# 学位論文

## MMP-7 reactivates latent VEGF and promotes angiogenesis in the tumor environment

（MMP-7によるVEGF活性化に伴うがん選択的血管新生機構の解析）

伊 藤 孝

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## Abbreviation

| | |
|---|---|
| Ab | Antibody |
| ADAMTS1 | A Disintegrin and Metalloproteinase with Thrombospondin motifs |
| CD | Cluster of Differentiation |
| CTGF | Connective tissue growth factor |
| DMEM | Dulbecco's modified Eagle's medium |
| ECM | extracellular matrix |
| EDTA | ethylenediaminetetraacetic acid |
| GFP | green fluorescent protein |
| HARP | heparin affin regulatory peptide |
| HUVECs | human umbilical vein endothelial cells |
| IGFBP-3 | Insulin-Like Growth Factor Binding Protein-3 |
| kDa | Kilo Dalton |
| MMPs | Matrix metalloproteinases |
| MPC | methacryloyloxyethyl phosphorylcholine |
| PF-4 | Platelet factor 4 |
| sVEGFR-1 | soluble Vascular endothelial growth factor receptor 1 |
| TIMP-1 | Tissue inhibitor of metalloproteinase-1 |
| TSP-1 | Thrombospondin-1 |
| VEGF | Vascular endothelial growth factor |
| VEGFR-2 | Vascular endothelial growth factor receptor-2 |
| vWF | von Willebrand factor |

# I Introduction

## Tumor and blood vessel

The blood vessel system is essential for tumor development as it provides tumor with oxygen and nutrient. It also becomes a path for tumor cells to spread from the original site to other organs, called metastasis, which mainly contributes to mortality from tumors. The formation of new blood vessel, called angiogenesis, accompanies tumor development and helps tumor cells to grow and metastasize (Carmeliet and Jain, 2000; Hanahan and Folkman, 1996). However, angiogenesis does not occur in normal adult tissue except during the female reproductive cycle (Hanahan and Folkman, 1996). These differences in angiogenesis have been mainly explained by up-regulation of angiogenic factors such as Vascular Endothelial Growth Factor (VEGF) in response to hypoxia, paracrine factors, or gene mutations in tumor tissue (Carmeliet and Jain, 2000; Ferrara, 1999). Paradoxically, however, all the normal tissues in the body also express angiogenic factors (Hanahan and Folkman, 1996). It remains unclear whether just the increased expression of angiogenic factors explains the "black and white" difference in angiogenesis between normal tissues and tumors.

## VEGF

VEGF is a major angiogenic factor that specifically targets endothelial cells, which constitute the inner surface of the blood vessel (Ferrara *et al.*, 2003). VEGF has several isoforms, among which $VEGF_{165}$ is the most active and predominant form (Ferrara *et al.*, 2003). VEGF promotes developmental, physiological, and several pathological angiogenesis, and is considered as a central mediator of angiogenesis (Ferrara *et al.*, 2003). In several cancer animal models, anti-VEGF antibodies and reagents are proved effective in decreasing tumor vessels, volume, and metastasis (Ferrara *et al.*, 2003). Anti-VEGF antibody has been effective in treating cancer patients who have metastasis (McCarthy, 2003). Thus, VEGF generally promotes angiogenesis in many types of cancer. However, a paradoxical feature of VEGF is its wide distribution in normal adult tissues, wherein vascular quiescence is maintained (Hanahan and Folkman, 1996). Also, though the VEGF level in the blood as well as in the tumor tissue of patients is generally increased (Ferrara *et al.*, 2003; Jelkmann, 2001; Takahashi and Shibuya, 2005), VEGF increase in the blood does not trigger angiogenesis outside the tumor tissue. These indicate that the mere presence or increase of VEGF protein is insufficient for the initiation of VEGF-driven angiogenesis. However, little is known about factors governing the bioavailability of VEGF *in vivo*.

### VEGF binding proteins

The interaction of VEGF with several cytokines inhibits VEGF activity at least *in vitro* (Gengrinovitch *et al.*, 1995; Gupta *et al.*, 1999; Heroult *et al.*, 2004; Inoki *et al.*, 2002; Kendall and Thomas, 1993; Luque *et al.*, 2003). Recently, one of these cytokines soluble VEGF receptor 1 (sVEGFR-1) has been proved to bind and inhibit VEGF in the cornea (Ambati *et al.*, 2006). This type of regulation could also account for vascular quiescence in the presence of VEGF in other normal tissues.

### MMPs

Matrix metalloproteinases (MMPs) represent a major family of extracellular proteases targeting a variety of extracellular molecules such as extracellular matrix (ECM) components, cytokines, and receptors (Overall and Kleifeld, 2006; Roy *et al.*, 2006). MMPs are generally upregulated in cancer as well as several conditions that accompany angiogenesis such as wound healing, menstruation, age-related macular degeneration, diabetic retinopathy, atherosclerosis, rheumatoid arthritis, microbial infection, and other inflammatory diseases (Burrage *et al.*, 2006; Curry and Osteen, 2003; Greenlee *et al.*, 2007; Raffetto and Khalil, 2008; Sivak and Fini, 2002). Some of the family members have been recognized as a pro-angiogenic factor that acts by

remodeling the perivascular ECM and liberating ECM-trapped angiogenic factors in pathologic conditions (Overall and Kleifeld, 2006; Roy *et al.*, 2006). MMP has been implicated in the increase of VEGF bioavailability in a mouse cancer model by a largely unknown mechanism (Bergers *et al.*, 2000). Human MMPs degrade natural VEGF binding inhibitors including Connective tissue growth factor (CTGF) (Dean *et al.*, 2007; Hashimoto *et al.*, 2002), Platelet factor 4 (PF-4) (Van den Steen *et al.*, 2000), and heparin affin regulatory peptide (HARP) (Dean *et al.*, 2007) *in vitro* . In contrast, human VEGF is resistant to human MMPs (Hashimoto *et al.*, 2002; Keyt *et al.*, 1996). The selective proteolysis of VEGF inhibitors may trigger the VEGF-driven angiogenesis by liberating VEGF from these inhibitors, and contribute to the clear distinction of angiogenesis in normal tissues of tumors.

**Fibroblasts and endothelial cells**

The specific characteristics of tumors and normal tissues are due to interactions between different cell types within each tissue. Common cellular components of both tissues are fibroblasts and blood vessel cells such as endothelial cells. Fibroblasts generate ECM components and paracrine factors (Kalluri and Zeisberg, 2006). In tumors, fibroblasts as well as cancer cells are a significant source of VEGF for tumor

angiogenesis (Dong *et al.*, 2004; Fukumura *et al.*, 1998; Hlatky *et al.*, 1994). Although the phenotypic changes in tumor fibroblasts are considered responsible for the angiogenic properties as distinct from those of normal fibroblasts (Kalluri and Zeisberg, 2006; Orimo *et al.*, 2005), fibroblasts from normal tissues also produce VEGF (Berse *et al.*, 1992; Hlatky *et al.*, 1994). This suggests that a mechanism other than the regulation of VEGF expression may modulate fibroblast VEGF activity, and subsequently affect whether angiogenesis is triggered in those tissues. Since angiogenesis is a tumor-specific phenomenon, this mechanism may involve an interaction between fibroblasts and cancer cells. Also, endothelial cells may receive a signal from cancer cells that allows endothelial cells to form a new blood vessel because endothelial cells do not promote angiogenesis in the presence of VEGF in normal tissues.

**MMP-7**

While most MMPs are produced by stromal cells such as fibroblasts, vascular cells, and inflammatory cells, MMP-7 is primarily expressed by cancer cells (Overall and Kleifeld, 2006). This may therefore implicate that MMP-7 acts as a specific signal from cancer cells to the stromal cell components necessary for tumor angiogenesis.

What we report

In this paper, we report the interaction of VEGF, VEGF binding inhibitors, and MMP-7 in regulating VEGF activity and tumor angiogenesis in three models. (1) We show that VEGF from human fibroblasts is endogenously inactivated by CTGF. Such inactive form of VEGF is reactivated by MMP-7 from cancer cells, resulting in tumor-selective utilization of fibroblast-derived VEGF. (2) We demonstrate that human endothelial cells inhibit VEGF by sVEGFR-1. MMP-7 liberates VEGF from sVEGFR-1 and promotes angiogenesis. (3) In human colorectal patients, the expression level of VEGF and MMP-7 affects tumor angiogenesis. These three models suggest that normal cells usually keep VEGF in an inactive state, and MMP-7 activate the VEGF from these cells and trigger angiogenesis selectively in tumor tissues.

## Ⅱ VEGF & MMP-7 in fibroblasts

Fibroblasts are a major cell type consisting of both normal tissues and tumors. They produce VEGF in both tissues (Hlatky *et al.*, 1994). Fibroblasts in a tumor model promote angiogenesis in a VEGF dependant manner (Dong *et al.*, 2004). However, their angiogenic activity in normal tissues has not been examined to our knowledge.

We have investigated the angiogenic activity of fibroblasts in tumor and nontumor environments using human pancreatic cancer cells and human fibroblast strains. Our data show that VEGF secreted from some fibroblasts is intrinsically inactive, and that cancer cells reactivate this inactive form of VEGF, making it selectively available within the tumor microenvironment. As the molecular mechanisms for this cancer-stroma interaction, we demonstrate that CTGF, which was shown to sequester VEGF *in vitro* (Inoki *et al.*, 2002), is involved in the inactivation of the VEGF from fibroblasts. We further show that MMP-7 from cancer cells is responsible for the reactivation of this inactive VEGF.

## Results

**Angiogenic activity of VEGF from fibroblasts in a tumor environment.**

We examined the effect of several human fibroblasts differently expressing VEGF (Figure 1a) on tumor xenografts in nude mice. Subcutaneous co-injection of human pancreas cancer Capan-1 cells with green fluorescent protein (GFP)-labeled human VA-13 fibroblasts or human WI-38 fibroblasts resulted in a significant increase in tumor growth (Figure 1b), whereas no tumors were formed by injecting any of the fibroblast strains alone (data not shown). Immunohistochemical analysis showed that all GFP-labeled human fibroblast strains examined contributed to tumor stroma formation (Figure 1c and data not shown). Macroscopic analysis and von Willebrand factor (vWF) immunostaining of the tumors containing VA-13 fibroblasts suggested that these fibroblasts promoted tumor angiogenesis (Figure 1c). Co-culture experiments of Capan-1 cells and VA-13 fibroblasts showed no effect on Capan-1 cell proliferation (Figure 1d), suggesting that VA-13 fibroblast-induced angiogenesis was due to a direct effect on vessels rather than an indirect effect that followed the acceleration of tumor growth by VA-13 fibroblasts.

To study the effect of VEGF from VA-13 fibroblasts on tumor angiogenesis, we used oligonucleotide siRNA. Introduction of VEGF siRNA into VA-13 fibroblasts blocked

VEGF mRNA and protein expression as compared with control siRNA (Figure 1e and f). VEGF suppression in VA-13 fibroblasts abolished the increased tumor growth (Figure 1g and h) and blocked the increased microvessel density induced by VA-13 fibroblasts (Figure 1i). These results show that VEGF from VA-13 fibroblasts contributes to tumor angiogenesis and growth.

**Angiogenic activity of VEGF from fibroblasts in a nontumor environment.**

We next examined the angiogenic activity of VEGF from VA-13 fibroblasts in a nontumor environment. Injection of VA-13 fibroblasts alone did not alter subcutaneous vascular structure compared with the noninjected left side (Figure 2a). Consistent with these observations, VA-13 fibroblasts alone did not stimulate angiogenesis in chick chorioallantoic membranes (Figure 2b, 2c), showing that VA-13 fibroblasts have no proangiogenic effect in nontumor environments. We also performed an *in vitro* tube formation assay of human umbilical vein endothelial cells (HUVECs) with culture supernatant from VA-13 fibroblasts. With or without an anti-VEGF antibody, the supernatant failed to alter endothelial tube formation, in contrast to the positive effect of serum-free medium with recombinant human VEGF (Figure 2d, 2e). Taking into consideration the VEGF production in the VA-13 fibroblast supernatant (Figure 1f),

these findings suggest that VEGF from VA-13 fibroblasts may be inactivated in the nontumor environment and activated in the tumor environment, thus leading to the different angiogenic responses of VA-13 fibroblasts according to prevailing conditions.

**Regulation of angiogenic activity of VEGF from fibroblasts by CTGF and MMP-7**

If so, we would expect that VEGF from VA-13 fibroblasts would be sequestered in an inactive form by a binding protein. Previous reports have shown that some secreted proteins, including sVEGFR-1, PF-4, thrombospondin (TSP-1), A Disintegrin And Metalloproteinase with Thrombospondin motifs (ADAMTS1), HARP, and CTGF, bind to VEGF and inhibit angiogenic activity (Gengrinovitch *et al.*, 1995; Gupta *et al.*, 1999; Heroult *et al.*, 2004; Inoki *et al.*, 2002; Jang *et al.*, 2004; Kendall and Thomas, 1993; Luque *et al.*, 2003). Because one of these proteins, CTGF is typically expressed in fibroblasts (Brigstock, 1999), we considered the possibility that CTGF might function as an inhibitor of VEGF from fibroblasts. It has also been reported that the angiogenic activity of VEGF suppressed by complex formation with CTGF is recovered through selective degradation of CTGF by some MMPs *in vitro* (Hashimoto *et al.*, 2002). Among such MMPs (MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, and MMP-13), MMP-7 expression is up-regulated selectively in cancer cells during tumor progression

(Crawford *et al.*, 2002). Quantitative real-time PCR analysis demonstrated CTGF expression in all fibroblast strains examined (Figure 3a) and MMP-7 expression exclusively in Capan-1 cells (Figure 3b and c). Taken together, these reports and data led us to hypothesize that VEGF from VA-13 fibroblasts is inactivated by CTGF, and that MMP-7 from Capan-1 cells restores the angiogenic activity.

To test this hypothesis, we performed tube formation assays with culture supernatant from VA-13 fibroblasts. MMP-7 treatment elicited the angiogenic activity in the supernatant, but MMP-7 itself had no angiogenic effect (Figure 3d). This elicited activity was abolished by an anti-VEGF antibody (Figure 3d), showing that MMP-7 activates the latent activity of the VEGF from VA-13 fibroblasts. This activity was also inhibited by the addition of CTGF after blocking MMP-7 activity with either tissue inhibitor of metalloproteinase-1 (TIMP-1) or an anti-MMP-7 antibody after the MMP-7 treatment to avoid the degradation of added CTGF (Figure 3d). In contrast, CTGF exhibited no significant activity when added to serum-free media or the supernatant without MMP-7 treatment (Figure 3d), suggesting that CTGF itself had no significant activity on endothelial tube formation. These data show that CTGF inactivates the angiogenic activity of VEGF from human fibroblasts.

We examined whether these mechanisms work in the tumor xenografts by using

CTGF siRNA for VA-13 fibroblasts (Figure 3e) and MMP-7 siRNA for Capan-1 cells (Figure 3f). We confirmed that introduction of CTGF siRNA did not alter VEGF expression in VA-13 fibroblasts (Supplementary Figure 1). The stimulating effects of VA-13 fibroblasts on tumor growth and angiogenesis were abrogated when MMP-7 expression in Capan-1 cells was suppressed (Figure 3g and h). CTGF suppression in VA-13 fibroblasts restored the tumor stimulating effects that otherwise would have been abolished by MMP-7 suppression in Capan-1 cells (Figure 3g and h). Because the effects of VA-13 fibroblasts were mediated by VEGF (Figure 1h and i), these results indicate that MMP-7 from human cancer cells activates CTGF-inactivated VEGF from human fibroblasts in a tumor environment.

We further investigated whether these mechanisms can also apply in primary fibroblasts isolated from human normal lung, lung cancer, and pancreas cancer. We analyzed VEGF, CTGF, and MMP-7 expression by real-time RT-PCR. Consistent with the results for VA-13 fibroblasts, there was high expression of VEGF (Figure 4a) and CTGF (Figure 4b), and no expression of MMP-7 (Figure 4c) in these primary fibroblasts. Using one strain of these fibroblasts, we subsequently performed tube formation assays. The angiogenic activity of the VEGF from the primary fibroblasts was activated by MMP-7 and inactivated by CTGF (Figure 4d), indicating that VEGF from primary

tumor-associated fibroblasts also contributes to tumor angiogenesis through these activation mechanisms.

