MMP16 mediates a proteolytic switch to promote cell-cell adhesion, collagen alignment, and lymphatic invasion in melanoma

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Supplementary Data

Supplementary Materials and Methods

Antibodies

Following antibodies were used: mouse antibodies against MMP14 catalytic domain (LEM2-15/8, Millipore), L1CAM ectodomain (Millipore and Covance), N-cadherin (Pharmingen), podoplanin (D2-40, Abcam), HA-Tag (6E2, Cell Signaling), human CD31 and CD34 (Dako), CD44 (Santa Cruz), GAPDH (Sigma); rabbit antibodies against L1CAM C-terminus (LSBio), mouse Lyve-1 (1), mouse collagens I and IV (Millipore), MMP16 (Abcam), MMP1 (Millipore), ZO-1 (Invitrogen), S100B (Dako), ADAM10, ADAM17 (Abcam), EphA2 (SantaCruz), and fibrinogen (Dako); goat antibodies against human collagen I (Millipore), VE-cadherin (Santa-Cruz) and L1CAM cytosolic domain (Santa Cruz); rat anti-mouse CD31 (BD Biosciences). Phalloidin-TRITC was from Sigma.

qPCR

The cDNAs were amplified on GeneAmp 7500 Sequence Detector thermal cycler (Applied Biosystems) using TaqMan Universal PCR Master Mix and validated primers (*MMP14*, Hs01037006_gH; *MMP16*, Hs00234676_m1; *L1CAM*, Hs01109748_m1; human col1A1, Hs00164004_m1; mouse col1A1, Mm00801666_g1; mouse col3A1, Mm01254476_m1; Applied Biosystems). The expression levels were normalized with *TBP* or *GAPDH*.

Clinical melanoma samples

Whole tumor sections or biopsies of archival paraffin-embedded human melanomas removed for diagnostic purposes during years 2008-2009 (n=10) and 2013 (n=9) were obtained from the Skin and Allergy Hospital of Helsinki University Central Hospital. Benign nevi were kindly provided by Dr. Paula Kujala, Fimlab, Tampere, Finland. The tissue for RNA isolation from clinical samples was obtained using 2 mm punch biopsy from the areas containing predominantly melanoma cells or nevus melanocytes without connective tissue or normal skin. Breslow thickness and information on metastases were obtained from the diagnostic pathology reports. Lymphatic invasion was assessed from tumor sections stained for podoplanin (D2-40) and S100B from the samples with whole tumor section available for representative immunohistochemistry. Collagen infiltration was defined as diffuse invasion of cell chains or single cells between fragmented and disordered collagen structures in the deeper dermis at the invasive edge of the tumor, as contrasted by expansive cell nest growth pushing the dermis in non-infiltrative tumors, and was assessed from HE- and Herovici-stained paraffin sections.

siRNA and cDNA constructs

Small interfering RNAs targeting *MMP16* (SI00083006, MMP16sIR1Q-1), *MMP14* (SI03648841, MMP14sIR1Q), *L1CAM* (SI00009275, SI00009282), *ADAM10* (SI00022029), *ADAM17* (SI02664501), *MMP1* (pool of SI00037492, SI03021802, SI03033772, SI05160141), *MMP2* (pool of SI02780666, SI02781079, SI00064974, SI00064995), and non-silencing control siRNA (siCtrl, Qiagen), and SMARTpool siRNA targeting *MMP14* (M-004145-00-0005, Dharmacon) were used. Short hairpin RNA (shRNAs; Open Biosystems) targeting *MMP16* (shMMP16-1, TRCN0000052249 and shMMP16, TRCN0000052250) or non-targeting scrambled shRNA were also used. shMMP16 refers to shMT3-2 in reference (2). *L1CAM* (Open Biosystems) was cloned to pCR3.1 vector. Expression vectors for *MMP16*, *MMP14* (2), and *L1CAM* were transfected using Fugene (Roche). Knockdown and overexpression efficiencies

