

# Principles and Therapeutic Implications of Angiogenesis, Vasculogenesis and Arteriogenesis

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**Abstract** The vasculature is the first organ to arise during development. Blood vessels run through virtually every organ in the body (except the avascular cornea and the cartilage), assuring metabolic homeostasis by supplying oxygen and nutrients and removing waste products. Not surprisingly therefore, vessels are critical for organ growth in the embryo and for repair of wounded tissue in the adult. Notably, however, an imbalance in angiogenesis (the growth of blood vessels) contributes to the pathogenesis of numerous malignant, inflammatory, ischaemic, infectious and immune disorders. During the last two decades, an explosive interest in angiogenesis research has generated the necessary insights to develop

the first clinically approved anti-angiogenic agents for cancer and blindness. This novel treatment is likely to change the face of medicine in the next decade, as over 500 million people worldwide are estimated to benefit from pro- or anti-angiogenesis treatment. In this following chapter, we discuss general key angiogenic mechanisms in health and disease, and highlight recent developments and perspectives of anti-angiogenic therapeutic strategies.

**Keywords** Angiogenesis · Vasculogenesis · Arteriogenesis · Angiogenic disorders · Vessel growth · Endothelial progenitors · Haematopoietic progenitors · Guided navigation · Anti-angiogenic therapy

## 1

### Angiogenic Disorders

After birth, angiogenesis still contributes to organ growth, but during adulthood most blood vessels remain quiescent; angiogenesis only occurs in the cycling ovary and placenta during pregnancy. However, endothelial cells (ECs) retain the remarkable ability of dividing rapidly in response to a physiological stimulus, such as hypoxia and inflammation. Angiogenesis is also reactivated during wound healing and repair. In many disorders, however, this stimulus becomes excessive, and the balance between stimulators and inhibitors is disturbed, resulting in an angiogenic switch. The best-known conditions in which angiogenesis is switched on are malignant, ocular and inflammatory disorders, but many additional processes are affected—such as atherosclerosis, asthma, diabetes, cirrhosis, multiple sclerosis, endometriosis, acquired immunodeficiency syndrome (AIDS), bacterial infections and autoimmune diseases (Table 1). In obesity, adipose tissue may also show excessive growth. A high-fat diet induces an angiogenic gene programme in fat (Li et al. 2002) and angiogenic factors stimulate adipogenesis, while treatment of obese mice with anti-angiogenic agents results in weight reduction and adipose tissue loss (Rupnick et al. 2002). Viral and bacterial pathogens carry angiogenic genes of their own (Meyer et al. 1999), or induce the expression of angiogenic genes in the host (Harada et al. 2000). The human herpesvirus-8 transforms ECs and causes Kaposi's sarcoma in human immunodeficiency virus (HIV)-1-infected AIDS patients (Barillari and Ensoli 2002).

In other diseases, such as ischaemic heart disease or pre-eclampsia, the angiogenic switch is insufficient, thereby causing EC dysfunction, vessel malformation and regression, or preventing revascularisation, healing and regeneration (Table 2). In the skin, age-dependent reductions in vessel density and maturation cause vessel fragility, leading to hair loss and the development of purpura, telangiectasia, angioma and venous lake formation (Chang et al. 2002). A progressive loss of the microvasculature in elderly people has been implicated in nephropathy (Kang et al. 2001), bone loss (Martinez et al. 2002) and impaired re-endothelialisation after arterial injury (Gennaro et al. 2003). Diabetes, atherosclerosis and hyperlipidaemia also impair vessel growth

