MT1-MMP regulates VEGF-A expression through a complex with VEGFR-2 and Src

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Summary

Membrane-type-1 matrix metalloproteinase (MT1-MMP) is a zinc-dependent type-I transmembrane metalloproteinase involved in pericellular proteolysis, migration and invasion, with elevated levels correlating with a poor prognosis in cancer. MT1-MMP-mediated transcriptional regulation of genes in cancer cells can contribute to tumour growth, although this is poorly understood at a mechanistic level. In this study, we investigated the mechanism by which MT1-MMP regulates the expression of VEGF-A in breast cancer cells. We discovered that MT1-MMP regulates VEGFR-2 cell surface localisation and forms a complex with VEGFR-2 and Src that is dependent on the MT1-MMP hemopexin domain and independent of its catalytic activity. Although the localisation of VEGFR-2 was independent of the catalytic and intracellular domain of MT1-MMP, intracellular signalling dependent on VEGFR-2 activity leading to VEGF-A transcription still required the MT1-MMP catalytic and intracellular domain, including residues Y573, C574 and DKV582. However, there was redundancy in the function of the catalytic activity of MT1-MMP, as this could be substituted with MMP-2 or MMP-7 in cells expressing inactive MT1-MMP. The signalling cascade dependent on the MT1-MMP–VEGFR-2–Src complex activated Akt and mTOR, ultimately leading to increased VEGF-A transcription.

Key words: MT1-MMP, VEGF-A, Src, VEGFR-2, KDR

Introduction

Matrix metalloproteinases (MMPs) are key modulators of normal physiological tissue homeostasis and pathologies, including cancer, cardiovascular disease and arthritis (Burrage et al., 2006; Egeblad and Werb, 2002; Janssens and Lijnen, 2006; Overall and Kleifeld, 2006). The MMPs have a broad substrate repertoire, ranging from components of the extracellular matrix (ECM) to cytokines, chemokines and membrane-anchored receptors. In many instances, there is redundancy in the proteinase network with respect to substrate cleavage, often with several MMPs as well as proteinases from other families contributing to proteolysis of the same substrate (Egeblad and Werb, 2002; Lopez-Otin and Matrisian, 2007; Mohamed and Sloane, 2006). The identification of novel and unique modes of regulation of the physiology and pathology by MMPs is therefore necessary for the identification of which MMPs are suitable therapeutic targets (Overall and Kleifeld, 2006).

One MMP that is of particular interest is membrane-type-1 MMP (MT1-MMP; also known as matrix metalloproteinase-14, MMP14). The importance of MT1-MMP is demonstrated by the unusually severe phenotype that occurs upon gene ablation in mice; MT1-MMP$^{-/-}$ mice show defects in vascularisation, connective tissue turnover and bone formation, leading to craniofacial abnormalities, dwarfism, osteopenia and arthritis (Holmbeck et al., 1999; Lehti et al., 2005; Zhou et al., 2000). The expression of MT1-MMP is frequently upregulated in cancer, and modulation of its expression in cancer models leads to the regulation of tumour growth, invasion, metastasis and angiogenesis, making MT1-MMP an important target for therapy or prognosis (Nomura et al., 1995; Soulie et al., 2005; Sounni et al., 2002; Tokuraku et al., 1995; Ueno et al., 1997; Yoshizaki et al., 1997; Zhang et al., 2006).

MT1-MMP has a broad repertoire of ECM substrates including type-I, -II and -III collagen, fibronectin and laminin (d'Ortho et al., 1997; Fosang et al., 1998; Koshikawa et al., 2000; Ohuchi et al., 1997) as well as non-ECM proteins, including CD44, syndecan-1, ICAM-1 and CTGF (Endo et al., 2003; Kajita et al., 2001; Sithu et al., 2007; Tam et al., 2004). MT1-MMP is important in regulating cellular migration and invasion, particularly through three-dimensional matrices in which it can also confer a proliferative advantage (Hotary et al., 2000; Hotary et al., 2003; Lafleur et al., 2002; Wolf and Friedl, 2009). MT1-MMP has also been shown to regulate transcriptional programmes in a number of cell lines. Overexpression of MT1-MMP in the normally poorly tumorigenic breast cancer cell line MCF-7 leads to increased tumour growth, angiogenesis and metastasis, which is in part due to increased transcription of vascular endothelial growth factor A (VEGF-A) (Sounni et al., 2002; Sounni et al., 2004). Overexpression of MT1-MMP also increased transcription of the gene encoding VEGF-A and tumour growth in U251 cells (Deryugina et al., 2002b). A recent microarray study in fibrosarcoma cells depleted of MT1-MMP also identified changes in a number of pro-angiogenic and pro-tumorigenic genes (Rozanov et al., 2008). MT1-MMP was found to regulate transcription of Dickkopf-related protein 3 (DKK3) in urothelial cells and Smad1 in various cancer cell lines (Freudenberg and Chen, 2007; Saeb-Parsy et al., 2008). However, little is known about the mechanisms by which MT1-MMP regulates transcription. In MCF-7 cells, MT1-MMP has been reported to regulate expression of VEGF-A through its catalytic activity, amino acid C574 in its intracellular domain (ICD) and Src kinase activity (Sounni et al., 2004). However, how MT1-MMP regulates intracellular signalling pathways through kinases such as Src and how this results in activation of gene transcription is not clearly understood.

As regulation of VEGF-A transcription by MT1-MMP appears to be a common observation, we investigated how it regulates transcription of VEGF-A in detail in MCF-7 cells. We found that MT1-MMP regulates the localisation of and signalling by vascular endothelial growth factor receptor 2 (VEGFR-2), which results in increased VEGF-A transcription.

Results

Expression of MT1-MMP increases VEGF-A transcription in MCF-7 cells

Our first aim was to determine whether the MT1-MMP-dependent increase in VEGF-A transcription previously reported by Sounni and colleagues (Sounni et al., 2004) could be reproduced using short-term transient expression of MT1-MMP, rather than through the selection of stably expressing clones. By using this approach, we aimed to identify whether the increase in VEGF-A had arisen in the original study from the adaptation to the selection process and whether the transcriptional upregulation of VEGF-A occurred rapidly. To this end, MT1-MMP was expressed in MCF-7 cells either by transient transfection (Fig. 1A) or by adenoviral transduction (see supplementary material Fig. S1). Transient expression of wild-type MT1-MMP (MT1-WT) in MCF-7 cells led to a 2.7-fold increase in VEGF-A mRNA level (P=0.0007, Student's t-test) compared with cells transfected with the pcDNA3.1 vector control (Fig. 1A). A similar dose-dependent increase was observed using adenoviral transduction of the MT1-WT cDNA (supplementary material Fig. S1A,B). Expression of VEGF-A mRNA was also found to correlate with MT1-MMP expression (supplementary material Fig. S1C). As expected, expression of MT1-WT increased VEGF-A protein expression in cell lysates as well as its secretion into the extracellular milieu (Fig. 1B). VEGF-A mRNA upregulation was found to be dependent on metalloproteinase (MP) activity, as demonstrated using the synthetic broad-spectrum MP inhibitor CT1746 (P<0.001 compared with DMSO control) or following the addition of the physiological recombinant human MP inhibitor Tissue inhibitor of metalloproteinases 2 (rh-TIMP-2) (P<0.0001, Fig. 1C). By contrast, rh-TIMP-1 increased the MT1-MMP-mediated VEGF-A mRNA expression slightly, although this was not significant (P>0.05, Fig. 1C). These data confirm previous findings that the enzymatic activity of a MP is required for the transcriptional upregulation of VEGF-A in MCF-7 cells (Sounni et al., 2004) and that VEGF-A mRNA and protein levels increase shortly after the expression of MT1-MMP independently of the expression system used.

MMP-2 can substitute for the catalytic activity of MT1-MMP and increase the expression of VEGF-A mRNA

Sounni and colleagues had previously demonstrated that MT1-MMP activity was required to induce expression of VEGF-A mRNA in MCF-7 cells by: (i) expressing an inactive MT1-MMP cDNA (MT1-E240A), (ii) by inhibition with rh-TIMP-2 or (iii) using a functional blocking antibody (Sounni et al., 2004). We also confirmed that, in contrast to MT1-WT-expressing cells, the expression of MT1-E240A in MCF-7 cells did not increase VEGF-A mRNA levels compared with those of mock transfectants (Fig. 2B and Fig. 6D). In order to confirm that MT1-MMP activity is required in tumour cells, we next investigated whether the activity of other MMPs, which might be expressed in the tumour microenvironment in vivo, could substitute for MT1-MMP. MCF-7 cells do not express MMP-2 (Sounni et al., 2004), although it is commonly expressed by several cell types within solid tumour stroma (Noel et al., 2008). The addition of rh-MMP-2 to MCF-7 cells did not alter VEGF-A mRNA levels in pcDNA3.1- or MT1-WT-transfected cells (Fig. 2B). Surprisingly, the addition of increasing amounts of purified rh-MMP-2 to cells expressing MT1-E240A was able to increase VEGF-A mRNA expression to levels similar to those observed upon transfection of MT1-WT alone (Fig. 2B). In order to assess whether this rescue was dependent on the MT1-MMP ICD, MCF-7 cells were transfected with MT1-E240A-ΔICD (Fig. 2A) and incubated with increasing amounts of rh-MMP-2. In this instance, we were unable to observe

