Soluble extracellular matrix metalloproteinase inducer (EMMPRIN, EMN) regulates cancer-related cellular functions by homotypic interactions with surface CD147

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EMMPRIN (extracellular matrix metalloproteinase inducer) is a widely expressed glycoprotein and a member of the immunoglobulin superfamily which exists in both a membrane-spanning and a soluble form. Homotypic interactions of EMMPRIN underlie its multiple roles in normal development and pathological situations such as viral infections, Alzheimer’s disease and cancer. This study employed a recombinant soluble, fully glycosylated EMMPRIN domain (rhsEMN) as a tool to characterize the structural basis of EMMPRIN-EMMPRIN receptor (EMNR) contacts and their functional effects on MCF-7 breast carcinoma cells. rhsEMN did not form dimers in solution but bound to surface EMMPRIN (EMN) on MCF-7 cells with high affinity and was readily internalized. The interaction interface for the homotypic contact was localized to the N-terminal Ig domain. rhsEMN exerted a stimulatory effect on proliferation of MCF-7 cells whereas it reduced cell migration in a dose-dependent manner. These effects were accompanied by an upregulation of endogenous EMMPRIN as well as of matrix metalloproteinase-14 (MMP-14), a membrane-bound protease involved in the extracellular release of soluble EMMPRIN, indicating a regulatory feedback mechanism. The proliferation-promoting activity of rhsEMN was mimicked by a novel functional antibody directed to EMMPRIN, underscoring that crosslinking of cell surface EMMPRIN (EMNR) is crucial for eliciting intracellular signalling. Addressing malignancy-related signal transduction in HEK-293 cells, we could show that rhsEMN triggers the oncogenic Wnt pathway.

Introduction

EMMPRIN (extracellular matrix metalloproteinase inducer; EMN; CD147) was initially described as a murine tumour-cell-derived collagenase stimulating factor showing affinity for target structures in the cell membrane of fibroblasts [1,2]. By sequence homology and functional similarities, EMN analogues were identified, although diversely designated, in different species, e.g. Basigin and M6 leukocyte activation antigen (human) [3], MRC-OX47 antigen/CE9 (rat) [4] and blood–brain barrier specific HT7 molecule in chicken [5,6].

EMN is a glycosylated protein belonging to the immunoglobulin superfamily [7] and exists both in a transmembrane and a soluble form [2]. It is widely expressed in a variety of normal and cancerous tissues and has been implicated in various physiological and pathological processes.

Abbreviations

APC, aliphococyanin; CD, cluster of differentiation; DMEM, Dulbecco’s minimum essential medium; EMMPRIN, EMN, extracellular matrix metalloproteinase inducer; EMNR, EMMPRIN receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HG, highly glycosylated; LG, less glycosylated; MMP, matrix metalloproteinase; PE, phycoerythrin; rhsEMN, recombinant human soluble EMMPRIN domain; TCF4, transcription factor 4.
expressed in various tissues and cell types [8] and is crucial for embryogenesis and developmental processes, e.g. EMN expression was observed during the whole process of mammary gland differentiation in mice [9]. EMN is also involved in various diseases, e.g. viral infections (HIV, hepatitis B virus) [10,11], Alzheimer’s disease [12] and ischaemia [13].

EMN has attracted much attention in tumour biology and oncology, since it is overexpressed and associated with poor prognosis in various malignant tumours [8,14–16]. It is also connected to cancer progression in various types of malignomes, e.g. of breast and brain [17]. Moreover, high abundance of EMN mRNA was linked to metastasis of lung, prostate and breast cancer [18,19]. On the cellular level, EMN expression was shown to promote matrix metalloproteinase (MMP) expression and malignancy parameters [10,11]. EMN is also involved in various diseased states, e.g. viral infections (HIV, hepatitis B virus) [10,11], Alzheimer’s disease [12] and ischaemia [13].

EMN has attracted much attention in tumour biology and oncology, since it is overexpressed and associated with poor prognosis in various malignant tumours [8,14–16]. It is also connected to cancer progression in various types of malignomes, e.g. of breast and brain [17]. Moreover, high abundance of EMN mRNA was linked to metastasis of lung, prostate and breast cancer [18,19]. On the cellular level, EMN expression was shown to promote matrix metalloproteinase (MMP) expression and malignancy parameters such as proliferation, migration, invasiveness and neoangiogenesis [14–16,20–23]. Molecular pathways underlying tumorigenic EMN activities are not thoroughly defined. Interestingly, oncogenic signalling by EMN in lung cancer cells has been reported to involve induction of the Wnt/β-catenin pathway [22].

