# Chapter 16
# *In Vitro* and *In Vivo* Models of Angiogenesis to Dissect MMP Functions

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**Abstract** Angiogenesis and lymphangiogenesis, the formation of new blood and lymphatic vessels from preexisting ones, are important processes associated with cancer growth and metastatic dissemination. It has become clear that matrix metalloproteinases contribute more to angiogenesis than by just degrading matrix components. They are capable to process a large array of extracellular and cell-surface proteins, and they contribute both in the onset and in the maintenance of angiogenesis. Their implication during lymphangiogenesis is expected, but not yet documented. This chapter describes *in vitro* and *in vivo* models which have proven suitability for investigating each step of (lymph)angiogenic processes. Their rationale and limitation is discussed and emerging functions of matrix metalloproteinases are reviewed.

## Introduction

Angiogenesis, the formation of new blood vessels from a preexisting vascular network, is associated with normal developmental processes, physiological tissue remodeling, and a wide range of pathologies, such as tumor development, metastasis, inflammation, and ocular illness (Carmeliet 2003). In tumors, angiogenesis is reinforced by vasculogenesis, the recruitment and functional incorporation of bone marrow-derived cells into the newly forming vessels (Carmeliet 2003). Both angiogenesis and vasculogenesis contribute to tumor growth by providing nutrients and oxygen, as well as to the formation of metastases by offering a route for dissemination. In addition, cancer cells can hijack the lymphatic vasculature which is amplified in tumor through lymphangiogenesis (Adams and Alitalo 2007).

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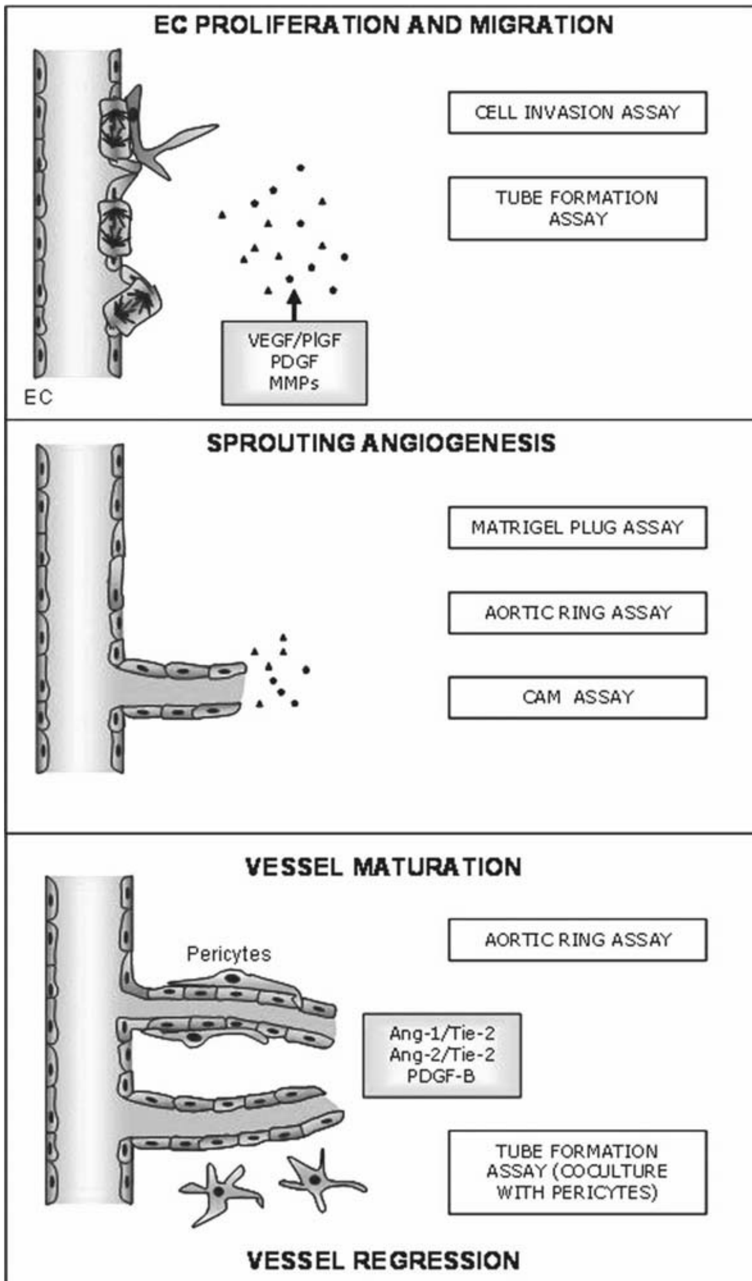