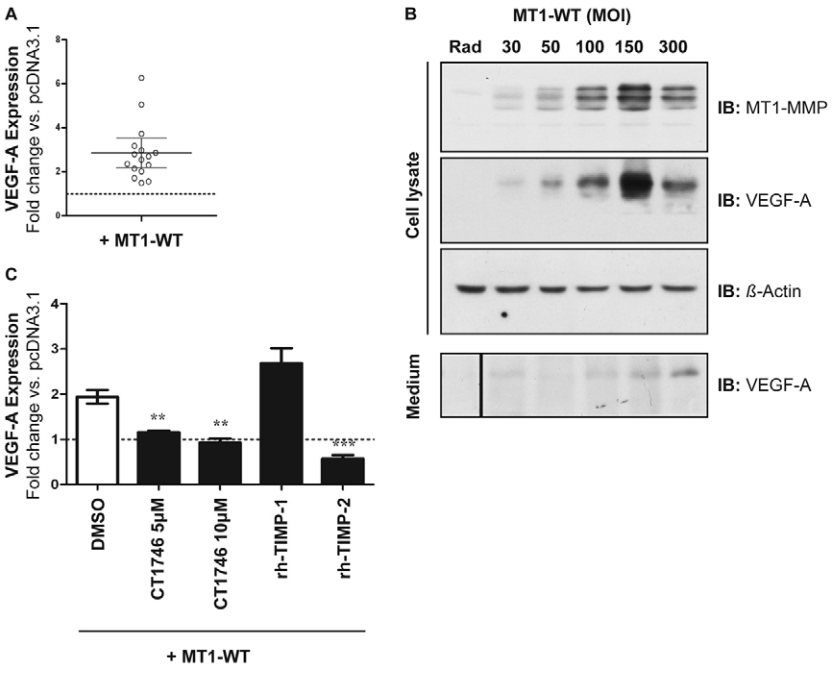

Fig. 1. MT1-MMP expression in MCF-7 cells increases VEGF-A expression. (**A**) MCF-7 cells were transiently transfected with MT1-WT cDNA, and VEGF-A mRNA levels were detected by real-time PCR. Relative expression levels of VEGF-A were normalised to GAPDH. Data represent the mean expression plotted as the fold change compared with the pcDNA3.1 vector control (dotted line, n=16, ±95% confidence intervals). (**B**) MCF-7 cells were transduced with increasing MOIs of control (Rad, 300 plaque-forming units per cell) or MT1-WT-expressing adenovirus, and cell lysates and conditioned medium were analysed by immunoblotting (IB) with an anti-MT1-MMP (LEM-2/15.8), an anti-VEGF-A or an anti-β-actin antibody. (**C**) MT1-WT-transfected cells were treated with 1 µM rh-TIMP-1, 1 µM rh-TIMP-2 and 5 µM or 10 µM CT1746 for 24 hours. VEGF-A mRNA expression levels were detected by real-time PCR relative to GAPDH expression levels. Data represent the mean VEGF-A mRNA expression plotted as the fold change compared with the pcDNA3.1 control (dotted line, n=3, ±s.e.m.), with $^{**}P$<0.001 and $^{***}P$<0.0001 compared with pcDNA3.1.

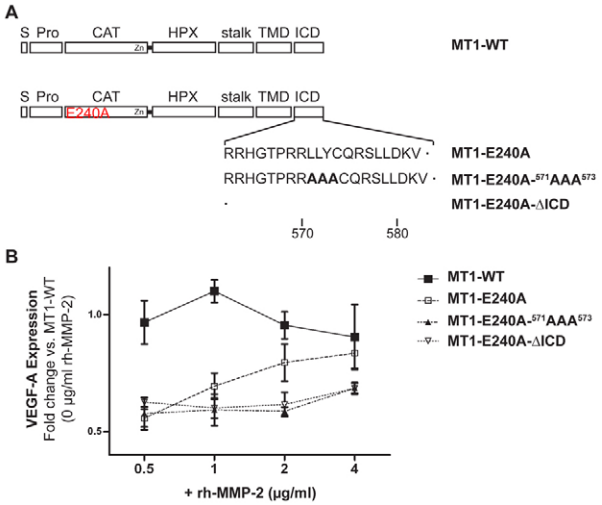

Fig. 2. MMP-2 rescues deficiency in the catalytic activity of MT1-MMP in order to increase the level of VEGF-A mRNA. (**A**) Schematic representation of the MT1-MMP mutants used. S, signal sequence; Pro, propeptide; CAT, catalytic domain; HPX, hemopexin domain; stalk, stalk region; TMD, transmembrane domain; ICD, intracellular domain. (**B**) MCF-7 cells were transfected with cDNAs expressing MT1-WT, MT1-E240A, MT1-E240A-^{571}AAA^{573} or MT1-E240A-ΔICD. Cells were incubated for 24 hours with increasing concentrations of rh-MMP-2, and VEGF-A mRNA expression levels were assessed by real-time PCR. Data represent VEGF-A mRNA levels plotted as the fold change (n=4, ±s.e.m.), compared with the mRNA levels of MT1-WT-transfected cells without addition of rh-MMP-2.

the MMP-2 rescue previously shown (Fig. 2B), demonstrating the importance of the MT1-MMP ICD in the increase in VEGF-A mRNA and redundancy of the MT1-MMP catalytic activity. Amino acid Y573 of the ICD has been shown to be phosphorylated by Src, and the region LLY573 has also been demonstrated to be required for the MT1-MMP-dependent increase in VEGF-A (Nyalendo et al., 2007; Sounni et al., 2004). To test the role of this motif, we transfected MCF-7 cells with MT1-E240A-^{571}AAA^{573} (Fig. 2A) and found that the addition of rh-MMP-2 did not increase VEGF-A mRNA. Furthermore, an increase in VEGF-A mRNA expression was observed following addition of rh-MMP-7 to MT1-E240A-expressing cells, confirming the redundancy of the MT1-MMP catalytic activity (supplementary material Fig. S2).

VEGFR-2, PI3K, Akt, mTOR and Src kinase activities are required for the MT1-MMP-induced transcription of VEGF-A

To obtain a better understanding of the cellular signalling mechanism underlying the MT1-MMP-induced upregulation of VEGF-A, MT1-WT-transfected MCF-7 cells were treated with inhibitors of key signalling pathways, and VEGF-A mRNA levels were assessed by real-time PCR. As previously reported, inhibition of members of the Src-kinase family with the pyrazolopyrimidine PP2 significantly ablated the MT1-MMP-induced upregulation of VEGF-A mRNA levels (Fig. 3A) (Sounni et al., 2004). A similar observation was obtained following addition of an inhibitor of the RAC-alpha serine/threonine-protein kinase (also known as Akt/PKB) (SH-5), phosphoinositide 3-kinase (Wortmannin, LY294002) or mTOR (rapamycin) (Fig. 3A, each P<0.0001 compared with DMSO control). Treatment of cells with SU4312 was also found to inhibit MT1-MMP-mediated expression of VEGF-A (Fig. 3A, P<0.0001 compared with DMSO control). As SU4312 inhibits both VEGFR and the Platelet-derived growth factor receptor PDGFR, we used AG-1296 to inhibit PDGFR more selectively. As shown in Fig. 3A, AG-1296 did not inhibit the MT1-MMP-induced VEGF-A mRNA expression, suggesting that VEGFR activity is required. Treatment of MT1-WT-transfected MCF-7 cells with inhibitors of the Mitogen-activated protein kinase kinase MEK (PD98059), the Mitogen-activated protein kinase 8 (also known as JNK1) (SP006125), the Epidermal growth factor receptor EGFR (gefitinib, tarceva/erlotinib), the Inhibitor of nuclear factor kappa-B kinase IKK (BMS-345541), the Insulin-like growth factor 1 receptor IGF1R (PPP), p38 (SB203580) or the proteasome (lactacystin) did not inhibit the MT1-MMP-driven increase in VEGF-A mRNA levels (P.A.E., unpublished observations). In order to identify which members of the Src and VEGFR families might be involved, we analysed the expression levels of Src, Fyn, VEGFR-1 and VEGFR-2 mRNA by real-time PCR in MCF-7 cells. Of these, only Src and VEGFR-2 were expressed detectably within their respective gene families (P.A.E., unpublished observations).

MT1-MMP expression induces activation of Akt and mTOR

As the inhibitors of Akt and mTOR inhibited the MT1-MMP-driven increase in VEGF-A mRNA levels, we next investigated whether MT1-MMP expression also regulated Akt and mTOR phosphorylation. As shown in Fig. 3B, expression of MT1-WT augmented the level of pS473-Akt and pT308-Akt in cell lysates compared with vector control transfectants without affecting the levels of total Akt. Transfection of increasing amounts of MT1-WT cDNA also led to a dose-dependent increase in Akt phosphorylation (Fig. 3C). We also found increased phosphorylation of mTOR (pS2448-mTOR) in MT1-WT-expressing cells compared with pcDNA3.1 transfected control cells (Fig. 3B). To further analyse the MT1-MMP-induced signalling pathway and to obtain information on the signalling hierarchy, MT1-WT-transfected MCF-7 cells were incubated for 24 hours with PP2, SH-5, LY294002 or SU4312 before analysis of Akt and mTOR phosphorylation. As shown in Fig. 3B, mTOR phosphorylation at S2448 and Akt phosphorylation at S473 and T308 was reduced with all the inhibitors used, suggesting that the mTOR and Akt phosphorylation events are downstream of Src, PI3K and VEGFR-2 activation. However, treatment of cells with SH-5 showed a reproducible decrease in MT1-MMP protein levels, which was also observed using triciribine (API-2), a different Akt inhibitor, making the interpretation of the role of Akt inhibition in our system difficult. The Akt-inhibitor-driven decrease in MT1-MMP protein expression was further confirmed by immunoblotting using two antibodies against MT1-MMP targeting the ICD or extracellular domain (ECD), respectively (supplementary material Fig. S3A). These antibodies also detected decreased protein levels of the 43–45 kDa autocatalytic cleavage fragment. By contrast, the MT1-MMP mRNA level was not affected by the treatment with SH-5 (supplementary material Fig. S3B). We next chose to investigate Akt phosphorylation by immunofluorescence to determine where MT1-MMP was regulating Akt. As expected, Akt phosphorylation at S473 increased in MT1-WT-expressing MCF-7 cells (Fig. 3D,*i*). Colocalisation between MT1-MMP and pS473-Akt was observed in intracellular vesicles as well as in membrane protrusions (Fig. 3D,*iv*), as demonstrated using an orthogonal *z*/*x*-section along the *y*-axis (Fig. 3D,*v*).