The transmembrane form of the EMN molecule (at the same time also the EMN receptor EMNR in the course of homotypic interactions) consists of an extracellular domain comprising two Ig domains, a transmembrane domain and a short cytoplasmic tail, 41 amino acid residues in length [1]. While the amino-terminal Ig domain (Ig1) mediates homotypic interaction between EMN molecules and is essential for induction of MMPs [24,25], the carboxy-terminal Ig2 domain is involved in contacts with caveolin-1 [26] and integrin β1 [23]. The transmembrane domain can interact with cyclophilin A, thereby contributing to the stimulation of MMP expression and the secretion of pro-inflammatory cytokines [21,27]. EMN, in particular its cytoplasmic portion, also acts as a chaperone for the lactate transporter MCT-1 [28].

Functions and mechanistic properties of soluble EMN in cancer are less well characterized. Soluble EMN is present in serum and is discussed as a marker for hepatocarcinoma [29]. Soluble EMN from breast cancer, epitheloid sarcoma and laryngeal epidermoid cells was shown to induce expression of MMP-2 in fibroblasts [30–32] and to interact with membrane EMN in a homotypic manner via the Ig1 domain [29,30]. Generation of soluble EMN proceeds through activity of the membrane-type MMP MT1-MMP. MT1-MMP, also termed MMP-14, has been associated with invasiveness of cancers [33] and cleaves soluble EMN off the membrane EMN. This proteolytic fragment can activate target cells by interaction with surface EMMPRIN/CD147 and participates in a positive feedback mechanism [34]. Moreover, it was shown to activate intracellular mitogen activated protein kinase pathways [14], to stimulate migration of cervical carcinoma cells [35] and to form an active complex with cyclophilin A [21]. There are controversial reports on the issue whether homophilic interactions between EMN molecules result in the abundance of soluble EMN dimers. Bacterially produced recombinant soluble EMN was convincingly shown by gel filtration, native gel analysis and NMR to exist as monomer [26], whereas other workers observed by native gel electrophoresis a certain fraction of dimeric soluble EMN [30].

Depending on tissue type or cell line investigated, EMN exists in a ‘less glycosylated’ (LG) and two different ‘highly glycosylated’ (HG45 and HG65) forms [8]. It has been stated that EMN glycosylation enhances both oligomerization in solution and functional activity of the protein, e.g. induction of MMP secretion [36], and that deglycosylated EMN even antagonizes signalling induced by fully glycosylated CD147 [29]. This notion, however, is not supported by reports on fully binding-competent and bioactive soluble EMN produced in Escherichia coli [30].

Mechanisms and cellular consequences of homophilic interactions between soluble EMN and the membranous EMN receptor (EMMPRIN/CD147) in cancer are potential targets for therapeutic interference, but are not yet consistently settled. Here we employed a recombinant fully glycosylated soluble EMN protein, studied its binding properties and characterized its effects on malignancy parameters in MCF-7 cells. This breast cancer cell line is known to secrete considerable amounts of soluble EMN [15] and to express predominantly the maximally glycosylated form (HG65) of membranous EMN [8].

**Results**

**Generation and characterization of a recombinant glycosylated soluble human EMN domain**

An expression construct encoding a soluble histidine- and epitope-tagged EMN fragment comprising the extracellular Ig1 and Ig2 domains of EMN (Fig. 1A) was stably introduced into HEK-293 cells to produce recombinant human soluble EMN (rhsEMN). The secreted protein was purified by Ni²⁺-affinity chromatography (Fig. 1B). Analysis by SDS/PAGE showed three forms of the eluted protein ranging in apparent size between roughly 30 and 45 kDa (Fig. 1C).
Treatment of transfected cells with glycosylation inhibitor tunicamycin dose- and time-dependently resulted in the preferential secretion of the smaller rhsEMN form(s) compared to products of non-treated cells (Fig. 1D). This result indicates that the major form of rhsEMN produced by HEK-293 cells is HG.

Characterization of homotypic interactions of soluble EMN

Since conflicting findings have been reported with regard to dimer formation of soluble EMN, we defined the predominant conformation of purified rhsEMN. Size exclusion chromatography showed no indication of dimerization (Fig. 2A), and neither did native PAGE (data not shown). We conclude that fully glycosylated rhsEMN exists virtually exclusively in a monomeric form.