**Fig. 16.1** Schematic representation of the different steps of angiogenesis and relevance of *in vitro* and *in vivo* models to study these events. Endothelial cells (*EC*) proliferation and migration is initiated by angiogenic factors [such as vascular endothelial growth factor (*VEGF*), placental-like growth factor (*PlGF*)] and by matrix metalloproteinases (*MMPs*). Relevant models to study the

During the angiogenic growth, some endothelial cells (EC) within the capillary vessel wall are activated for sprouting (Fig. 16.1). Sprouting is controlled by a balance between proangiogenic signals such as vascular endothelial growth factor (VEGF) family members (VEGF, placental-like growth factor, PlGF) and elements that promote quiescence such as the presence of covering pericytes. Expansion of endothelial sprouts requires the induction of proliferation, motile and invasive activities, as well as the modulation of cell–cell interactions and local matrix degradation (Fig. 16.1). Further steps are then required to convert endothelial sprouts into functional and blood-carrying vessels. Strong adhesive interactions and EC–EC junctional contacts need to be established and blood flow requires the formation of a vascular lumen. Vessel stabilization is dependent on the recruitment of perivascular covering cells (pericytes and/or smooth muscle cells). Although proteases were initially viewed as simple regulators of matrix destruction, they are now recognized as active players in the different steps of the angiogenic process. The different proteolytic systems involved comprise serine proteases (Noel et al. 2004), cathepsins, and metalloproteinases, as metalloproteinases (MMPs) and related enzymes (a disintegrin and metalloprotease or ADAM, ADAM with thrombospondin-like domain or ADAMTS) (Handsley and Edwards 2005, Noel et al. 2007). The majority of MMPs are secreted and some are membrane anchored (MT-MMP-1, -2, -3, -5) or bound with a glycosyl phosphatidyl inositol link to the cell surface (MT4-MMP and MT6-MMP) (Hernandez-Barrantes et al. 2002). Their enzymatic activities are regulated by a class of natural inhibitors named TIMP-1 to -4 for tissue inhibitor of metalloproteinases (Brew et al. 2000). A number of studies including gene deletions in mice have pinpointed the role of MMP-2, MMP-9, and MT1-MMP (MMP-14) in the onset of angiogenesis in tumors and in development and in bone formation (Itoh et al. 1998, Holmbeck et al. 1999, Bergers et al. 2000, Zhou et al. 2000, Masson et al. 2005). Well-coordinated extracellular and pericellular proteolytic activities control the extracellular matrix (ECM) remodeling and modulate the bioavailability and the activity of regulatory proteins such as growth factors, growth factor-binding proteins, cytokines, chemokines, membrane receptors and cell adhesion molecules (Overall and Dean 2006, van Hinsbergh et al. 2006, Cauwe et al. 2007, Hu et al. 2007, Noel et al. 2007). The major mechanisms of action of MMPs in angiogenesis and vasculogenesis are summarized in Table 16.1.

In the present chapter, we focus on MMP-related proteolytic activities involved in angiogenesis. We discuss the most relevant models of angiogenesis which have proven valuable for unravelling the multiple roles of MMPs in this complex biological process. For a general description of MMPs, the reader is referred to other chapters of the present volume, as well as to reviews published previously

**Fig. 16.1** (Continued) onset of angiogenesis (activation of EC) are the cell invasion and the tube formation assays. Vessel sprouting can be mimicked in the Matrigel plug assay, the aortic ring and the chick chorioallantoic membrane (*CAM*) assays. Vessel maturation relying on perivascular cell recruitment and vessel coverage can be studied in the aortic ring assay and the tube assay in coculture with pericytes or smooth muscle cells

**Table 16.1** Implication of MMPs in different steps of angiogenesis

| Steps of angiogenesis | Mechanisms | Representative examples |
|---|---|---|
| Angiogenic switch | Production, activation of angiogenic factors | MT1-MMP enhances VEGF gene expression in tumor cells (Deryugina et al. 2002, Sounni et al. 2004). |
| | or enhancement of their bioavailability | MMP-9 mobilizes VEGF sequestered in ECM (Bergers et al. 2000). |
| | | VEGF is released after proteolytic cleavage of CTGF engaged in CTGF/VEGF complex (Hashimoto et al. 2002). |
| | | The cleavage of VEGF 165 by MMP-3 or MMP-9 results in the generation of a smaller molecule with properties similar to VEGF 121 (Lee et al. 2005). |
| Cell migration and pericellular proteolysis | Degradation of matrix components | MT1-MMP acts as a fibrinolysin and facilitates capillary outgrowth (Hiraoka et al. 1998, Hotary et al. 2002). |
| | Cell–cell and cell–matrix interactions | MT1-MMP colocalizes with β1 integrin in cell–cell contact, and is associated with αvβ3 integrins in migrating EC (Galvez et al. 2002). |
| | | MMP-9 interacts with the cell adhesion molecule CD44, and CD44 cleavage by MT1-MMP promotes cell migration (Mori et al. 2002). |
| | Pericellular proteolysis | Internalization via caveolae is involved in MT1-MMP-mediated migration of EC. (Galvez et al. 2004). |
| | | The localization of MMP-2 on the cell membrane is associated with αvβ3 integrin which aids in focusing the proteolytic activity pericellularly (Brooks et al. 1998, Silletti et al. 2001). |
| | | MT1-MMP can process several membrane proteins (integrin subunit, t-transglutaminase, syndecan-1) Deryugina et al. 2002; Belkin et al. 2000, Endo et al. 2003. |
| Angiogenesis inhibition | Generation of angiogenic inhibitors by the cleavage of matrix components | MMP-9 cleaves type-IV collagen and generates Tumstatin (Hamano and Kalluri 2005). |
| | | The cleavage of collagen type-VIII by MMP-12 generates endostatin which inhibits VEGF-induced EC migration and promotes EC apoptosis (Dixelius et al. 2000, Rehn et al. 2001). |
| Vessel maturation/ stabilization | Mobilization of pericytes to cover EC | MMP-9 plays a crucial role in the recruitment of bone marrow-derived cells and vessel coverage by pericytes (Jodele et al. 2005). |