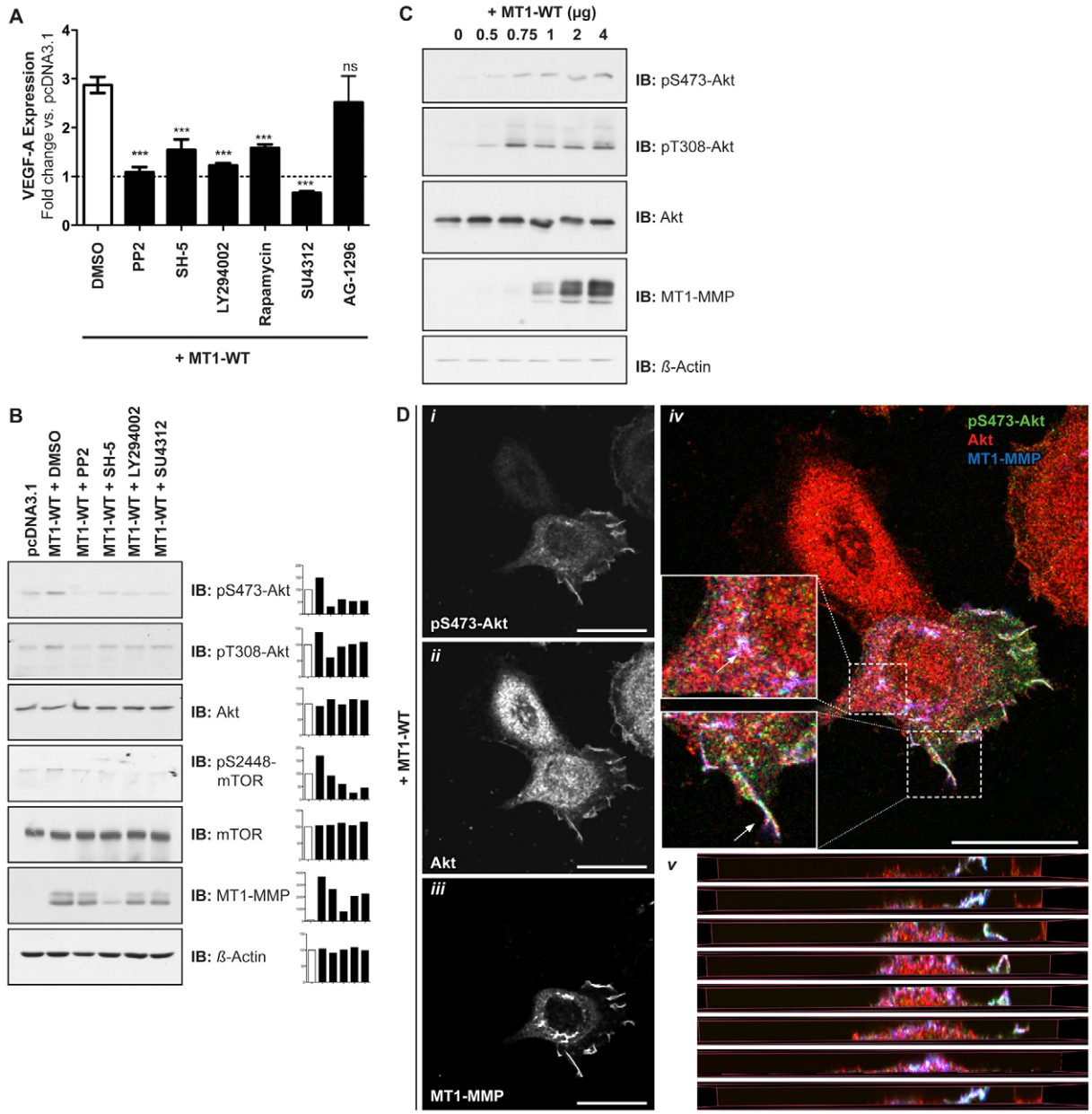

Fig. 3. MT1-MMP-mediated VEGF-A expression requires VEGFR-2, Src, PI3K, Akt and mTOR activities. (**A**) MCF-7 cells were transiently transfected with pcDNA3.1 or MT1-WT cDNA and treated for 24 hours with either DMSO, PP2, SH-5, LY294002, rapamycin, SU4312 or AG-1296. VEGF-A mRNA levels were quantified by real-time PCR and were normalised to GAPDH mRNA levels. Data represent the mean VEGF-A mRNA expression plotted as the fold change compared with the pcDNA3.1 control (dotted line, n=3, ±s.e.m.) with ***P<0.0001 compared with the DMSO control. n.s., not significant. (**B**) MCF-7 cells were transfected with either pcDNA3.1 or MT1-WT cDNA. DMSO, PP2, SH-5, LY294002 or SU4312 were added for 24 hours. Lysates were immunoblotted (IB) with an anti-pS473-Akt, anti-pT308-Akt, anti-Akt, anti-pS2448-mTOR, anti-mTOR, anti-MT1-MMP (LEM-2/15.8) or anti-β-actin antibody. The semi-quantitative analyses of band intensities of the immunoblots are shown on the right-hand side. (**C**) MCF-7 cells were transfected with increasing concentrations of MT1-WT cDNA and lysates were immunoblotted with an anti-pS473-Akt, an anti-pT308-Akt, an anti-Akt, an anti-MT1-MMP (LEM-2/15.8) or an anti-β-actin antibody. (**D**) MT1-WT-transfected MCF-7 cells were immunostained with an anti-pS473-Akt (green, Alexa Fluor 488 secondary, *i*, *iv*, *v*), anti-Akt (red, Alexa Fluor 546 secondary, *ii*, *iv*, *v*) and an anti-MT1-MMP antibody (blue, N175/6; Cy5 secondary, *iii*, *iv*, *v*). Arrows in the merged image indicate colocalisation of Akt, phosphorylated Akt and MT1-MMP within the perinuclear region and at membrane protrusions (*iv*). Inset panels show magnified portions of each merged image, as indicated (dashed rectangles). Serial images present orthogonal sections along the *y*-axis of the merged confocal laser scanning micrograph (*v*). Scale bars: 25 μm.

MT1-MMP expression induces Src phosphorylation and its peripheral targeting

As we had identified Src activity as an important mediator of the increased levels of VEGF-A mRNA observed upon expression of MT1-MMP, we next investigated the role of MT1-MMP expression in the phosphorylation and localisation of Src. Immunological detection of endogenous Src and particularly its Y416-phosphorylated form (pY416-Src) was found to be low and therefore poorly detectable in MCF-7 cells (P.A.E., unpublished observations). In order to increase the level of pY416-Src detection, we transfected

