}^{-1}$ ) was added to the serum-free medium in the upper Transwell chamber and the amount of migrated MMP-8 + HSC-3 and control cells was measured as absorbance at 650 nm (B). \* $P < 0.05$ , \*\* $P < 0.01$ . ELISA assay was performed using samples from three individual experiments and Transwell assay using five (48 h) and three (72 h) samples.

VEGF-C expression in OTSCC cells is corroborated by the negative association of MMP-8 and VEGF-C in human OTSCC samples. In line with previous studies (O-charoenrat *et al*, 2001; Yanase *et al*, 2014), VEGF-C expression was associated with lymph node metastasis. Low MMP-8 and high VEGF-C have been separately linked to more aggressive OTSCC (Korpi *et al*, 2008; Siriwardena *et al*, 2008; Yanase *et al*, 2014), but the association of MMP-8 and VEGF-C and the strong and independent prognostic value of their combination have not been described before in OTSCC.

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#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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