**Table 1** Diseases characterised or caused by abnormal or excessive angiogenesis

Organ	Disease in mice or humans
Numerous organs	Cancer (activation of oncogenes; loss of tumour suppressors) and metastasis; infectious diseases (pathogens that express angiogenic genes <sup>(Meyer et al. 1999)</sup> , induce angiogenic programmes <sup>(Harada et al. 2000)</sup> or transform ECs <sup>(Barillari and Enzoli 2002; Wang et al. 2004)</sup> ); vasculitis and angiogenesis in auto-immune diseases such as systemic sclerosis, multiple sclerosis and Sjögren's syndrome <sup>(Kirk and Karlik 2003; Ohno et al. 2004; Storkebaum et al. 2004)</sup>
Vasculature	Vascular malformations (Tie-2 mutation <sup>(Vikkula et al. 1996)</sup> ); DiGeorge syndrome (low VEGF/Nrp-1 expression, <sup>(Stalmans et al. 2003)</sup> ); hereditary haemorrhagic telangiectasia (mutation of endoglin or ALK <sup>(van den Driesche et al. 2003; Lebrin et al. 2005)</sup> ); cavernous haemangioma (loss of Cx37/40 <sup>(Simon and McWhorter 2002)</sup> ); cutaneous haemangioma (VG5Q mutation <sup>(Lambrechts and Carmeliet 2004; Tian et al. 2004)</sup> ); transplant arteriopathy and atherosclerosis <sup>(Kahlon et al. 1992; Khurano et al. 2005; Nakano et al. 2005)</sup>
Skin	Psoriasis (high VEGF and Tie2 <sup>(Xia et al. 2003; Leong et al. 2005; Voskas et al. 2005)</sup> ); warts <sup>(Harada et al. 2000)</sup> ; allergic dermatitis (high VEGF and PlGF <sup>(Oura et al. 2003; Agha-Majzoub et al. 2005)</sup> ); scar keloids <sup>(Yang et al. 2003; Gira et al. 2004)</sup> ; pyogenic granulomas; blistering disease <sup>(Brown et al. 1995)</sup> ; Kaposi's sarcoma in AIDS patients <sup>(Barillari and Enzoli 2002)</sup> ; systemic sclerosis <sup>(Distler et al. 2004)</sup>
Adipose tissue	Obesity (angiogenesis induced by fat diet); weight loss by angiogenesis inhibitors; anti-VEGFR2 inhibits preadipocyte differentiation via effects on ECs <sup>(Fukumura et al. 2003)</sup> ; adipocytokines stimulate angiogenesis <sup>(Shibata et al. 2004)</sup>
Eye	Persistent hyperplastic vitreous syndrome (loss of Ang-2 <sup>(Hackett et al. 2000; Gale et al. 2003)</sup> or VEGF <sup>164</sup> <sup>(Stalmans et al. 2002)</sup> ); diabetic retinopathy; retinopathy of prematurity <sup>(Campochiaro 2004)</sup> ; choroidal neovascularisation <sup>(Campochiaro 2004)</sup> (TIMP-3 mutation <sup>(Qi et al. 2003)</sup> )
Bone, joints	Arthritis and synovitis <sup>(Arima et al. 2005; Lainer and Brahn 2005; Szekanecz et al. 2005; Taylor and Sivakumar 2005)</sup> ; osteomyelitis <sup>(Hausman and Rinker 2004)</sup> ; osteophyte formation <sup>(Luttun et al. 2002)</sup> ; HIV-induced bone marrow angiogenesis <sup>(Patsouris et al. 2004)</sup>

**Table 1** (continued)

Organ	Disease in mice or humans
Lung	Primary pulmonary hypertension (BMPR-2 mutation; somatic EC mutations (Yeager et al. 2001; Humbert and Trembath 2002; Voelkel et al. 2002)); asthma (Bai and Knight 2005), nasal polyps (Gosepath et al. 2005); rhinitis (Kirmaz et al. 2004); chronic airway inflammation (Baluk et al. 2005), cystic fibrosis (Shute et al. 2003)
Gastro-intestinal tract	Inflammatory bowel disease (ulcerative colitis (Konno et al. 2004)); liver cirrhosis (Ward et al. 2004; Fernandez et al. 2005; Medina et al. 2005)
Reproductive system	Endometriosis (Hull et al. 2003; Groothuis et al. 2005); uterine bleeding, ovarian cysts (Abd el Aal et al. 2005); ovarian hyperstimulation (LeCouter et al. 2001)
Kidney	Diabetic nephropathy (Yamamoto et al. 2004; Schrijvers et al. 2005)

