The protease MT1-MMP drives a combinatorial proteolytic program in activated endothelial cells

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ABSTRACT The mechanism by which proteolytic events translate into biological responses is not well understood. To explore the link of pericellular proteolysis to events relevant to capillary sprouting within the inflammatory context, we aimed at the identification of the collection of substrates of the protease MT1-MMP in endothelial tip cells induced by inflammatory stimuli. We applied quantitative proteomics to endothelial cells (ECs) derived from wild-type and MT1-MMP-null mice to identify the substrate repertoire of this protease in TNF-α-activated ECs. Bioinformatics analysis revealed a combinatorial MT1-MMP proteolytic program, in which combined rather than single substrate processing would determine biological decisions by activated ECs, including chemotaxis, cell motility and adhesion, and vasculature development. MT1-MMP-deficient ECs inefficiently processed several of these substrates (TSP1, CYR61, NID1, and SEM3C), validating the model. This novel concept of MT1-MMP-driven combinatorial proteolysis in angiogenesis might be extendable to proteolytic actions in other cellular contexts.—Koziol, A., Gonzalo, P., Mota, A., Pollán, A., Lorenzo, C., Colomé, N., Montaner, D., Dopazo, J., Arribas, J., Canals, F., Arroyo, A. G. The protease MT1-MMP drives a combinatorial proteolytic program in activated endothelial cells. FASEBJ. 26, 000–000 (2012). www.fasebj.org

Key Words: angiogenesis · degradome · inflammation · SILAC

Proteolysis governs biological processes such as angiogenesis, the formation of new vessels from preexisting ones; however, the mechanisms by which proteolytic events actually translate into defined cellular responses remain poorly characterized. Angiogenesis requires the presence of invasive endothelial tip cells at the nascent sprout. The endothelial tip cell transcriptome during postnatal retinal vascularization was reported recently (1, 2), but the proteome has not been characterized. Angiogenesis in adults is often linked to inflammation, and factors such as tumor necrosis factor α (TNF-α), sphingosine-1-phosphate (S1P), and bradykinin induce formation of endothelial tip-like cells in vitro (3–5). The protease membrane type-1 matrix metalloproteinase (MT1-MMP) is likely active at the developing sprout during inflammation, since it is required for capillary formation induced by nitric oxide (a downstream effector of bradykinin), prostaglandin E2 (PGE2), and the chemokine monocyte chemoattractant protein-1 (MCP-1)/chemokine (C-C motif) ligand 2 (CCL2) (3). Moreover, MT1-MMP is expressed in endothelial tip cells in vitro (6), and mathematical models suggest that MT1-MMP contributes to endothelial tip cell guidance (7). Thus, a global analysis of MT1-MMP proteolytic activity in endothelial tip cells

Abbreviations: 2D, 2-dimensional; 3D, 3-dimensional; ADAM, a disintegrin and metalloproteinase; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; BP, biological process; CCL2, chemokine (C-C motif) ligand 2; CSUP, culture supernatant; CXCR4, CXC chemokine receptor type 4; CYR61, cysteine-rich angiogenic inducer of endothelial cell; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; GO, Gene Ontology; H/L, heavy/light; HUVEC, human umbilical vein endothelial cell; ICAM, intercellular adhesion molecule; iMLEC, immortalized mouse lung endothelial cell; Jag1, Jagged 1; MCP-1, monocyte chemoattractant protein-1; MLEC, mouse lung endothelial cell; MMP, matrix metalloproteinase; MS/MS, tandem mass spectrometry; MT1-MMP, membrane type-1 matrix metalloproteinase; NID1, nidogen 1; P, postnatal day; PECAM-1, platelet endothelial cell adhesion molecule 1; PFA, paraformaldehyde; pMLEC, primary mouse lung endothelial cell; S1P, sphingosine-1-phosphate; SEM3C, semaphorin 3C; SILAC, stable isotope labeling of amino acids in cell culture; siRNA, small interfering RNA; TNF-α, tumor necrosis factor α; TSP1, thrombospondin 1; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; VCAM-1, vascular cell adhesion molecule 1; WT, wild type

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should provide deeper understanding of the link between proteolysis and angiogenesis.

The fingerprint of a protease is its full set of substrates in a given cellular context (also known as the protease degradome; ref. 8). Shotgun and quantitative proteomics approaches have significantly advanced this field. Stable isotope labeling of amino acids in cell culture (SILAC) is a powerful quantitative proteomics approach (9), but the need for several cell doublings limits its use in slowly proliferating primary cells. Bioinformatic analysis of identified substrate sets can provide additional information about protease action in specific cell contexts. We have used an inflammatory tip cell model system to integrate SILAC-identified MT1-MMP substrates in TNF-α-activated endothelial cells (ECs) with bioinformatics analysis. This global analysis reveals a combinatorial MT1-MMP proteolytic program that governs EC chemotaxis, motility, and adhesion, ultimately leading to angiogenesis.

MATERIALS AND METHODS

Mice

MT1-MMP-deficient mice were generated as described previously (10). MT1-MMP heterozygotes on the C57BL/6 background were crossed, and wild-type (WT) and null littermates were used for experiments. Mice were kept in the Centro Nacional de Investigaciones Cardiovasculares (CNIC) Animal Facility under pathogen-free conditions and according to institutional guidelines.