**Table 16.1** (continued)

| Steps of angiogenesis | Mechanisms | Representative examples |
|---|---|---|
| | | Pericyte-derived TIMP-2 inhibits MT1-MMP dependent activation of EC (Anand-Apte et al. 1997, Lafleur et al. 2001). |
| Vasculogenesis | Recruitment of hematopoetic/ endothelial precursor cells (EPCs) from the bone marrow | MMP-9 recruits EPCs from the vascular niche to the proliferation compartment in the bone marrow via the release of soluble c-kit ligand (Heissig et al. 2002). |

(Egeblad and Werb 2002, Overall and Lopez-Otin 2002, Handsley and Edwards 2005, Overall and Kleifeld 2006). General descriptions of *in vitro* and *in vivo* models of angiogenesis are also available (Desbaillets et al. 2000, Auerbach et al. 2003, Norrby 2006, Wartenberg et al. 2006, Noel et al. 2007b). The models of angiogenesis used for MMP investigation and presented here include cell migration, endothelial tube formation, and aortic ring assays (for the *in vitro* assays) and chick chorioallantoic membrane (CAM), Matrigel plug assay, and zebrafish (for the *in vivo* assays). We will critically present their advantages, limitations, and interests in evaluating MMP implication in the angiogenic process. Since lymphangiogenesis (i.e., the formation of new lymphatic vessels) is emerging as an important process contributing to metastatic dissemination, a brief section will focus on *in vitro* and *in vivo* models of lymphangiogenesis.

## Relevance of Models of Angiogenesis

It is obvious that no single model is able to elucidate the entire process of angiogenesis. Because of the complexity of the cellular and molecular mechanisms underlying the angiogenic reaction, *in vivo* studies are more informative and more relevant than *in vitro* investigations. However, *in vivo* assays are time-consuming, expensive, and the implication of inflammatory reactions in these systems renders complex the interpretation of the cellular and molecular mechanisms. *In vitro* studies allowing defined experimental conditions are therefore a necessary complement to the *in vivo* experiments. An “ideal” model of angiogenesis should fulfill several requirements. It should (1) be easy, rapid to use, reproducible, and reliable; (2) identify which EC function is affected by the experimental condition (cell proliferation, migration, invasion, survival); (3) provide a quantitative measure of the vasculature and its complexity (number, length and surface of vessels, number of branchings); and (4) give information on the functional characteristic of the new vasculature (permeability, blood flow) and its level of maturation/stabilization (coverage with perivascular cells, regression in the absence of angiogenic stimuli). Any response seen *in vitro* should be confirmed *in vivo*. Unfortunately, no single

assay can fulfill all these criteria and a panel of complementary models is required to address this issue.

## Cell Invasion Assay

Both macrovascular (human umbilical vascular endothelial cells) and microvascular endothelial cells (HDMEC or human dermal microvascular endothelial cells, BAEC or bovine aortic endothelial cells, PAEC or porcine aortic endothelial cells, HBMEC or human brain microvascular endothelial cells) are used in vitro (McLaughlin et al. 2006, Albini and Benelli 2007). There is also a batch of commercial microvascular EC isolated from various organs (aorta, coronary artery, dermis, lung, bladder, pulmonary artery, saphenous vein, lymphatic origin).