BMPR-2, bone morphogenic protein-2

(Van Belle et al. 1997; Waltenberger 2001; Tepper et al. 2002), whereas hypertension causes microvascular rarefaction (Boudier 1999). Reduced angiogenic signalling causes pulmonary fibrosis (Koyama et al. 2002) and emphysema (Kasahara et al. 2000). The delayed healing of gastric or aphthous oral ulcerations has been attributed to the ability of invading pathogens to produce angiogenesis inhibitors-in particular after *Helicobacter pylori* infections (Jenkinson et al. 2002). Besides its vascular activity, vascular endothelial growth factor (VEGF) is also trophic for nerve cells, cardiac muscle fibres and lung epithelial cells, further explaining why insufficient VEGF levels contribute to cardiac failure, respiratory distress and motor neuron degeneration, reminiscent of amyotrophic lateral sclerosis (Table 2). At present, angiogenesis has been implicated in more than 70 disorders, and the list is ever-growing.

## 2

### Modes of Vessel Growth

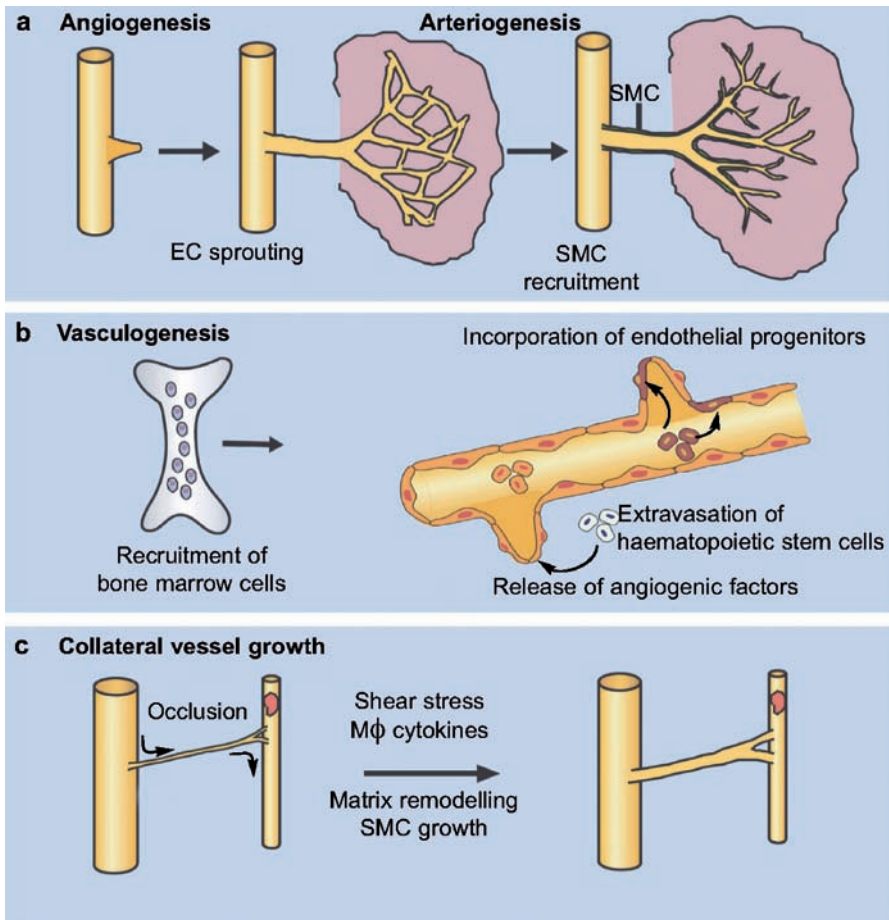
In the developing embryo as well as in adult tissues, key events and distinct mechanisms exist to establish and maintain a functional vascular network (Fig. 1). Endothelial progenitor cells (EPCs) arising from various embryonic regions or from adult bone marrow can form vessels in a process referred to as vasculogenesis. Angiogenesis denotes the process in which budding from pre-existing vessels gives rise to sprouts of new blood vessels, while arteriogenesis refers to the stabilisation of these new sprouts by mural cells such as pericytes and smooth muscle cells (SMCs)-arteriogenesis is critical for the new vasculature to become stable, mature and functional. Collateral vessel growth repre-