## Discussion

We report that the interaction between human cancer cells and fibroblasts triggers the VEGF angiogenic switch in fibroblasts in experimental tumors in nude mice (Our model is illustrated in Figure 5). We have demonstrated that VA-13 fibroblasts expressing a high level of VEGF do not trigger angiogenesis within nontumor tissues, whereas they promote angiogenesis in a VEGF-dependent manner in Capan-1 tumor xenografts. VEGF released from such fibroblasts is intrinsically inactive, whereas Capan-1 cancer cells are involved in making the VEGF available, leading to tumor-selective exploitation of VEGF. We identified CTGF as the factor produced by VA-13 fibroblasts that inactivates VEGF, and MMP-7 as the factor produced by Capan-1 cancer cells that reactivates the inactive form of VEGF. We also showed that MMP-7 activates and CTGF inactivates the VEGF released from primary fibroblasts isolated from human pancreas cancer in an *in vitro* assay. The concept that a cancer-cell-specific proteinase regulates VEGF activity within the tumor stroma provides another mechanism by which tumor-associated fibroblasts reveal angiogenic activity not normally present in fibroblasts.

MMP-7 was reported to induce HUVEC proliferation (Huo *et al.*, 2002). As a possible underlying mechanism, this effect could be mediated by degradation of VE-cadherin and

nuclear accumulation of beta-catenin in HUVECs (Ichikawa *et al.*, 2006). Additionally, in light of the secretion of VEGF (Huo *et al.*, 2002) and CTGF (Brigstock. 1999) by HUVECs, the HUVEC proliferation induced by MMP-7 could also be explained by liberation of CTGF-sequestered VEGF, helping endothelial cells use the self-made VEGF.

Similar to tumor formation, wound healing is often characterized by angiogenesis and the recruitment of fibroblasts (Dvorak, 1986). Fibroblasts synthesize ECM components and paracrine factors, including VEGF and CTGF. These molecules would probably be sequestered in the ECM because they bind to fibronectin (Chen *et al.*, 2004; Wijelath *et al.*, 2002) and heparin (Brigstock *et al.*, 1997; Ferrara and Henzel, 1989). Furthermore, MMP-7 or other MMPs are up-regulated in injured tissues (Parks, 1999), suggesting the potential application of our results in wound healing. Our study indicates the potential role of fibroblasts as a reservoir of angiogenic factors, to keep them inactive in the extracellular environment in case of an emergency that requires quick regenerative responses, and thus providing rigorous regulation of angiogenesis.

## Ⅲ VEGF & MMP-7 around endothelial cells

Endothelial cells express (Kendall *et al.*, 1996) and deposits sVEGFR-1, one of the VEGF binding inhibitors, in the adjacent extracellular matrix (Orecchia *et al.*, 2003). Endothelial-derived sVEGFR-1 sequesters exogenous VEGF (Hornig *et al.*, 2000), suggestive of its role as a natural inhibitor of paracrine VEGF signaling on endothelial cells to maintain normal vascular quiescence. Since VEGF-dependent activation of endothelial cells is essential for VEGF-driven angiogenesis (Ferrara *et al.*, 2003), paracrine VEGF needs to pass through the barrier of sVEGFR-1 to VEGF receptor-2 (VEGFR-2) on endothelial cells in the shift towards a pro-angiogenic state. However, the mechanism that regulates the balance between VEGF and sVEGFR-1 in the endothelial microenvironment remains unknown. The fate of sVEGFR-1-trapped VEGF, whether to be discarded or recycled, has also remained elusive.

We demonstrate that human MMP-7 degrades human recombinant and native sVEGFR-1, resulting in the escape of VEGF from sequestration by sVEGFR-1. Using *in vitro* assays, we demonstrate that MMP-7 initiates VEGF-driven angiogenesis that otherwise would have been blocked by sVEGFR-1. Furthermore, we show that MMP-7 liberates VEGF from sVEGFR-1 secreted from human endothelial cells, and thereby provide a mechanism for the regulation of VEGF bioavailability within the local

endothelial microenvironment.

## Results

### sVEGFR-1 is degraded by MMP-7

We first tested if sVEGFR-1 is a substrate of MMP-7. Incubation with MMP-7 resulted in degradation of sVEGFR-1 as detected by silver staining (Figure 6a). This effect was abrogated in the presence of EDTA (ethylenediaminetetraacetic acid), an MMP inhibitor (Figure 6a), showing that the degradation was due to the proteolytic activity of MMP-7. MMP-2 displayed a comparable activity to that of MMP-7, whereas MMP-9 had much less effect as detected by Western blotting (Figure 6b). Insulin-Like Growth Factor Binding Protein-3 (IGFBP-3), a known substrate for these MMPs (Miyamoto *et al.*, 2004; Nakamura *et al.*, 2005), was degraded by these three MMPs (Supplementary Figure 2). Human $VEGF_{165}$ was refractory to MMP-7, -2, and -9 in our experimental condition (Figure 6c and Supplementary Figure 3), which was consistent with previous reports (Hashimoto *et al.*, 2002; Keyt *et al.*, 1996). MMP-7 promoted sVEGFR-1 degradation in a time dependent manner (Figure 6d), confirming the result of Figure 6a.

We could not detect proteolytic fragments of sVEGFR-1 in the experiments above (Figure 6a, 6d) probably because sVEGFR-1 degradation products were unstable. To determine the cleavage sites, we tried to detect fragments using a less effective buffer in sVEGFR-1 degradation. In this condition, sVEGFR-1 degradation products became

visible time-dependently in Western blotting (Figure 7a) and silver staining (Supplementary Figure 4). We collected proteolytic samples, and analyzed N-terminal amino acid sequences of proteins that could be visualized with SYPRO Ruby stain (Figure 7b). We analyzed three bands. One at around 15 kDa was the N-terminal sequence of MMP-7 (Figure 7b: P-2), whose molecular weight was comparable to full length MMP-7. Another band at about 30 kDa had the intact N-terminal sequence of sVEGFR-1 (Figure 7b: P-1). This band should be cleaved by MMP-7 specifically at the C-terminal side of sVEGFR-1. The other band at 10 kDa had the sequence starting from $Leu^{420}$ of sVEGFR-1 (Figure 7b: P-3). Thus, we could confirm the degradation of sVEGFR-1 by MMP-7 and determine one cleavage site.

To negate the possibility that this phenomenon was artificial and limited to recombinant protein, we tested the susceptibility to MMP-7 of native sVEGFR-1 produced by human umbilical vein endothelial cells (HUVECs). MMP-7 treatment of the supernatant of HUVECs decreased the amount of endogenous sVEGFR-1 dose-dependently (Figure 8). This reduction was accompanied by concomitant increase of smaller molecular weight bands, presumably proteolytic fragments of native sVEGFR-1 (Figure 8). Thus, MMP-7 also degraded native sVEGFR-1 from human endothelium. Notably, human endothelial cells also express CTGF (Brigstock. 1999). In

addition to sVEGFR-1, MMP-7 degraded native CTGF from HUVECs (Supplementary Figure 5), suggesting that the reactivation of VEGF by MMP-7 around endothelial cells is not limited to the inactivation by sVEGFR-1 and work in a broader range.

**MMP-7 reactivates sVEGFR-1-inactivated VEGF**

To determine if the degradation of sVEGFR-1 by MMP-7 affects VEGF-stimulated angiogenesis, we performed tube formation and migration assays. sVEGFR-1 blocked both $VEGF_{165}$-induced tube formation and migration of HUVECs (Figure 9a-c). The inhibitory activity of sVEGFR-1 was counteracted by subsequent treatment with MMP-7, whereas MMP-7 itself showed no significant activity in these assays (Figure 9a-c). This restored activity was blocked by anti-VEGF antibody (Figure 9b and 9c), indicating that MMP-7 revitalized the inactive VEGF. These data suggest that MMP-7 liberates VEGF from sequestration by sVEGFR-1 and triggers angiogenesis.

**MMP-7 allows VEGF access to endothelial cells**

Endothelial cells express sVEGFR-1 (Kendall *et al.*, 1996), which would trap and prevent paracrine VEGF from binding to its membrane receptors on endothelial cells. We assume that in pathological conditions accompanied by MMP-7 expression, MMP-7

degrades sVEGFR-1 around endothelial cells and helps paracrine VEGF to access membrane receptors, triggering VEGF-driven angiogenesis that is suppressed in normal conditions despite a broad distribution of VEGF. To assess this possibility, we tested the ability of sVEGFR-1 to block VEGF-binding to its receptor in the presence or absence of MMP-7. sVEGFR-1 completely blocked VEGF-induced phosphorylation of VEGF receptor 2 (VEGFR-2) (Figure 10a). In clear contrast, after incubation with MMP-7, sVEGFR-1 failed to block VEGF access to endothelial cell receptor, resulting in phosphorylation of VEGFR-2 on HUVECs (Figure 10a).

To mimic the endothelial microenvironment, we administered exogenous VEGF to the supernatant of HUVECs as a model of paracrine VEGF moving towards endothelial cells. Coimmunoprecipitation assay showed that exogenous VEGF was immunoprecipitated with native sVEGFR-1, whereas without addition of exogenous VEGF, sVEGFR-1 was detected exclusively in the flowthrough fraction (Figure 10b). These results show that native sVEGFR-1 from endothelial cells traps exogenous VEGF. Subsequent incubation with MMP-7 caused selective degradation of native sVEGFR-1 with VEGF intact (Figure 10b). These data suggest that paracrine VEGF passes through the barrier of sVEGFR-1 to endothelial cells in the presence of MMP-7, resulting in selective utilization of VEGF depending on MMP availability in the

endothelial microenvironment.

## Discussion

Our data support a model whereby human MMP-7 increases the bioavailability of human $VEGF_{165}$ in the endothelial microenvironment (Figure 11). Selective degradation of sVEGFR-1 by MMP-7 leads to VEGF exploitation by endothelial cells that stimulates migration, tube network formation, and phosphorylation of VEGFR-2. These effects do not occur in the absence of MMP-7 and the presence of sVEGFR-1. These findings suggest that MMP-7 serves as a modulator of VEGF activity by changing the balance between VEGF and sVEGFR-1.

Notably, MMP-7 treatment after the VEGF-sVEGFR-1 complex formation by pre-incubation of the two proteins did not recover the phosphorylation of endothelial VEGFR-2 by the VEGF that theoretically would have been liberated (Data not shown). This is in clear contrast to the result of the MMP-7 treatment with sVEGFR-1 before the complex formation (Figure 10a). These results indicate that MMP-7 alone may not be able to fully reactivate the sVEGFR-1-bound VEGF. However, MMP-7 treatment after the complex formation recovered VEGF activity and promoted angiogenesis in the tube formation assay and migration assay using HUVECs (Figure 9a-c), assuring that MMP-7 can liberate VEGF from sVEGFR-1 in these assays. Moreover, MMP-7 selectively degraded sVEGFR-1 after the complex formation in the supernatant of HUVECs (Figure 10b). A possible explanation for these apparently conflicting results is

as follows. In case sVEGFR-1 had been bound to VEGF, proteolytic fragments of sVEGFR-1 produced by MMP-7 that might have not been detected in our experiments still inhibited VEGF activity. However, in hours of incubation with HUVECs, proteases secreted by HUVECs worked secondarily and supplementarily to digest the fragments completely. This led to VEGF-activation in the two angiogenesis assays, but these effects were too weak in five minutes for the phosphorylation experiment. In support of this hypothesis, endothelial cells undergoing angiogenesis predominantly express MMPs such as MMP-2 (Musso *et al.*, 1997), membrane type 1-MMP (MT1-MMP) (Yana *et al.*, 2007), and MMP-7 (Sier *et al.*, 2008). Thus, sprouting endothelial cells may function to use VEGF more actively and thereby make distinction from static endothelial cells.

The present study identifies for the first time, to our knowledge, a protease that degrades sVEGFR-1. Our results demonstrate the reversibility of the VEGF activity inactivated by sVEGFR-1, and provide a novel insight into the regulation of VEGF availability around endothelial cells that affects the switch from normal vascular quiescence to angiogenesis.

## Ⅳ **VEGF & MMP-7 in colorectal tissue**

As fibroblasts and endothelial cells generally exist in tissues, we speculated that these mechanisms that trigger angiogenesis may also function at the tissue level. We have investigated whether the molecular mechanisms in the experimental models above can apply to human colorectal tissues.

## Results

**Regulation of angiogenic activity of VEGF in colorectal tissue by MMP-7 and other MMPs.**

We analyzed the expression of VEGF, CTGF, sVEGFR-1, MMP-7, and other MMPs in normal and tumor samples from 37 colorectal cancer patients. We analyzed colorectal cancers because angiogenesis in colorectal cancers is thought to be VEGF dependent to some extent, because of the therapeutic effectiveness of an anti-VEGF monoclonal antibody (McCarthy, 2003), and therefore expression patterns of these molecules might involve a shift from a normal to a malignant phenotype with newly developing vasculature. There was persistent expression of VEGF in normal tissues, although it was up-regulated 2.5-fold in tumors (Figure 12a). CTGF and sVEGFR-1 were constitutively expressed, and no significant change was observed (Figure 12b and 12c). The most marked difference was observed in MMP-7 expression. MMP-7 was barely detected in normal tissues, but was 138-fold in tumors (Figure 12d). There was also a sharp increase in MMP-1 and MMP-3 levels in tumors, no significant change in MMP-2 and MMP-9 levels (Supplementary Figure 6), and almost no detectable amounts of MMP-13 (data not shown). These expression patterns suggest that, in addition to the increased expression of VEGF, up-regulated MMP-7, MMP-1, and MMP-3 may regulate

the switch from vascular quiescence to angiogenesis in the human colorectum.

Finally, we studied the relevance of these molecules to tumor angiogenesis in these patients. As vWF expression is induced by VEGF in endothelial cells and reflects tumor angiogenesis in the colorectum (Zanetta *et al.*, 2000), we evaluated vWF mRNA expression in tumor samples as a measure of tumor angiogenesis. We divided colorectal cancer patients into four groups according to the expression patterns of VEGF and MMP-7. We did not consider the CTGF and sVEGFR-1 expression level because they were expressed at a similar level in both tumors and normal tissues (Figure 12b and 12c). We found that only the group with high VEGF and high MMP-7 expression revealed elevated expression of vWF (Figure 12e). This was confirmed by examining the expression of another endothelial marker, CD31 (Figure 12f). We also analyzed MMP-1 and MMP-3, and obtained similar results as for MMP-7 (Supplementary Figure 7), although neither the expression of MMP-1 nor MMP-3 correlated with that of MMP-7 (Spearman's correlation coefficient = 0.20 and 0.11, respectively; data not shown). The tumors that are VEGF-positive and positive for all three MMPs showed a stronger association with vWF and CD31 expression (Supplementary Figure 8), which suggests that these MMPs may work in a cooperative and compensatory manner. Furthermore, immunohistochemical analysis revealed that vWF+ clusters were observed more

frequently in tumors with high VEGF and MMP-7 expression than those with high VEGF and low MMP-7 expression (Figure 12g). Collectively, these studies indicate that MMP-7 and other MMPs may trigger VEGF-induced angiogenesis in tumor tissue, supporting the idea that these MMPs activate CTGF or sVEGFR-1-sequestered VEGF within the tissue.

## Discussion

We have shown correlative data in which high levels of expression of MMP-7, MMP-1, and MMP-3 along with VEGF are associated with increased expression of angiogenesis markers in the human colorectum, where CTGF and sVEGFR-1 are constitutively expressed. In addition, whereas these three MMPs were up-regulated in tumor tissues, the expression of MMP-2 and MMP-9 was similar to that seen in normal tissues. The proteolytic activity of MMP-2 and -9 to CTGF has been described as extremely low *in vitro* (Hashimoto *et al.*, 2002). Our data also showed that the proteolytic activity of MMP-9 to sVEGFR-1 was extremely weak, suggesting that these MMPs may not be active in CTGF and sVEGFR-1 cleavage in tissues. As well as in the colorectum, VEGF, CTGF, sVEGFR-1 are widely expressed in tumors and normal tissues of many organs (Brigstock, 1999; Hanahan and Folkman, 1996; Toi *et al.*, 2002). MMP-7 is selectively expressed in many tumors, including gastric (McDonnell *et al.*, 1991), pancreatic (Crawford *et al.*, 2002), breast (Basset *et al.*, 1990), prostate (Pajouh *et al.*, 1991), liver (Yamamoto *et al.*, 1997), lung, head, and neck carcinomas (Muller *et al.*, 1991). Although the interaction between these factors is yet to be demonstrated, the extracellular communication in VEGF regulation reported here may also operate at the tissue level.

## V General Discussion

Taken together, we suggest that (1) normal cells such as fibroblasts and endothelial cells have machinery that inactivates VEGF, (2) the inactivation of VEGF by normal cells contribute to vascular quiescence in normal tissues, and (3) MMP-7 reactivated inactive forms of VEGF, resulting in tumor-selective angiogenesis.