Among the tests that have been used for evaluating the migrative properties of a specific cell population in response to several factors is the chemoinvasion chambers (so-called Boyden chambers) (Albini and Benelli 2007). Cells are seeded on the top of a cell-permeable filter coated with a matrix component (collagen, fibronectin, laminin) or a reconstituted matrix such as Matrigel [a crude extract of Engelbreth-Holm-Swarm (EHS) tumors mainly composed of laminin (Norrby 2006)]. Chemoattractant is added in the culture medium below the filter to promote cell migration. Measurements are carried out by counting cells that have migrated on the lower side of the filter. This assay is largely used to study the migrative properties of endothelial cells transfected or not transfected with the MMP of interest. Natural or synthetic inhibitors of MMPs such as galardin (Roeb et al. 2005), depsipeptide (Klisovic et al. 2005), marimastat (Wagner et al. 1998), IP6 (inositol hexaphosphate) (Tantivejkul et al. 2003) and short-chain fatty acids (Emenaker and Basson 1998) have shown an antimigrative effect in this model. Recently, MT1-MMP expressed by several head and neck squamous cell carcinoma cell lines has been shown to be required for the processing and the release of semaphorin 4D into its soluble form from these cells, thereby inducing endothelial cell chemotaxis *in vitro* (Basile et al. 2007). Such *in vitro* system has the following advantages: (i) defined experimental conditions can be achieved, (ii) the EC population is relatively uniform, (iii) the function of individual genes or protein can be addressed, and (iv) the quantification is easy. However, this model reflects only the migrative properties of EC and/or chemotactic response of EC to specific attractants.

## Tube Formation Assay

A reliable test to investigate EC morphogenesis is based on the ability of endothelial cells to form three-dimensional (3D) structures (tube formation) on an appropriate ECM environment (Madri et al. 1988). *In vitro* EC organization into tube-like structures, also called capillaries, has been studied for decades on 2D-coated plates or on 3D gels (Davis et al. 2002). In the initial assay established by Montesano et al. (1992), EC are seeded as a monolayer onto the surface of collagen or fibrin gels, and

some EC invade the matrix to form tube structures. In more recent assays, EC are suspended as single cell in 3D-matrix (Davis et al. 2002). Alternatively, HUVEC cell monolayer can be seeded between two layers of collagen (Deroanne et al. 2001). In fact, the most widely used matrix is Matrigel. Although tube formation assay on Matrigel has gained a proeminent place in the angiogenesis field, it is worth noting that some cultured cells of nonendothelial origin such as fibroblasts may also respond to Matrigel by forming tube-like structures (Noel et al. 1991). One critical concern when using Matrigel is to standardize the protein concentration that may lead to discrepancy in the results generated. Furthermore, a strong word of caution is that these tube formation assays, by creating a *de novo* vascular-like network from isolated EC or EC monolayer, do not mimic the sprouting process of angiogenesis but rather mimic vasculogenesis (Davis et al. 2002). In addition, one crucial limitation is the lack of a standardized quantification method, measurements being often made manually.

The advantages of these "vasculogenic assays" are to offer the possibility to investigate the mechanisms underlying EC morphogenesis, lumen formation, and tube stabilization or regression. In this context, matrix-integrin-cytoskeletal signaling appears as a major pathway (Davis et al. 2002). EC tubulogenesis is sensitive to TIMP-2 and TIMP-4, but not to TIMP-1 (Lafleur et al. 2002, Davis and Saunders 2006). Inhibition of MMP-9 reduced tube formation (Jadhav et al. 2004). MT1-MMP can act as a fibrinolysin and promote capillary formation in a fibrin gel (Hiraoka et al. 1998). Overexpression of MT1-, MT2-, or MT3-MMP, but not MT4-MMP, enhances the fibrin-invasive activity of EC (Hotary et al. 2002, Plaisier et al. 2004). MT1-MMP also colocalizes with NO synthase in migratory endothelial cells, and thus appears to be a key molecular effector of NO during EC migration (Genis et al. 2007). While some MMPs such as MT-MMPs can stimulate tube formation (Jeong et al. 1999, Davis and Saunders 2006), others regulate tube regression (e.g., MMP-1, MMP-10, and MMP-13) in 3D collagen matrices (Davis et al. 2001, Bayless and Davis 2003). Interestingly, when pericytes are added to EC during the regression phase, they strongly inhibit MMP-1 and MMP-10-dependent regression (Saunders et al. 2006). In this model, EC-derived TIMP-2 and pericyte-derived TIMP-3 are responsible, in concert, for tube regression (Saunders et al. 2006). Although TIMP-1 does not affect tube formation, it strongly inhibits tube regression (Davis and Saunders 2006). Altogether, these observations led to the concept that distinct MMPs primarly act as promorphogenic (i.e., tube formation) or proregression agents (Davis et al. 2002, Davis and Senger 2005, Handsley and Edwards 2005, Saunders et al. 2005).