We then analysed homotypic interactions of rhsEMN with membranous EMN presented on MCF-7 breast carcinoma cells by studying its interference with epitope accessibility. This issue was addressed by the employment of two different antibodies with specificity for one or the other of the two Ig domains of EMN. In flow cytometric studies, short-term incubation of rhsEMN (3 h) selectively and dose-dependently inhibited cell staining by an antibody to the amino-terminal Ig domain (Ig1) of cell surface EMN (EMNR), while no effects were seen on the binding of an Ig2 (C-terminal) domain specific antibody (Fig. 2B). The highest tested concentration of rhsEMN (30 µg·mL−1) completely occupied EMN receptors within 30 min.
(data not shown). These results localize homotypic interaction between EMN molecules to Ig1.

We next addressed the fate of rhsEMN upon binding to the EMNR on MCF-7 cells. Cell lysates probed for rhsEMN after 4 h of incubation showed significant amounts of soluble EMMPRIN accumulated within cells, indicating a profound degree of rhsEMN internalization as a result of binding to the EMNR (Fig. 2C). Intracellular localization of soluble EMN upon uptake was confirmed by probing cytoplasmic cellular fractions for His-tagged rhsEMN (Fig. 2D). To further support the notion that the EMN receptor is responsible for this effect, MCF-7 cells with an shRNA-mediated stable knockdown of EMN were generated. Cytometric analysis proved a considerable decrease of surface EMN expression in MCF-7 shEMN cells compared to reference cells (MCF-7 sh scr) transfected with a scrambled control shRNA (Fig. 2E). As shown in Fig. 2F, cellular uptake of rhsEMN was observed only in EMN positive control
cells but was virtually absent in EMN knockdown cells.

**Adverse effects of rhsEMR on proliferation and migration of breast cancer cells**

To address the cellular consequences of the rhsEMN–EMNR interaction, we analysed the influence of rhsEMN on MCF-7 cell proliferation. A long-term proliferation test revealed a significant proliferative effect of an rhsEMN concentration as low as 0.01 µg·mL\(^{-1}\) which was only slightly increased by application of 1 µg·mL\(^{-1}\) rhsEMN (Fig. 3A). In contrast, migratory activity of MCF-7 cells, as quantified by a wound healing assay, was dose-dependently decreased by rhsEMN (Fig. 3B). These results indicate that soluble EMN alters the pattern of malignancy-related parameters in a specific manner.

**Proliferative effect of EMN receptor activation as a result of ligand-mediated crosslinking**

rhsEMN was employed as an antigen for the immunization of mice and generation of hybridoma clones. The aim was to obtain functional monoclonal antibodies as tools for the further characterization of the sEMN-EMNR system. Of nine purified antibodies positively tested for binding to denatured immobilized antigen (data not shown), two mAbs (10C5 and 6G9) were conjugated to FITC and were shown to bind specifically to native surface EMNR on MCF-7 cells by flow cytometry (Fig. 4A). Next, the two mAbs were tested for their effects on MCF-7 cell proliferation and were found to induce long-term cell proliferation (Fig. 4B). Notably, the degree of proliferative activity of the two antibodies correlated with their respective binding affinities for the EMNR. These results strongly suggest that bivalent interaction of mAbs with the EMNR mimics the proliferation-inducing effects of rhsEMN and indicates that EMNR crosslinking on MCF-7 cells is mitogenic.

**Upregulation of endogenous EMN in cells exposed to soluble EMN**

Having shown the malignancy-related effects of rhsEMN, we next addressed potential feedback mechanisms based on alterations in EMN receptor expression. To this end, we performed studies on EMNR expression in MCF-7 cells in dependence on stimulation with rhsEMN. As shown in Fig. 5A, treatment of MCF-7 cells with rhsEMN resulted in increased surface expression of EMNR as analysed by flow cytometry.
Western blot analysis of total cell lysates confirmed dose-dependent rhsEMN-induced upregulation of endogenous EMN, in both its HG and LG forms (Fig. 5B). Furthermore, treatment of MCF-7 cells with rhsEMN resulted in a dose-dependent but transient stimulation of EMN transcription as shown by quantitative real-time RT-PCR (Fig. 5C). Interestingly, the stimulatory effect on transcription was observed only after 6 h of cell exposure to rhsEMN, while it ceased and even turned into a repression after 24 h.