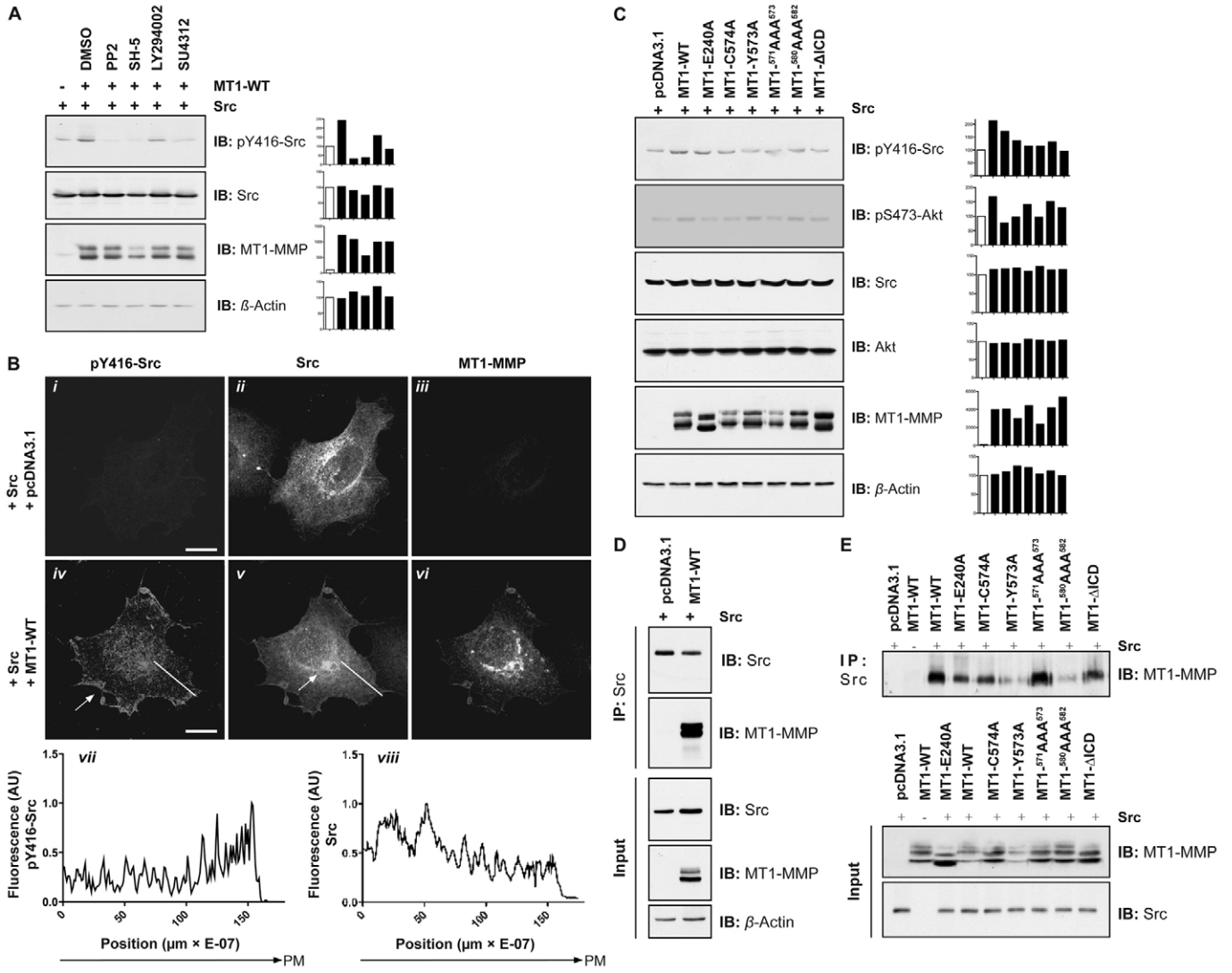

Fig. 4. MT1-MMP is found in the same complex with Src and modulates its phosphorylation. (**A**) MCF-7 cells were transfected with either Src and pcDNA3.1 or Src and MT1-WT, as indicated. DMSO, PP2, SH-5, LY294002 or SU4312 were added for 24 hours. Lysates were immunoblotted (IB) with an anti-pY416-Src, anti-Src, anti-MT1-MMP (LEM-2/15.8) or an anti-β-actin antibody. The semiquantitative analyses of band intensities of the immunoblots are shown on the right-hand side. (**B**) MCF-7 cells were either transfected with Src and pcDNA3.1 (*i–iii*) or with Src and MT1-WT (*iv–vi*). Localisation of active Src was detected using an anti-pY416-Src antibody (Alexa Fluor 488 secondary), total Src using an anti-Src antibody (Alexa Fluor 546 secondary), and MT1-MMP was detected with an antibody raised to the MT1-MMP ECD (N175/6, Cy5 secondary). Arrows indicate pY416-Src at the cell periphery and total Src localising at the perinuclear region of the cell, showing a Src phosphorylation gradient. Orthogonal sections along the indicated line from the nucleus to the plasma membrane (PM) demonstrate the distribution of both pY416-Src and total Src (*vii*, *viii*). Scale bars: 10 µm. (**C**) MCF-7 cells were transfected with either pcDNA3.1, MT1-WT, MT1-E240A, MT1-C574A, MT1-Y573A, MT1-^{571}AAA^{573}, MT1-^{580}AAA^{582} or MT1-ΔICD, as indicated. Lysates were immunoblotted with an anti-pY416-Src, an anti-pS473-Akt, an anti-Src, an anti-Akt, an anti-MT1-MMP (LEM-2/15.8) or an anti-β-actin antibody. The semiquantitative analyses of band intensities of the immunoblots are shown on the right-hand side. (**D**) Either Src- and pcDNA3.1- or Src- and MT1-WT-cotransfected MCF-7 cells were coimmunoprecipitated with an anti-Src antibody. Immunoprecipitates were detected with an anti-Src or an anti-MT1-MMP (LEM-2/15.8) antibody. Input controls were immunoblotted with an anti-Src, an anti-MT1-MMP (LEM-2/15.8) or an anti-β-actin antibody. (**E**) MCF-7 cells were transfected with pcDNA3.1, MT1-WT or different MT1-MMP extracellular and intracellular domain mutants as indicated and co-immunoprecipitated with an anti-Src antibody. Input controls were immunoblotted with an anti-Src antibody and both immunoprecipitates and input controls were immunoblotted with an anti-MT1-MMP (LEM-2/15.8) antibody.

the Src cDNA into MCF-7 cells (Fig. 4A). We observed that transfection of Src itself did not affect the levels of VEGF-A mRNA (supplementary material Fig. S4). Expression of MT1-WT in Src-transfected cells increased Src phosphorylation (pY416-Src) without affecting the levels of Src expression (Fig. 4A). A similar increase in Src phosphorylation was observed by immunoprecipitating endogenous Src and subsequently immunoblotting for pY416-Src, indicating that this observation is not an artefact of Src transfection (P.A.E., unpublished observations). Treatment of cells transfected with both Src and MT1-WT with the inhibitors PP2, LY294002 or SU4312 revealed that Src phosphorylation was inhibited by PP2 and to a lesser extent by SU4312, suggesting that Src potentially is activated downstream of VEGFR-2. Although the Akt inhibitor SH-5 inhibited Src phosphorylation, it also decreased MT1-MMP expression; hence the role of Akt in this signalling cascade is not certain.

The induction by MT1-MMP of Src phosphorylation was also observed by immunofluorescence. Transient transfection of Src alone resulted in the localisation of the kinase throughout the cell cytoplasm, with a higher concentration within the perinuclear region and little in the region of the cell periphery (Fig. 4B,*ii*). In Src-transfected cells, we were unable to detect pY416-Src above the background levels of fluorescence seen in nontransfected cells (Fig. 4B,*i*). By contrast, in cells transiently expressing Src and MT1-WT, staining of pY416-Src was readily detectable and increased at or close to the plasma membrane, where it also colocalised with MT1-MMP (Fig. 4B,*iv*, white arrow). Plots of the relative fluorescence intensity of total Src and pY416-Src from an optical orthogonal section taken along the *x*–*y* axis of the cell revealed a Src phosphorylation gradient across the cytoplasm, with an enrichment of pY416-Src towards the plasma membrane. By contrast, Src was detected mainly in the perinuclear region of the cell (Fig. 4B,*vii*,*viii*).

In order to determine which domain of MT1-MMP is required for the induction of Src phosphorylation, cells were transfected with pcDNA3.1 or MT1-MMP mutants, as indicated (Fig. 4C). Src phosphorylation at Y416 was induced following expression of MT1-WT, MT1-E240A and to a lesser extent in MT1-C574A and MT1-^{580}AAA^{582} but not in MT1-MMP Y573-mutant-expressing cells (MT1-Y573A, MT1-^{571}AAA^{573} and MT1-ΔICD), suggesting that Src phosphorylation depends on the MT1-MMP ICD but not on its catalytic activity. By contrast, Akt phosphorylation at S473 was reduced in cells expressing the catalytic inactive form as well as in the MT1-C574A, MT1-^{571}AAA^{573} and to a lesser extent in the remaining MT1-MMP ICD mutants (Fig. 4C). The reduction of Akt phosphorylation at S473 in MT1-E240A-expressing cells compared with MT1-WT-transfected cells was also visualised by immunostaining (supplementary material Fig. S5). However, Akt phosphorylation was restored after addition of rh-MMP-2 or rh-MMP-7 to MT1-E240A-expressing cells, confirming the redundancy of the MT1-MMP catalytic activity, as shown in the transcriptional activation of VEGF-A (Fig. 2B; supplementary material Fig. S2).

MT1-MMP and Src are in the same complex

As Src and in particular pY416-Src showed increased colocalisation with MT1-MMP, we next tested whether MT1-MMP and Src were present in the same protein complex. Cell extracts that were prepared from MCF-7 cells expressing Src alone, or cotransfected with MT1-WT, were immunoprecipitated with antibody against Src, and immunocomplexes were immunoblotted with antibodies against Src and MT1-MMP. As shown in Fig. 4D, MT1-MMP and Src were co-immunoprecipitated, demonstrating that both proteins are present in the same complex.

We next tested whether the MT1-MMP ICD was required for the formation of the complex between MT1-MMP and Src. Whole-cell extracts prepared from MCF-7 cells coexpressing Src and various cDNAs expressing MT1-MMP ICD mutants (MT1-C574A, MT1-Y573A, MT1-^{571}AAA^{573}, MT1-^{580}AAA^{582}), an ICD deletion (MT1-ΔICD) or the MT1-E240A catalytically inactive mutant were co-immunoprecipitated as previously described. As presented in Fig. 4E, none of the cDNAs tested altered the presence of the MT1-MMP–Src complex, suggesting that this interaction does not depend on the MT1-MMP ICD and might involve additional molecules. Unfortunately, the relative amount of MT1-MMP present in the different complexes proved not to be quantifiable owing to the variation of transfection efficiencies and MT1-MMP expression observed for the various cDNAs used.

The spatial distribution and the colocalisation between pY416-Src and various MT1-MMP mutants were further assessed and quantified using immunofluorescence staining (supplementary material Figs S6 and S7A). Consistent with the data obtained in the immunoblot analysis (Fig. 4C), very little pY416-Src was detected in cells expressing MT1-ΔICD, MT1-Y573A or MT1-^{571}AAA^{573} mutants (supplementary material Fig. S6), whereas mutations of the C574, E240 and DKV582 motifs did not affect the phosphorylation and cellular localisation of pY416-Src with MT1-MMP. Quantification of colocalisation between pY416-Src and MT1-MMP based on confocal data revealed a colocalisation between both proteins (supplementary material Fig. S7A, colocalisation coefficient ~62%). Mutation of the MT1-MMP Y573 residue (MT1-Y573A and MT1-^{571}AAA^{573}) or deletion of the ICD led to a reduction of Src phosphorylation at Y416 (Fig. 4C; supplementary material Fig. S6). In order to analyse whether constitutive levels of pY416-Src in MT1-ΔICD-, MT1-Y573A- or MT1-^{571}AAA^{573}-expressing cells still colocalise with MT1-MMP, the colocalisation coefficient of pY416-Src versus MT1-MMP was calculated. As shown in supplementary material Fig. S7A, MT1-MMP mutation at Y573 or deletion of the ICD reduced the amount of colocalisation between pY416-Src and MT1-MMP (P<0.0001, Student's t-test).