The physiological action of MMPs varies between species and target organs (Overall and Kleifeld, 2006). Our data suggest a regulation of VEGF activity by CTGF and MMP-7 in human fibroblasts and by sVEGFR-1 and MMP-7 around human endothelial cells. Human MMP-7 and other MMPs do not cleave the predominant isoform of VEGF ($VEGF_{165}$) (Figure 6c and Supplementary figure 3)(Hashimoto *et al.*, 2002; Keyt *et al.*, 1996), which leads to selective degradation of CTGF and sVEGFR-1 and the release of VEGF activity. In contrast, mouse MMP-7 and other MMPs modulate bioactivity of the corresponding isoform of mouse VEGF through the processing of VEGF itself (Lee *et al.*, 2005). Thus, it is likely that VEGF regulation by MMPs is quite different in mice and humans. MMP-9 from leukocytes has been described as relevant for VEGF release from the ECM and/or tumor angiogenesis in several mouse models of cancer such as cervical cancer, islet cell tumor, skin cancer, and neuroblastoma (Coussens *et al.*, 1999; Giraudo *et al.*, 2004; Jodele *et al.*, 2005; Nozawa *et al.*, 2006). In these models, VEGF may not be

sequestered in the ECM with CTGF or sVEGFR-1 because MMP-9 is not competent in the degradation of CTGF and sVEGFR-1 in our experiment (Figure 6b) and the previous report (Hashimoto et al., 2002), indicating alternative mechanisms according to the type of tumor. The relevance of other molecules that bind VEGF to angiogenesis triggered by MMPs should be tested for better understanding of VEGF regulation in the body. Our experiments indicate that another VEGF binding inhibitor TSP-1 is also degraded by MMP-7 at a similar sensitivity of sVEGFR-1 (Data not shown), implicating more general application of our model.

Several pathological states upregulate MMP-7 such as wound healing, bacterial infection, cancer, and inflammatory disorders (Wielockx *et al.*, 2004). MMP-7 is also induced in the female reproductive cycle (Hulboy *et al.*, 1997). We speculate that MMP-7 contributes to these angiogenesis-related conditions by liberating VEGF from sVEGFR-1(Figure 9, 10), CTGF (Figure 3d, 3g, 3h, 4d; Hashimoto *et al.*, 2002), and possibly other VEGF binding inhibitors. Notably, MMP-7 is expressed in cancer cells predominantly at the invasive front of tumors (Liu *et al.*, 2002; Ougolkov *et al.*, 2002; Yamamoto *et al.*, 2001). Furthermore, endothelial cells adjacent to MMP-7-expressing cancer cells (Nagashima *et al.*, 1997) and sprouting endothelial cells (Sier *et al.*, 2008) specifically express MMP-7. The invasive front is a site of particularly intense

angiogenesis in tumor tissues (Belien *et al.*, 1999; Takagi *et al.*, 2005), implicating a potential application of our model in this context. Our immunostaining at the invasive front of colorectal cancer (Figure 12g) supports this hypothesis.

Degradation of sVEGFR-1 by MMP-7 was relatively slow in comparison to that of other VEGF binding proteins such as CTGF (Dean *et al.*, 2007; Hashimoto *et al.*, 2002) and HARP (Dean *et al.*, 2007), and PF-4 (Van den Steen *et al.*, 2000) by MMPs. This may indicate that sVEGFR1-bound VEGF is less easily accessible and a concentrated amount of MMP-7 is required to liberate VEGF from sVEGFR-1. sVEGFR-1 is expressed in the cornea (Ambati *et al.*, 2006) or endothelium (Kendall *et al.*, 1996), where angiogenesis does not and "must not" occur in normal conditions. Or in the placental trophoblasts (Clark *et al.*, 1998) where fetal and maternal blood must not be intermingled. In these sites, VEGF activity may be more rigorously controlled. In other sites, VEGF may be more easily reachable for use by bone marrow-derived cells or neuronal cells in non-angiogenic phenomena such as cell survival, migration, differentiation, and stem cell homeostasis (Ferrara *et al.*, 2003; Takahashi and Shibuya, 2005).

Degradation of sVEGFR-1 also occurred with MMP-2 but much less effectively with MMP-9 (Figure 6b). These results indicate that some other MMPs also liberate VEGF

by degrading sVEGFR-1. Since various MMPs are concomitantly upregulated under most angiogenic conditions (Burrage *et al.*, 2006; Curry and Osteen, 2003; Greenlee *et al.*, 2007; Raffetto and Khalil, 2008; Sivak and Fini, 2002), multiple MMPs along with MMP-7 may act cooperatively in utilizing VEGF for angiogenesis. This model is supported by the finding that colorectal cancer patients with high expression of MMP-7, -1, and -3 along with high VEGF level have exhibited increased angiogenesis compared to those with high expression of either MMP together with high VEGF (Supplementary Figure 8). Thus, participation of multiple MMPs may reinforce the proteolytic activity on sVEGFR-1 and/or CTGF that might be insufficient with each MMP alone. Also, the robustness of human $VEGF_{165}$ to MMPs in our experimental conditions and others (Hashimoto *et al.*, 2002; Keyt *et al.*, 1996) may make it a major contributor in physiological and pathological angiogenesis (Ferrara *et al.*, 2003).

Our data provide for the first time, to our knowledge, the models whereby normal cells have two faces in angiogenesis according to the environment there are in: the anti-angiogenic character in normal tissue and the pro-angiogenic character in tumors. Our findings contribute to the understanding of clear and rigorous regulation of angiogenesis in the body.

## Ⅵ Materials and Methods

### Human cell lines

Human SV40-transformed fibroblast cell lines VA-13, IMR-90SV, MRC-5SV1TG1 (RIKEN, Tsukuba, Japan), and KM-104 (a gift from Dr. K. Harigaya, Chiba University, Japan) were transfected with the pMX-GFP vector (a gift from Dr. T. Kitamura, University of Tokyo, Japan), and GFP-positive cells were sorted as previously described(Ishii *et al.*, 2005). These cell lines and the nontransformed human fibroblast cell line WI-38 (RIKEN) were cultured in αMEM medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS). Human pancreas cancer cell line Capan-1 (ATCC, Manassas, VA) was cultured in DMEM (Sigma, St. Luis, MO) with 20% FBS. HUVECs (Dainippon Pharmaceutical, Osaka, Japan) were maintained in MCDB131 (Invitrogen) supplemented with 10% FBS, 10 ng/ml bFGF, and 10 mmol/l glutamine on a collagen-coated dish. For all experiments, HUVECs were used at passage 5–9. All cells were cultured in a humidified atmosphere of 95% air and 5% $CO_2$ at 37 °C.

### Tumor xenografts in nude mice

Capan-1 cells alone ($5 \times 10^6$) or $5 \times 10^6$ Capan-1 cells and $5 \times 10^6$ fibroblasts were suspended in 200 μl DMEM (Dulbecco's modified Eagle's medium), and injected

subcutaneously into 7–8-week-old female nude mice (Clea Japan, Inc, Tokyo, Japan). The mice were sacrificed two weeks later. Tumors were excised for volume measurements and immunohistochemical analyses. Tumor volume was calculated with the formula $V = 0.52 \times \text{length} \times \text{width} \times \text{height}$.

## Immunohistochemistry

Paraffin tumor sections (2 μm) were stained for GFP and vWF using the EnVision + System (Dako, Glostrup, Denmark). Human fibroblasts were detected with an antibody to GFP (Molecular Probes, Eugene, OR), and endothelial cells with an antibody to vWF (Dako). For GFP staining, tumor sections were incubated with primary antibody diluted 1:400 for 2 h, and with labeled polymer for 30 min at room temperature. For vWF staining, sections were incubated in 0.1% trypsin for 1 h at room temperature for tumor xenografts, or were heated at 95 °C for 10 min in a microwave oven for human tumor tissues for antigen retrieval. Tumor sections were incubated with primary antibody for 30 min and labeled polymer for 30 min at room temperature.

## Microvessel density analysis

Tumor sections were stained for vWF as described above. Highly vascularized areas

were searched and vessels in more than five fields (×400) were counted in each section. The three fields with the highest vascular density were averaged. Vessels were counted by two observers, one of whom was blind to experimental conditions.

**Co-culture assay**

Capan-1 cells ($1 \times 10^5$) were cultured together with fibroblasts ($1 \times 10^5$) in a 6-well transwell plate with 3.0-μm pore size membrane (Becton Dickinson Labware, Bedford, MA) in DMEM with 20% FBS. After 72 h, the number of Capan-1 cells was counted. The experiments were performed in sextuplicate for each data point.

**Quantitative real-time RT-PCR**

Total RNA was extracted from cultured cells or tissue samples of cancer patients with TRIzol reagent (Invitrogen), and purified using a PCR purification kit (Qiagen K.K, Tokyo, Japan). A reverse transcription kit (Invitrogen) was used to construct the template cDNA for real-time PCR with LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics, Indianapolis, IN). The primer sequences for human VEGF were: forward, 5′-ctacctccaccatgccaagt-3′; and reverse, 5′-gcagtagctgcgctgataga-3′. The primer sequences for human CTGF were: forward, 5′-caagggcctcttctgtgact-3′; and reverse,

5′-acgtgcactggtacttgcag-3′. The primer sequences for human MMP-1 were: forward, 5′-ctggccacaactgccaaatg-3′; and reverse, 5′-ctgtccctgaacagcccagtactta-3′. The primer sequences for human MMP-2 were: forward, 5′-tctcctgacattgaccttggc-3′; and reverse, 5′-caaggtgctggctgagtagatc-3′. The primer sequences for human MMP-3 were: forward, 5′-attccatggagccaggctttc-3′; and reverse, 5′-catttgggtcaaactccaactgtg-3′. The primer sequences for human MMP-7 were: forward, 5′-tgagctacagtgggaacagg-3′; and reverse, 5′-tcatcgaagtgagcatctcc-3′. The primer sequences for human MMP-9 were: forward, 5′-ttgacagcgacaagaagtgg-3′; and reverse, 5′-gccattcacgtcgtccttat-3′. The primer sequences for human MMP-13 were: forward, 5′-tcccaggaattggtgataaagtaga-3′; and reverse, 5′-ctggcatgacgcgaacaata-3′. The primer sequences for human vWF were: forward, 5′-cggcttgcaccattcagcta-3′; and reverse, 5′-tgcagaagtgagtatcacagccatc-3′. The primer sequences for human CD31 were: forward, 5′-attgcagtggttatcatcggagtg-3′; and reverse, 5′-ctcgttgttggagttcagaagtgg-3′. The primer sequences for human GAPDH were: forward, 5′-agggctgcttttaactctggt-3′; and reverse, 5′-ccccacttgattttggaggga-3′. Each analysis was performed in triplicate.

**RNA interference**

The siRNA oligonucleotide for the nonspecific control and VEGF

(5′–GGAGUACCCUGAUGAGAUCdTdT–3′)(Takei *et al.*, 2004), and the target siRNA SMARTpools for CTGF and MMP-7 were synthesized by Dharmacon Research Inc (Chicago, IL). CTGF and MMP-7 siRNAs were designed according to PubMed accession numbers NM_001901 and NM_002423, respectively. Target or control siRNAs were transfected at a final concentration of 50 nM into VA-13 fibroblasts at 30%–40% confluence, or at a final concentration of 100 nM into Capan-1 cells at 50%–60% confluence using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. At 48 h after transfection, cells were used for experiments.

VEGF ELISA

The medium on VA-13 fibroblasts was replaced with αMEM containing 10% FBS after transfection with siRNAs for 48 h. Conditioned medium was collected after 24 h of incubation, centrifuged at 800 × g for 10 min, and passed through a 0.22 μm filter. ELISA was performed using a human VEGF Quantikine kit (R&D, Minneapolis, MN) in accordance with the manufacturer's protocol. Each assay condition was measured in triplicate.

**Chick chorioallantoic membrane assay**

Capan-1 or VA-13 cells were suspended at $2 \times 10^7$ cells/ml in DMEM containing 1.5 mg/ml pig type I collagen (Nitta Gelatin, Osaka, Japan). Five microliters of the mixture was loaded onto a quarter of a 13 mm Thermanox coverslip (NALGE NUNC International, Rochester, NY) and polymerized at 37 °C. Each coverslip was applied to the chorioallantoic membrane of 10-day-old embryos at 37 °C for 72 h. The eggs were photographed after injection of a fat emulsion under the membranes to clearly visualize the vessels. The number of newly formed vessels was counted by two observers. The experiments were carried out using 9–10 eggs for each data point.

**Tube formation assay for the supernatant of fibroblasts**

Matrigel (80 μl; Becton Dickinson) was applied onto 96-well culture dishes on ice and polymerized for 30 min at 37 °C. HUVECs ($1 \times 10^4$) in 50 μl of MCDB131 were seeded onto the layer of Matrigel, and 50 μl of the culture supernatants from VA-13 or primary fibroblasts under various conditions was added. After 18 h at 37 °C, the wells were photographed. The length of the endothelial tube networks was quantified using NIH image software. Each assay condition was assessed in octuplicate. Regarding the conditioned media, the supernatant was collected after VA-13 or primary fibroblasts

were cultured with MCDB131 with 0.1% bovine serum albumin (BSA) for 16 h. After centrifugation at 800 × g for 10 min and passage through a 0.22 μm filter, the supernatant was incubated with human MMP-7 (6.7 ng/ml; Chemicon, Temecula, CA) at 37 °C for 24 h. MMP-7-treated supernatant was incubated with a neutralizing anti-human VEGF antibody (1000 ng/ml; R&D) at 37 °C for 1 h. Alternatively, MMP-7-treated supernatant was incubated with human TIMP-1 (20 ng/ml; Chemicon) or a neutralizing anti-human MMP-7 antibody (400 ng/ml; R&D) at 37 °C for 30 min, and then with human CTGF (500 ng/ml; BioVendor, Brno, Czech Republic) at 37 °C for 4 h. TIMP-1 or anti-MMP-7 antibody was added to avoid the degradation of added CTGF by blocking the proteolytic activity of MMP-7.

**Tube formation assay for the experiment of sVEGFR-1**

HUVECs were incubated in MCDB131 with 0.5% FBS for 6h before experiment. Matrigel (50 μl; Becton Dickinson) was applied onto 96-well culture dishes on ice and polymerized for 30 min at 37 °C. HUVECs ($1 \times 10^4$) in 50 μl of MCDB131 with 0.1% BSA and 10 mM glutamine were seeded onto the layer of Matrigel, and 50 μl of the medium with VEGF, sVEGFR-1, and MMP-7. After 18 h at 37 °C, the wells were photographed. The length of the endothelial tube networks was quantified using NIH image software.

Each assay condition was assessed in quadruplet. Regarding the conditioned media, VEGF (final concentration, 20 ng/ml) was incubated with sVEGFR-1 at 4 °C for 24 h, and subsequently with MMP-7 at 37 °C for 24 h at a molar ratio of 1:2:1. MMP-7-treated supernatant was incubated with a neutralizing anti-human VEGF antibody (1000 ng/ml; R&D systems) at 37 °C for 30 min before the assay.

**Degradation assays**

Recombinant human sVEGFR-1 (Cell Sciences, Canton, MA), $VEGF_{165}$ (R&D systems, Minneapolis, MN), and IGFBP-3 (R&D systems) were incubated with active human MMP-7 (Millipore, Bedford, MA), human MMP-2 (R&D systems), or human MMP-9 (R&D systems) in buffer (10 mM Hepes, pH 7.4, 150mM NaCl, and 5 mM $CaCl_2$) using a 0.5 ml 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer-coated tube (Assist, Tokyo, Japan) to prevent protein absorption. Pro-MMP-2 and pro-MMP-9 were activated by p-aminophenylmercuric acetate (Sigma-Aldrich, St Louis, MO) before use according to the manufacturer's protocol. Unless specified, substrates were incubated with MMPs at a 1:1 substrate/enzyme molar ratio at 37 °C for 24 h. For time course experiment of sVEGFR-1 with MMP-7, the molar ratio was 2:1. For degradation of human native sVEGFR-1 from HUVECs, the supernatant of HUVECs was collected

after incubation with 5 ml of MCDB131 containing 10 mM glutamine at 37 °C for 48 h. After centrifugation at 800 × *g* for 10 min, the supernatant (500 μl) was concentrated with Microcon (Millipore), and incubated with MMP-7 at 37 °C for 24 h. When specified, 20 mM EDTA was added before incubation with MMPs. The reactions were terminated with the addition of Laemmli buffer, boiled for 5 min, and subjected to SDS-PAGE.

**$NH_2$-terminal sequence analysis**

Recombinant human sVEGFR-1 (3.6 μg) was incubated with active human MMP-7 (375 ng) at a 2:1 substrate/enzyme molar ratio in buffer (10 mM Hepes, pH 7.4, 150mM NaCl, 5 mM $CaCl_2$, and 0.05% Brij-35) at 37 °C for 24 h. The reactions were terminated with the addition of 100mM EDTA, 10 % glycerol, Laemmli buffer, boiled for 5 min, subjected to 12 % SDS-PAGE, and blotted on a PVDF membrane. Proteins were visualized with SYPRO Ruby stain (Molecular Probes, Eugene, OR, USA). N-terminal amino acid sequences were determined with a Procise cLC protein sequencer (Applied Biosystems).