**Effects of soluble EMN on MMP expression and activity**

We analysed, on the levels of both mRNA and cell surface appearance, the expression of matrix metalloproteinase 14 (MMP-14), an established modulator of EMMPRIN activity, in dependence on rhsEMN stimulation (Fig. 6A,B). The finding that MMP-14 mRNA as well as surface expression was enhanced by rhsEMN suggests that the observed increased release of soluble EMN, probably by shedding of membrane EMN through MMP-14, contributes to EMN function via positive feedback. Reasoning that MMP-14 activity, on the one hand, is regulated by rhsEMN stimulation and, on the other hand, is an important mediator for the maturation of MMPs in general, we studied the effect of EMN knockdown on MMP activity released by MCP-7 cells. Zymographic analysis showed that MCF-7 knockdown cells (MCF-7 shEMN) secrete significantly less MMP activity than EMN positive control cells, in particular with regard to MMP-2 and MMP-9 (Fig. 6C).

**Induction of cellular Wnt/β-catenin signalling by soluble EMN**

As an obvious candidate pathway for proliferation control, we studied Wnt/β-catenin signalling in cells exposed to rhsEMN. HEK-293 cells were employed for initial investigations since (a) this cell line represents a well characterized model for the analysis of Wnt/β-catenin-driven gene regulation [37] and (b) we had previously generated a stable HEK-293 derivative overexpressing rhsEMN (Fig. 1) which could be conveniently compared with its non-transfected counterpart.

Transfection of HEK-293 cells with a Wnt/β-catenin-activatable, TCF4 (transcription factor)-controlled reporter gene construct and subsequent incubation with different concentrations of rhsEMN resulted in a dose-dependent stimulation of reporter gene expression (Fig. 7A). Furthermore, HEK-293 cells overexpressing rhsEMN showed a 5-fold increase of Wnt/β-catenin-dependent reporter activity compared to non-transfected cells (Fig. 7B). These results strongly suggest that the influence of soluble EMN on malignancy-related cell behaviour involves signal transduction via the Wnt/β-catenin pathway.

**Discussion**

The rhsEMN domain generated for this study showed homotypic interaction with EMN molecules present in
the membrane of breast cancer cells with the binding interface localized to the N-terminal Ig domain. These findings are largely in line with results by Belton et al. [38] who showed specific interaction of a bacterially produced EMN exodomain with surface EMMPRIN on uterine fibroblasts and cervical adenocarcinoma cells. The non-glycosylated soluble EMN domain from E. coli [38], however, bound with about five times lower affinity to cells compared to the eukaryotic rhsEMN protein used in this study, pointing at a functional contribution of glycosylation to protein–protein interaction.

Our results clearly show that biologically active rhsEMN has no tendency to form dimers, supporting the earlier notion that soluble EMMPRIN is monomeric [21] and that the observed association of extracellular EMN domains [38] might result from non-physiological disulfide bridges occurring between recombinant proteins of bacterial origin.

Upon interaction with membranous EMN of breast cancer cells, rhsEMN becomes quickly internalized. A similar process was also described by Belton et al. [38] who have demonstrated uptake of bacterially produced soluble EMN by fibroblasts as a consequence of interaction with the EMN/basigin splice variant 3. Intracellular reactions initiated upon cellular entry of soluble EMN have not yet been defined. Sidhu et al. [22] provided evidence for a functional connection between EMN and Wnt/β-catenin signalling in lung cancer cells. We have been able to show that the soluble form of EMN can trigger Wnt/β-catenin-related TCF4-associated transcriptional regulation. While we have not yet...
characterized reactions connecting the initial contact of rhsEMN with membranous EMN to transcription control, our results suggest that enhancement of the endogenous EMN level by rhsEMN is at least to some extent attributable to TCF4 activity on the EMN promoter. Gene regulation experiments employing an EMN promoter based reporter gene construct revealed that rhsEMN treatment of cells had only a minor stimulatory effect (data not shown). In contrast, significant upregulation of EMN mRNA was determined by real-