were assessed by immunoblotting and qPCR (2). To generate stable cell pools, transfected Bowes cells were selected using G418 (400 ug/ml; Calbiochem). Rescue constructs for MMP16 with double silent mutations in shMMP16 target sequence MMP16-r1 (T822C-T825C) and MMP16-r2 (T831C-A836C) were generated with primers (5'CTACAGTGAATTAGAAAATGGCAAACGCGACGTGGATATAACC'3 and 5'GGTTATATCCACGTCGCGTTTGCCATTTTCTAATTCACTGTAT'3) and (5'GGCAAACGTGATGTGGACATCACCATTATTTTTGCATCTGG'3 and 5'CCAGATGCAAAAATAATGGTGATGTCCACATCACGTTTGCC'3), respectively, using QuikChangeH Site-Directed Mutagenesis Kit (Stratagene).

Immunohistochemistry

Paraffin-embedded xenografts and archival human melanoma sections were subjected to immunohistochemistry. Tissue sections were deparaffinized in TissueClear (Tissue-Tek) and rehydrated in graded ethanol series. After antigen retrieval by sodium citrate treatment (10 mM sodium citrate, 0.05% Tween-20, pH 6), sections were incubated for 10 min in 0.6% (v/v) hydrogen peroxide, and with 2.5% normal horse serum (blocking buffer, Vector Laboratories) for 30 min. Primary antibodies were incubated o/n, followed by incubation with peroxidase-conjugated secondary antibodies for 30 min and detection using Vectastain DAB kit. Alternatively, TSA™ Biotin System Kit (PerkinElmer) was used. After washes the slides were mounted using Cytoseal mounting medium (Thermo Scientific), and photographed using Leica DM LB microscope. Cells and frozen sections (20 μm) were subjected to immunofluorescence using Alexa Fluor 488 and 594 secondary antibodies (3).

Quantification of immunohistochemistry and immunofluorescence images

For quantification of percentage of lymphatic or blood vessels containing tumor cells in mouse xenografts and human samples, the number of intratumoral and peritumoral vessels containing tumor cells was quantified from whole tumor sections, one-three sections per tumor, stained with

CD31, LYVE-1 or podoplanin (D2-40), and MMP14 or S100B, and divided by total number of respective vessels in the same tumor section. For quantification of collagen content, color masks recognizing red pixels from the images of Herovici-stained sections were created using Corel Photo-Paint software. The coverage of red area/microscopic field was then quantified using ImageJ software. For quantification of lymphatic vessel density (LVD) or blood vessel density (BVD), coverage of CD31/CD34- or podoplanin (D2-40) immunohistochemistry staining/microscopic field was calculated. Coverage of fibrinogen, S100B and L1CAM immunohistochemistry staining were calculated in the same manner. Briefly, the image pixels were classified in three different colors: red, blue and white. The coverage is expressed as the ratio of red pixels in the image. For quantification of MMP14 staining saturation, the images were white balanced and converted to HSV color space. The hue values were discretized by using k-means clustering (k=3) for each image individually. The center with hue nearest to zero (red) was selected as the object-of-interest color. The staining saturation is expressed as the hue independent color difference from white. Derived from HSV values, the difference is defined as: $E = ((1-V)^2 + S^2)^{1/2}$. Both of the quantifications were implemented in the freely available workflow framework Anduril (4). For quantification of melanoma cells inside endothelial spheroids, a spheroid boundary was determined by finding the convex hull around the endothelial cells for each confocal slice using Anduril software. Melanoma cells were determined based on green fluorescent signal. The melanoma cells were then counted and classified as internal or external to the spheroid. The result is presented as the average number of melanoma cells inside the spheroid quantified from 20 slices with the biggest spheroid area. All image analysis methods are available as a part of the freely available Anduril software.

In vitro morphology, proliferation, migration, and invasion assays

For cell morphology assessment, 5.000 melanoma cells/well in 24-well-plate overlaid with 1.7 mg/mL bovine collagen or 3 mg/ml fibrin were fixed after 2 days or 5 days, respectively, and photographed using Axiovert microscope (Zeiss; 2). For proliferation assay, cells were

quantified using BioRad TC10 Automated Cell Counter. For wound healing, WM852 monolayers were scratched, and the distance migrated by cells was measured. Collagen invasion assay was performed as described (5).