## Aortic Ring Assay

Since angiogenesis involves not only EC but also perivascular cells, an *ex vivo* vascular tissue culture method has been developed (Nicosia and Ottinetti 1990). When aortic fragments isolated either from rat or from mice are cultured in a type-I collagen gel, they spontaneously give rise to a microvascular network within 7–9 days. Microvessels originated mostly from the two wounded edges of aortic

fragments, with only a few growing out from the intimal zone (Villaschi and Nicosia 1993). Growth factors or inhibitors can be added onto the medium in order to evaluate their pro- or antiangiogenic impact. Previously, quantification used to be done manually by blinded observers. Nowadays, quantification is often performed by computer-assisted methods (Masson 2002). Main parameters measured are the length, the number of vessels, and their branchings. Perivascular cells which do not associate with the forming vascular network and their distribution around the aortic explant can also be quantified (Blacher et al. 2001).

This model has gained broad acceptance (Masson 2002) since it bridges the gap between *in vitro* and *in vivo* models. The aortic ring assay mimics the sprouting of EC from a preexisting vessel and takes into account the importance of perivascular cells (Zhu and Nicosia 2002, Li et al. 2005). In addition, in this system, EC are not preselected by passaging and thus are not in a proliferative state (Auerbach et al. 2003). Other advantages are (i) the possibility of generating many assays per animal, (ii) the lack of inflammatory complications to unravel molecular mechanisms of angiogenesis, and (iii) the unique opportunity to exploit the recent generation of MMP-deficient mice. However, one point of caution must be paid to the variability of the angiogenic responses between different mice strains and aging (Burbridge et al. 2002, Zhu et al. 2003).

In this model, MMP expression levels increased gradually during the angiogenic growth phase and remained high when vessels regressed and collagen is lysed around the aortic rings. The profile of MMP expression is modulated by both matrix composition and exogenous addition of growth factors. For example, while MMP-2 and MMP-3 are present in large amount in fibrin cultures, MMP-11 and MT1-MMP are more highly expressed during vessel formation in collagen gels. The angiogenic bFGF (basic fibroblast growth factor) upregulates the expression of MMP-2, MMP-3, MMP-9, MMP-10, MMP-11, and MMP-13 (Burbridge et al. 2002). Synthetic MMP inhibitors such as Ro-28-2653 (Maquoi et al. 2004), batimastat, and marimastat (Zhu et al. 2000, Burbridge et al. 2002) block the formation of microvessels when added in the culture medium at the beginning of the experiment (Zhu et al. 2000). However, batimastat and marimastat stabilized the microvessels and prevented vascular regression after the angiogenic growth phase. MMPs are thus implicated in the microvascular outgrowth phase, in the regression process as well as in the degradation of the neovasculature in latter stages (Zhu et al. 2000).

The aortic ring assay has been recently applied to different MMP-deficient mice (Masson et al. 2002). MMP-11 and MMP-19 are not required for EC spreading out from the aortic rings (Masson et al. 2002, Pendas et al. 2004). Similarly, a single or combined lack of MMP-2 and MMP-9 does not impair the *in vitro* capillary outgrowth from aortic rings (Masson et al. 2005). In sharp contrast, aortic explants isolated from MT1-MMP-null mice display defective capillary sprouting in collagen gels compared with wild-type counterparts. However, wild-type and MT1-MMP-null explants display comparable neovessel outgrowth when embedded in a 3D-gel of cross-linked fibrin, revealing matrix-dependent effect (Chun et al. 2004). MT1-MMP may contribute to the angiogenic process through different mechanisms including at least ECM remodeling and processing of cell-surface molecules

(Sounni et al. 2003, Handsley and Edwards 2005, van Hinsbergh et al. 2006). Indirect effects of MT1-MMP on angiogenesis can also rely on the enhancement of VEGF gene expression by tumor cells (Deryugina et al. 2002, Sounni et al. 2002). Indeed, conditioned media of MT1-MMP overexpressing MCF-7 clones upregulates microvessel outgrowth from aortic rings and this effect can be abrogated by blocking VEGF (Sounni et al. 2002). Therefore, among different individual MMPs investigated in the aortic ring assay, MT1-MMP appears as a key regulator of angiogenic sprouting.

## The CAM Assay

The CAM assay was set up by Folkman et al. in 1974 (Auerbach et al. 1974, Ausprunk et al. 1974). First used by embryologists, it has been transposed for the study of tumor angiogenesis and the screening of anti- or proangiogenic factors. CAM are highly vascularized membranes whose EC display morphological characteristics of immature and undifferentiated cells with a high mitotic rate until day 10 of development (Ausprunk et al. 1975). The angiogenic process can be divided into three phases. In the early phase (Day 5–7), the majority of the angiogenic process is achieved by sprouting. The intermediate phase (Day 8–12) is characterized by an intussusception growth process that replaces the sprouts: intussusception involves the formation of transluminal pillars that expand and modify vessel form and function (Patan et al. 1992, Schlatter et al. 1997). By Day 12 or 13, the chorioallantois encircles the entire shell membrane and its expansion is complete (Ausprunk et al. 1974). The CAM assay is carried out *in ovo* by placing growth factors directly onto the CAM through an opening in the eggshell. Test molecules are prepared in carriers (such as slow-release polymer pellets, gelatin sponges, or air-dried on plastic discs). The quantification of angiogenesis is made after 3–4 days of engraftment. Recently, Blacher et al. (2005) have reported an accurate method for assessment of microvascular parameters.