Fig. 6. MMP expression and activity in MCF-7 cells in dependence on EMN. (A) Quantification of MMP-14 mRNA abundance in MCF-7 cells by RT-PCR upon stimulation with the indicated rhsEMN concentrations for 24 h. Reactions were performed as described in Materials and methods. Data represent means of three independent experiments with standard deviation; the asterisk indicates the significance of change relative to non-stimulated cells ($P < 0.05$). (B) Increase of MMP-14 surface expression on MCF-7 cells in response to stimulation with 1 µg·mL$^{-1}$ (left-hand side) or 10 µg·mL$^{-1}$ (right-hand side) rhsEMN. Flow cytometric analysis of PE-coupled anti-MMP-14 binding to MCF-7 cells as described in Materials and methods. Black curves represent signals from non-treated cells; grey curves represent signals from rhsEMN-treated cells. Shown is one representative out of three independent experiments. Cells incubated with FITC-conjugated anti-mouse IgG served as a control. (C) MMP activity secreted by MCF-7 sh scr (left) and MCF-7 shEMN cells (right) determined by gelatine zymography. Samples of 20 µL from supernatants derived from the indicated cells were separated through an 8% SDS/PAGE gel containing 0.5% gelatine. After staining and destaining, the gel was photographed. Molecular masses of marker proteins are given on the left-hand side; pro-MMPs and mature MMPs were identified by their molecular masses according to [46]. Results from a representative out of three independent experiments are shown.
time RT-PCR. These findings do not provide a full picture yet; however, they leave open the possibility that, through complex transcriptional regulation of the EMN gene and involvement of β-catenin/TCF, a positive feedback loop based on EMN expression and activity is triggering cellular malignancy parameters.

Our results are consistent with the notion that soluble EMN elicits a specific pattern of changes in malignancy-related parameters of tumour cells. In MCF-7 breast cancer cells, activation of the EMN receptor (both by rhsEMN and a crosslinking antibody) was found to favour cell proliferation on the one hand and to dampen cell migration on the other hand. This is in line with findings by Sato et al. [35] who reported that reciprocal interaction of EMN receptors via their second Ig domains impaired migration of cervical carcinoma cells. Cell migration is closely connected to cell invasiveness. It is thus interesting, in the light of our findings, that an inhibitory effect of a recombinant EMMPRIN domain was reported to inhibit expression of invasiveness-promoting MMP-2 [25]. Published findings suggest the possibility that this effect of soluble EMN is connected to functions of integrins. Specific interactions of membraneous EMMPRIN, i.e. EMNR, with integrins expressed on adjacent cancer cells were found to be associated with pronounced tumour growth and invasive behaviour [23,39]. We hypothesize that soluble EMN, through homotypic binding to the EMNR, facilitates the interaction of the EMNR with integrins and homotypic interaction of EMN receptors by inducing EMN receptor levels on the cell surface. This results in decreased cellular motility but an increased proliferation rate. It remains to be elucidated whether the integrin-associated focal adhesion kinase signalling pathway is involved in these malignancy modulating processes. Strong hints to this connection come from findings on reduced focal adhesion kinase activity in EMN-knockdown hepatocellular carcinoma cells [23].

This study provides evidence that the multimerization status of EMNR on the cell surface is an important determinant for malignancy of MCF-7 breast cancer cells, probably with involvement of β-catenin-mediated signalling. Our results suggest that rhsEMN-mediated induction of MCF-7 proliferation is connected to the stimulation of the Wnt/β-catenin target protein MMP-14. Studies on glioma cells showed pro-proliferative effects of MMP-14 [8]. Moreover, phosphorylation of the cytoplasmatic portion of MMP-14 was demonstrated to affect growth of breast cancer cells [40]. Another possible explanation is positive feedback of EMN-governed cellular reactions proceeding through increased generation of soluble EMMPRIN via enhanced MMP-14 expression. Such a mechanism could result in an EMN-ligand-mediated increase of EMN receptors on adjacent tumour cells without obligatory participation of cell types other than tumour cells. Our results are also consistent with the possibility that EMN-mediated upregulation of MMP-14 activity...
might result in enhanced activation of other MMPs and thus contribute to cancer cell malignancy. Interesting in this context are recent findings showing that selective inhibition of MMP-14 has the potential to interfere with the progression of breast cancer and other solid tumours [41].

It is important to note, however, that cancer cells in a physiological setting are exposed to a complex environment, consisting not only of soluble factors but also of stromal cells such as fibroblasts. Complex mechanisms, most probably controlled by intercellular communication, participate in the regulation of EMN expression in cancer cells as suggested by the variety of transcription factors (Sp1, HIF-1α and c-myc) which contribute to EMN promoter regulation [3,11,41]. The role of EMMPRIN as a component of crosstalk between cancer and stromal cells is exemplified by positive feedback regulation of soluble EMMPRIN and MMP expression in cocultures of breast cancer cells and fibroblasts [31]. It will be very interesting to study the role of soluble EMN in settings that more closely resemble the natural tumour situation in order to more thoroughly understand its role in the control of oncogenic parameters.