**Endothelial cell migration assay**

HUVECs ($2 \times 10^4$) in 100 μl of MCDB131 with 0.1% BSA and 10 mmol/l glutamine were seeded onto polycarbonate culture inserts (8-μm pore size, BD Biosciences, Bedford,

MA). Three μl of FBS (final concentration, 0.5 %) and 600 μl of MCDB131 containing 0.1% BSA and 10 mmol/l glutamine incubated with VEGF (20 ng/ml) and other cytokines were added to the lower compartment of 24-well plates. Incubation of cytokines was done as described above, at a molar ratio VEGF:sVEGFR-1:MMP-7 = 1:2:3. After 6 h of incubation at 37 °C, cell culture inserts were fixed and stained with H&E. The upper surface of the insert was wiped with a cotton swab. Migrated cells to the lower side of the insert were counted. Each assay condition was measured in triplicate.

**Western blot analysis**

The reaction solutions were denatured by boiling in Laemmli buffer. Or cells were lysed in a buffer containing 50 mM Tris, pH 7.6, 150 mM NaCl, 1 mM $MgCl_2$, 2 mM EDTA, 1 mM sodium orthovanadate, 10% Glycerol, 1% NP-40, and Complete protease inhibitor (Roche Diagnostic) and boiled in Laemmli buffer. Samples were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore). The membrane was blocked with 5% non-fat milk and 1% BSA in TBS-T [20 mM Tris (pH 7.4), 137 mM NaCl, and 0.1% Tween 20] for 1 h at room temperature, incubated with primary antibody overnight at 4 °C, and with secondary antibody for 1 h at room temperature.

Antibodies used were the extracellular region of VEGF receptor 1 to detect sVEGFR-1 (R&D systems), VEGF (Santa Cruz Biotechnology, Santa Cruz, CA), VEGFR-2 (R&D systems), VEGFR-2 Tyr1054/Tyr1059 phospho-specific antibody (Biosource, Camarillo, CA), CTGF (Santa Cruz Biotechnology), IGFBP3 (Santa Cruz Biotechnology), and actin (Santa Cruz Biotechnology). Secondary antibody was HRP-conjugated antibodies (Zymed, San Francisco, CA). Proteins were detected using ECL (GE Healthcare, Buckinghamshire, UK) and film (GE Healthcare).

**Silver staining analysis**

Samples were prepared as described above. Silver staining was performed using a silver staining kit (Sekisui Medical, Tokyo, Japan) in accordance with the manufacturer's protocol.

**Phosphorylation assay**

HUVECs were cultured on collagen-coated 35 mm dishes. The cells were serum-starved for 20 h before the assay, washed with MCDB131, and incubated for 5 minutes with sample solutions. After washed with PBS, cells were extracted and analyzed by Western blot with antibody of phosphorylated VEGFR-2 and VEGFR-2 as described above.

**Co-immunoprecipitation**

Semi-confluent 10-cm dishes of HUVECs were washed with MCDB131 and incubated with 5 ml of MCDB131 with 10 mM glutamine at 37 °C for 48 h. Conditioned media were centrifuged 10 min at 800 *g*. Conditioned media (0.5 ml) from HUVECs were incubated with VEGF (50 ng) in a 0.5 ml MPC polymer-coated tube at 4 °C for 24 h, or additionally with MMP-7 (300 ng) for 24 h. Media were incubated with anti-VEGF antibody (800 ng/ml; R&D Systems, Minneapolis, MN) for 1 h and subsequently with 10 μl of protein G (Invitrogen, Carlsbad, CA) overnight at 4 °C. After centrifugation at 7700 × *g* for 1 min, the flowthrough fractions were collected. Immunoprecipitates were washed three times with PBS buffer containing 0.1% NP-40, dissolved in 30 μl of 1.5× Laemmli buffer, boiled for 5 min, centrifuged at 7700 × *g* for 1 min, and the sample solutions were collected. Western blot analysis of immunoprecipitated VEGF and sVEGFR-1 was performed.

**Tissue samples**

Primary fibroblasts were obtained from surgical specimens from pancreas cancer patients, and were cultured in αMEM with 10% FBS. Normal and tumor tissues were obtained from surgical specimens from colorectal cancer patients. The tissues were

collected at the time of surgery and immediately stored in TRIzol at -80 °C until they were used for RNA extraction. These patients had undergone surgical resection in the National Cancer Center Hospital East. All tissue samples were collected after obtaining informed consent according to institutional ethical guidelines.

**Statistical analysis**

Data are expressed as means ± s.d. Data were tested for normality using the Kolmogorov-Smirnov test. Equality of variance was tested using the F-test for a single pairwise comparison and Bartlett's test for multiple pairwise comparisons. Statistical significance was assessed using two-tailed Student's *t*-tests or Mann-Whitney *U* test according to the normality of the data for a single pairwise comparison, and post hoc Dunnett's test following ANOVA or Kruskal-Wallis test according to the variance of the data for multiple pairwise comparisons. Differences were considered significant if $P < 0.05$.

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# Ⅸ Figures



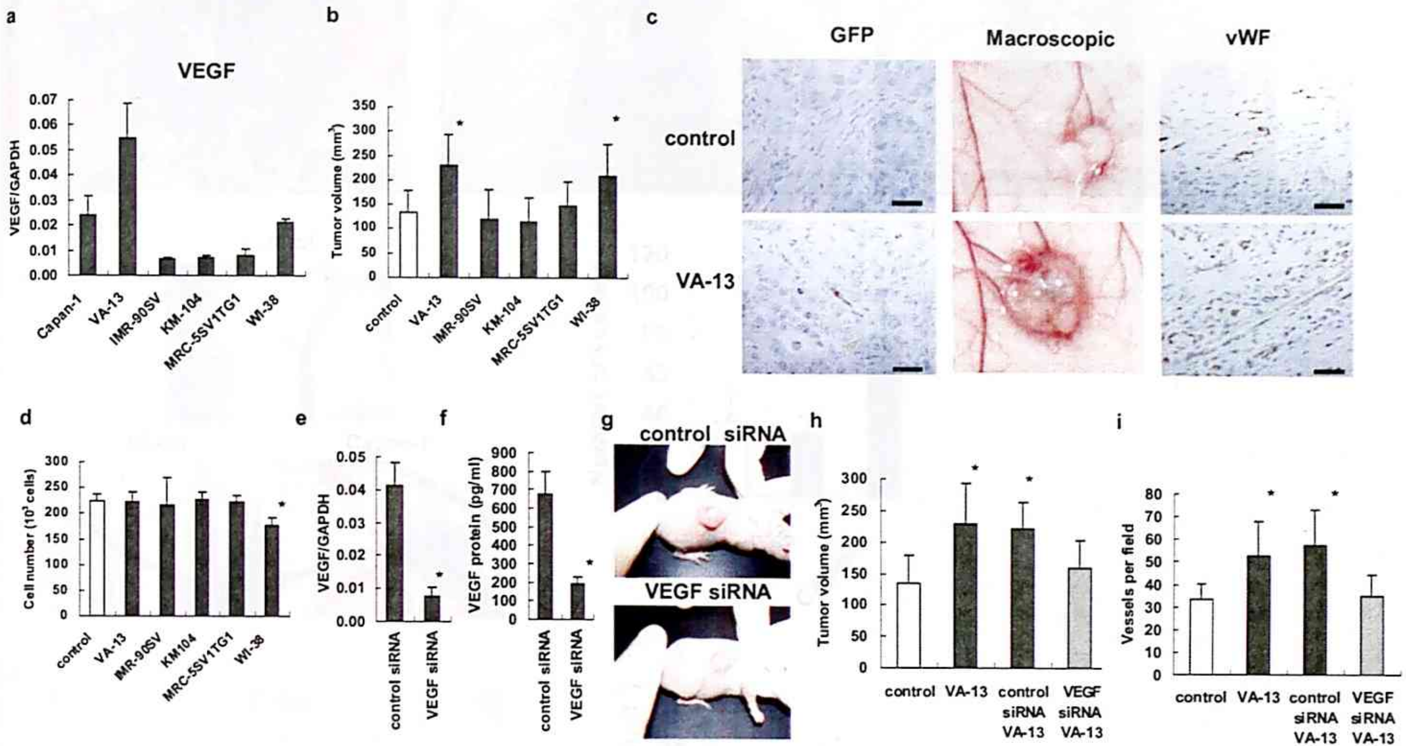



**Figure 1. Angiogenic activity of VEGF from fibroblasts in a tumor environment.**
(a) Quantitative real-time RT-PCR of VEGF in human cancer and fibroblast cell lines. n = 3. (b) Volume measurements of tumor xenografts formed after 14 d by Capan-1 cells alone (control) or Capan-1 cells and human fibroblasts. *$P < 0.01$, n = 9−13. (c) Left, GFP immunostaining of Capan-1 tumor xenografts with or without VA-13 fibroblasts. Bars represent 50 μm. Middle, microscopic analysis of the xenografts. Right, vWF immunostaining for endothelial cells in the xenografts. Bars represent 50 μm. (d) Co-culture of Capan-1 cells and fibroblasts for 72 h. Control represents the culture of Capan-1 cells alone. *$P < 0.05$, n = 6. (e) Effect of siRNA on VEGF mRNA expression in VA-13 fibroblasts by real-time RT-PCR. *$P < 0.005$, n = 3. (f) Effect of siRNA on VEGF protein expression in culture supernatants from VA-13 fibroblasts by ELISA. *$P < 0.005$, n = 3. (g) Photographs of tumor xenografts with siRNA-treated VA-13 fibroblasts. (h) Effect of siRNA-treated VA-13 fibroblasts on tumor growth. *$P < 0.01$, n = 10−13. (i) Effect of siRNA-treated VA-13 fibroblasts on tumor microvessel density. *$P < 0.01$, n = 10−13.

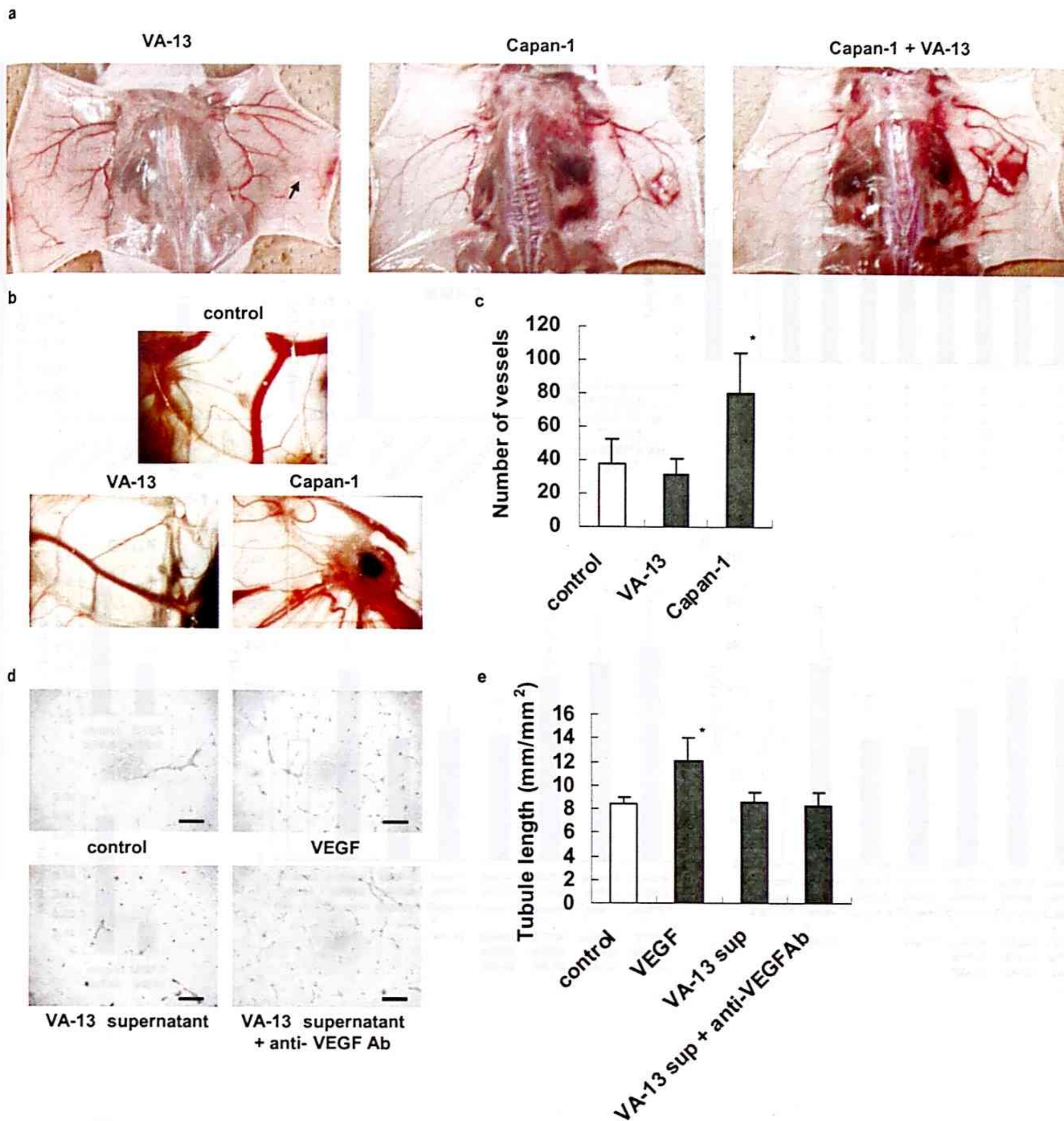



**Figure 2. Angiogenic activity of VEGF from fibroblasts in a nontumor environment.**
(a) Macroscopic analysis of the subcutaneous tissues of nude mice 14 d after injection of VA-13 fibroblasts (arrow) or Capan-1 cells with or without VA-13 fibroblasts (right side). (b) Photographs of the chick chorioallantoic membranes after 72 h with a collagen gel alone (control) or collagen-embedded cells. (c) Quantification of the angiogenic activity in the chick chorioallantoic assay. $^*P < 0.01$, $n = 9–10$. (d) Micrographs of the tube formation assay. HUVECs were incubated for 18 h in media with 0.1% BSA (control), 0.1% BSA and VEGF (10 ng/ml), or culture supernatants from VA-13 fibroblasts with or without anti-VEGF antibody. Bars represent 500 μm. (e) Quantification of the angiogenic activity in the tube formation assay. $^*P < 0.01$, $n = 8$.