MT1-MMP is in a complex with active Src and VEGFR-2

Given that MT1-ΔICD and Src co-immunoprecipitated within the same complex (Fig. 4E), our data indicate an indirect interaction between MT1-MMP and Src. As we had found that VEGFR-2 activity was required for the MT1-MMP-induced increase in VEGF-A (Fig. 3A), and VEGFR-2 has been shown to associate with phosphorylated Src (Chou et al., 2002; Olsson et al., 2006), we hypothesised that MT1-MMP could be in a complex with VEGFR-2. To test this, lysates prepared from cells transfected with either pcDNA3.1 or MT1-WT were immunoprecipitated with an antibody against VEGFR-2. As shown in Fig. 5B, MT1-MMP was found to co-immunoprecipitate with VEGFR-2. We next characterised the domains of MT1-MMP involved in the interaction with VEGFR-2. MCF-7 cells transiently expressing MT1-MMP cDNAs with various ECD, hemopexin domain or ICD mutations were tested by immunoprecipitation (summarised in Fig. 5A). The MT1-E240A mutant and all the ICD mutants tested co-immunoprecipitated with VEGFR-2 (Fig. 5B). By contrast, deletion of regions of the MT1-MMP ECD (MT1-ΔECD-FLAG and MT1-ΔECD-EGFP) ablated the formation of a complex between MT1-MMP and VEGFR-2 (Fig. 5C). A similar result was observed when the MT1-MMP hemopexin domain was replaced by EGFP (MT1-ΔHPX, Fig. 5D). These findings suggest that the MT1-MMP hemopexin domain is required for formation of a complex between MT1-MMP and VEGFR-2.

In order to confirm the role of the MT1-MMP ECD in the formation of the complex between MT1-MMP and VEGFR-2 and to establish whether there can be a direct interaction, we performed an ELISA using purified recombinant proteins. Either immobilised rh-VEGFR-2-Fc or milk control protein was incubated with purified rh-MT1-Ecto, rh-MT1-Ecto-E240A or rh-MT1-Cat. Incubation with rh-VEGF$_{165}$ was used as a positive control (Fig. 5E). As expected, rh-MT1-Ecto and rh-MT1-Ecto-E240A were found to bind to rh-VEGFR-2-Fc significantly over the control coating (P<0.0001; Fig. 5E). By contrast, no interaction between rh-VEGFR-2-Fc and rh-MT1-Cat was detected in this assay. The rh-MT1-Cat was able to bind to TIMP-2, confirming that it was

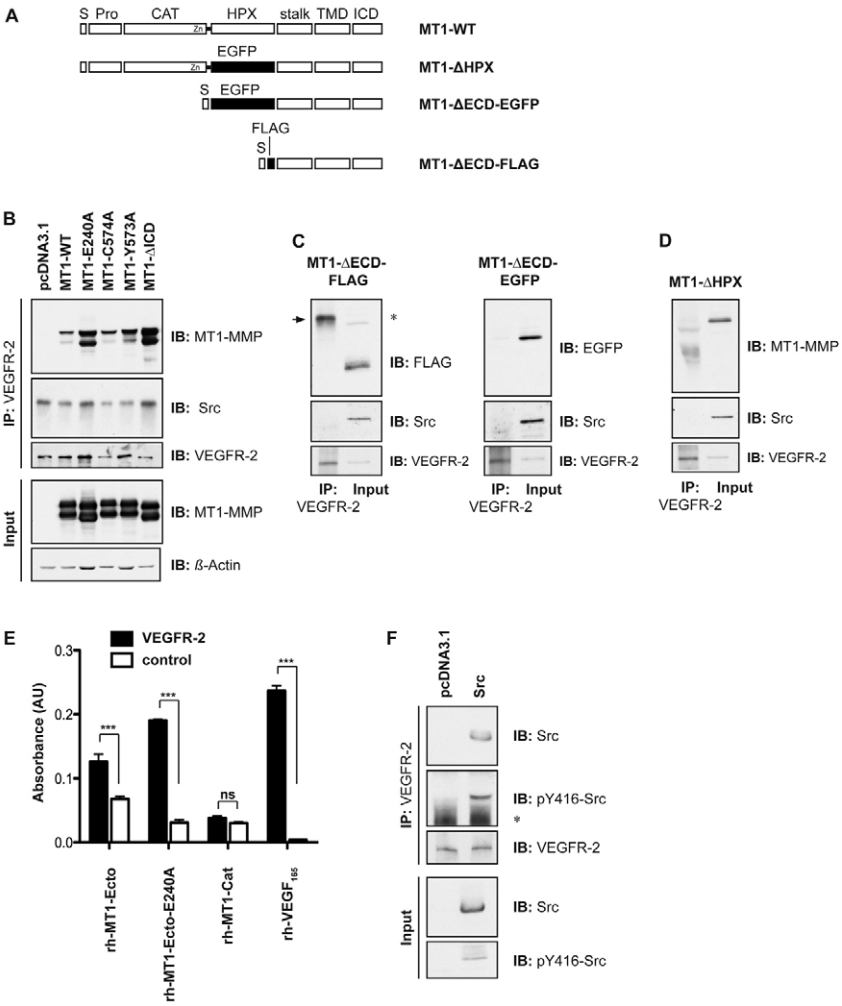

Fig. 5. MT1-MMP is found in the same complex with VEGFR-2. (**A**) Schematic representation of the MT1-MMP mutants used. S, signal sequence; Pro, propeptide; CAT, catalytic domain; HPX, hemopexin domain; stalk, stalk region; TMD, transmembrane domain; ICD, intracellular domain; FLAG, DYKDDDDK; EGFP, enhanced green fluorescent protein. (**B**) MCF-7 cells were transfected with either pcDNA3.1, MT1-WT or MT1-MMP extracellular and intracellular domain mutants, and cell extracts were immunoprecipitated with an anti-VEGFR-2 antibody. Immunoprecipitates and input controls were immunoblotted (IB) with an anti-MT1-MMP (LEM-2/15.8), an anti-Src, an anti-VEGFR-2 or an anti-β-actin antibody. (**C**,**D**) Cells were transfected with either MT1-ΔECD-FLAG, MT1-ΔECD-EGFP (C) or MT1-ΔHPX (D). Lysates were immunoprecipitated with an anti-VEGFR-2 antibody, and immunoprecipitates and input samples were immunoblotted with an anti-FLAG, an anti-EGFP, an anti-MT1-MMP (LEM-2/15.8), an anti-Src or an anti-VEGFR-2 antibody. The asterisk indicates a nonspecific band, and the arrow shows the IgG light chain. (**E**) A microtiter plate was coated with either 10 μg/ml rh-VEGFR-2 or milk control protein. rh-MT1-Ecto, rh-MT1-Ecto-E240A, rh-MT1-Cat or rh-$VEGF_{165}$ as a positive control were added in an ELISA. Data represent the mean $AU405_{nm}$ ±s.e.m. with ***P<0.0001. n.s., not significant. (**F**) Cells were transfected with either pcDNA3.1 or Src cDNA. Protein lysates were immunoprecipitated with an anti-VEGFR-2 antibody and immunoblotted with an anti-Src, an anti-pY416-Src or an anti-VEGFR-2 antibody. Input control samples were immunoblotted with an anti-Src or an anti-pY416-Src antibody. The asterisk indicates the IgG heavy chain.

correctly folded (M.F., unpublished observations). This suggests that the MT1-MMP hemopexin domain interacts directly with the VEGFR-2 ECD. The difference in binding observed between rh-MT1-Ecto and rh-MT1-Ecto-E240A to rh-VEGFR-2 resulted from the partial autocatalytic degradation of rh-MT1-Ecto that occurs during the preparation of the recombinant protein (P.A.E., unpublished observations). Degradation products of the MT1-MMP ECD, which are not detected by the antibody against the MT1-MMP catalytic domain, are likely to compete with the binding of the intact MT1-MMP ECD with VEGFR-2.

We next tested whether the complex of pY416-Src with MT1-MMP identified previously included VEGFR-2. Endogenous Src and pY416-Src co-immunoprecipitated at low levels with VEGFR-2, which were increased upon transfection of Src, indicating its association with VEGFR-2 in MCF-7 cells (Fig. 5F). We also examined the colocalisation between VEGFR-2, pY416-Src and MT1-MMP in cells transfected with Src and MT1-WT, MT1-E240A and ICD mutants (supplementary material Figs S6 and S7). As mentioned previously, MT1-ΔICD, MT1-Y573A or MT1-^{571}AAA^{573} mutants showed decreased pY416-Src staining as well as decreased colocalisation of pY416-Src with VEGFR-2, whereas the inactive MT1-E240A mutation did not result in a significant decrease in colocalisation of VEGFR-2 with pY416-Src, indicating that the ICD in particular regulates association of pY416-Src with VEGFR-2–MT1-MMP (supplementary material Fig. S7C).