Cells

Isolation of mouse lung ECs (MLECs) was described previously (11). Briefly, lungs from WT or MT1-MMP-null mice were excised, disaggregated, and digested in 0.1% collageously (11). Briefly, lungs from WT or MT1-MMP-null mice

Flow cytometry

Immortalized MLECs were left untreated or treated with 20 ng/ml of the angiogenic factors vascular endothelial growth factor (VEGF) or TNF-α (both from PeproTech, Rocky Hill, NJ, USA). For surface expression analysis, cells were harvested using Cell Dissociation Buffer (Gibco, Carlsbad, CA, USA) and stained with rat anti-mouse vascular cell adhesion molecule 1 (VCAM-1; BD Biosciences), biotinylated hamster anti-mouse ICAM-1 (BD Biosciences), hamster anti-mouse platelet EC adhesion molecule 1 (PECAM-1; Millipore, Bedford, MA, USA), rabbit anti-mouse Jagged 1 (Jag1; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-mouse VEGF receptor 2 (VEGFR2; Abcam, Cambridge, MA, USA), rabbit anti-mouse delta-like ligand 4 (Dll4; Santa Cruz Biotechnology, Santa Cruz, CA, USA), hamster anti-mouse β3 integrin (BD Biosciences), rat anti-mouse C-X-C chemokine receptor type 4 (CXCR4; BD Biosciences), and rabbit anti-mouse MT1-MMP (HR; Abcam), followed by incubation with appropriate fluorescent-labeled secondary antibodies. Cell surface expression was determined in a flow cytometer (FACSscan; Beckton Dickinson, Franklin Lakes, NJ, USA), and data were analyzed with FACSdiva software (Beckton Dickinson).

Hierarchical clustering analysis

Regulatory patterns were compared using the platform-independent Java suite Genesis (13).

Immunofluorescence staining

MLECs were plated on matrix-coated glass coverslips. Cells were then fixed with 4% paraformaldehyde (PFA) for 10 min. After blocking with 2% BSA for 30 min at room temperature, coverslips were incubated with Texas Red-phalloidin, rabbit anti-mouse thrombospondin 1 (clone 7280; kindly provided by Dr. M. Luisa Iruela-Arispe, University of California, Los Angeles, CA, USA), goat anti-mouse cytosine-rich angiogenic inducer 61 (CYR61), goat anti-mouse nidogen 1 (NID1), or goat anti-mouse semaphorin 3C (SEM3C; Santa Cruz Biotechnology), followed by fluorescent-labeled secondary antibodies. Mouse anti-human TSP1 (Thermo Fisher Scientific, Waltham, MA, USA), rabbit anti-human CYR61, and rabbit anti-human NID1 or goat anti-human SEM3C (Santa Cruz Biotechnology) were used for immunostaining of human ECs. Coverslips were mounted with ProLong Gold antifade reagent (Invitrogen). Samples were examined under a Zeiss LSM700 confocal microscope (Plan-Apochromat 63x1.4 or 40x1.3 Oil DIC M27; Carl Zeiss, Oberkochen, Germany) and images were analyzed with ZEN software (Zeiss).

Three-dimensional (3D) endothelial tip-like cell sprouting assay

MLECs were plated at confluence in Ibidi angiogenic chambers with Matrigel (BD Biosciences) on top; medium containing TNF-α was then added, and the chamber was kept at 37°C for ≥18 h. At the end of incubation, medium was removed, and the cells were fixed with freshly prepared 4% PFA for 20 min, followed by permeabilization in 0.3% Triton X-100. Samples were washed with PBS/Triton X-100 and stained with phalloidin, anti-PECAM-1, anti-TSP1, anti-CYR61, anti-NID1, or anti-SEM3C antibodies. Samples were examined under a Zeiss LSM700 confocal microscope, and images were analyzed with ZEN software.
MLECs were grown in complete SILAC Advance D-MEM F-12 Flex Medium (Invitrogen) supplemented with "light" and "heavy" amino acids and dialyzed serum to exclude the possibility of incorporating unlabeled amino acids. We used nonlabeled lysine and the 13C6 form of arginine (light conditions) for cell cultures derived from MT1-MMP-null mice, and isotopes of lysine (13C6) and arginine (15N4, 15N2) for cells derived from WT mice (heavy conditions). Cells were cultured for 7 d, corresponding to 7 d of culture for cells derived from WT mice (heavy conditions). Cells were incubated with 20 ng/ml TNF-α. At the end of the 7-d period, conditioned medium from null and WT cultures was collected, and the cells were harvested with cold PBS and 10 mM ethylenediaminetetraacetic acid (EDTA). Supernatants from null (light condition) and WT (heavy condition) were pooled 1:1 after bicinchoninic acid protein determination (BCA Protein Assay; Pierce, Rockford, IL, USA). The pooled media were incubated for 3 h at 4°C with wheat germ agglutinin (WGA)-agarose beads to extract glycoproteins, and the glycoproteins were eluted by incubation with 0.5 M N-acetyl-D-glucosamine. The glycoprotein mixture was concentrated on a 3-kDa cutoff centrifugal filter (Amicon Ultra-4; Millipore) to a final volume of 200 μl. Urea was added to a final concentration of 8 M (pH 8.5). Proteins were reduced by addition of 50 mM dithiothreitol (DTT; Amersham Biosciences, Piscataway, NJ, USA), followed by carbamidomethylation with 125 mM iodoacetamide (Sigma). Finally, the protein mixture was purified by acetone–trichloroacetic acid precipitation (2D-CleanUp kit; GE Healthcare, Piscataway, NJ, USA). For microsomal fraction analysis, cells were detached nonenzymatically with cold PBS and 10 mM EDTA, and equal amounts of cells from WT and MT1-MMP-null culture were pooled, left in sucrose buffer, and cracked by sequential passages through a 25-gauge needle. The pooled fraction was spun to pellet nuclei, and the supernatant was ultracentrifuged for 2 h at 55,000 rpm to separate cytosol from the microsomal fraction. The microsomal pellet was resuspended in guadinium chloride (6 M; Sigma). Supernatant and microsomal fractions were resuspended in loading buffer, and proteins were separated by 10% SDS-PAGE.