Advantages of the CAM are that it is technically very simple and inexpensive and thus suitable for large scale screening within a short response period (2–3 days). A major limitation of this assay is the standardization of the method used to apply the compound to be tested. In addition, attention should be paid not to misinterpret the de novo angiogenic process since molecules of interest are placed onto preexisting vessels that could appear artifactually to be increased following contraction of the membrane (Ribatti and Vacca 1999) and thus lead to difficulty in discrimination between new capillaries and already existing ones. As the immune system of the CAM is not fully developed, this model also allows the study of tumor-induced angiogenesis by tumor engraftment and subsequent metastasis to chick organs (Auerbach et al. 1976, Ausprunk et al. 1975, Gordon and Quigley 1986, Hagedorn et al. 2005, Zijlstra et al. 2006).

The CAM has been helpful to investigate tumor-derived MMPs. Application of this model to MMP-tumor-secreted studies had been extensively reported (Baum

et al. 2007, Schneiderhan et al. 2007). A model of 3D collagen engraftment on the CAM has been developed to analyze spatial and temporal associations *in vivo* between inflammatory cell-derived MMPs and the angiogenesis induced by tumor cells. The onset of angiogenesis is critically dependent on the stromal collagenase MMP13 (chMMP-13), supplied mainly by a hematopoietic lineage (monocytes-macrophages). Initiation of HT1080 cell-induced angiogenesis onto the CAM is dependent on an initial influx of MMP9-containing heterophils (avian counterparts of mammalian neutrophils) followed by an accumulation of chMMP-9 protein in the collagen engraftment and the later arrival of monocytes/macrophages (Zijlstra et al. 2004). Accordingly, disruption of this inflammatory cell influx by anti-inflammmatory drugs significantly reduced angiogenesis. This indicates a possible role of inflammatory cells in the CAM angiogenic process (Zijlstra et al. 2006).

The CAM is also suitable for studying intravasation, a critical step of the metastatic process. MMP-9 expression in human cell lines including HT-1080 cells correlates with the ability of human cells to intravasate and a synthetic inhibitor (marimastat) inhibits significantly tumor cell intravasation and metastasis (Kim et al. 1998). However, MMP-9 downregulation by siRNA in HT-1080 cells showed an unexpected two- to threefold increase in levels of intravasation and metastasis, while intravasation was sensitive to a broad-range MMP inhibitor (Deryugina et al. 2005).

## The Matrigel Plug Assay

Matrigel supplemented with either cells or angiogenic molecules (bFGF, VEGF) is injected subcutaneously into mice and allowed to solidify where it forms a plug (Akhtar et al. 2002). This plug can be removed after 7–21 days from the animal and examined histologically to determine the blood vessel infiltration. Plugs can also be quantified for their hemoglobin contents (Passaniti et al. 1992) or fluorescein measurements of plasma volume can be assessed using FITC-dextran (Johns et al. 1996). The Matrigel plug assay has been modified to permit a clear delineation of the neovascularization zone. In this sponge/Matrigel plug assay, Matrigel is first injected alone into the mouse followed by an insertion of a tissue fragment or a sponge into the plug. Measurements of new vessels are then achieved by FITC-dextran injection (Akhtar et al. 2002).

Although this *in vivo* model does not require any surgical procedure and is easy to administer, it suffers from several drawbacks. First, the histological quantification on sections is quite tedious (Passaniti et al. 1992). Second, it is somewhat an artificial model because Matrigel is a reconstituted matrix, not chemically defined and which contains a large variety of growth factors that can influence results. In more recent studies, growth factor-depleted Matrigel is used (Norrby 2006). Finally, it is subject to considerable variability because of the difficulty to obtain similar 3D plugs, even though total Matrigel volume is kept constant (Auerbach et al. 2000). To overcome this problem, a modification can be introduced in the assay in