**Table 2** Diseases characterised or caused by insufficient angiogenesis or vessel regression

Organ	Disease in mice or humans	Angiogenic mechanism
Nervous system	Alzheimer's disease	Vasoconstriction, microvascular degeneration and cerebral angiopathy due to EC toxicity by amyloid- $\beta$ (de la Torre 2004; Zlokovic 2005)
	Amyotrophic lateral sclerosis; diabetic neuropathy	Impaired perfusion and neuroprotection, causing motoneurone or axon degeneration due to insufficient VEGF production (Oosthuysen et al. 2001; Lambrechts et al. 2003; Azzouz et al. 2004; Storkebaum and Carmeliet 2004; Storkebaum et al. 2005)
Vasculature	Stroke	Correlation of survival with angiogenesis in brain (Krupinski et al. 1994); stroke due to arteriopathy (Notch-3 mutations (Kalimo et al. 2002))
	Diabetes	Characterised by impaired collateral growth (Waltenberger 2001) and angiogenesis in ischaemic limbs (Rivard et al. 1999), but enhanced retinal neovascularisation secondary to pericyte drop-out (Caldwell et al. 2005)
	Hypertension	Microvessel rarefaction due to impaired vasodilatation or angiogenesis (Boudier 1999; Kubis et al. 2002; Sane et al. 2004)
	Atherosclerosis	Characterised by impaired collateral vessel development (Van Belle et al. 1997)
Heart	Restenosis	Impaired re-endothelialisation after arterial injury (Gennaro et al. 2003)
	Ischaemic heart disease, cardiac failure	Imbalance in capillary-to-cardiomyocyte fibre ratio due to reduced VEGF levels (Jesmin et al. 2005; Shiojima et al. 2005)
Gastro-intestinal tract	Gastric or oral ulcerations	Delayed healing due to production of angiogenesis inhibitors by pathogens ( <i>H. pylori</i> ) (Jenkinson et al. 2002; Kim et al. 2004)
	Crohn's disease	Characterised by mucosal ischaemia (Konno et al. 2004; Hatoum et al. 2005)

**Table 2** (continued)

Organ	Disease in mice or humans	Angiogenic mechanism
Bone	Osteoporosis, impaired bone fracture healing	Impaired bone formation due to age-dependent decline of VEGF-driven angiogenesis (Martinez et al. 2002); angiogenesis inhibitors prevent fracture healing (Yin et al. 2002); osteoporosis due to low VEGF (Pufe et al. 2003); healing of fracture non-union is impaired by insufficient angiogenesis (Hausman and Rinker 2004)
Skin	Hair loss Skin purpura, telangiectasia, and venous lake formation Systemic sclerosis, lupus Pre-eclampsia	Retarded hair growth by angiogenesis inhibitors (Yano et al. 2001) Age-dependent reduction of vessel number and maturation (SMC drop-out) due to EC telomere shortening (Chang et al. 2002) Insufficient compensatory angiogenic response (Mackiewicz et al. 2002) EC dysfunction, resulting in organ failure, thrombosis and hypertension due to deprivation of VEGF by soluble Flt1 (Maynard et al. 2003; Levine et al. 2004) Fragility of SMC-poor vessels due to low Ang-1 production (Hewett et al. 2002)
Reproductive system		Insufficient lung maturation and surfactant production in premature mice with low HIF-2/VEGF (Compernelle et al. 2002); low VEGF levels in human neonates also correlate with RDS (Tsao et al. 2005)
Lung	Menorrhagia (uterine bleeding) Neonatal respiratory distress syndrome (RDS) Pulmonary fibrosis, emphysema	Alveolar EC apoptosis upon VEGF inhibition (Kasahara et al. 2000; Tang et al. 2004b; McGrath-Morrow et al. 2005)
Kidney	Nephropathy (ageing; metabolic syndrome); glomerulosclerosis; tubulointerstitial fibrosis	Characterised by vessel dropout, microvasculopathy and EC dysfunction (low VEGF; high TSP1) (Kang et al. 2001; Gealekman et al. 2004; Long et al. 2005); recovery of glomerular/peritubular ECs in glomerulonephritis, thrombotic microangiopathy and nephrotoxicity is VEGF-dependent (Schrijvers et al. 2004)