MT1-MMP expression increases VEGFR-2 cell surface levels

The identification of the interaction between MT1-MMP and VEGFR-2 led us to test whether MT1-MMP expression could also affect the subcellular distribution of VEGFR-2. Src-expressing MCF-7 cells were transfected with MT1-WT or a vector control, and the localisation of VEGFR-2 was assessed. In Src-expressing cells, VEGFR-2 was mainly localised in intracellular vesicles scattered throughout the cell cytoplasm (Fig. 6A,*ii,* arrowhead), as seen in control-vector-transfected cells. By contrast, expression of MT1-WT and Src induced the phosphorylation of Src at Y416, as previously observed (Fig. 4B,*iv* and Fig. 6A,*iv*), and a clear cellular redistribution of VEGFR-2 in these cells (Fig. 6A,*v*). VEGFR-2 was found to colocalise with pY416-Src (Fig. 6A,*iv*, arrows) and MT1-MMP (Fig. 6A,*vi*, arrows). Similar results were obtained for the MT1-E240A and all ICD mutants tested (supplementary material Fig. S6), although the staining intensity of pY416-Src was reduced in cells expressing MT1-MMP Y573 mutants (supplementary material Fig. S7A,C). Transfection of the MT1-ΔICD cDNA led to a VEGFR-2 staining pattern similar to that of MT1-WT-expressing cells; however, the colocalisation coefficient of VEGFR-2 versus MT1-MMP was slightly decreased (supplementary material Fig. S7B). Interestingly, expression of the MT1-MMP ECD deletion mutant (MT1-ΔECD-FLAG) did

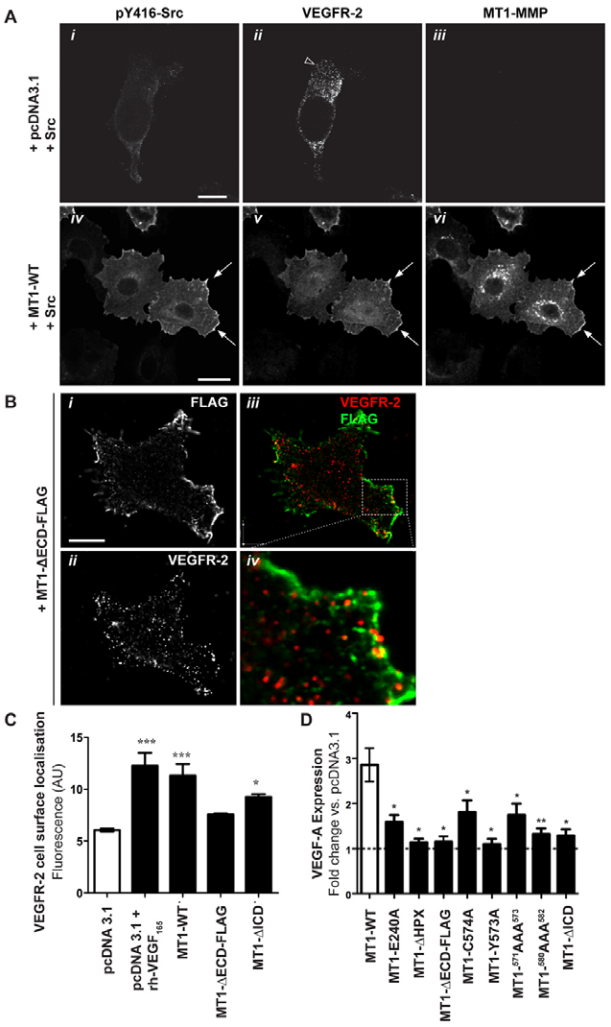

Fig. 6. The MT1-MMP extracellular domain modulates the surface localisation of VEGFR-2. (**A**) MCF-7 cells were transiently transfected with either pcDNA3.1 and Src cDNA (*i–iii*) or MT1-WT and Src cDNA (*iv–vi*), and the localisation of pY416-Src (Alexa Fluor 488 secondary, *i*, *iv*), VEGFR-2 (Alexa Fluor 546 secondary, *ii*, *v*) and MT1-MMP (N175/6, Cy5 secondary, *iii*, *vi*) was detected. The hollow arrowhead indicates the vesicular staining pattern of VEGFR-2 in cells expressing Src only, whereas arrows show the colocalisation of pY416-Src, MT1-MMP and VEGFR-2 at or close to the cell surface of cells expressing MT1-WT and Src. Scale bars: 10 µm (*i–iii*) or 25 µm (*iv–vi*). (**B**) MCF-7 cells were transfected with MT1-ΔECD-FLAG cDNA, and the localisation of VEGFR-2 and MT1-MMP was assessed using an anti-FLAG (Cy5 secondary, *i*) and an anti-VEGFR-2 (Alexa Fluor 488 secondary, *ii*) antibody. Panel *iv* shows magnified portion of the image, as indicated by the dashed rectangle. Scale bar: 10 µm. (**C**) Cells were transfected with pcDNA3.1, MT1-WT, MT1-ΔECD-FLAG or MT1-ΔICD, and the cell surface localisation of VEGFR-2 was assessed by flow cytometry. As a positive control, pcDNA3.1-transfected cells were stimulated for 30 minutes with 100 ng/ml rh-$VEGF_{165}$. Data represent the mean fluorescence (n=4, ±s.e.m.) with *P<0.05 and ***P<0.0001 compared with the pcDNA3.1 control. A total of 10^4 events was counted per sample. (**D**) MT1-MMP mutant cDNAs were transiently transfected in MCF-7 cells, and VEGF-A mRNA levels were detected by real-time PCR, with expression levels being normalised to GAPDH mRNA levels. Data represent the mean VEGF-A expression plotted as the fold change compared with the pcDNA3.1 control (dotted line, ±s.e.m.) with *P<0.05 and **P<0.001 compared with MT1-WT-transfected cells (MT1-WT, n=16; MT1-E240A, n=11; MT1-ΔHPX, n=6; MT1-ΔECD-FLAG, n=5; MT1-C574A, n=14; MT1-Y573A, n=5; MT1-^{571}AAA^{573}, n=14; MT1-^{580}AAA^{582}, n=9; MT1-ΔICD, n=5).

not significantly affect the VEGFR-2 localisation pattern when compared with vector-control-transfected cells (Fig. 6B,*ii*).

The MT1-MMP ECD-dependent staining of VEGFR-2 observed at, or close to, the plasma membrane led us to test whether MT1-MMP increases the levels of VEGFR-2 at the cell surface. Therefore, MT1-WT, MT1-ΔECD-FLAG or MT1-ΔICD (Fig. 5A) cDNAs were transfected into MCF-7 cells, and the cell surface levels of VEGFR-2 were analysed by flow cytometry. As a positive control for cell surface VEGFR-2, cells transfected with pcDNA3.1 were stimulated for 30 minutes with 100 ng/ml rh-$VEGF_{165}$ to induce VEGFR-2 traffic to the cell surface, as has been described in endothelial cells (Gampel et al., 2006). As expected, treatment with rh-$VEGF_{165}$ significantly increased the staining of VEGFR-2 at the cell surface by 50% (Fig. 6C, P<0.0001, compared with pcDNA3.1-transfected cells). VEGFR-2 cell surface localisation was also increased in MT1-WT- and MT1-ΔICD-transfected cells by 40% (P<0.0001) and 33% (P<0.05), respectively, compared with cells transfected with pcDNA3.1. By contrast, expression of MT1-ΔECD-FLAG did not increase the cell surface staining of VEGFR-2 compared with vector control transfectants (Fig. 6C), suggesting that the increased cell surface level of VEGFR-2 is dependent on the ECD of MT1-MMP and not on the intracellular or catalytic domain.

The MT1-MMP extracellular domains are important for expression of VEGF-A

The role of the MT1-MMP extracellular domains in the redistribution of VEGFR-2 led us to test whether these domains also had an effect on VEGF-A mRNA expression. As presented in Fig. 6D, transient expression of MT1-ΔHPX or MT1-ΔECD-FLAG did not increase VEGF-A mRNA expression significantly over the levels of vector controls, supporting our previous observations that the hemopexin domain is crucial. Similar results were observed with mutations of the E240, C574, Y573, LLY573 and DKV582 motifs or complete deletion of the MT1-MMP ICD (MT1-ΔICD). This was also observed with adenoviral delivery of the key MT1-MMP ICD mutant cDNAs (P.A.E., unpublished observations).

Discussion

MT1-MMP has been shown to regulate gene transcription of a number of proteins, including the regulation of VEGF-A in MCF-7 and U251 cells (Deryugina et al., 2002b; Freudenberg and Chen, 2007; Rozanov et al., 2008; Saeb-Parsy et al., 2008; Sounni et al., 2004). In addition, we have found that transient expression of MT1-MMP in MDA-MB-453 cells also increases VEGF-A mRNA levels (P.A.E., unpublished observations), suggesting that MT1-MMP regulation of VEGF-A expression might be widespread in cancer cells. Here, we have investigated how MT1-MMP regulates VEGF-A transcription in MCF-7 cells and have identified a novel role for MT1-MMP in the modulation of Src activity as well as in the regulation of the function and localisation of VEGFR-2.