Figure 3

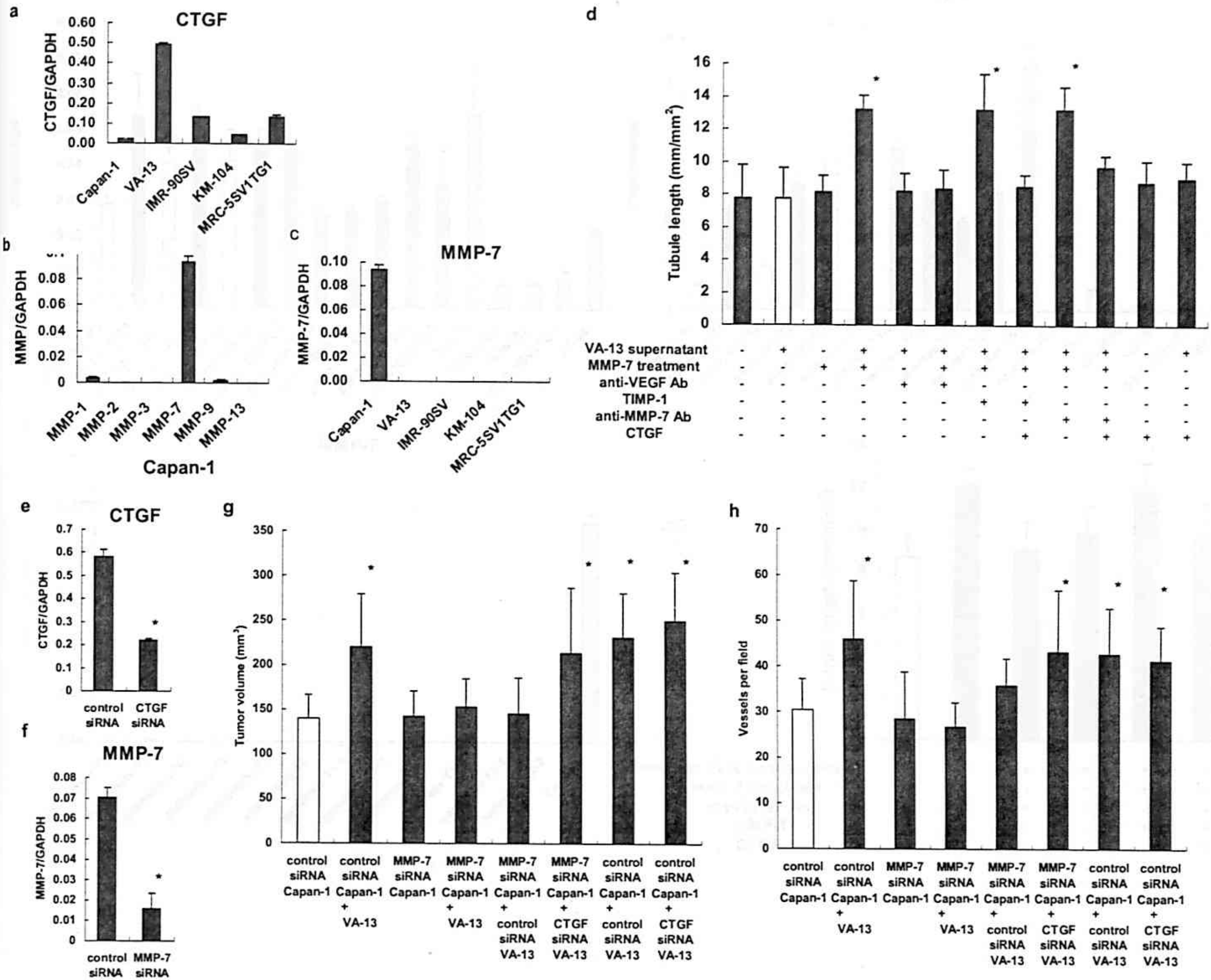


**Figure 3. Regulation of angiogenic activity of VEGF from fibroblasts by CTGF and MMP-7.** (a) Quantitative real-time RT-PCR of CTGF in human cancer and fibroblast cell lines. n = 3. (b) Quantitative real-time RT-PCR of MMPs in human cancer cells. n = 3. (c) Quantitative real-time RT-PCR of MMP-7 in human cancer and fibroblast cell lines. n = 3. (d) Effect of CTGF and MMP-7 on the angiogenic activity of VEGF from fibroblasts in the tube formation assay. MMP-7 treatment represents the incubation of medium with MMP-7 at 37 °C for 24 h before adding other proteins. $*P < 0.01$, n = 8. (e) Effect of siRNA on CTGF mRNA expression in VA-13 fibroblasts. $*P < 0.0001$, n = 3. (f) Effect of siRNA on MMP-7 mRNA expression in Capan-1 cells. $*P < 0.0005$, n = 3. (g) Effect of gene suppressions of CTGF and MMP-7 in each cell type on tumor growth 14 d after transplantation into nude mice. $*P < 0.05$, n = 8–10. (h) Effect of gene suppressions of CTGF and MMP-7 in each cell type on tumor microvessel density 14 d after transplantation into nude mice. $*P < 0.05$, n = 8–10.

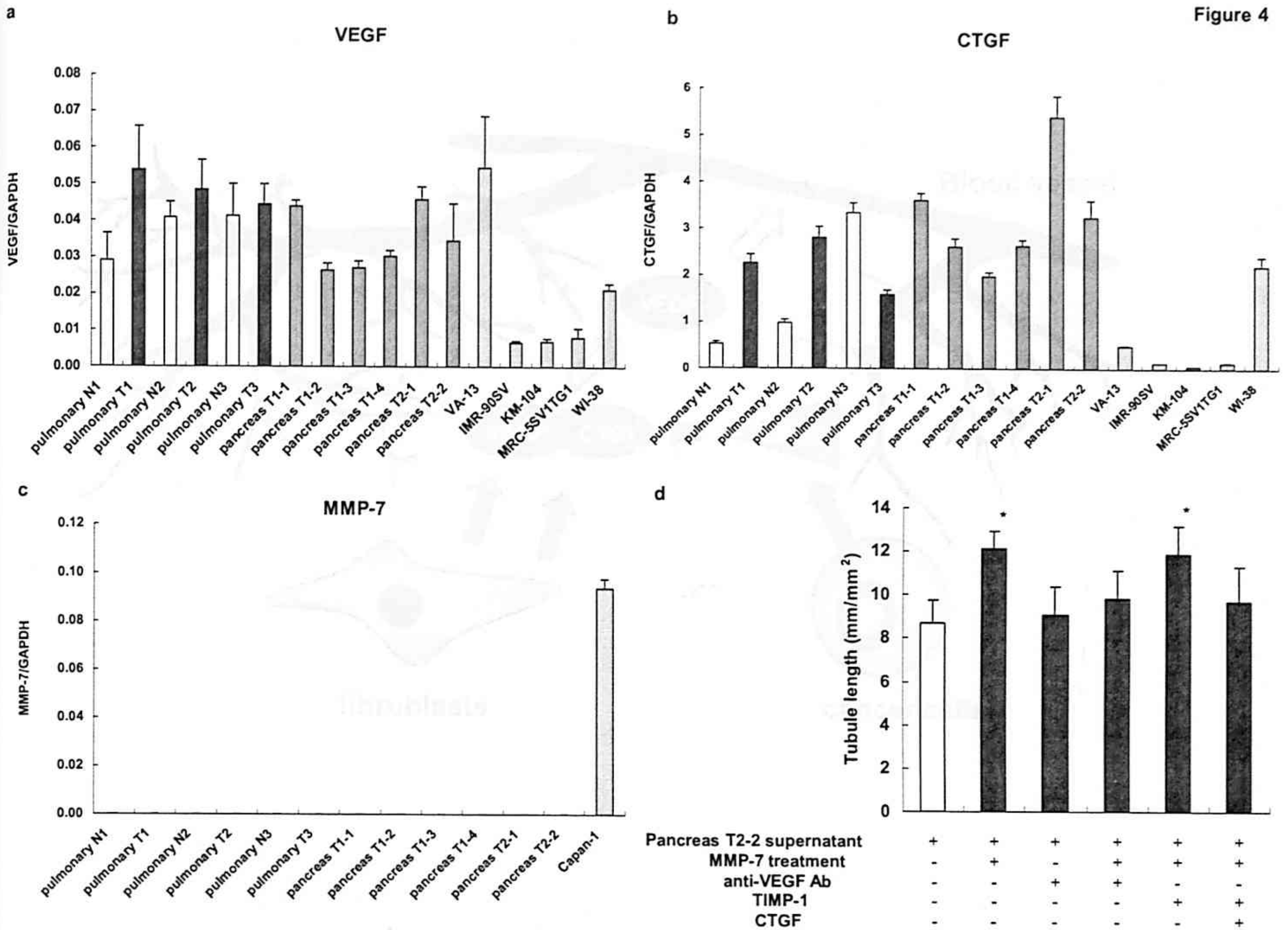



**Figure 4. Regulation of angiogenic activity of VEGF in primary fibroblasts by CTGF and MMP-7.**

(a) Quantitative real-time RT-PCR of VEGF in primary fibroblasts from human normal lung (white), lung cancer (black), pancreas cancer (dark gray), and cell lines (light gray). n = 3. (b) Quantitative real-time RT-PCR of CTGF in primary fibroblasts and cell lines. n = 3. (c) Quantitative real-time RT-PCR of MMP-7 in primary fibroblasts and cell lines. n = 3. (d) Effect of CTGF and MMP-7 on the angiogenic activity of VEGF from primary fibroblasts in the tube formation assay. TIMP-1 and other proteins were added after the MMP-7 treatment for 24 h. *$P$ < 0.01, n = 8.

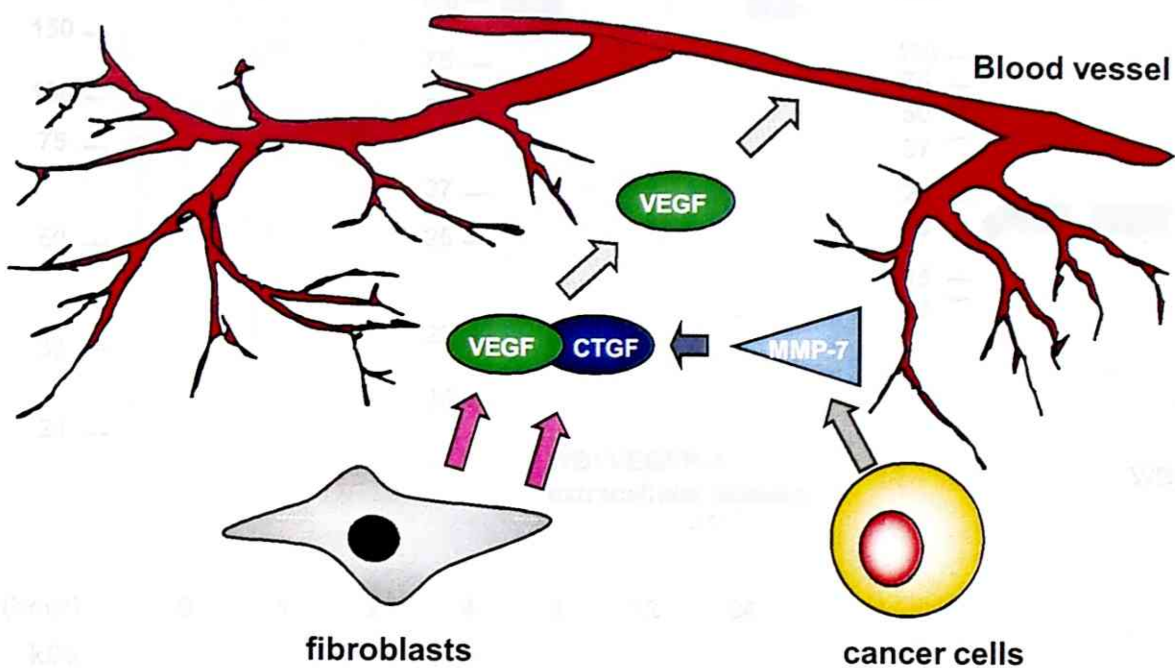



**Figure 5. Model of Regulation of VEGF from fibroblasts by CTGF and MMP-7.**
Some human fibroblasts secrete VEGF in a latent state due to the action of CTGF in normal tissue.
MMP-7secreted by human cancer cells activates this inactive form of VEGF. Activated VEGF promotes new blood vessel formation selectively in tumor tissue.

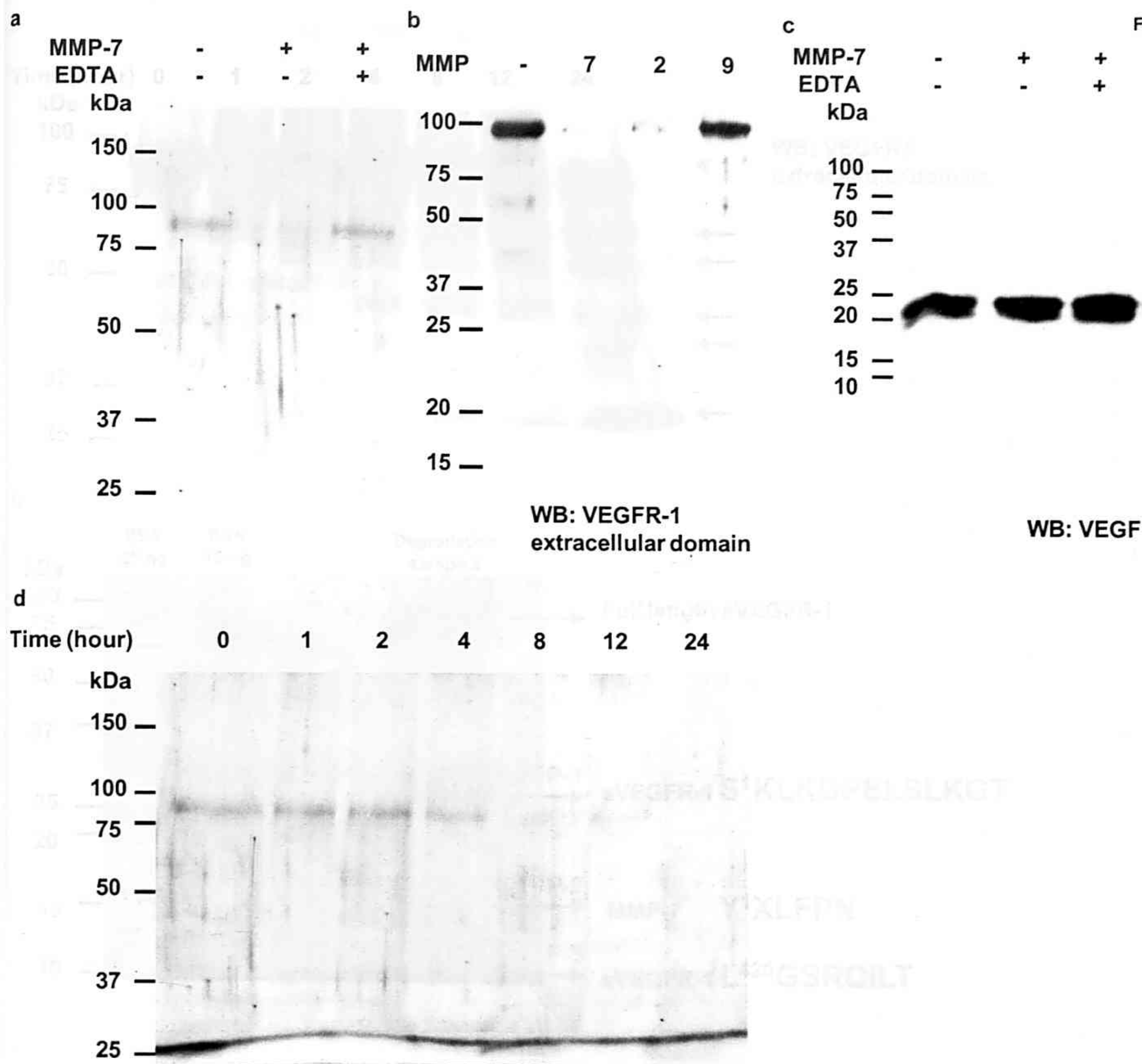



**Figure 6. MMP-7 degrades human recombinant sVEGFR-1, but not VEGF.**
(a) Human recombinant sVEGFR-1 was incubated with MMP-7 in the presence or absence of MMP inhibitor EDTA. The digestion products were analyzed by SDS-PAGE and silver staining. (b) Human recombinant sVEGFR-1 was incubated with MMP-7, -2, or -9 and analyzed by SDS-PAGE and Western blot. sVEGFR-1 was detected by antibody directed against the extracellular region of VEGFR-1, which shares identical extracellular domains with sVEGFR-1. (c) Human recombinant $VEGF_{165}$ was incubated with MMP-7 in the presence or absence of EDTA and analyzed by SDS-PAGE and western blot. (d) Human recombinant sVEGFR-1 was incubated with MMP-7 at different time points and analyzed by SDS-PAGE and silver staining.