Mass spectrometry

After separation of glycoproteins by 10% SDS-PAGE, Comassie-stained gel was cut into 20 slices, and each one was in-gel digested with modified porcine trypsin (Promega, Madison, WI, USA). The digests were dried in a vacuum centrifuge, extracted with formic acid solution, and analyzed on an Esquire Ultra IT mass spectrometer (Bruker, Bremen, Germany) coupled to a nano-HPLC system (Ultimate; LC Packings, Amsterdam, The Netherlands). Peptide mixtures were first concentrated on a 300-mm i.d. and 1-mm PepMap nanotrapping column and then loaded onto a 75-mm i.d., 15-cm PepMap nanoseparation column (LC Packings). Peptides were then eluted with an acetonitrile (ACN) gradient (0–60% B in 150 min, where B is 80% ACN, 0.1% formic acid in water; flow rate ~300 nL/min) through a PicoTip emitter nanospray needle (New Objective, Woburn, MA, USA) onto the nanospray ionization source of the IT mass spectrometer. Tandem mass spectrometry (MS/MS) fragmentation (1.9 s, 100–2800 m/z) was performed on three of the most intense ions, as determined from a 1.2 s MS survey scan (310–1500 m/z), using a dynamic exclusion time of 1.2 min for precursor selection and excluding single-charged ions.

Protein identification and data analysis

Data were processed for protein identification and quantification with Protein Scape 2.1 and WARP-LC 1.2 (Bruker), a software platform that integrates processing of LC-MS run data, protein identification through database search of MS/MS spectra, and protein quantification based on the integration of the chromatographic peaks of MS-extracted ion chromatograms for each precursor. Proteins were identified using Mascot (Matrix Science, London, UK) to search the SwissProt database, restricting the search to mouse proteins. MS/MS spectra were searched with a precursor mass tolerance of 1.5 Da, fragment tolerance of 0.5 Da, trypsin specificity with a maximum of 1 missed cleavage, cysteine carbamidomethylation as a fixed modification, and methionine oxidation and the N-terminal and corresponding Lys and Arg SILAC labels as variable modifications. A positive identification criterion was set as an individual Mascot score for each peptide MS/MS spectrum higher than the corresponding homology threshold score. The false-positive rate for Mascot protein identification was measured by searching a randomized decoy database, as described previously (14), and estimated to be <4%. For protein quantification, heavy/light (H/L) ratios were calculated by averaging the measured H/L ratio for the observed peptides, after discarding outliers. For selected proteins of interest, quantitative data obtained from
the automated Protein Scape software analysis were manually reviewed. Further protein analysis was conducted with Protein Knowledgebase UniProtKB (http://www.uniprot.org) and the GeneCards database (http://www.genecards.org).

Bioinformatic data analysis with Babelomics

The Babelomics Web tool (http://www.babelomics.org) is a freely available program suited to analysis of large-scale experiments (microarrays, proteomics) with functional annotation (15). The Fatigo module (16) was used for enrichment analysis of selected proteins. This functional profiling was performed by comparing two lists of genes, the first being the genes encoding the proteins with an H/L ratio > 1.5 in the SILAC analysis unless otherwise stated, and the second being the rest of the genome.

Overlap analysis

Overlap analysis and generation of Venn diagrams was performed using an online application (http://www.pangloss.com/seidel/Protocols/venn4.cgi).

Microarrays

Primary MLECs were treated with 20 ng/ml MCP-1 for 6 h. The experiments were conducted using 1-color mouse whole-genome oligomicroarrays (Applied Biosystems, Foster City, CA, USA), and differential expression analyses was performed using the linear modeling features of the Limma package (Bioconductor; http://www.bioconductor.org/).

RT-PCR

For relative quantification of expression, quantitative real-time PCR analysis was performed using the AB7900 FAST 384 Detection System (Applied Biosystems), according to the manufacturer’s instructions. Total cellular RNA was extracted from cultured confluent MLECs using TRIzol reagent (Invitrogen), according to the manufacturer’s protocol, and quantified using a Nanodrop ND-1000 (Thermo Fisher Scientific). cDNA synthesis was performed with 1 µg of total RNA using an RNA synthesis kit (RNase Plus mini kit; Qiagen, Valencia, CA, USA). Predesigned qPCR primers for mouse Mmp14, Thbs1, Cyr61, Nid1, Sema3c, Tbp, and Hrpt1 were selected from TaqMan Gene Expression Assay database (Applied Biosystems). Relative quantification of each target gene was performed using comparative threshold multiplex PCR in gene expression relative to the Tbp and Hrpt1. The data were analyzed by the qBASE program (http://www.biogazelle.com), obtaining the C of the amplification products.