mice and rats using subcutaneous chambers that allow constant 3D form and volume of the Matrigel plug which increases reproducibility (Kragh et al. 2003, Ley et al. 2004). To minimize the amount of Matrigel used, angioreactors have been set up, which consist of semiclosed silicone cylinders that are implanted subcutaneously into nude mice (Guedez et al. 2003). The Matrigel plug assay is viewed as a valuable *in vivo* model for the rapid screening of potential pro- and antiangiogenic agents. In this assay, the oral administration of an MMP inhibitor (BAY12-9566) inhibits FGF-induced angiogenesis. Injection of MMP-9 antisense in mice also decreases EC migration and Matrigel vascularization (London et al. 2003). Surprisingly, angiogenesis in Matrigel plugs is increased rather than decreased in MMP-$19^{-/-}$ mice (Jost et al. 2006). This observation further supports the emerging opposite effects of various MMPs during the process of angiogenesis, some being proangiogenic agents (e.g., MMP-9) and other acting as negative regulators (e.g., MMP-19) of angiogenesis. Chantrain et al. (2004) have adapted the Matrigel plug assay by incorporating tumor cells into the matrix. A significant inhibition of angiogenesis is then observed in immunodeficient RAG1/MMP-9 double-deficient mice orthotopically implanted with a mixture of neuroblastoma cells and Matrigel. In this system, stromal-derived MMP-9 contributes to angiogenesis by promoting blood vessel morphogenesis and pericyte recruitment (Chantrain et al. 2004). Altogether, these data in accordance with previous ones (Coussens et al. 2000) have underlined the key contribution of MMP-9 in angiogenesis and in the mobilization of bone marrow-derived cells.

## Zebrafish

In 1999, zebrafish was depicted as a whole animal model for screening drugs that affect the angiogenic process (Serbedzija et al. 1999). These tropical freshwater fish have a short generation time (∼3 months) and can be housed in large numbers and in a small space. A striking organ similarity is observed between zebrafish and mammals at the anatomical, physiological and molecular levels despite their phylogenetic lineage differences (more than 400 million years) (Ny et al. 2006a). The optical transparency of embryos makes them easy to study for diverse developmental processes, from gastrulation to organogenesis. Small test molecules are directly added to the water and diffuse into the embryos. Anti- and proangiogenic molecules already tested in mammals have been shown to exert similar effects in the zebrafish (Norrby 2006). One major advantage is the use of fluorescent labels that can stain a single cell population (e.g., endothelial cells). This assay is useful for embryonic and organogenic angiogenesis. A very large catalog of genetic tools is now available to act on the zebrafish genome. A strong way to understand molecular events in angiogenesis or vasculogenesis is based on the morpholino (MO) knock-down technology which permits reverse genetic analysis of gene function (Ober et al. 2004, Chen et al. 2005, Kajimura et al. 2006).

This model has recently enlightened the evolutionary pattern of the metzincin family. In the zebrafish genome, 83 metzincin genes have been identified. Further

phylogenetic analyses reveal that the expansion of the metzincin gene superfamily in vertebrates has occurred predominantly by the simple duplication of preexisting genes rather than the appearance and subsequent expansion of new metzincin subtypes. Evolution of the related TIMP gene family identifies four zebrafish TIMP genes (Huxley-Jones et al. 2007). A study conducted on zebrafish MMP-9 and its developmental expression pattern suggests that zMMP-9 serves as a useful marker of mature myeloid cells (Yoong et al. 2007). The role of MMP-2 during embryogenesis is assessed by an *in situ* analysis showing zMMP2 expression at one-cell stage until 72 h stage of development (Zhang et al. 2003b). Injection of zMMP2 antisense MO oligonucleotides into the embryo resulted in a truncated axis, indicating that this MMP plays an important role in zebrafish embryogenesis (Zhang et al. 2003). In contrast, knockout studies indicate that MMP-2 does not play a key role in mouse embryogenesis (Itoh et al. 1998). Concerning the MT-MMPs, two isoforms isolated from the zebrafish are structurally similar to MT1-MMP (named zebrafish MT-MMP alpha and beta). These two metalloproteinases are expressed through at least the first 72 h of development and this expression is triggered at the cell surface (Zhang et al. 2003c). In addition, TIMP-2 appears to be required for the normal development of zebrafish embryos (Zhang et al. 2003a). By employing flurorescent MMP substrates, an *in vivo* model of zymography has been developed. MMP activity is primarily depicted in ECM-rich structures predicted to undergo active remodeling, such as the pericordal sheath and somite boundaries (Crawford and Pilgrim 2005).

## Lymphangiogenesis Models

Only a few lymphatic culture systems have been developed. Initial attempts used 2D cultures of human dermal lymphatic cells isolated either by immunopurification with fluorescence-activated cell sorting (FACS) or by magnetic beads (Davison et al. 1980, Kriehuber et al. 2001). Lymphatic EC can be isolated from the thoracic duct of different species (rat, mouse, dog, cow) by enzymatic digestion (Gnepp and Chandler 1985, Pepper et al. 1994, Tan 1998, Mizuno et al. 2003). They can also be generated by culturing cells induced by the intraperitoneal injection of incomplete Freund's adjuvant into mice (Gnepp and Chandler 1985, Tan 1998, Pepper et al. 1994, Mizuno et al. 2003). Isolated EC have been immortalized with human telomerase reverse transcriptase (hTERT-HDLEC) (Nisato et al. 2004). Furthermore, lymphatic EC differentiation can be induced in embryoid bodies (Liersch et al. 2006). These culture systems suffer from limitations that include (1) the limited number of cells that can be obtained by isolating nontransformed cells, (2) the nonphysiological features of immortalized cells, and (3) the putative dedifferentiation of cells in 2D cultures (Tammela et al. 2005). In addition, none of these culture systems adequately represent the 3D growth of lymphatic microvessels with a lumen. The adaptation of the aortic ring assay to the lymphatic ring assay using the thoracic duct issued from rat led to disappointing results, generating two types of tube-like structures