**Fig. 1 a–c** Mechanism of vessel growth. **a** Angiogenesis denotes the sprouting of new endothelial cell-lined vessels from pre-existing vessels; arteriogenesis refers to the subsequent stabilisation of nascent vessels via recruitment of smooth muscle cells. **b** Vasculogenesis refers to the recruitment of bone marrow-derived endothelial progenitors, which are incorporated into nascent vessels or stimulate new vessel growth by releasing pro-angiogenic factors. **c** Collateral vessel growth denotes the expansive growth of pre-existing collateral vessels upon occlusion of a supply vessel, for instance by a thrombus. Recruitment of macrophages and monocytes to the shear stress-activated endothelium plays a critical role in this process

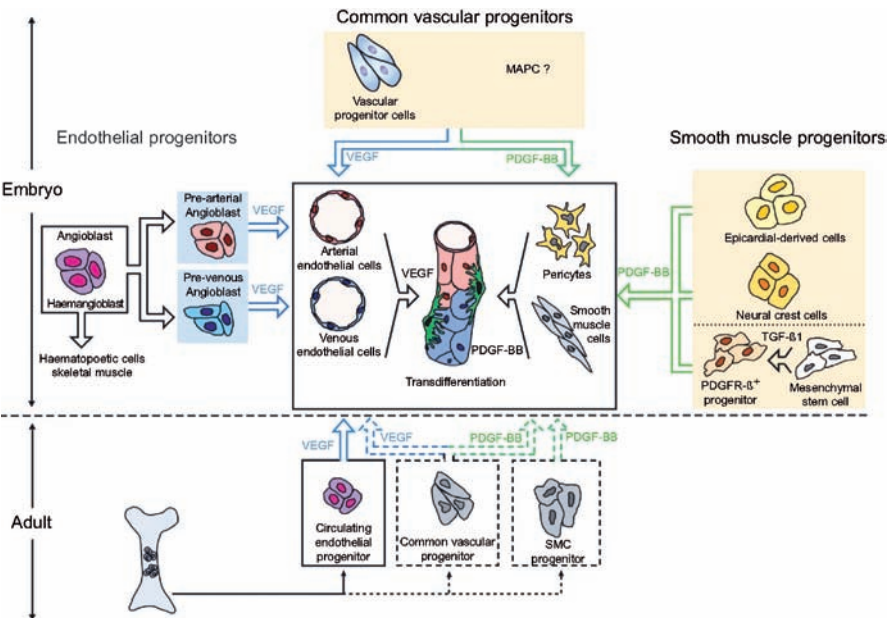
sents the formation of collateral bridges between arterial networks and remodelling of pre-existing vessels after occlusion of a main artery—this type of vessel growth is of major therapeutic importance. A fine-tuned interplay between molecular signals in a spatial and temporal manner is necessary for these essential events to occur. We will now discuss these individual steps in more detail.

### 3 Vasculogenesis

Vasculogenesis has now been documented in the embryo and adult. In the embryo, mesoderm-derived EPCs give rise to the first embryonic blood vessels. In the adult, EPCs originating from the bone marrow enter the circulation and are recruited to sites of neovascularisation. These two different events will be discussed separately in this chapter. In addition, we will discuss the exciting recent insights that haematopoietic progenitors also contribute to the formation of new vessels in the embryo and adult.