For HUVECs, after RNA extraction, cDNA synthesis was also performed with 1 µg of total RNA using an RNA synthesis kit (RNase Plus mini kit; Qiagen). cDNAs were subjected to PCR amplification using the following primer pairs for human MT1-MMP: 5'-CGCTACGCCATCCAGGGTCTC-3' and 5'-CGGTCATCATCGGGCAGCAAAAA-3' (Isogen Bioscience, Maarssen, The Netherlands). The PCR products were visualized using ethidium bromide in 1% agarose gel.

Western blot

Cells were lysed in RIPA buffer containing protease inhibitors, and culture supernatants (CSUPs) were collected and enriched for glycoproteins as described above. Culture (50 µg) supernatant and 20 µg of total cell lysate were loaded and separated by SDS-PAGE, and proteins were transferred to nitrocellulose membrane; protein loading of CSUPs was checked by Ponceau red staining of the blotted membrane. Blots were incubated with anti-mouse or anti-human antibodies overnight at 4°C, washed, and followed by secondary HRP-conjugated antibody. Signal was detected by chemiluminescence (Amersham ECL Signal Detection Reagent; GE Healthcare) or enhanced chemiluminescence (Immobilon Western, Chemiluminescent HRP Substrate; Millipore). Protein amount in the supernatants was quantified by densitometry using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA). All the experiments were performed at the same time using the same lot of antibodies.

**Figure 2.** iMLECs respond similarly to pMLECs to TNF-α stimulation. A) Morphology of pMLECs and iMLECs (left panels); levels of PECAM-1 and MT1-MMP in pMLECs and iMLECs assessed by flow cytometry (middle panels) and immunostaining (right panels). B) iMLECs were left untreated (0 h) or treated with 20 ng/ml of VEGF or TNF-α, and the expression of the tip cell markers VEGFR2, Jag1, DI4, MT1-MMP, integrin β3, and CXCR4 was analyzed at the cell surface by flow cytometry at 4, 24, and 48 h (n=3). Left panel: hierarchical schemes were obtained. Right panel: average percentages of positive population for each marker and condition were analyzed by the high-content analysis software Genesis on normalization of data. C) Cell surface levels of ICAM-1 and VCAM-1 were assessed by flow cytometry in pMLECs and iMLECs left untreated or treated with 20 ng/ml TNF-α for 24h; D) TNF-α-stimulated pMLECs or iMLECs were cultured in 2D or 3D conditions and stained with phalloidin or anti-PECAM-1 antibody, respectively.
least in triplicate. Data are represented as means ± se, and unpaired Student’s t test was applied. Differences were considered significant at values of $P < 0.05$.

**Whole-mount retina staining**

Eyes from postnatal day 6–8 (P6–P8) mice were removed and fixed with 4% PFA at 4°C. Fixed retinas were flat-mounted and blocked in 2% BSA, PBS, and 0.3% Triton X-100, followed by overnight incubation with primary antibodies (anti-CYR61 or rat anti-mouse NID1; Abcam) and isoelectin B4 (Sigma) to visualize vasculature. The next day, retinas were extensively washed and incubated with fluorescent-conjugated secondary antibodies. Images were acquired with a Zeiss LSM700 confocal microscope (Plan-Apochromat 63×/1.4 Oil DIC M27), and images were analyzed with ZEN software.

**Transfection of small interfering RNA (siRNA)**

Silencer siRNA constructs were purchased from Ambion (Applied Biosystems). Specific oligonucleotide sequences targeting human MT1-MMP were as follows: 5'-CAUCUGUGACGGGAACUUU-3' and 5'-GGAUAUGAGGAUCUAGG-3' and a nontargeting siRNA control, which bears no homology with relevant human genes. For siRNA transfection, cells were seeded in 6-well plates at $2.5 \times 10^5$ cells/well and grown to reach 60–70% confluence. The different amounts of siRNAs and Oligofectamine reagent (Invitrogen) were diluted in Opti-MEM I (Gibco; Invitrogen). The diluted siRNA-liposome complex was added to cells and incubated for 4 h. Following the transfection, fresh Opti-MEM I containing 30% FBS was added, and cells were grown for 24 h. After that, cells were rinsed with fresh M199 medium and cultured with TNF-α for 24 h for analysis.

**RESULTS**

**Pericellular proteolysis in inflammation-induced endothelial tip cells**

To understand the link of pericellular proteolysis to events relevant to capillary sprouting within the

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**Figure 3. SILAC in TNF-α-stimulated iMLECs.** A) SILAC strategy for comparing the supernatant proteome of WT (MT1$^{+/+}$) and MT1-MMP-null (MT1$^{-/-}$) iMLECs stimulated with TNF-α (20 ng/ml) for 24 h. B) Peptide mass spectra for NID1 and TSP1, with relative abundance of heavy and light labeled forms. C) Venn diagram showing proteins identified in both microsomal proteome and supernatant glycoproteome. D) Normal distribution of H/L ratios of labeled peptides in microsomal proteome and supernatant glycoproteome. Histogram shows the percentage of quantified proteins with H/L ratio = 1, and <1.5- or >1.5-fold change in the microsomal proteome and the supernatant glycoproteome.
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inflammatory context, we aimed at the identification of the collection of MT1-MMP substrates in endothelial tip cells induced by inflammatory stimuli. **Figure 1** outlines the experimental design undertaken in this study.