(LLC or lymphatic-like channels and HLC or hematic-like channels) (Nicosia 1987). The recent setting up of 3D-lymphatic ring cultures from mouse thoracic duct overcomes the main obstacle of the 2D systems and offers the possibility to exploit the panel of MMP-deficient mice recently generated (Bruyère et al. 2008).

Several *in vivo* models of lymphangiogenesis have been developed and mainly consist in the induction of tumor-associated lymphangiogenesis by VEGF-C overexpression in tumor cells (Skobe et al. 2001, Pepper and Skobe 2003) or in transgenic mice (Mandriota et al. 2001). Furthermore, lymphatic endothelial benign tumors (lymphangioma) are induced by intraperitoneal injection of incomplete Freund's adjuvant (Mancardi et al. 1999, Nakamura et al. 2004). This lymphatic cell hyperplasia is formed by VEGFR-3 and podoplanin-positive lesions growing on the surface of the diaphragm and liver, with leukocyte infiltration (Mancardi et al. 1999, Nakamura et al. 2004). Lymphedema are also induced by the excision of a circumferential band of skin in mouse tail (Rutkowski et al. 2006) or microsurgical ablation of tail lymph vessels (Tabibiazar et al. 2006). The corneal assay permits to study lymphangiogenesis by implanting growth factors containing pellets into corneal micropockets (Cao et al. 2004). In contrast to zebrafish, Xenopus develops a lymphatic system, and therefore, the Xenopus tadpole appears as a new genetic model to investigate lymphangiogenesis (Ny et al. 2006). One of the main limitations of these *in vivo* models to identify key regulators of lymphangiogenesis is the important implication of the inflammatory reaction, which does not allow discriminating between a direct effect on lymphatic endothelial cells and an indirect effect through a modulation of inflammation.

## Comments and Conclusions

No single model can mimic the entire angiogenic process. Major differences are seen between species (including also animal strains, gender), the specific environments (organ, tissue), the stage of development and age (embryonic versus adult including age-related differences) and the mode of administration of molecules of interest. Undoubtedly, there is a hierarchy of complexity between the different models (Fig. 16.1). The simplest *in vitro* ones focus on one single cell type and address specific EC function (e.g., EC proliferation, migration, chemotaxis). More complex systems (aortic ring assay and *in vivo* models) take into account several cell properties and several cell types (EC, pericytes, fibroblasts-like cells, smooth muscle cells). In these systems, the different events occurring during the angiogenic process are regulated sequentially and spatially, and thus better mimic the *in vivo* situation. Since quantification analysis may lead to some discrepancies when it is done manually, (semi)automatic computer-assisted analysis is required to allow a rapid, objective evaluation of pro- and antiangiogenic molecules/genes of interest (Blacher et al. 2001, 2005).

In the 1990s, clinical trials with MMP inhibitors were based on the concept that MMPs are mainly produced by cancer cells and contribute to tumor progression by degrading matrix components. However, it is now widely accepted that different

cell types such as EC, fibroblasts, inflammatory cells and adipocytes are the main source of MMPs. Initially viewed as major regulators of tissue destruction or remodeling, MMPs were expected to regulate angiogenesis by controlling EC migration. Recent studies underlined their key contribution in all steps of angiogenesis including the activation of EC and the promotion of their sprouting, migration, survival, differentiation, coverage by perivascular cells recruited from adjacent tissue or from the bone marrow and even by mediating the regression of tube-like structures in the absence of continuous angiogenic stimuli. MMPs are recognized as modulators of a large panel of molecules which generate new biologically active fragments from the matrix, cell surface-associated proteins and soluble factors (Overall and Lopez-Otin 2002, Handsley and Edwards 2005, Overall and Dean 2006, van Hinsbergh et al. 2006). It became apparent that MMPs have multiple functions, sometime opposite ones. Therefore, a better understanding of their mechanisms of action at different steps of the angiogenic, vasculogenic and lymphangiogenic processes is urgently needed. Instead of directly targeting MMPs, it is possible that substrates and products of MMPs will be preferred targets for treating angiogenesis-related disease. The success of such applications depends on knowledge of how proteases are acting in different contexts and require a panel of complementary models of angiogenesis.

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