Materials and methods

Cells and cell culture

HEK-293 and MCF-7 cells were purchased from the German Collection of Microorganisms and Cell Lines (DSMZ, Braunschweig, Germany). Cells were routinely cultured in Dulbecco’s minimum essential medium (DMEM) supplemented with phenol red, 4 mM stabilized L-glutamine, 4.5 g·L⁻¹ D-glucose, 10% FBS (Biochrom, Berlin, Germany) and 1% gentamycin (Ratiopharm, Ulm, Germany) at 37 °C and 5% CO₂. Media were obtained from Sigma-Aldrich (Steinheim, Germany). Every 2–3 days cells were passaged after incubation for 5 min with trypsin/EDTA (0.05%/0.02%) (Biochrom, Berlin, Germany) to disrupt the adherent monolayer of cells.

Antibodies and reagents

EMN (Ig1) goat polyclonal Ab (N19), horseradish peroxidase (HRP) conjugated secondary antibodies and FITC phycoerythrin (PE) coupled goat anti-mouse secondary antibodies were purchased from Santa Cruz Biotechnologies (Dallas, TX, USA). EMN mouse allophycocyanin (APC)- and PE-coupled mAbs were from ImmunoTools (Friesoythe, Germany), anti-EMN (Ig2) mouse mAb was from EXBIO Antibodies (Prague, Czech Republic), anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) mouse mAb (MAB374) was obtained from Merck Millipore (Darmstadt, Germany), anti-α-tubulin rabbit antibody was from Cell Signalling Technologies (Cambridge, UK), anti-His-tag mouse mAb was from Qiagen (Hilden, Germany) and PE-coupled anti-MMP-14 mAb was from R&D Systems (Minneapolis, MD, USA).

mAbs to the EMN extracellular domain were raised in BALB/c mice and tested for specific binding to the antigen by ELISA as described elsewhere [37]. mAbs from individual clones were purified by affinity chromatography and FITC-labelled for flow cytometric analyses as detailed before [37]. Specific binding of mAbs to cell surface EMN was tested using MCF-7 cells.

Tunicamycin was purchased from Sigma-Aldrich (Taufkirchen, Germany), zeocin from Genaxxon BioScience GmbH (Ulm, Germany). Imidazole and G418 were obtained from Carl Roth (Karlsruhe, Germany).

Recombinant DNA techniques

A cDNA construct encoding human EMN (kindly provided by A. Till, Institute for Clinical Molecular Biology, University Hospital of Schleswig Holstein, Kiel, Germany) was used as a template to generate a PCR product representing the extracellular domain of EMN. Primers for amplification and addition of a C-terminal 6xHis-tag were EMN-sense (5’-gcctagactatcaatgtgctgttgggctc-3’) and EMN antisense (5’-agatggecgcaggggtgcgtg-3’). The PCR product was digested with XhoI and XbaI and ligated into the XhoI/XbaI large vector fragment of pcDNA-IL13RεxAcyt [4,5]. This expression plasmid provides the coding sequence for an amino-terminally located signal peptide sequence originating from the human interleukin-4 receptor ε subunit and a P5D4 epitope-tag originating from vesicular stomatitis virus and approved for detection purposes [5,42]. The resulting expression construct was termed pcDNA3.1EMN-Ecto-domain-His₆. All constructs were verified by DNA sequencing.

Expression in HEK-293 cells and purification of recombinant protein

Plasmid pcDNA3.1EMN-Ecto-domain-His₆ was transfected into HEK-293 cells via electroporation using the Nucleofector™ device and Transfection Kit V (Lonza, Bergisch-Gladbach, Germany). Cells were selected with 200 μg·mL⁻¹ zeocin (Genaxxon BioScience) for 3 weeks to generate a stable HEK-293 cell line secreting rhsEMN. Conditioned medium was collected and stored at −20 °C until protein purification. For purification, conditioned medium was dialysed against a buffer containing 300 mM NaCl, 20 mM Na₂HPO₄, 12 mM H₂O, pH 7.4. His-tagged rhsEMN was purified from conditioned medium via Ni²⁺-affinity chromatography by employing an ÄKTA purifier™ FPLC system (GE Healthcare, Munich, Germany) and using an
Ni$^{2+}$-sepharose matrix (GE Healthcare). rhsEMN was eluted by a stepwise imidazole gradient. The purified protein was dialysed against PBS and stored in aliquots at −80 °C until use.