We first compared the behavior of primary MLECs (pMLECs; ref. 17) vs. polyoma virus middle T-immortalized MLECs (iMLECs) from WT mice. iMLECs maintained EC morphology and similar expression of PECAM-1 (95%) in both iMLECs and pMLECs; MT1-MMP cell surface levels were slightly higher in iMLECs (Fig. 2A). To choose the appropriate inflammatory stimulus for inducing endothelial tip cells, we evaluated the effect of the main angiogenic factor VEGF and of the proangiogenic cytokine TNF-α on the expression of the endothelial tip cell markers Jag1, VEGFR2, Dll4, MT1-MMP, integrin β3, and CXCR4 in iMLECs (Fig. 2B). Hierarchical clustering analysis of these data performed with Genesis (13) showed that TNF-α regulated the expression of the tip cell markers Dll4 + MT1-MMP and Jag1 + CXCR4 but in a different manner than VEGF, which mainly regulated Dll4 + VEGFR2, as reported previously (5). This analysis confirmed TNF-α as an inflammatory inducer of endothelial tip cells (4). TNF-α induced a similar increase of the endothelial activation markers ICAM-1 and VCAM-1 and enhanced MT1-MMP expression in both pMLECs and iMLECs (Fig. 2C and not shown). TNF-α stimulation also resulted in similar elongation and induction of filopodia-like membrane protrusions in both pMLECs and iMLECs cultured in 2D or 3D conditions (Fig. 2D). Since iMLECs responded similarly to TNF-α and displayed higher MT1-MMP levels and proliferative capacity than pMLECs, iMLECs were chosen as the proper cellular model to perform quantitative proteomics of MT1-MMP activity in inflammation-activated ECs.

**SILAC identification of MT1-MMP degradome in TNF-α-induced endothelial tip cells**

Proteolysis by transmembrane proteases mainly affects other transmembrane or secreted glycoproteins (18). We therefore used SILAC to analyze lectin-enriched glycoproteins in mixed supernatants of TNF-α-activated WT (labeled with heavy amino acids) and MT1-MMP-null iMLECs (labeled with light isotopes). Proteins in mixed microsomal fractions were also analyzed; for details of the SILAC procedure and examples of mass spectra, see **Fig. 3A, B** and Materials and Methods. Totals of 157 glycoproteins in conditioned medium and 797 proteins in the microsomal fraction were identified and quantified (relative H/L ratios; complete list of proteins available at http://www.cnic.es/doc/metaloproteinasas/SILACproteinlist.pdf), with only 28 proteins found in both proteomes (Fig. 3C). Interpretation of H/L ratios in the supernatant glycoproteome is complex; a transmembrane protein or a matrix-bound secreted protein shed by MT1-MMP will yield an H/L ratio frequencies show a gaussian distribution, with a peak at log H/L = 0, indicating equivalent labeling of WT and MT1-MMP-null cells (Fig. 3D). Notably, lack of MT1-MMP altered H/L ratios more in the supernatant glycoproteome than in the microsomal proteome (31.85 vs. 17.02% H/L >1.5; Fig. 3D). Since H/L > 1 is the more likely outcome for protease action on the supernatant glycoproteome (Supplemental Fig. S1), we defined the MT1-MMP endothelial degradome as the set of glycoproteins with H/L > 1.5 (Fig. 3D). Categorization of these identified proteins based on their generic assigned function [Database for Annotation, Visualization, and Integrated Discovery (DAVID); ref. 19] and PubMed search defined a group of angiogen-
...esis-related proteins and a smaller group of unrelated proteins (Table 1). Most identified proteins are exclusively MT1-MMP substrates, including extracellular matrix (ECM)/matricellular proteins (CYR61, FBLN2, SNED1), and non-ECM proteins (PR2C2, SLIT2, EGFR, SFRP1) (Table 1). Nonexclusive substrates include TSP1, NID1, and SEM3C.

Bioinformatic analysis reveals a combinatorial MT1-MMP proteolytic program in angiogenesis

Gene Ontology (GO) enrichment analysis of the MT1-MMP endothelial degradome (H/L > 1.5) was carried out with the FatiGO module (16) in the open-source bioinformatics Web tool Babelomics (15), which compares genes of interest with the rest of the mouse genome. This set of proteins was enriched in cellular components of extracellular and matrix-related compartments (Fig. 4A) correlating with exposure of these proteins to the protease. The MT1-MMP endothelial degradome was also enriched in molecular functions related to binding, particularly matrix binding, and enzyme activity (Fig. 4B) and was highly-enriched in biological processes (BPs) related to cellular adhesion, motility, and chemotaxis, key steps in endothelial tip cell function, and to vasculature development (Fig. 4D).