#### 3.1 Endothelial Progenitors in the Embryo

In the yet-avascular embryo, blood vessels emerge through recruitment of separate mesodermal precursors at distinct locations in the mesoderm (Fig. 2). In amniotes in particular, the first blood vessels arise in the extra-embryonic



**Fig. 2** Vascular progenitors in the embryo and adult. Endothelial, smooth muscle and common vascular progenitors contribute to vascular development in the embryo. Smooth muscle cells have different origins, as indicated. Recently, endothelial and smooth muscle progenitors, derived from the bone marrow, have also been demonstrated in the adult. The effect of VEGF, platelet-derived growth factor (PDGF)-BB and other growth factors on these progenitors is indicated



mesoderm of the yolk sac, when mesenchymal cells aggregate to form blood islands. The inner cells form primitive blood cells, while the outer boundaries give rise to precursors for ECs. Vascular progenitors that contribute to the formation of the major embryonic vascular system also derive from the intra-embryonic mesoderm, and differentiate to form the dorsal aorta, cardinal veins and vitelline plexus. Within the embryo, the different mesodermal compartments vary in their vasculogenic capacity; the splanchnopleural and the paraxial mesoderm are the richest in endothelial precursor cells. Grafting experiments in quail and chick embryos suggest the existence of two distinct lineages of EPCs (Pardanaud et al. 1996). A first lineage, derived from the paraxial mesoderm, is known to have solely angioblastic capacity. A second bipotential haemangioblastic precursor cell line is derived from the splanchnopleural mesoderm, and differentiates to both endothelial and haematopoietic cells. Although no ultimate proof has been provided, the close proximity of differentiating haematopoietic and ECs at sites of both extra- and intra-embryonic vasculogenesis (de Bruijn et al. 2000) suggests the existence of a bipotential mesodermal precursor cell for both systems, the haemangioblast (Choi 2002; Ribatti et al. 2002; Rafii and Lyden 2003).

A common origin for blood and ECs is further suggested by molecular links between the embryonic precursors of the angiogenic and haematopoietic lineages, which share expression of a number of different genes, such as *CD34*, *CD133*, *PECAM-1* (encoding platelet EC adhesion molecule-1), *c-Kit* and *Sca-1*. In addition, in vitro experiments have shown that a transient population of so-called blast colony-forming cells (BL-CFC) can be derived from embryonic stem cell cultures (Kennedy et al. 1997; Nishikawa et al. 1998). BL-CFCs are responsive to VEGF and contain both endothelial and haematopoietic precursors. Strikingly, expression of mesodermal genes precedes the expression of genes marking early stage endothelial and haematopoietic development in these embryonic stem cell lines (Kennedy et al. 1997; Fehling et al. 2003), thus recapitulating the gene expression sequence observed in the yolk sac in vivo. Moreover, isolation of single cells expressing VEGF-receptor 2 (VEGFR-2; also foetal liver kinase 1, Flk-1) that can give rise to both endothelial and haematopoietic cells in vitro strongly suggests the existence of a common progenitor for the two lineages, even though it does not rule out the possibility that this progenitor is actually a more primitive multipotent precursor.

Although the molecular players determining the critical early steps of haemangioblast differentiation in the blood islands are not yet fully elucidated, several genes have so far been implicated in this process, including *Ets-1*, *Fli-1*, *Vezf-1* and *VEGFR-2*, along with the transcription factor Tal-1 (T cell acute leukaemia, also *Scf*) and members of the GATA, Hox and inhibitor of differentiation (Id) protein families (Rafii and Lyden 2003).

VEGFR-2 is expressed on both mature ECs and endothelial precursors, and embryos deficient in VEGFR-2 display an early arrest in haemangioblast

differentiation (Shalaby et al. 1995). In contrast, loss of VEGF induces severe vascular defects, but ECs still differentiate in the absence of VEGF (Carmeliet et al. 1996). Whether this implies that VEGF-C, which also binds to VEGFR-2, might be involved in haemangioblast differentiation remains to be determined. Tal-1 is involved in early cell fate determination of the haemangioblast, most likely by exerting combinatorial effects with VEGFR-2 (Visvader et al. 1998; Ema et al. 2003). In contrast, targeted inactivation of VEGFR-1 (also fms-like tyrosine kinase 1, Flt-1) does not prevent haemangioblast differentiation, but leads to vascular disorganisation, most likely due to an excess in EPCs (Fong et al. 1995; Kearney et al. 2002).