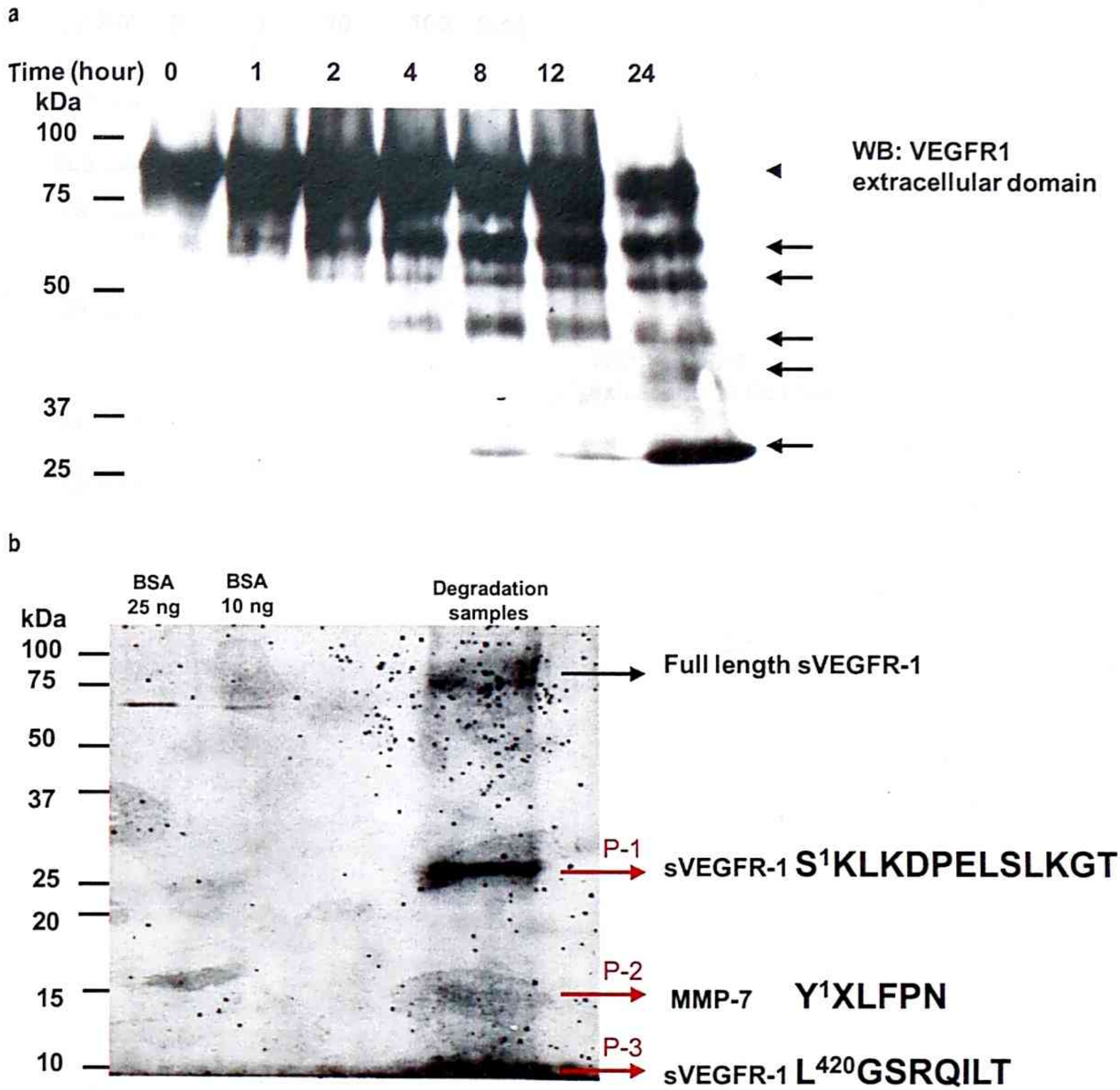



**Figure 7. Determination of cleavage sites in sVEGFR-1 by MMP-7.**

(a) Human recombinant sVEGFR-1 was incubated with MMP-7 at different time points in the original buffer to which 0.05% Brij-35 had been added, and analyzed by SDS-PAGE and silver staining. Arrowhead indicates full length recombinant sVEGFR-1 (96 kDa). Arrows indicate proteolytic fragments of recombinant sVEGFR1. (b) Degradation samples were subjected to 12 % SDS-PAGE followed by blotting and SYPRO Ruby staining. N-terminal amino acid sequences of visualized bands (P1-P3) were analyzed with a protein sequencer.

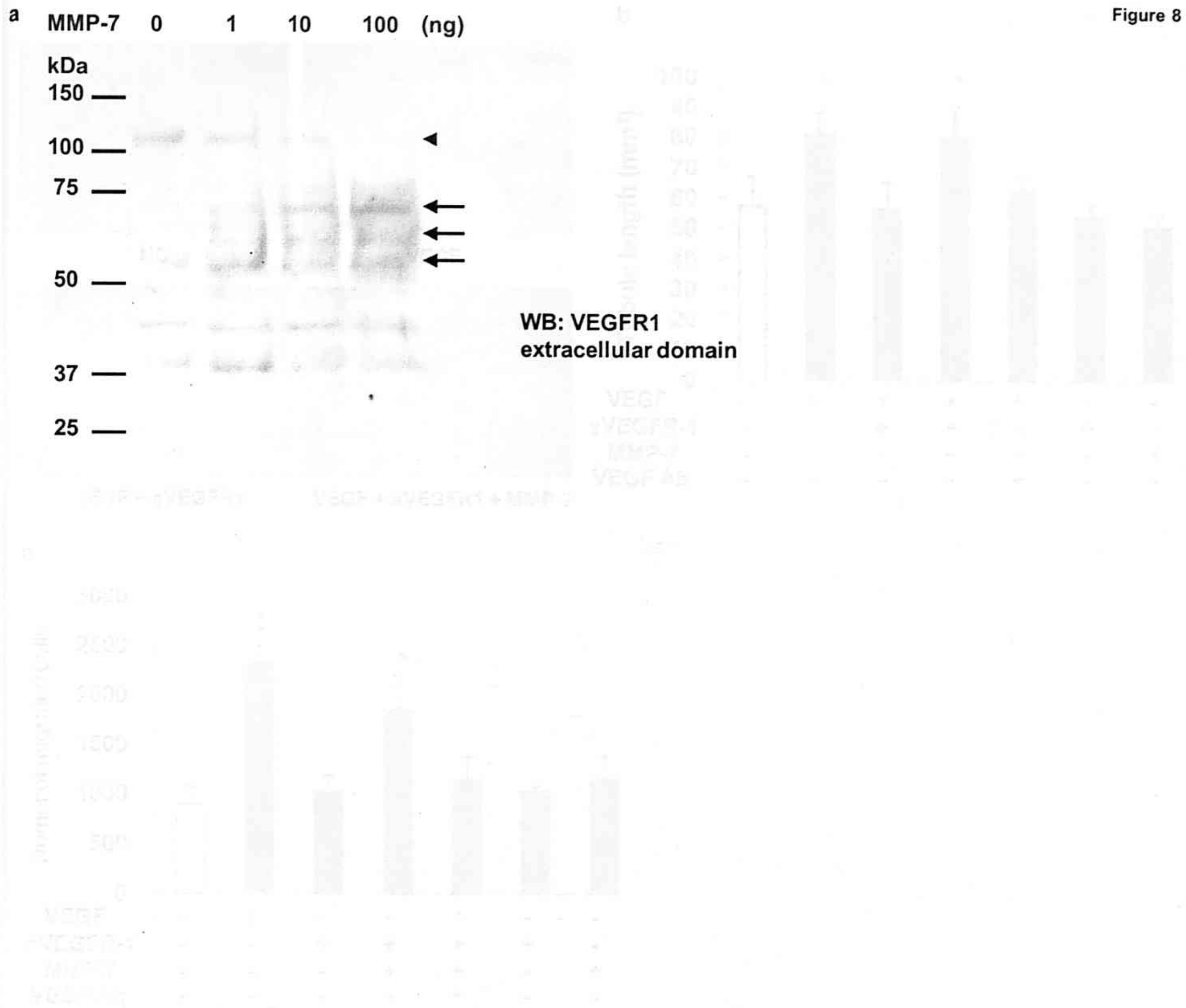



**Figure 8. MMP-7 degrades human native sVEGFR-1 from endothelial cells.**
Supernatant of HUVECs was incubated with MMP-7 at indicated doses. The digestion products were analyzed by SDS-PAGE and Western blot using antibody against the extracellular region of VEGFR-1. Arrowhead indicates the putative band of full length sVEGFR-1 that corresponds with the molecular mass of human native sVEGFR-1 from endothelial cells (110 kDa) (Hornig et al., 2000). Arrows indicate putative proteolytic fragments of sVEGFR1 from endothelial cells.

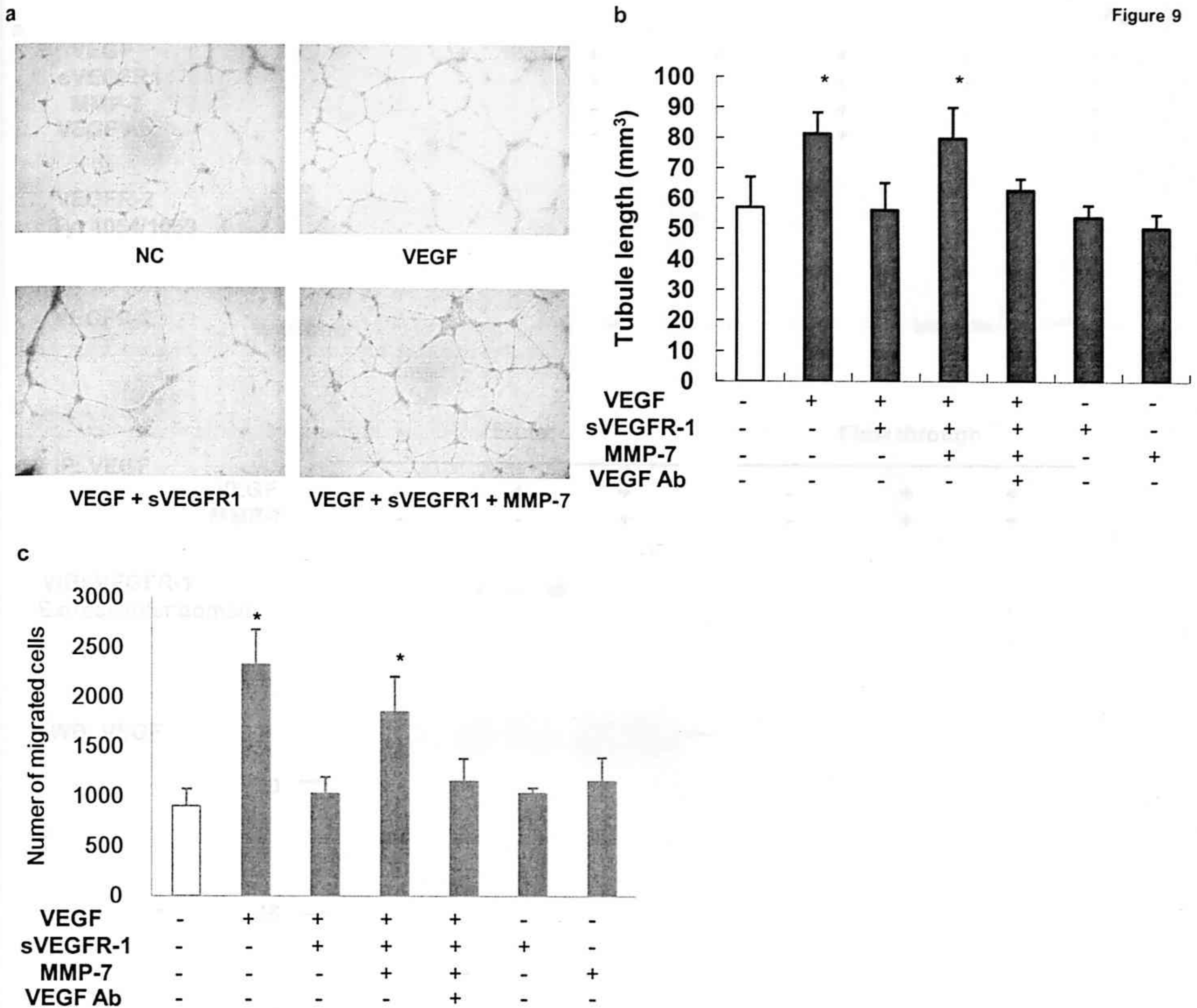



**Figure 9. MMP-7 reactivates sVEGFR-1-inactevated VEGF in angiogenesis assays.**
(a) Microscopic photographs of the HUVEC network formation in the tube formation assay.
NC indicates incubation of HUVECs with MCDB131 medium with 0.1% BSA in the assay. VEGF indicates the medium containing 20 ng/ml VEGF. VEGF + sVEGFR-1 indicates the medium with VEGF and sVEGFR-1 incubated for 24 h before addition to HUVECs. VEGF + sVEGFR-1 + MMP-7 indicates the medium with VEGF and sVEGFR-1 incubated for 24 h followed by incubation with MMP-7 for 24 h before addition to HUVECs. (b) Quantification of tube length in the tube formation assay. $*P < 0.01$, n =4. (c) Quantification of migrated HUVECs in the migration assay. $*P < 0.01$, n =3

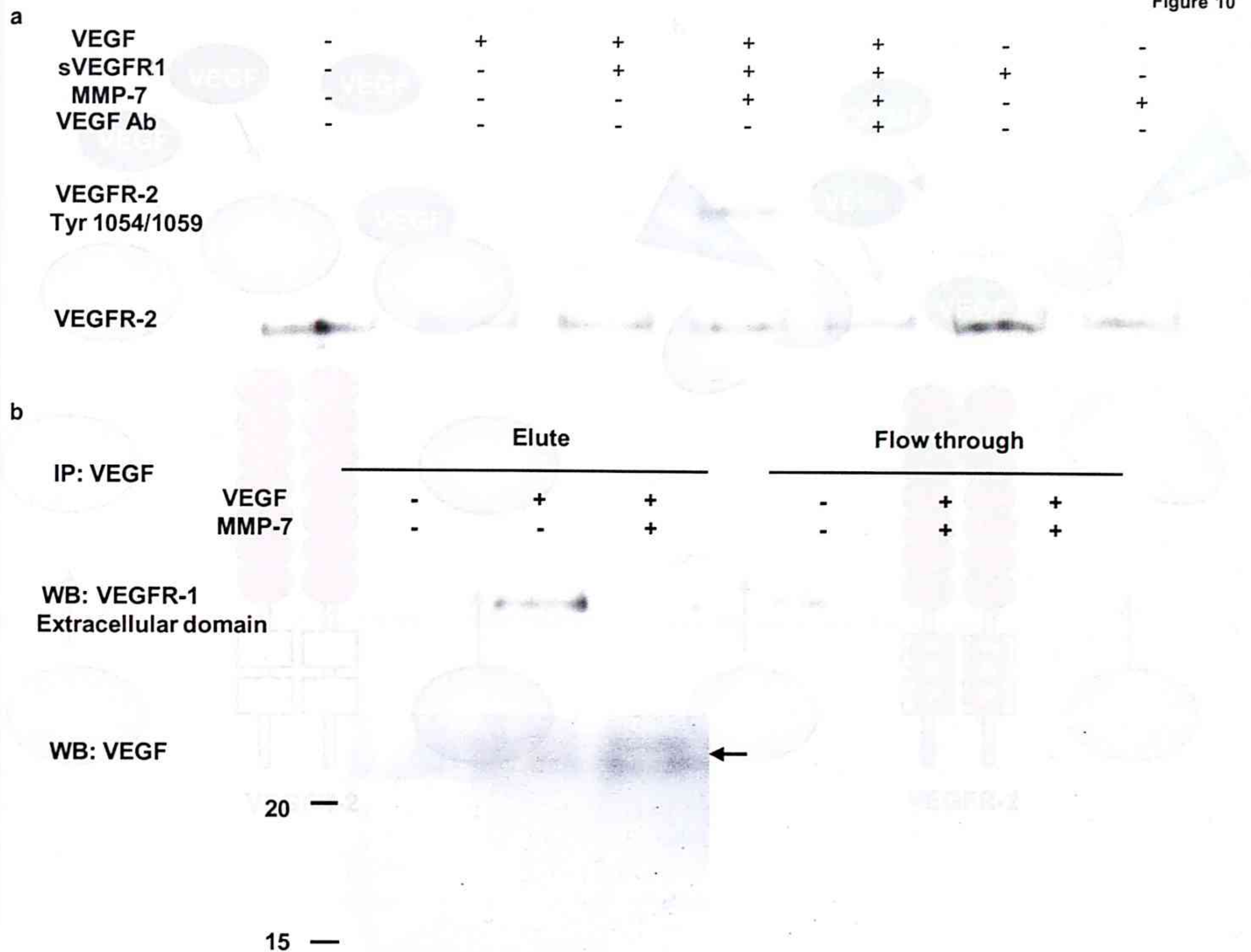



**Figure 10. VEGF evades sVEGFR-1 and access endothelial cells in the presence of MMP-7.** (a) Phosphorylation on VEGFR-2 following VEGF/sVEGFR-1/MMP-7 treatment of HUVECs. sVEGFR-1 (91.4 ng/ml) was incubated with MMP-7 (28.6 ng/nl) for 24 h, with VEGF (20 ng/ml) for 24 h, and with anti-VEGF antibody for 30 min. The sample solutions were added onto HUVECs for 5 min and the cells were analyzed by Western blot with antibody against phospho-VEGFR-2 (Tyr1054/1059). Total amount of VEGFR-2 was also analyzed. Total amount of VEGFR-2 was also analyzed. (b) Co-immunoprecipitation assay of exogenous VEGF and endothelial sVEGFR-1. Recombinant VEGF165 was added to the supernatant of HUVECs for 24 h. Subsequently, the supernatant was incubated with MMP-7 for 24 h and immunoprecipated with anti-VEGF antibody. The precipitations and unbound supernatant fluids were analyzed for sVEGFR-1 and VEGF (arrow) by Western blot.