As in the original study of Sounni and colleagues (Sounni et al., 2004), we have found that the MT1-MMP-dependent increase in VEGF-A mRNA and protein required the ICD and the catalytic activity of MT1-MMP. However, the catalytic activity of MT1-MMP was found to be redundant upon the addition of rh-MMP-2 or rh-MMP-7 in cells expressing an inactive form of MT1-MMP. VEGF-A is known to be sequestered as an inactive form in the extracellular milieu through several means, including through CTGF, resulting in reduced bioavailability (Inoki et al., 2002). CTGF has been demonstrated to be a substrate for several MMPs,

including MMP-1, -2, -3, -7 and -13 as well as MT1-MMP, and hydrolysis of CTGF releases bioactive $VEGF_{165}$ (Dean et al., 2007; Hashimoto et al., 2002). MCF-7 cells express CTGF, and we have found that overexpression of MT1-MMP in MCF-7 cells leads to hydrolysis of CTGF, as detected in the conditioned medium (supplementary material Fig. S8A), without affecting CTGF mRNA levels (supplementary material Fig. S8C). Our data indicate that, although MMP activity might be required for the release of bioactive $VEGF_{165}$ from the extracellular milieu, with CTGF cleavage being one possible mechanism, the activity of MT1-MMP is not exclusively required for signal transduction pathways leading to the increase in VEGF-A expression. This raises the interesting question of how significant the contribution of the catalytic activity of MT1-MMP might be to tumour progression in vivo when expressed in cancer cells. Expression of inactive MT1-MMP has been shown recently to cause increased tumour growth in some in vivo models, including upon overexpression in MCF-7 cells (D'Alessio et al., 2008; Rozanov et al., 2008). Although a function-blocking antibody raised to the catalytic domain of MT1-MMP has proven effective in some preclinical tumour models (Devy et al., 2009), our data and those of others suggest that targeting the catalytic activity of MT1-MMP in this manner might not inhibit all tumorigenic functions of MT1-MMP and emphasise its non-proteolytic functions.

Our findings indicate that MT1-MMP causes an increase in VEGF-A transcription, which is dependent on VEGFR-2 activity. Our data further show that MT1-MMP regulates VEGFR-2 in two distinct ways. First, MT1-MMP modulates VEGFR-2 cellular localisation. VEGFR-2 is localised predominantly in intracellular vesicles in endothelial cells and translocates to the plasma membrane upon the addition of $VEGF_{165}$ (Gampel et al., 2006), which we also observed in MCF-7 cells. VEGFR-2 cell surface localisation was also increased by MT1-MMP independently of the addition of exogenous $VEGF_{165}$, and dissection of the domains of MT1-MMP involved showed that this was dependent on the hemopexin domain but not the catalytic domain. We also observed an interaction between the entire recombinant ECD of MT1-MMP and VEGFR-2. This is consistent with findings showing that MT1-MMP interacts with other proteins, including CD44, CD151 and CD63, through its hemopexin domain (Mori et al., 2002; Takino et al., 2003; Yanez-Mo et al., 2008). MT1-MMP can be proteolytically processed to the 43–45 kDa forms that lack the catalytic domain (Lehti et al., 1998; Stanton et al., 1998; Toth et al., 2002). It is possible that accumulation of the 43–45 kDa forms of MT1-MMP will also lead to regulation of the function of VEGFR-2.

The second manner in which MT1-MMP modulates VEGFR-2 function in MCF-7 cells is in regulating VEGFR-2- and Src-dependent activation of intracellular kinases, including Akt and mTOR, leading to VEGF-A transcription. We have shown that MT1-MMP-induced Src phosphorylation is dependent on the MT1-MMP ICD and that active Src is required, but not sufficient, to induce VEGF-A transcription through the PI3K–Akt pathway. Y573, LLY573 and DKV582 have been identified as being particularly important residues of the MT1-MMP ICD required for VEGF-A expression. The MT1-MMP Y573 residue has been shown to be a Src phosphorylation site (Nyalendo et al., 2007). It is also part of the motif LLY573 that regulates clathrin-dependent endocytosis through binding to the μ2 subunit of AP-2 and *O*-glycosylation of the MT1-MMP hinge region, implicating intracellular traffic and/or maturation of MT1-MMP (Ludwig et al., 2008; Uekita et al., 2001). How exactly the ICD of MT1-MMP regulates the phosphorylation of Src and the VEGFR-2-dependent activation of Akt is not clear and will require further analyses in future studies. Dissection of the role MT1-MMP plays in the activation of Akt was impeded by the finding that Akt inhibition led to a decrease in MT1-MMP expression. Given that the MT1-WT cDNA is expressed from the CMV promoter and that a reduction of MT1-MMP protein has also been observed for the 43–45 kDa form (supplementary material Fig. S3A), it is presumed that Akt regulates MT1-MMP protein turnover or translation. These observations are consistent with recent findings showing that MT1-MMP protein expression was decreased in Akt1 knockout mice (Ulici et al., 2009). Although MT1-MMP has been implicated in the activation of the mitogen-activated protein kinase ERK in a number of systems, MEK–ERK inhibition in our system did not inhibit the increase of VEGF-A mRNA expression (D'Alessio et al., 2008; Gingras et al., 2001).

We have found that MT1-MMP can form a complex with VEGFR-2 in MCF-7, MDA-MB-468 and MDA-MB-231 cells and increases the transcription of VEGF-A on transfection in MCF-7 or MDA-MB-453 cells (P.A.E., unpublished observations). Increased MT1-MMP expression is more frequently associated with poor outcome in patients who are lymph node and metastases positive for disease (Figueira et al., 2009; Jiang et al., 2006; Kim et al., 2006; Ueno et al., 1997), and increased VEGF-A is also associated with poor prognosis and correlates with MT1-MMP expression (Linderholm et al., 2003; Munaut et al., 2003), suggesting the MT1-MMP–VEGF-A axis might be a key element in tumour progression. An improvement in our understanding of how the VEGF-A–VEGFR-2 axis functions in pathology is of particular importance as inhibitors of the VEGF axis have recently been shown to increase tumorigenicity at metastatic sites in murine models of cancer, which parallels observations emerging from clinical data (Ebos et al., 2009; Paez-Ribes et al., 2009). MT1-MMP has been shown to process the integrin αvβ3 as well as to interact with Src, Cav-1 and PDGFRβ (Deryugina et al., 2002a; Labrecque et al., 2004; Lehti et al., 2005; Yanez-Mo et al., 2008), all of which have also been demonstrated to interact with and regulate VEGFR-2 (Greenberg et al., 2008; Labrecque et al., 2003; Matsumoto et al., 2005; Soldi et al., 1999). Differences in regulation and expression of these kinase family members and their VEGFR-2 adaptors in different cell types might provide further levels of regulation of the MT1-MMP–VEGFR-2 complex and VEGFR-2 signalling (Holmqvist et al., 2004; Kroll and Waltenberger, 1997; Matsumoto et al., 2005; Warner et al., 2000; Yamaoka-Tojo et al., 2004).

In conclusion, we have shown that MT1-MMP regulates the function of VEGFR-2 in cancer cells independently of its catalytic activity through the formation of an MT1-MMP–VEGFR-2 complex that associates with Src and induces the phosphorylation of Akt and mTOR, ultimately leading to increased autocrine production of VEGF-A. The regulation of receptor tyrosine kinase function by MT1-MMP independently of its catalytic domain is an area of investigation that requires further study before the impact on tumour growth and metastasis of MT1-MMP can be fully understood.

Materials and Methods

All chemicals and reagents were purchased from Calbiochem (Nottingham, UK), unless stated otherwise.

Cell culture, cell treatments and reagents

The human breast cancer cell line MCF-7 was obtained from Cancer Research UK (London, UK) and was routinely cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Paisley, UK) supplemented with 10% (v/v) foetal calf serum

(FCS; Perbio, Northumberland, UK) and 2 mM glutamine (Invitrogen). For treatment with various inhibitors, 5×10^5 cells were seeded and transfected with 1 μg cDNA of various MT1-MMP cDNAs using FuGene6™ (Roche Applied Science, Welwyn, UK) according to manufacturer's instructions. Three hours after transfection, inhibitors were added at following concentrations: PP2 (5 μM), lactacystin (1 μM), wortmannin (250 nM), JNK inhibitor II (SP006125; 1 μM), SU4312 (1.5 nM), IKK inhibitor III (BMS-345541; 4 μM), PD98059 (50 μM), LY294002 (2 μM), gefitinib (5 μM), tarceva (5 μM), Akt inhibitor II (SH-5, 20 μM), IGF-IR inhibitor PPP (100 nM), rapamycin (25 nM), AG-1296 (10 μM), SB 203580 (20 μM) or CT1746 (kindly provided by A. Docherty, Celltech; 5 or 10 μM).

Source of antibodies and recombinant proteins

Recombinant TIMPs were prepared as described previously (Murphy & Willenbrock, 1995) and used at the concentrations indicated in the figure legends. The sheep polyclonal antibody to MT1-MMP (N175/6) was prepared as described by d'Ortho et al. (d'Ortho et al., 1997). rh-MMP-2 and rh-MMP-7 have been described previously, and were used at concentrations indicated in the figure legends (Crabbe et al., 1992; Murphy et al., 1992). The purified soluble and catalytic active MT1-MMP extracellular domain (amino acids 21–542; rh-MT1-Ecto), the catalytic inactive MT1-MMP extracellular domain E240A (rh-MT1-Ecto-E240A) and the MT1-MMP catalytic domain (amino acids 21–283; rh-MT1-Cat) were prepared as previously described (d'Ortho et al., 1997). The rh-VEGFR-2/KDR-Fc chimera and the anti-VEGF monoclonal antibody (mAb) (clone 26503, WB: 2 μg/ml; ELISA: 0.5 μg/ml) were from R&D Systems (Abingdon, UK), rh-$VEGF_{165}$ was from Autogen Bioclear UK (Nottingham, UK). Anti-MT1-MMP mAb (clone LEM-2/15.8; WB: 0.5 μg/ml; FACS: 10 μg/ml; ELISA: 1 μg/ml) and anti-phospho(p)Y416-Src mAb (clone 9A6; WB: 2 μg/ml; ICC: 5 μg/ml) were purchased from Millipore (Watford, UK). N175/6 sheep anti-MT1-MMP polylonal antibody (pAb) (WB: 2.5 μg/ml; ICC: 5 μg/ml) was previously described (d'Ortho et al., 1998). Anti-mTOR, anti-pS2448-mTOR, anti-Akt, anti-pS473-Akt, anti-pT308-Akt, anti-VEGFR-2 (55B11) pAbs and anti-Akt (clone 5G3) mAb (all applications: according to manufacturer's recommendation) were from Cell Signaling Technology (Hitchin, UK). Anti-β-Actin pAb (WB: 0.2 μg/ml), anti-Src mAb (clone 327; WB: 2.5 μg/ml), anti-MT1-MMP intracellular domain (ICD) pAb (WB: 0.5 μg/ml) and anti-CTGF pAb (WB: 0.75 μg/ml) were from Abcam plc (Cambridge, UK). Anti-FLAG M2 mAb (WB: 2.5 μg/ml) was from Sigma. Rabbit and mouse control IgG antibodies were purchased from Dako (Ely, UK).