Kaplan-Meier survival analysis

The clinical and segmented copy-number data for 192 skin cutaneous melanoma samples were downloaded from The Cancer Genome Atlas (TCGA, 6). The duplicated and metastasis samples as well as the samples with T0 stage were removed. Survival times were calculated as the days between diagnosis and date of death, or censored at the time of last follow-up. The samples had been preprocessed by TCGA (6, 7). The gain threshold was calculated as the median of all segment means plus one standard deviation over all segment means in all samples. Significance was calculated with Logrank test.

Cell surface biotinylation

Cell surface biotinylation was performed as described (2). Briefly, cells were rinsed twice with PBS and incubated with 0.5 mg/ml biotin (Pierce) in PBS on ice for 1 h. The reaction was terminated by washing 3 times for 10 min with 150 mM glycine/TBS. The cells were then lysed and subjected to immunoprecipitation with rabbit polyclonal MMP16 antibody and G protein sepharose (GE Healthcare). The immunoprecipitated material was resolved by SDS-PAGE, and detected with horseradish-peroxidase-conjugated streptavidin (Dako).

References

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Supporting figure legends

Figure S1. *MMP14* which is overexpressed in melanoma cell lines was not associated with patient survival. (A) *MMP14* mRNA is highly expressed in melanoma cell lines as compared to other cancer cell types (8; www.oncomine.org). *, $p = 2.74x10^{-17}$. (B) *MMP14* mRNA expression in primary melanomas was plotted against overall melanoma patient survival (9; www.oncomine.org; median follow-up time, 40 months; $n = 81$). (C) *MMP14* mRNA expression in melanoma metastases was plotted against overall melanoma patient survival. The outlier line (90%) marks significant *MMP14* overexpression (10; $p = 0.0001$; www.oncomine.org; median follow-up time, 20 months; $n = 44$).

Figure S2. Vasculature and morphology of human melanoma tumors. Light micrographs of human melanoma paraffin sections visualize collagen fibers (Herovici staining, red), blood vessels (CD31 and CD34 staining, red) and lymphatic vessels (podoplanin, brown). M6 and M9 were stained with CD31 antibody, and the rest of the tumors with CD34 antibody. Blood vessel invasion was challenging to score since mainly single melanoma cells were seen inside the blood vessels in few samples.

Figure S3. *MMP16* expression in WM852 cells and its correlation with tumor weight in melanoma xenografts. (A) *MMP16* mRNA expression in WM852 cells lentivirally transduced with scrambled shRNA (shScr) and shRNA targeting *MMP16* (shMMP16-1, TRCN0000052249; shMMP16, TRCN0000052250). The mean expression shScr cells was set to 100. (B) WM852 cells transduced with indicated shRNAs were subjected to cell-surface biotinylation and immunoprecipitation with MMP16 antibody. (C) Correlation between *MMP16* mRNA expression and tumor weight of shScr and shMMP16 (shMMP16, TRCN0000052250) tumor xenografts. Average weight, shScr: 0.24 ± 0.1 g, $n = 14$; shMMP16: 0.55 ± 0.2 g, $n = 13$.

Figure S4. No significant difference in the blood and lymphatic vessel densities was observed between shScr and shMMP16 tumors. (A, B, C) Light micrographs visualize representative fields of paraffin sections stained for mouse CD31 (A) and Lyve-1 (B, C). The coverage/microscope field of intratumoral CD31 (A) and intratumoral Lyve-1 (B), as well as relative area of peritumoral Lyve-1/peritumoral length (C) were calculated from light micrographs using Anduril software, $n = 14$ and 13.

Figure S5. *MMP16*-silencing does not affect mouse collagen type I in xenografts. Fluorescent micrographs visualize mouse collagen type I (mCol-I) and CD31 stained from frozen sections of mouse xenografts. The mean intensity in control xenografts (shScr) was set to 1. Mouse collagen type I intensity was quantified using ImageJ, $n = 7$.