Several substrates appeared in more than one BP (Supplemental Table S1). Overlap analysis and Venn diagrams of proteins annotated in these BPs revealed a proteolytic program of 14 substrates, in which combined rather than single substrate processing by MT1-MMP would control key steps of the angiogenic response, in particular cell motility, chemotaxis, cell adhesion, and vasculature development (Fig. 4D). In this combinatorial program, processing of TSP1 is common to these 4 BPs; CYR61 processing is the second branch point, excluding motility and steering toward one of the other fates. Processing of TSP1 and CYR61, combined with CCL2 and SLIT2, determines chemotaxis. Within the MT1-MMP-combinatorial program, adhesion and vasculature development would be more closely related, with motility showing the least overlap, being determined by combined processing of TSP1, SEM3C, EGFR, PR2C2, and CSF1.

The SILAC-analyzed microsomal fraction lacked most MT1-MMP substrates (Fig. 3C). However, the 24 h of iMLEC TNF-α stimulation is enough for transcrip-
tional regulation to occur, and genes encoding 4 of the 14 proteins in the combinatorial model (CO3A1, FBLN2, protein-lysine 6-oxidase and MMP19) are found up-regulated in MCP-1-stimulated WT but not MT1-MMP-null pMLECs (Supplemental Table S2 and Supplemental Fig. S2); thus, their H/L > 1 in iMLEC supernatant might partly reflect transcriptional regulation.

**Biological validation of MT1-MMP combinatorial proteolysis in TNF-α-stimulated primary ECs**

For further validation, we selected substrates within each of the 4 main BPs. *In vitro* digestion assays had previously shown the ability of MT1-MMP to cleave TSP1, CYR61, and NID1 (20, 21), and we further confirmed by *in silico* analysis that TSP1, CYR61, and NID1 and also SEM3C contain putative MT1-MMP cleavage sequences [R(F/K)-nonP-X(polar)-X(hydrophobic); ref. 22] at positions that predict the peptides identified by SILAC (Supplemental Fig. S3).

Based on the SILAC analysis performed in CSUPs and on the ability of the identified proteins TSP1, CYR61, NID1, and SEM3C to bind the ECM and/or the basement membrane, we next hypothesized that the absence of MT1-MMP might affect cleavage of these proteins from the ECM and therefore their release to the supernatant and their cellular distribution. No major differences were observed in mRNA and protein levels of TSP1, CYR61, NID1, and SEM3C in total lysates of WT and MT1-MMP-null pMLECs treated with TNF-α (24 h), pointing to similar abundance of these substrates (Fig. 5A, B). In accordance with SILAC data, lower levels of TSP1, NID1, and SEM3C were detected in the supernatants of MT1-MMP-null MLECs, indicating that MT1-MMP proteolytic action releases TSP1, NID1, and SEM3C from the ECM; consistent with cleavage of NID1 at the N or C terminus, the molecular mass of NID1 in supernatants from WT MLECs was slightly smaller (Fig. 5A). Higher levels of CYR61 were, however, found in supernatants from MT1-MMP-deficient pMLECs, correlating with a more complex SILAC peptide profile and pointing to an active processing of the intact soluble form of CYR61 by MT1-MMP. Impaired processing in the absence of MT1-MMP led to accumulation of TSP1, CYR61, NID1, and SEM3C in

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**Figure 5.** Impaired processing of TSP1, CYR61, NID1, and SEM3C in MT1-MMP-null pMLECs. A) Bar charts show mRNA levels of Thbs1, Cyr61, Nid1, and Sema3c in TNF-α-stimulated WT (MT1+/+) and null (MT1−/−) pMLECs analyzed by quantitative PCR (n=5). B) Immunoblots show detection of TSP1, CYR61, NID1, and SEM3C in total lysates (TL) and CSUPs of TNF-α-stimulated WT (MT1+/+) and null (MT1−/−) pMLECs; bar charts show densitometric quantification of proteins in supernatants (n=3). C) Confocal microscopy analysis was performed in TNF-α-stimulated WT and MT1-MMP-null pMLECs immunostained for TSP1, CYR61, NID1, or SEM3C; representative maximal projections are shown (n=5). D) Confocal immunofluorescence of TSP1, CYR61, NID1, or SEM3C in TNF-α-stimulated WT and null pMLECs grown in Ibidi chambers covered by 3D-Matrigel; representative maximal projections are shown (n=5). Insets: F-actin staining.
pericellular clusters in contact with the matrix and/or adhesion sites in MT1-MMP-null pMLECs (Fig. 5C).

TSP1, CYR61, NID1, and SEM3C have been related to angiogenesis and vascular development (23–26), and we therefore analyzed the effect of MT1-MMP-mediated processing of these substrates in models better mimicking vascular development, such as 3D-Matrigel MLEC cultures and retina postnatal vascularization. Although no major differences were observed in TSP1, CYR61, NID1, and SEM3C signal along EC 3D tubes, enrichment and/or pericellular clusters of these substrates, especially of CYR61 and NID1, were observed along the endothelial tip cells formed by MT1-MMP-strates, especially of CYR61 and NID1, were observed along the endothelial tip cells formed by MT1-MMP-null pMLECs compared with WT (Fig. 5D). Accordingly, accumulation of CYR61 and NID1 was also detected along endothelial tip cells at the vascular front of retinas from MT1-MMP-null neonates, but not their WT counterparts, indicating that this newly identified MT1-MMP processing axis is also relevant to in vivo angiogenesis (Fig. 6). SEM3C and TSP1 were undetectable in the vasculature of mouse neonate retinas (not shown).