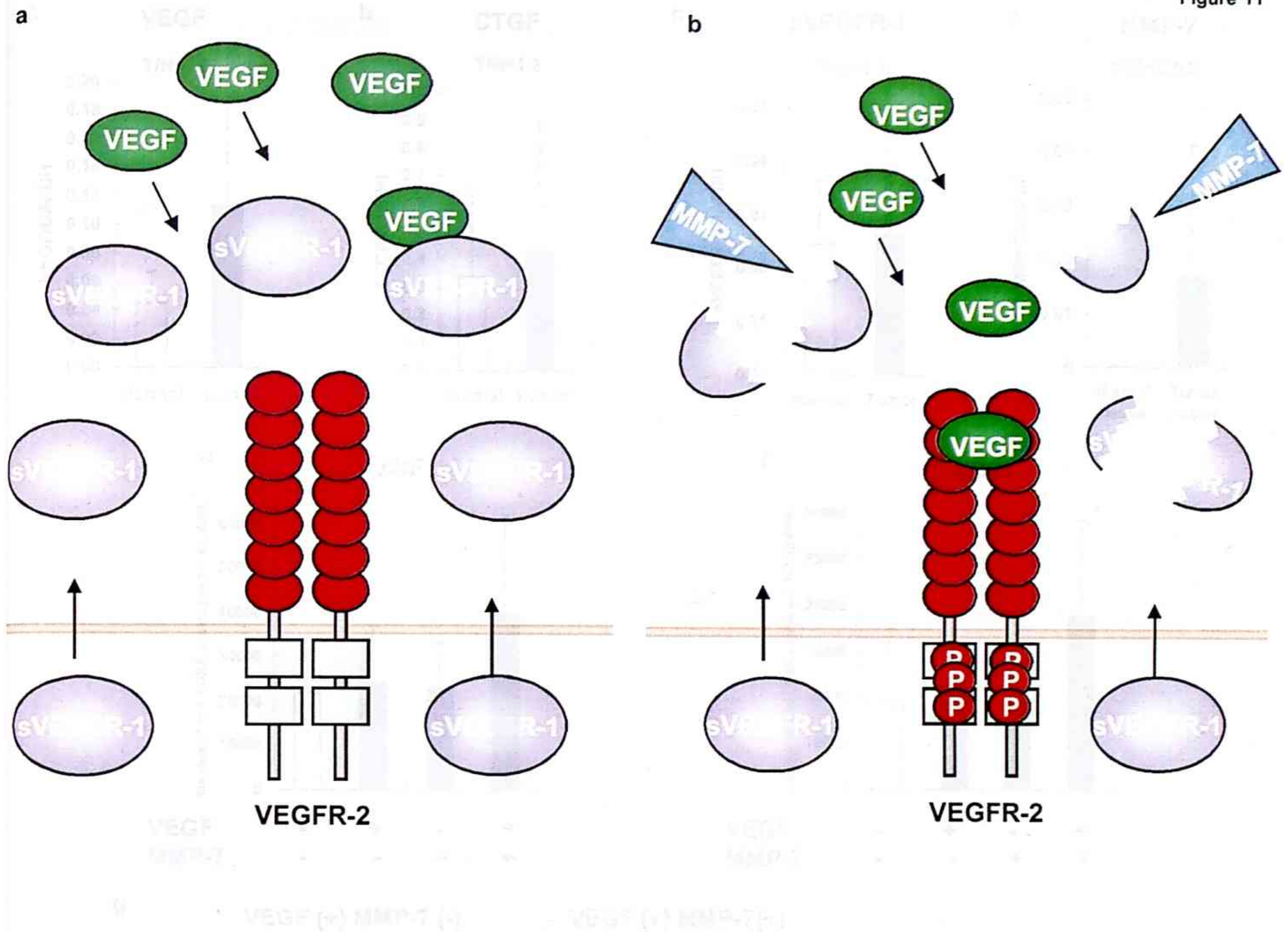



**Figure 11. Model of Regulation of VEGF around endothelial cells by sVEGFR-1 and MMP-7.**
(a) Human endothelial cells secrete sVEGFR-1 and block the access of VEGF to its receptor in normal tissue.
(b) MMP-7 liberates VEGF by degradation of sVEGFR-1. Activated VEGF promotes new blood vessel formation selectively in tumor tissue.

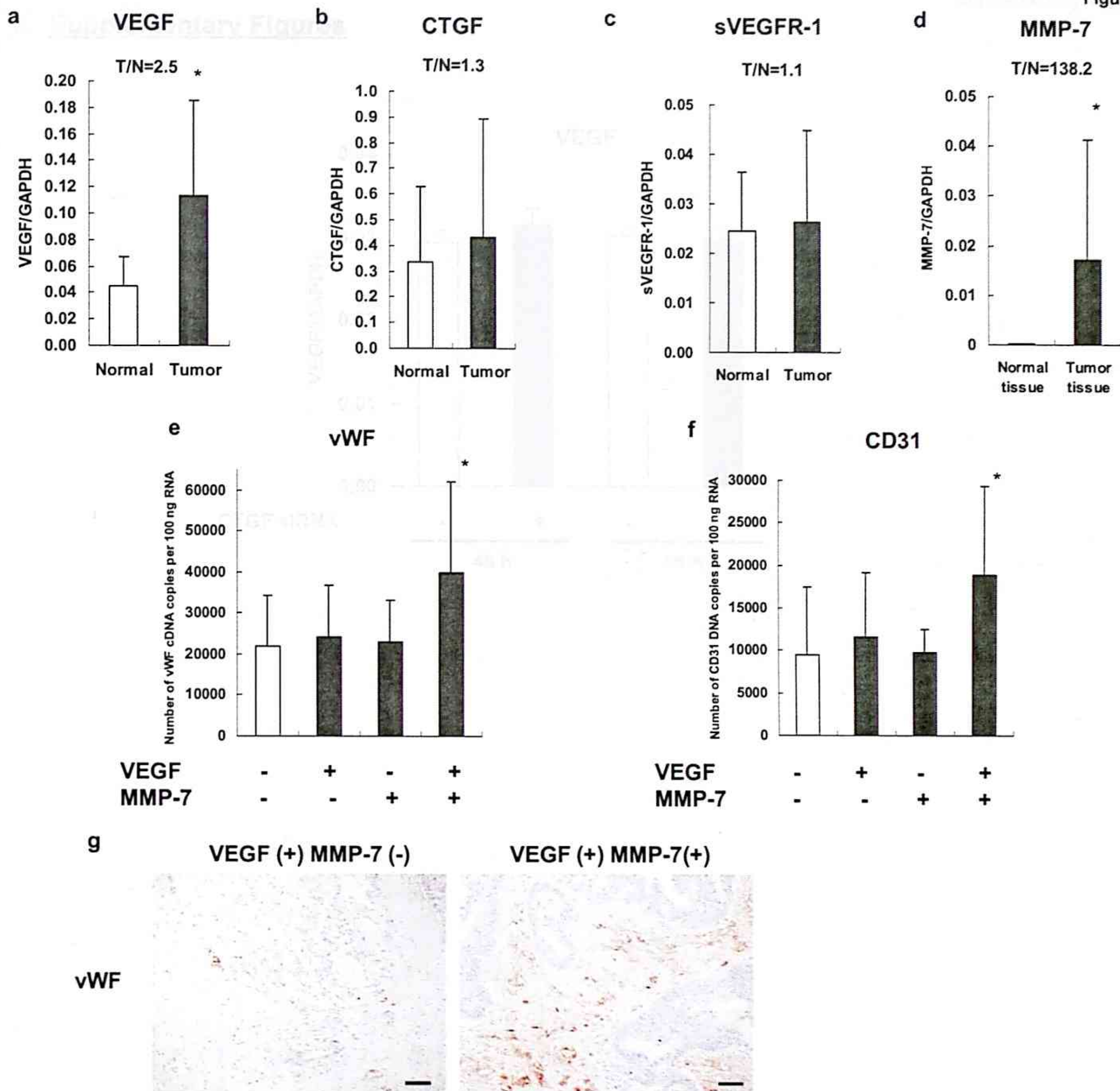
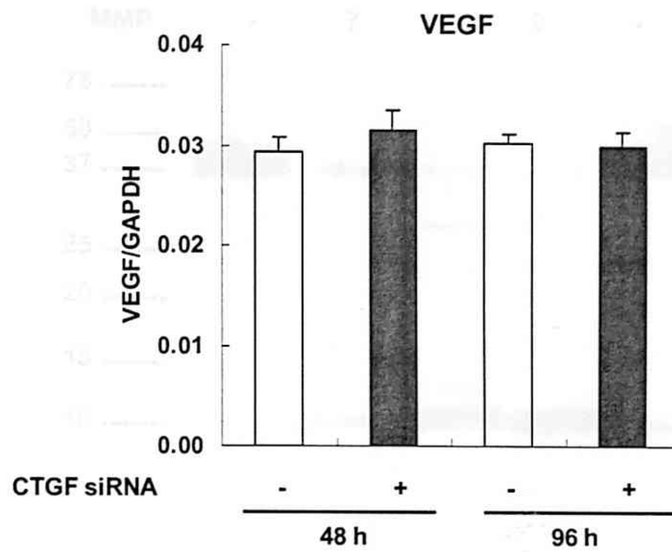
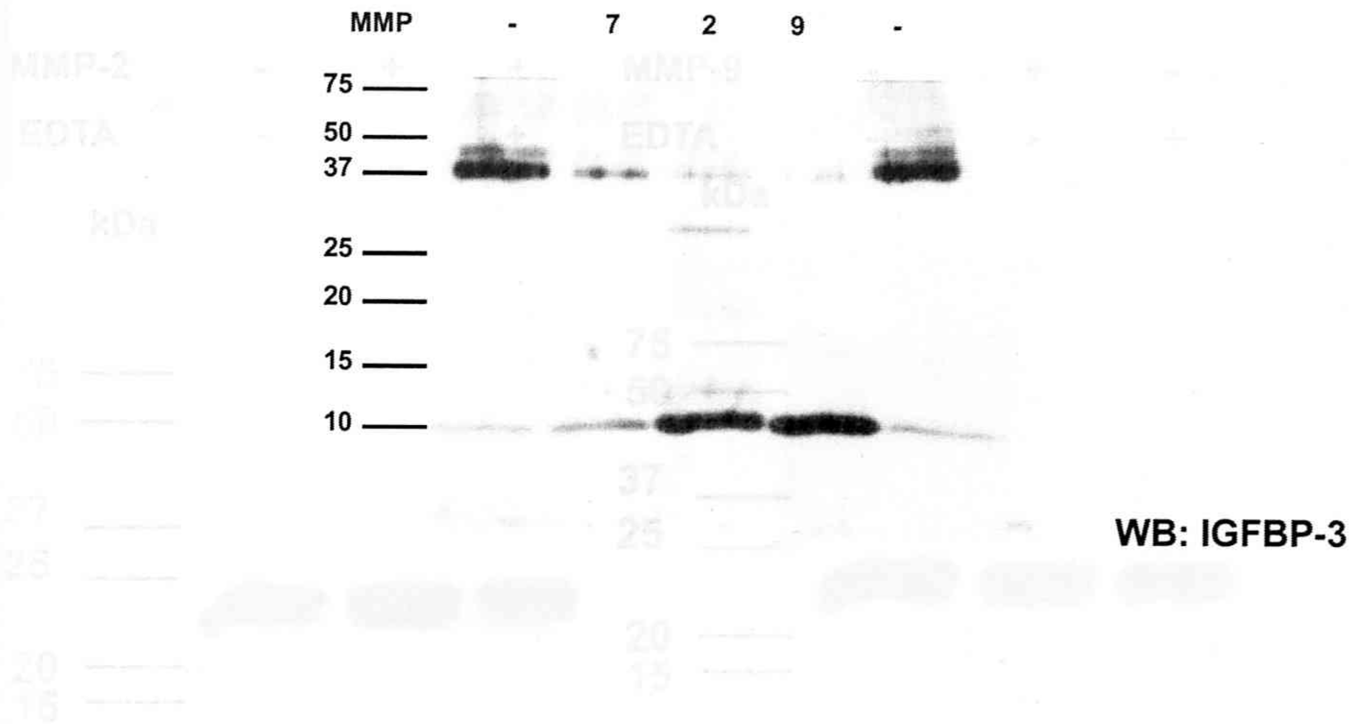



**Figure 12. Regulation of angiogenic activity of VEGF in colorectal tissues.**
(a) Quantitative real-time RT-PCR of VEGF in normal and tumor tissue samples from colorectal cancer patients. *$P < 0.0005$, n = 37. (b) Quantitative real-time RT-PCR of CTGF in normal and tumor tissue samples from colorectal cancer patients. n = 37. (c) Quantitative real-time RT-PCR of sVEGFR-1 in normal and tumor tissue samples from colorectal cancer patients. n = 10. (d) Quantitative real-time RT-PCR of MMP-7 in normal and tumor tissue samples from colorectal cancer patients. *$P < 0.0005$, n = 37. (e) Quantitative real-time RT-PCR of vWF in tumor tissue samples from 37 colorectal cancer patients divided into four groups according to VEGF and MMP-7 expression. The median value of VEGF and MMP-7 expression was used as the cutoff value. *$P < 0.05$, n = 12 for the group with low VEGF and low MMP-7 expression and the group with high VEGF and high MMP-7 expression, n = 6 for the group with high VEGF and low MMP-7 expression, and n = 7 for the group with low VEGF and high MMP-7 expression. (f) Quantitative real-time RT-PCR of CD31 in tumor tissue samples from 37 colorectal cancer patients divided into four groups according to VEGF and MMP-7 expression. *$P < 0.05$. (g) vWF immunostaining of invasive front of colorectal tumor tissues with high VEGF and low MMP-7 expression (left panel and high VEGF and high MMP-7 expression (right panel). Bars represent 100 μm.

# X Supplementary Figures

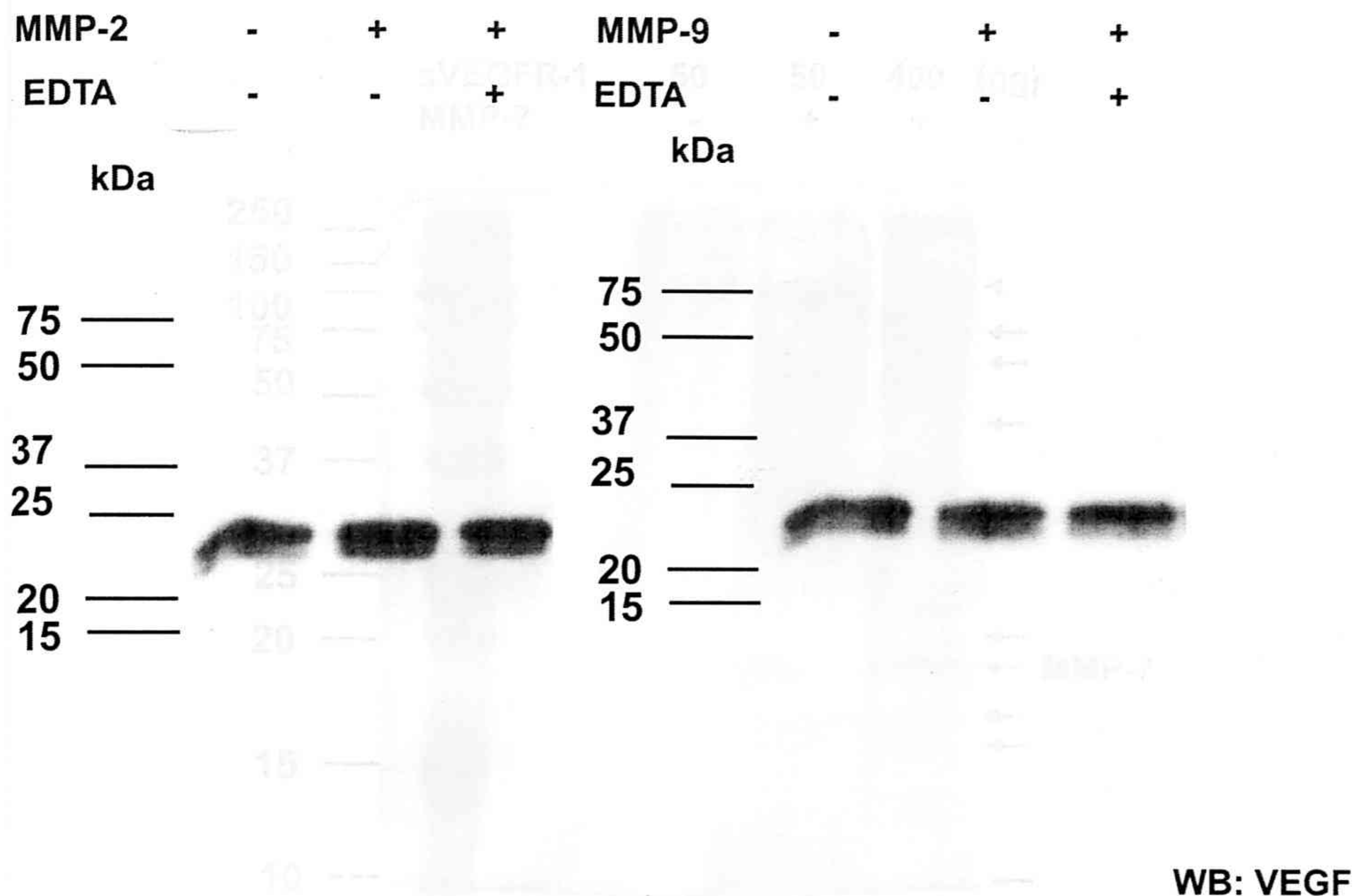



**Supplementary Figure 1. No effect of CTGF siRNA on VEGF expression in VA-13 fibroblasts**
Quantitative real-time RT-PCR of VEGF in VA-13 fibroblasts treated with transfection reagent (lipofectamin2000) and/or CTGF siRNA for 48 h or 96 h. n = 3.