Endodermal signals may also regulate vasculogenesis in the adjacent mesoderm. Recent studies in *Xenopus* and in avian embryos suggest that the endoderm regulates the assembly of angioblasts to vascular tubes-rather than the specification of the haemangioblast lineage-and that sonic hedgehog (Shh) signalling is the key mediator involved in this interaction (Vokes and Krieg 2002; Vokes et al. 2004). By contrast, genetic studies in zebrafish show that the endoderm regulates the directional migration of the angioblasts to the midline (Jin et al. 2005).

### 3.2

#### **Endothelial Progenitors in the Adult**

Until recently, neovascularisation in the adult has been primarily attributed to sprouting angiogenesis. However, the isolation of putative EPCs from circulating mononuclear cells in the peripheral blood of adult humans and the demonstration that such EPCs home to sites of neovascularisation and contribute to this process introduced the concept of “post-natal vasculogenesis” (Figs. 1 and 2). EPCs, isolated from human peripheral blood mononuclear cells (PB-MNCs), express VEGFR-2 and CD34 (Asahara et al. 1997). Various other cell surface markers [c-kit, CXCR4, von Willebrand’s factor (vWF), CD31, CD146] have been identified on EPCs (Rafii and Lyden 2003). Expression of AC133, an orphan receptor that is specifically expressed on immature EPCs, is lost upon differentiation into more mature ECs (Iwami et al. 2004).

EPCs should be distinguished from circulating ECs (CECs) in the peripheral blood, which are sloughed off due to shedding from the existing vasculature and enter the circulation as a result of traumatic or infectious vascular injury, or tumour growth. Unlike EPCs, mature CECs do not express the stem cell marker AC133 (Rafii and Lyden 2003). Not all circulating CECs are viable, and the fraction of apoptotic CECs in the peripheral blood increases upon treatment of tumour-bearing mice with anti-angiogenic agents. Besides bone marrow-derived EPCs, multipotent adult progenitor cells (MAPCs) with angioblastic potency have been identified in the bone marrow (Reyes et al. 2002), while tissue-specific stem cells might also exist. In skeletal muscle, myo-endothelial progenitors might differentiate locally into muscle or ECs (Tamaki et al. 2002).

In the bone marrow niche, EPCs are likely to reside in close association with haematopoietic stem cells and stromal cells. Though not yet fully elucidated, these cells are possibly involved in promoting local EPC proliferation and transmigration across the bone marrow/blood barrier via secretion of VEGF, placental growth factor (PlGF) and other angiogenic factors (Tordjman et al. 2001). Mobilisation of EPCs from the bone marrow, as well as their recruitment to sites of adult vasculogenesis, involves a number of similar cues that also regulate EC sprouting (angiogenesis), such as VEGF (Asahara et al. 1999), fibroblast growth factor-2 (FGF-2), PlGF (Carmeliet et al. 2001; Rafii and Lyden 2003) platelet-derived growth factor-CC (PDGF-CC; Li et al. 2005), and angiopoietin-1 (Ang-1; Hattori et al. 2001), in addition to other factors such as metalloproteinases and adhesion molecules (Rafii and Lyden 2003). A chemoattractant for haematopoietic progenitor cells (HPCs), the chemokine stromal cell-derived factor-1 $\alpha$  (SDF-1 $\alpha$ ), also induces migration of EPCs (some of which express the SDF-1 $\alpha$  receptor CXCR-4; Yamaguchi et al. 2003) and enhances VEGF-mediated proliferation of ECs (Peichev et al. 2000; Neuhaus et al. 2003), while inhibition of SDF-1 $\alpha$  blocks EPC recruitment to tumours (Guleng et al. 2005).

To what extent EPCs contribute to vascular growth remains an outstanding question. Apart from differentiating to mature ECs, which are incorporated as building blocks in the endothelial layer (Peichev et al. 2000) in nascent vessels, mononuclear cells might also create, together with accessory cells derived from the bone marrow, a pro-angiogenic microenvironment to facilitate neovascularisation. For instance, CD34-expressing precursor cells, mobilised from the bone marrow, stimulate vascularisation in myocardial infarcts both via vasculogenic in situ vessel formation and via stimulation of angiogenic sprouting by local induction of angiogenic growth factor secretion (Kocher et al. 2001).