We next investigated whether the MT1-MMP proteolytic program identified in mouse ECs could be extendable to the human model. To this purpose, MT1-MMP expression was inhibited by two independent siRNA oligonucleotides in HUVECs; the efficiency of MT1-MMP interference was >70% assessed by RT-PCR and Western blot (Fig. 7A). TSP1, CYR61, NID1, and SEM3C levels in total lysates from negative siRNA and MT1-MMP-inhibited HUVECs were similar (not shown). Lower levels of TSP1, CYR61, and NID1 were, however, found in supernatants from MT1-MMP-inhibited compared with negative siRNA-inhibited HUVECs, pointing to decreased processing of these substrates in cells with reduced levels of MT1-MMP (Fig. 7B). No differences could be detected in supernatant levels of SEM3C (Fig. 7B). In a complementary approach, we compared substrate subcellular distribution in MT1-MMP-inhibited HUVECs. As shown in Fig. 7C, the subcellular distribution of TSP1, CYR61, NID1, and SEM3C was changed in HUVECs with reduced expression of MT1-MMP; in particular, CYR61 and SEM3C appeared more intense and NID1 formed pericellular clusters in HUVECs with lower expression of MT1-MMP. These data support the hypothesis that the identified MT1-MMP combinatorial program is also operative in human ECs.

**DISCUSSION**

SILAC performed in TNF-α-activated primary ECs has provided an integrated view of the contribution of the protease MT1-MMP to inflammatory capillary sprouting through combinatorial cleavage of defined sets of substrates and a mechanistic link between proteolytic events and biological responses by ECs during angiogenesis (Fig. 8).

Largely known for a long time, the link between angiogenesis and inflammation is not well understood. TNF-α is an early tissue-damage signal for angiogenesis and can induce morphological and molecular changes compatible with induction of the endothelial tip cell phenotype (4, 27); we confirmed this ability in iMLECs. Endothelial tip cells are crucial to initiate an efficient angiogenic response (5). The molecular fingerprint of these tip cells has started to be elucidated by recent transcriptomics analysis in retinal tip cells; matrix and matrix-related genes as well as secreted proteins, such as apelin, seemed to be particularly represented (2). MT1-MMP gene was not found to be enriched in the microarray assays performed in retinal tip cells (1, 2); low levels of MT1-MMP might have been missed, or MT1-MMP might be especially relevant in inflammatory angiogenesis. We then hypothesized that identification of MT1-MMP collection of substrates in inflammatory tip cells will provide insights into their function and into the link between proteolytic events and biological responses during angiogenesis.

We have circumvented SILAC limited use in primary cells by immortalizing mouse lung ECs, allowing us to perform this approach for the first time in ECs obtained from genetically modified mice. Our results showed larger variations in the supernatant glycoproteome than in the microsomal proteome in the absence of MT1-MMP; this highlights the power of performing

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Accumulation of CYR61 and NID1 in endothelial tip cells of retinas from MT1-MMP-null mouse neonates. Whole-mount confocal immunofluorescence of CYR61 or NID1 (red) and isolectin B4 (green) in retinas of P6 WT (MT1-MMP+/−) and null (MT1-MMP−/−) mice; representative maximal projections are shown (n=4).
SILAC in CSUPs for protease analysis, since catalytic activity mostly affects secreted glycoproteins that are a small fraction of the total proteome (28). A few SILAC MT1-MMP substrates were identified previously by isobaric tags for relative and absolute quantitation (iTRAQ)/isotope-coded affinity tag (ICAT) in tumor cells (29) and by differential gel electrophoresis (DIGE) in human plasma incubated with the MT1-MMP catalytic domain (30), supporting the robustness of these techniques and pointing to MT1-MMP cleavage of a given substrate in different contexts. MT1-MMP can affect angiogenesis through the processing of matrix components (31) but also of transmembrane molecules such as semaphorin 4D and endoglin (32, 33). Although we used SILAC to identify transmembrane glycoproteins, most of the identified MT1-MMP substrates in inflammatory endothelial tip cells are ECM and secreted proteins, in accordance with reported retinal endothelial tip cell transcriptome (2), and reinforcing the idea that matrix and its remodeling are essential for modulating endothelial tip cells. Further optimization of cell surface protein labeling (34) might still expand MT1-MMP repertoire to include endothelial transmembrane proteins. SILAC also provided insight into redundancy vs. specificity of substrate cleavage by MMP/a disintegrin and metalloproteinase (ADAM) family members. MT1-MMP mainly shares substrates such as TSP1, NID1, and SEM3C with ADAM with thrombospondin motifs 1 (ADAMTS1), suggesting cooperation among these proteolytic pathways in angiogenesis. Accordingly, ADAMTS1 is up-regulated in the transcriptome of retinal endothelial tip cells (2) and down-regulated in MCP-1-stimulated MT1-MMP-null pMLECs (Supplemental Table S2).