**Supplementary Figure 2. Degradation of IGFBP-3 by MMP-7, MMP-2, and MMP-9**

Human recombinant IGFBP-3 (50 ng) was incubated with MMP-7, MMP-2, and MMP-9 for 24 h. The digestion products were analyzed by SDS-PAGE and Western blot. IGFBP-3 migrates as an apparent mass of 41 kD.



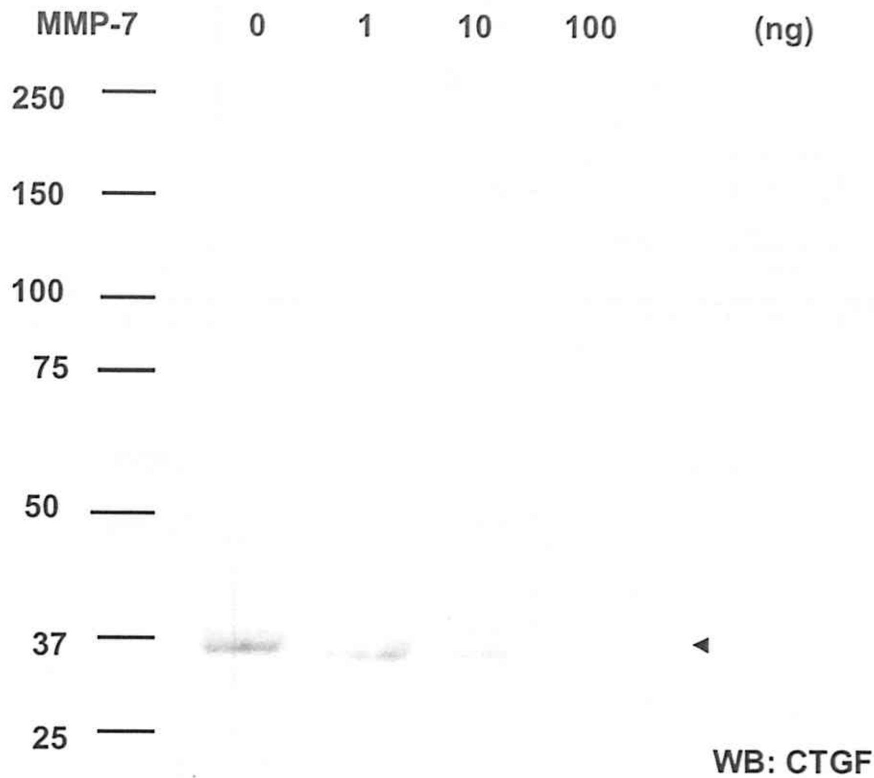

**Supplementary Figure 3. Resistance of VEGF to MMP-2 and MMP-9**
Human recombinant VEGF (50 ng) was incubated with MMP-2 and MMP-9 (VEGF:MMP=1:1) for 24 h with or without MMP inhibitor EDTA. The digestion products were analyzed by SDS-PAGE and Western blot. VEGF migrates as an apparent mass of 21 kD.

sVEGFR-1 50 50 400 (ng)
MMP-7 - + +

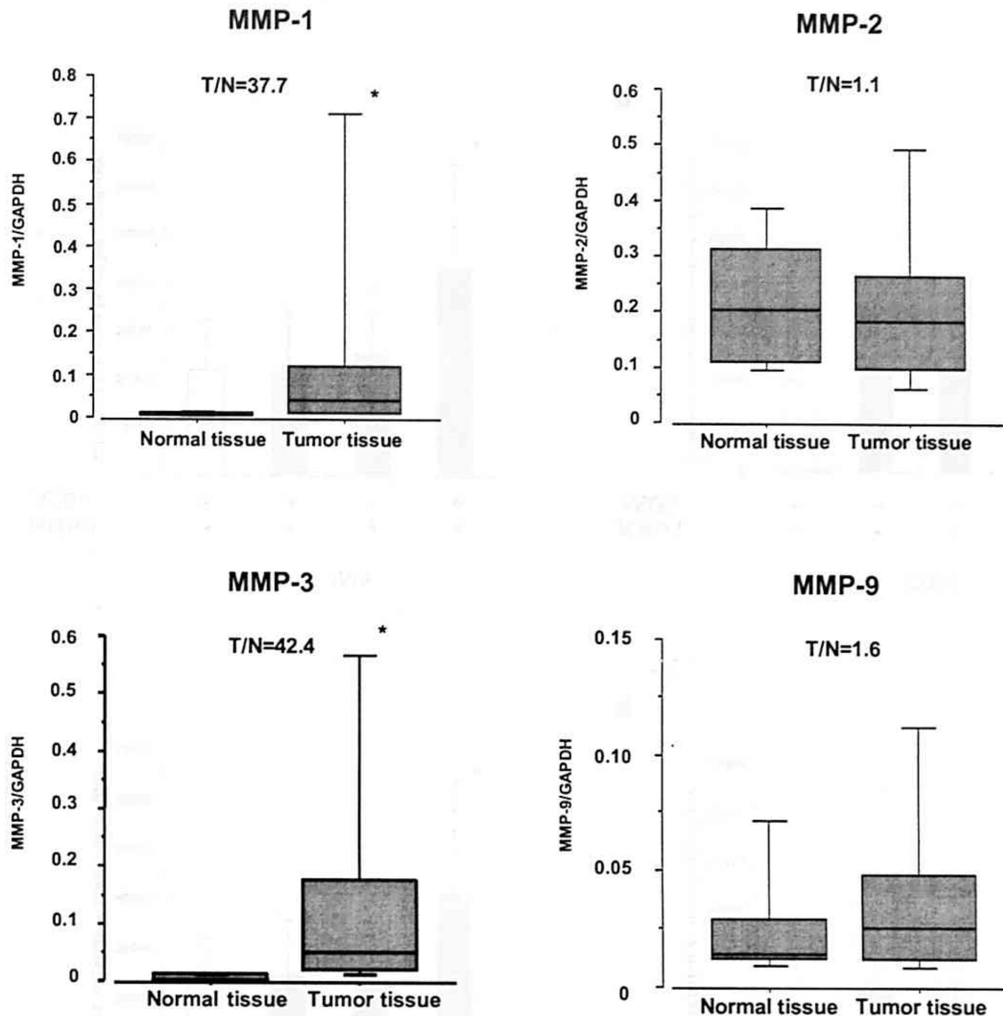
250
150
100
75
50
37
25
20
15
10

MMP-7

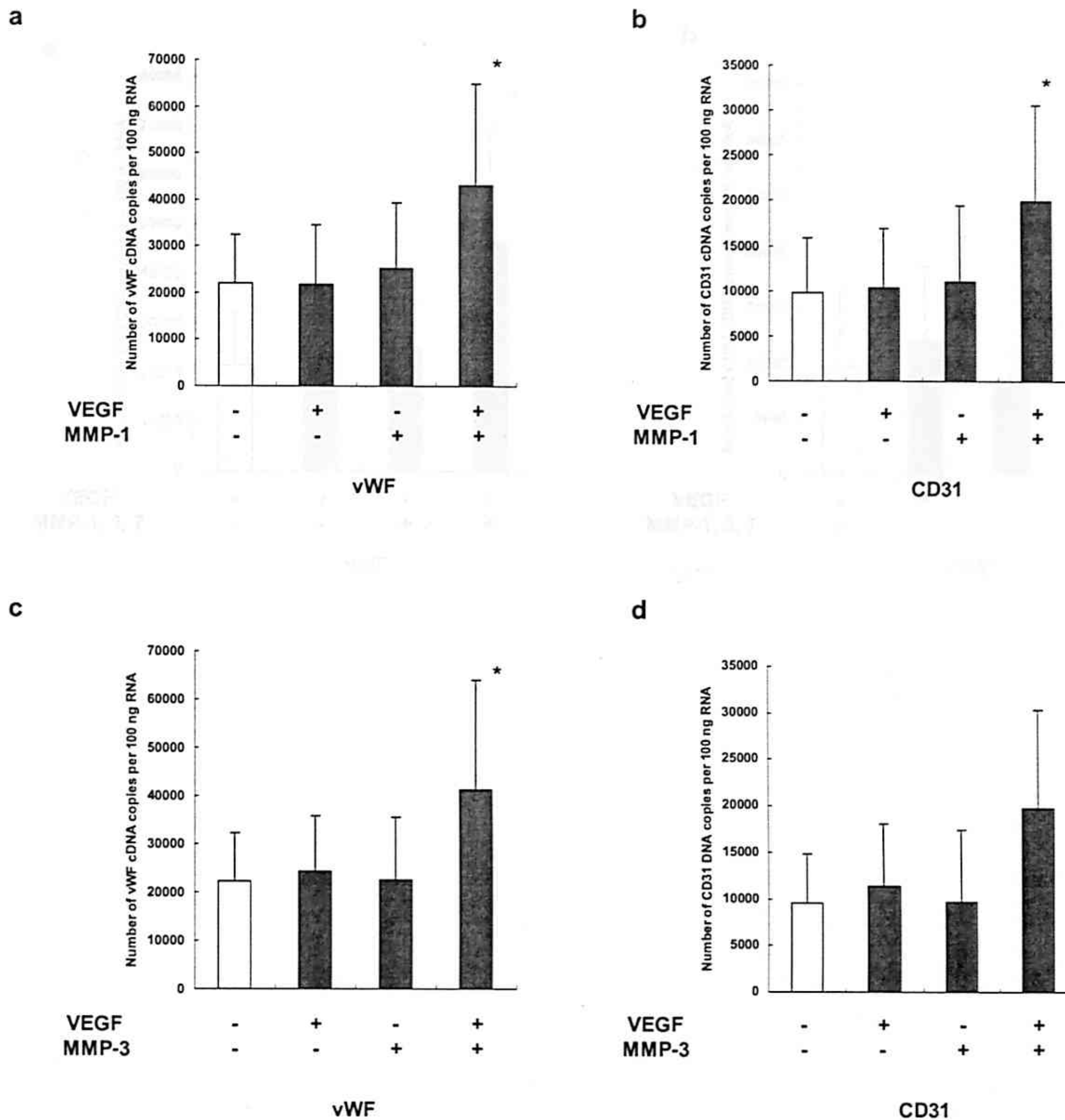
**Supplementary Figure 4. Detection of proteolytic fragments of sVEGFR-1 by MMP-7**
Human recombinant sVEGFR-1 (50 ng or 400 ng) was incubated with MMP-7 at a substrate/enzyme molar ratio 2:1 for 24 h. The digestion products were analyzed by SDS-PAGE and silver staining. Arrowhead indicates full length recombinant sVEGFR-1 (96kD). Arrows indicate degradation products of sVEGFR-1. Dotted arrow represents MMP-7 that corresponds with predicted molecular weight and the sample of recombinant MMP-7 only (data not shown).




**Supplementary Figure 5. MMP-7 degrades human native CTGF from endothelial cells.**
Supernatant of HUVECs was incubated with MMP-7 at indicated doses. The digestion products were analyzed by SDS-PAGE and Western blot using antibody against CTGF. Arrowhead indicates the putative band of full length CTGF that corresponds with the molecular mass of human native sVEGFR-1 from endothelial cells (38 kDa) (Brigstock , 1999).

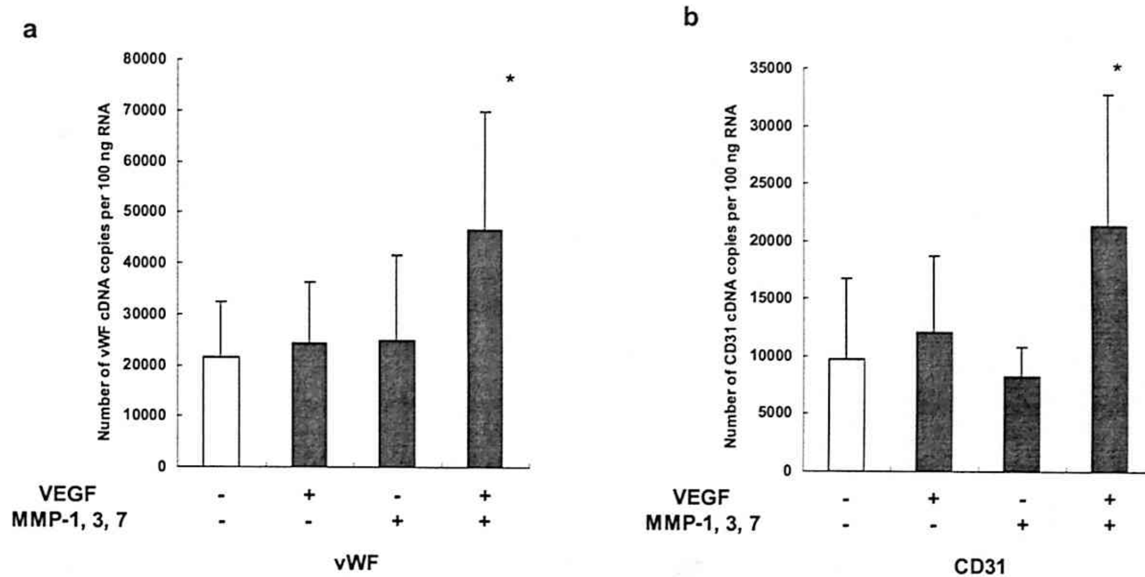



**Supplementary Figure 6. Expression of MMPs in human colorectal cancer patients.**
Quantitative real-time RT-PCR of MMP-1, MMP-2, MMP-3, and MMP-9 in normal and tumor tissue samples from colorectal cancer patients. Because some data values exhibited asymmetrical distributions, box plots were used. The 10th, 25th, 50th (median), 75th, and 90th percentiles of the variables are shown. T/N represents the ratio of tumor/normal expression. $*P < 0.0005$, Mann–Whitney $U$ test, n = 37 for MMP-1 and MMP-3 and n = 22 for MMP-2 and MMP-9.




**Supplementary Figure 7. Correlation of endothelial markers with VEGF and MMP-1 or MMP-3 expression in human colorectal cancer patients.**

(a), quantitative real-time RT-PCR of vWF in tumor tissue samples from 37 colorectal cancer patients divided into four groups according to VEGF and MMP-1 expression. *$P < 0.05$, n = 12 for the group with low VEGF and low MMP-1 expression, n = 7 for the group with high VEGF and low MMP-1 expression, n = 8 for the group with low VEGF and high MMP-1 expression, and n = 10 for the group with high VEGF and high MMP-1 expression. (b), quantitative real-time RT-PCR of CD31 in tumor tissue samples from 37 colorectal cancer patients divided into four groups according to VEGF and MMP-1 expression. *$P < 0.05$. (c), quantitative real-time RT-PCR of vWF in tumor tissue samples from 37 colorectal cancer patients divided into four groups according to VEGF and MMP-3 expression. *$P < 0.05$, n = 12 for the group with low VEGF and low MMP-3 expression, n = 6 for the group with high VEGF and low MMP-3 expression, n = 8 for the group with low VEGF and high MMP-3 expression, and n = 11 for the group with high VEGF and high MMP-3 expression. (d), quantitative real-time RT-PCR of CD31 in tumor tissue samples from 37 colorectal cancer patients divided into four groups according to VEGF and MMP-3 expression.




**Supplementary Figure 8. Correlation of endothelial markers with VEGF and all of MMP-1, -3, and -7 expression in human colorectal cancer patients.**

Quantitative real-time RT-PCR of endothelial markers in tumor tissue samples from 37 colorectal cancer patients divided into four groups according to VEGF and MMPs expression. The MMP-1, 3, 7 positive groups include the tumors with high expression of all of the three MMPs. The MMP-1, 3, 7 negative groups include the tumors with low expression of at least one of the MMPs (a) Quantitative real-time RT-PCR of vWF. $P < 0.01$, $n = 15$ for the group with low VEGF and low MMP-1, 3, 7 expression, $n = 11$ for the group with high VEGF and low MMP-1, 3, 7 expression, $n = 3$ for the group with low VEGF and high MMP-1, 3, 7 expression, and $n = 8$ for the group with high VEGF and high MMP-1, 3, 7 expression. (b) Quantitative real-time RT-PCR of CD31. *$P < 0.01$.