**Size exclusion chromatography**

Size exclusion chromatography was performed using an AKTA purifier FPLC system. Test protein (330 µg) and standard proteins of known molecular mass (Blue Dextran 2000 kDa, aldolase 158 kDa, BSA 67 kDa, ovalbumin 43 kDa, chymotrypsinogen 25 kDa; Amersham Pharmacia Biotech, Vienna, Austria) were loaded onto a Superdex200™ HR 10/30 column (gel volume 24 mL, diameter 1 cm; Amersham) with a dextran/agarose matrix at a flow rate of 0.5 mL/min$^{-1}$. Tris buffer (pH 8.0) was used for column equilibration and washing. Proteins were monitored by UV absorbance at 280 nm.

**SDS/PAGE and western blot**

SDS/PAGE and western blot analysis were performed as described previously [4]. Briefly, whole cell extract or cytoplasmic fractions obtained by removal of membrane debris through 30 min centrifugation at 16 000 g were solubilized in gel loading buffer (62.5 mM Tris/HCl pH 6.8; 2% SDS; 25% glycerol; 1% phenol blue; 5% β-mercaptoethanol), boiled for 10 min and separated through 10% acrylamide SDS gels. Proteins were silver or Coomassie stained as described previously [43] or blotted onto nitrocellulose membranes (GE Healthcare). Non-specific binding sites on blot membranes were blocked with 5% milk protein in 68 mM Tris buffer (pH 8.0) was used for gel loading buffer (62.5 mM Tris/HCl pH 6.8; 2% SDS; 25% glycerol; 1% phenol blue; 5% β-mercaptoethanol), boiled for 10 min and separated through 10% acrylamide SDS gels. Proteins were silver or Coomassie stained as described previously [43] or blotted onto nitrocellulose membranes (GE Healthcare). Non-specific binding sites on blot membranes were blocked with 5% milk protein in 68 mM Tris buffer (pH 8.0) was used for gel loading buffer (62.5 mM Tris/HCl pH 6.8; 2% SDS; 25% glycerol; 1% phenol blue; 5% β-mercaptoethanol), boiled for 10 min and separated through 10% acrylamide SDS gels.

**Flow cytometry**

Cells were harvested and washed in PBS. Treatment with rhsEMN was carried out for 3 h in PBS on an orbital shaker (for short-term experiments) at room temperature or for 24 h in DMEM at 37 °C. Cells were washed in PBS and mAbs MMP-14-PE, CD147-APC, CD147-PE (10 µg/mL$^{-1}$) (Ig1 domain) and EMN-Ig2 (20 µg/mL$^{-1}$) were incubated for 1 h on an orbital shaker. Secondary antibodies (FITC-, PE-coupled) were diluted 1:300 and applied for another 30 min to primary-antibody-treated and non-treated samples (negative control). After another washing step in PBS, cells were analysed on a CyFlo™ Space Cytometer (Partec, Münster, Germany). Data analysis was performed using the CYFLOGIC SOFTWARE (CyFlo Ltd, freeware).

**Cell proliferation assays**

Cell proliferation was measured either using the xCELLigence™ system (Roche, Mannheim, Germany) or the Cell-Titer-Glo™ assay (Promega, Mannheim, Germany). xCELLigence is based on the measurement of impedance triggered by cell attachment. The dimensionless cell index (CI) quantifies cell attachment resulting from cell growth and proliferation. The relative cell index (rcI) represents the ratio between the CI at a specific time point and the CI determined at the start of the measurement ($t_0$). Samples of 1.5 × 10⁵ MCF-7 cells were seeded into each well of an E-plate (ACEA Biosciences, San Diego, CA, USA) and grown overnight in an xCELLigence/RTCA DP instrument (Roche). Stimulus was optionally added to wells diluted in fresh 200 µL DMEM/10% FBS on the next day, thus defining $t_0$.

The CellTiter-Glo assay (Promega) was applied following instructions by the manufacturer. MCF-7 cells were seeded into 96-well plates (1000 cells-well$^{-1}$) and optionally supplemented with effectors on the following day in DMEM/10% FBS. Measurements were performed according to the manufacturer’s instructions by adding 40 µL of the assay reagent directly to the cells for 10 min followed by quantification of luminescence using a Glo-Max™ 96 Microplate Luminometer (Promega).