One of the most novel aspects of our study involves the identification of a combinatorial proteolytic program by overlap analysis and Venn diagram representation of the MT1-MMP endothelial degradome. In this combinatorial program, TSP1 and/or CYR61 processing is central to processes such as cell adhesion, motility, chemotaxis, and vasculature development (35). Matricellular proteins, such as TSP1 and CYR61, are interesting because they act as a bridge between cells and the ECM, thus modulating cell behavior; therefore, it is not unexpected that MT1-MMP action relies mainly on processing of members of this family. Outcome of angiogenesis will then depend on combined rather than individual processing of substrates, whose fragments might act in a competitive, synergistic, or sequential manner to fine-tune the angiogenic response; for example, TSP1 and CYR61 share domains that directly or indirectly bind integrin αβ3, an adhesion receptor expressed in endothelial tip cells that cooperates with endothelial MT1-MMP (2, 12). Overlap analysis also showed that EC adhesion and vasculature development are molecularly closer and that cell motility is the most unique in the combination of processed substrates.

Increased abundance of a given protein in the supernatant of WT cells might also be related to higher expression of the protein. Although no major changes were found in the microsomal fraction, 4 of the 14 genes encoding for proteins represented in the combinatorial proteolytic program were up-regulated in MCP-1-stimulated pMLECs expressing MT1-MMP vs.
null cells; in particular, genes and proteins related to cell adhesion and vasculature development, complex responses that likely require activation of a given transcriptional program. These data indicate a bimodal contribution of MT1-MMP to inflammatory angiogenesis, including regulation of a restricted gene set but dominated by combinatorial processing of defined substrates related to motility, chemotaxis, and adhesion.

We next biologically validated the processing of selected substrates involved in the four main BPs, cell motility, chemotaxis, cell adhesion, and vasculature development (TSP1, CYR61, NID1, and SEM3C), whose cleavage would likely occur within the same time frame, cooperating in driving cellular responses leading to inflammatory capillary sprouting. Conversely, as we demonstrated in TNF-α-stimulated MT1-MMP-null pMLECs, in the absence of MT1-MMP simultaneous accumulation of these substrates in ECs might contribute to defective angiogenesis (10, 17). Whether the proteolytic effect of MT1-MMP on the identified substrates in cells is only due to direct processing or to additional indirect effects, for instance, through the combined action of other proteases, such as ADAMTS1, remains to be defined. Notably, we have also confirmed that the combinatorial program driven by MT1-MMP identified in mouse cells is also acting in human ECs, since decreasing MT1-MMP expression affects the processing and subcellular distribution of TSP1, CYR61, NID1, and SEM3C in HUVECs; however, milder effects were observed and were likely related to the experimental approach and possibly to a different repertoire of proteases expressed by human ECs. The multidomain structure of matricellular proteins contributes to their pleiotropic functions in angiogenesis. Thus, TSP1 effects on angiogenesis largely depend on whether it is soluble or matrix-bound (25); during inflammatory angiogenesis, MT1-MMP could modulate these angiogenic properties by cooperating with ADAMTS1 (35) or acting at a different cleavage site and possibly affecting interaction with integrin αβ3. Matrix-bound TSP1 accumulation in the absence of MT1-MMP might contribute to the reduced angiogenesis reported in MT1-MMP-null cells (11, 17). Moreover, accumulation of CYR61/CCN1 might impair its induction of EC adhesion, migration, and proliferation (24) likely by altering its binding to integrins αβ1 or αβ3 (36). CYR61 was also accumulated in MT1-MMP-deficient retinas pointing to a key role for MT1-MMP in CYR61 processing in vivo and suggesting that loss of this processing might mimic vascular defects observed in the absence of CYR61 (24). However, since CYR61 can play dual roles in physiological and pathological vascularization in the retina (37), functional consequences of CYR61 processing would deserve further investigation. Likewise, pericellular deposits of NID1, a protein normally associated to laminin and fibulin at the basement membrane (25) might confer basement membrane-like “quiescence” signals hindering EC active sprouting. We also observed NID1 deposition along the filopodia of endothelial tip cells at the retinal vascular front of MT1-MMP-null neonates in contrast to the restricted distribution of NID1 to the posterior pole of retinal tip cells in WT (2); this points to an active role of MT1-MMP in NID1 processing during vascular sprouting also in vivo. Finally, SEM3C processing by ADAMTS1 can contribute to tumor cell migration (26), and therefore we can envision that MT1-MMP-processing of SEM3C might affect EC migration required for capillary sprouting; in the absence of this processing, impaired migration might lead to defective angiogenesis. In sum, TSP1, CYR61, NID1, and SEM3C can modulate EC adhesive and migratory behavior through complementary mechanisms including binding to integrins; the synergistic or competitive action of these accumulated substrates in the absence of MT1-MMP would impair migration and adhesion, yielding a more quiescent endothelial state and defective angiogenesis (ref. 17 and Fig. 8).

We have established a valid cell model system for studying the mechanistic link between proteolysis and biological responses in the context of inflammation-activated ECs. SILAC-based proteomics identified the supernatant glycoproteome as the main protein set targeted by the protease MT1-MMP. Combination of SILAC with bioinformatics revealed that the main con-
tribution of MT1-MMP to inflammatory angiogenesis is a combinatorial processing of defined substrates impacting early and rapid responses such as motility, chemotaxis, and adhesion, finally leading to new vessel formation. Profiling of MT1-MMP-processed substrates in plasma could be used as a surrogate marker of angiogenic activity in chronic inflammatory disease, as rheumatoid arthritis, inflammatory bowel disease, psoriasis, and atherosclerosis, potentially identifying patients who might benefit from antiangiogenic therapies.

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