Source, cloning and mutagenesis of cDNAs

The murine Src cDNA was kindly provided by Richard Béliveau (University of Québec, Montréal, Canada). Full-length MT1-MMP (MT1-WT), EGFP-tagged MT1-MMP (MT1-ΔECD-EGFP), L571A/L572A/Y573A MT1-MMP (MT1-^{571}AAA^{573}), the intracellular domain deleted MT1-MMP mutant (MT1-ΔICD), the hemopexin domain deletion mutant (MT1-ΔHPX) and catalytically inactive MT1-MMP (MT1-E240A) were described elsewhere (Atkinson et al., 2006; Nyalendo et al., 2007; Remacle et al., 2003; Sounni et al., 2004). MT1-C574A, MT1-Y573A, D580A/K581A/V582A (MT1-^{580}AAA^{582}), MT1-E240A-^{571}AAA^{573} and MT1-E240A-ΔICD mutants were generated by site-directed mutagenesis as previously described (Labrecque et al., 2004). The FLAG-tagged truncated MT1-MMP cDNA (MT1-ΔECD-FLAG), comprising an extracellular FLAG tag followed by MT1-MMP stalk, transmembrane domain and intracellular domain, was generated from MT1-ΔECD-EGFP and a Myc-tagged full-length MT1-MMP, in which the Myc tag was fused to the C terminus (C.R., unpublished data). The Myc tag, intracellular domain, transmembrane domain and stalk region (MT1-MMP amino acids 512–582) were amplified by PCR from MT1-Myc, inserting a 5′ *Age*I restriction site and a FLAG tag. Restricted PCR fragment and pEGFP-C1 vector, containing the MT1-MMP signal sequence, were purified, ligated and a stop codon was inserted before the Myc tag by PCR. All cDNAs were verified by sequencing.

Adenovirus expression and transduction

Recombinant adenovirus (Ad5 ΔE1/E3) expressing MT1-WT was prepared as described previously (Krubasik et al., 2008). Infection of MCF-7 cells (3.5×10^5 cells) was carried out by adding the virus at various multiplicities of infection (MOIs) to the cell culture medium. After 2 hours, the medium was replaced by fresh medium and the cells were cultured for 72 hours.

Real-time PCR

RNA isolation, quantification and reverse transcription were performed as previously described (Krubasik et al., 2008). TaqMan real-time PCR gene expression profiling was done by using the real-time PCR Applied Biosystems 7900HT platform (Applied Biosystems, Warrington, UK). Technical triplicates were used for each of the three biological replicates in 384-well plates. Reactions were performed in a 12.5 μl final volume containing 2.5 ng cDNA, 100 nM of forward and reverse primer, 200 nM fluorogenic probe (Applied Biosystems) and TaqMan Universal PCR Master Mix (Applied Biosystems). Fluorescent signal detection used ROX as the internal passive reference dye. Human GAPDH was used as a housekeeping gene to normalise cellular RNA amounts. The relative expression of each sample was calculated using the $2^{-\Delta\Delta CT}$ method. The TaqMan assay identification numbers (Applied Biosystems) are: MT1-MMP (Hs00237119_m1), VEGF-A (Hs00173626_m1), Src (Hs00178494_m1), Fyn (Hs00176628_m1), VEGFR-1 (Hs01052936_m1), VEGFR-2 (Hs00176676_m1), CTGF (Hs00170014_m1), HARP (Hs00383235_m1), MMP-2 (Hs00234422_m1) and human GAPDH (Hs00266705_g1).

Immunoprecipitation and protein immunoblotting

Cells were lysed either in sample buffer [1% (w/v) SDS, 50 mM Tris-HCl, pH 6.8, 8% (v/v) glycerol and 4% (v/v) β-mercaptoethanol] for immunoblotting or in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, 0.5% (v/v) Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate for immunoprecipitation. Antibodies (3 μg) were bound to Dynabeads protein G (Invitrogen) at 4°C for 16 hours in 5 mg/ml BSA in PBS (137 mM NaCl, 4.3 mM Na_2HPO_4, 2.7 mM KCl, 1.47 mM KH_2PO_4). Cell lysates were then incubated with antibody-bound Dynabeads for 2 hours at 4°C under constant rotation. Beads were washed four times for 10 minutes with immunoprecipitation buffer and resuspended in 2× Laemmli buffer. Denatured proteins were separated on 10% SDS-polyacrylamide gel electrophoresis (PAGE) and electro-transferred onto nitrocellulose membrane (GE Healthcare, Bucks, UK). Membranes were blocked in 3% (w/v) low-fat milk in TBS (136.9 mM NaCl, 2.68 mM KCl, 24.76 mM Tris Base, pH 8.0) containing 0.1% (v/v) Tween20 for 1 hour at room temperature (RT) and probed with the primary antibodies diluted in blocking buffer at 4°C for 16 hours. After washing, membranes were incubated for 1 hour at RT with horseradish-peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, Newmarket, UK) and immunoreactive bands were detected by enhanced chemiluminescence (ECL Detection Kit, GE Healthcare). Band intensities were quantified using the ImageJ image processing software.

Immunocytochemistry

For immunostaining, 10^5 cells were seeded on glass cover slips and transfected as previously described. After 24 hours, cells were washed in PBS and fixed at RT for 20 minutes with 4% (w/v) PFA in PBS. PFA was quenched using 50 mM glycine (pH 8.0). Cells were subsequently washed in PBS, permeabilised in PBS containing 0.1% (v/v) Triton X-100 and incubated with the primary antibody for 1 hour at RT. Cells were washed three times for 5 minutes with PBS and incubated with species-specific Alexa-Fluor-488, Alexa-Fluor-546 (Invitrogen) or Cy5 (Jackson ImmunoResearch) conjugated secondary antibodies. Slides were mounted using ProLong Gold antifade reagent with DAPI (Invitrogen). Series of optical sections were acquired using the Leica Tandem SP5 Confocal laser microscope (Leica Microsystems, Germany) with a 63× oil immersion objective. Colocalisation coefficients were calculated using the Volocity 3D imaging and analysis software (Improvision, Perkin Elmer, Coventry, UK).

MT1-MMP–VEGFR-2 ELISA

Wells of a microtitre plate were coated with either VEGFR-2-Fc (10 μg/ml) or low-fat milk (10 μg/ml) for 16 hours at 4°C and blocked with 3% (w/v) low-fat milk in wash buffer [50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 0.05% (v/v) Tween20] for 1 hour at RT. After three washes in wash buffer, rh-MT1-Ecto, rh-MT1-Ecto-E240A, rh-MT1-Cat (all at 2 μM) or rh-$VEGF_{165}$ (1 μM) were added in wash buffer for 1 hour at RT. Protein complexes were washed three times in wash buffer. Bound rh-MT1 domains or rh-$VEGF_{165}$ were detected using the LEM-2/15.8 or VEGF-A antibodies (1 hour at RT), respectively, followed by an HRP-conjugated anti-mouse secondary antibody (1 hour at RT) and incubation with TMB High Sensitivity Substrate Solution (BioLegend, Cambridge, UK) according to manufacturer's instructions. TMB was measured by absorbance at 450 nm after addition of 1 M H_2SO_4. The OD_{450nm} obtained from wells without rh-MT1-MMP domain or rh-$VEGF_{165}$ incubation was subtracted from the OD_{450nm} of each sample well. Samples were assayed in triplicate.

Flow cytometry

MCF-7 cells were transfected as previously described. rh-$VEGF_{165}$ (100 ng/ml) was added to the cells for 30 minutes where indicated. Cells were washed with PBS and detached from the plastic with PBS containing 5 mM EDTA. After 15 minutes, fixation in PFA [4% (w/v) in 5 mM EDTA/PBS], cells were incubated with anti-MT1-MMP and anti-VEGFR-2 antibodies in PBS for 1 hour at RT. After three washes, cells were incubated for 1 hour at RT with 10 μg/ml PE-conjugated anti-mouse (Abcam, UK) or allophycocyanine (APC; Invitrogen)-conjugated anti-rabbit secondary antibodies. Cells were washed, sieved through a 70 μm filter and analysed on a FACSCalibur II flow cytometer (BD Biosciences, Oxford, UK). 10^4 events were acquired per sample and the mean fluorescence intensity of four independent experiments was determined using FlowJo flow cytometric analysis software (v.8.8.4, Tree Star Inc., Olten, Switzerland).

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 5 Software (GraphPad Software, San Diego, CA). Statistical significance was assessed by one-way ANOVA with a Student's Newman-Keuls post *t*-test, unless indicated otherwise. All numerical values shown are the means ± s.e.m.

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