Figure S6. Endogenous or exogenous MMP16 regulates collagen invasion and cell-cell contacts in WM852 and Bowes melanoma cells. (A and B) MMP16 was silenced using shRNA (A) or siRNA (B). Charts represent the number of WM852 cells that invaded into 3D collagen within 5 days, $n = 3$. (C) MMP16 overexpression in Bowes cells decreases collagen invasion, $n = 3$. Invasion of the control cells (Mock) was set to 100 (D) Relative distances migrated by the cells during 6 and 24 h wound migration. The mean distance migrated by shScr cells in 6 h was set to 1. (E) Cell proliferation in 2D cultures. (F) Fluorescent micrographs of cells transfected with indicated siRNAs and stained for ZO-1, N-cadherin, and EphA2 visualize the reduction of cell-cell contacts after *MMP16* silencing. Scale bar, 25 μm. (G) Stable MMP16 overexpression in Bowes cells leads to increased N-cadherin staining. *, $p = 0.02$.

Figure S7. Endogenous or recombinant MMP16 regulates LEC spheroid intravasation of WM852 and Bowes melanoma cells. (A) Representative confocal images visualize WM852 cells (green) co-cultured with LEC or BEC spheroids (red) in 3D fibrin for 3 days, $n = 5$-10, $p =$

0.001. See quantification in Fig. 5D. (B) Representative confocal images visualize Bowes cells (green) stably transfected with MMP16 or mock vector, and co-cultured with LEC spheroids (red) in 3D fibrin for 3 days. (C) Quantification of relative number of intravasated Bowes melanoma cells per spheroid. The mean number of intravasated control cells (Bowes+mock) was set to 1, n = 13-23; *, p = 0.001.

Figure S8. Silencing of *L1CAM* cleaved by MMP16 rescued the transmigration across BECs but not the cell junction disassembly of shMMP16 cells. (A) Silencing efficiency of indicated siRNAs as assessed by qPCR (n = 3). The mean expression of *MMP16*, *MMP14* and *L1CAM* in control (siCtrl) cells was set to 1. (B and C) WM852 (B) and WM165 (C) cells were transfected with indicated siRNAs and subjected to immunoblotting. (D) WM852 shScr or shMMP16 cells were transfected with indicated siRNAs and subjected to immunoblotting. (E) WM852 cells were transfected with siRNA targeting *L1CAM*, *MMP14* and *MMP16*, and L1CAM was detected from the cell lysates. Only silencing of *MMP16* increased full-length L1CAM in the cell lysates. (F) WM165 shScr and shMMP16 cells were transfected with rescue MMP16 constructs (MMP16-r1 and MMP16-r2) and subjected to immunoblotting. Conditioned media were immunoblotted for L1CAM ectodomain (ecto), and lysates for L1CAM intracellular domain (intra). (G) L1CAM was expressed alone or in combination with MMP14 or MMP16 in COS1 cells, followed by immunoblotting. Note the appearance of ~30 kDa intracellular fragment after MMP16 co-expression and ~90 kDa C-terminal fragment after MMP14 co-expression. Longer exposure visualizes additional ~160 kDa L1CAM ectodomain fragment increased after MMP14 co-expression. Conditioned media were immunoblotted for L1CAM ectodomain (ecto), and lysates for L1CAM intracellular domain (intra). (H) ShMMP16 cells transfected with the siL1CAM were stained for filamentous actin. Silencing of *L1CAM* did not affect the morphology of shMMP16 cells. (I) WM852 cells transfected with indicated siRNAs transmigrated across LEC or BEC monolayers for 12 h, n = 3. Recombinant soluble L1CAM-Fc corresponding to N-

terminal fragment of L1CAM (L1-Fc, 1 µg/ml) was added to the upper and lower wells where indicated. The relative transmigration of control (siCtrl) cells across BECs was set to 1. (J) *L1CAM* and *MMP14* were silenced using siRNAs and *MMP16* using shRNA, and the conditioned media of cell cultures were subjected to gelatin zymography. The intensities of MMP2 bands are expressed below each lane. *, $p < 0.01$.

Figure S9. L1CAM immunohistochemistry of xenografts and human samples. Immunohistochemistry visualizes larger tumor areas of L1CAM-stained tissues presented in Figure 6L-M.