**Cell migration assay**

Cell motility was quantified by monitoring the closing of a wound scratched into a monolayer of MCF-7 cells. Samples of 2 × 10⁵ cells-well$^{-1}$ were grown in 24-well plates for 24 h before generating cross-shaped scratches with a pipette tip. The degree of wound healing was determined after 24 h incubation by taking microscope pictures. Photographs were analysed for the percentage of scratch closure using the TSCRATCH software developed by Tobias Gebick and Martin Schulz and provided as freeware by the Computational Science and Engineering Laboratory of the Eidgenössische Technische Hochschule, Zurich, Switzerland.

**Reporter gene assay**

HEK-293 cells were transfected with the TCF/LEF-responsive firefly luciferase reporter gene construct pGL4.26BAR-Luc [44] along with the renilla luciferase reference construct pRL-TK (Promega) via electroporation using the Nuclear factor device and Transfection Kit V (Lonza). 24 h post transfection, cells were treated with stimuli for another 24 h before luciferase activity was quantified by chemiluminescence measurement employing a Glo-Max 96 Microplate Luminometer from Promega.
RNA extraction, cDNA synthesis and RT-PCR

Extraction of total mRNA was performed using an RNA isolation kit from Macherey-Nagel (Düren, Germany) following the manufacturer’s protocol. 5 μg of RNA was reverse transcribed with RevertAid™ reverse transcriptase from Thermo Scientific (St Leon-Rot, Germany) employing random hexamer primers and oligo-(dT)18 primers (Thermo Scientific, Schwerte, Germany). Q-real-time-PCR was performed by using the Universal ProbeLibrary (UPL)™ technology by Roche (Mannheim, Germany). Target-specific probes and primers were identified employing the Universal ProbeLibrary Assay Design Center (Roche). Primer–probe combinations used in this study were GAPDH sense (5’-agccacatcgaccaac-3’), GAPDH antisense (5’-gccaacta cgaccaaatcc-3’), probe #60; EMN sense (5’-ctggtgctcaagata ctct-3’), EMN antisense (5’-gctggagctcactga-3’), probe #68; MMP-14 sense (5’-gcttgtgactctgaattgga-3’), MMP-14 antisense (5’-aggggtctactggtc-3’), probe #37. PCR products were generated by 40 cycles of 15 s 95 °C, 60 s 60 °C, 90 s 72 °C in MicroAmp™ optical 96-well reaction plates using the StepOne™ real-time PCR procedure (Applied Biosystems, Darmstadt, Germany). Relative fold changes were calculated with the comparative Ct method. Gene expression levels of treated samples were related to the non-treated control (control = 1).

Stable EMN knockdown in MCF-7 cells by shRNA

EMN knockdown was performed using the pSUPER.GFP/Neo-EMN-shRNA and the pSUPER.GFP/Neo-scrambled RNA vector (specificity control) which are described elsewhere [45] and were kindly provided by A. Till, University Hospital Kiel, Germany. MCF-7 cells were transfected with 2 μg DNA per well using the TurboFect™ reagent (Thermo Fisher, Schwerte, Germany) according to the manufacturer’s instructions. Selection of G418 resistant clone mixtures started 3 days post transfection by cultivation of transfecants in the presence of 200 μg·mL⁻¹ G418. After cytometric verification of stable expression of green fluorescent protein, resulting cell populations were designated MCF-7 shEMN and MCF-7 sh scr, respectively, routinely grown in the presence of G418 (100 μg·mL⁻¹) and subjected to functional investigations.

Gelatin zymography

Samples were mixed with a non-reducing sample buffer (4.7 mL H2O, 4 mL glycerol, 1 mL 0.5 m Tris/HCL (pH 6.8), 1 mL SDS (10%), 0.05% bromophenol blue) and separated through 8% SDS/PAGE gels containing 0.5% gelatin. Gels were developed as described previously [46]; the identity of pro- and mature forms of proteases was deduced from apparent molecular masses according to zymographic MMP patterns given in [47].

Statistics

Distribution of values was analysed for normality. All values were normally distributed and determination of statistical significance was performed using analysis of equality of variances (Levene’s test) and the t-test (spss software). Values of $P < 0.05$ were considered as statistically significant.

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Author contributions

Nadine Knutti performed and documented all experiments except for the generation of monoclonal antibodies which were raised and isolated by Michael Kuepper. Karlheinz Friedrich designed the study, continuously discussed experiments and results and wrote the paper.

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