The relative numeric contribution of bone marrow-derived EPCs to adult organ and tumour neovascularisation is highly variable. In different experimental settings of pathological angiogenesis, incorporation of EPCs into the growing vasculature has been reported to be remarkably high (Garcia-Barros et al. 2003) or negligibly low (Rajantie et al. 2004; Ziegelhoeffer et al. 2004). Apart from differences in the genetic background of mouse strains used for those studies, the variability might also reflect differences of spontaneous and xenografted tumours in their dependence on bone marrow-derived endothelial precursors (Ruzinova et al. 2003). Mathematical models have been suggested to calculate-and possibly predict-the contribution of EPCs to tumour neovascularisation (Stoll et al. 2003).

Despite these unresolved questions, the concept of post-natal vasculogenesis offers challenging clinical perspectives for the treatment of cardiovascular disorders and cancer. Mobilisation of EPCs from the bone marrow is enhanced in patients with ischaemic conditions (Shintani et al. 2001), and levels of circulating EPCs have been introduced as a valuable clinical parameter for cardiovascular risk assessment (Hill et al. 2003). In tumour-bearing mice, EPC

(and CEC) levels in peripheral blood correlate with the anti-angiogenic effect of angiogenesis inhibitors on tumour angiogenesis and growth (Shaked et al. 2005), suggesting EPCs (and CECs) as a useful biomarker for dose finding and monitoring the effect of anti-angiogenic treatment in cancer (see Sect. 6.1).

### 3.3

#### **The Endothelial/Haematopoietic Connection: An Emerging Theme**

In the embryo, haematopoietic stem cells (HSCs) migrate into avascular areas and attract sprouting ECs by releasing angiogenic factors such as Ang-1 (Takakura et al. 2000). In the adult, bone marrow-derived haematopoietic cells-expressing markers such as Sca-1, c-Kit, CXCR4 and VEGFR1-become recruited, often together with EPCs, to tumours or ischaemic tissues in response to VEGF and PlGF (Hattori et al. 2002; Rafii and Lyden 2003; Grunewald et al. 2005; Orimo et al. 2005). These angio-competent cells extravasate around nascent vessels, where they are retained by SDF-1 $\alpha$ , and stimulate growth of resident vessels by releasing angiogenic factors such as VEGF, PlGF and angiopoietin-2 (Ang-2; Ceradini et al. 2004; Butler et al. 2005; Okamoto et al. 2005). In other cases, these cells function as haemangioblasts, producing both haematopoietic and endothelial progenitors, which give rise to new blood vessels (Rafii and Lyden 2003). Furthermore, in response to PlGF released by tumour cells, VEGFR-1-expressing haematopoietic bone marrow progenitors home to tumour-specific pre-metastatic sites, where they recruit tumour cells and EPCs; anti-VEGFR1 antibodies prevent the formation of such pre-metastatic niches (Riba et al. 2005).

### 3.4

#### **Arterial and Venous Cell Fate Specification**

Arteries and veins have evolved as anatomically distinct but closely interconnected blood vessel types. The structural differences between arteries and veins were attributed to different flow dynamics and distinct physiological requirements. But, evidence has recently emerged that molecular differences between arterial and venous ECs already exist even before blood vessels are formed, and that complex genetic pathways are responsible for this arterial versus venous specification. The expression of the ligand ephrinB2 in arteries and of the Eph receptor tyrosine kinase EphB4 in veins occurs before the onset of circulation (Gerety et al. 1999; Gerety and Anderson 2002). This indicates that while ephrins are essential for proper distinction between arterial and venous cells, they are not required for the initial fate decision that distinguishes arterial and venous endothelial progenitors.

Lineage tracking in zebrafish embryos indicates that angioblasts in the lateral posterior mesoderm receive signals from the notochord and the ventral

endoderm, and become restricted to the aorta or trunk vein (Zhong et al. 2001). Studies in zebrafish and *Xenopus* indicate that Shh, produced by the notochord, specifies arterial EC fate (Lawson and Weinstein 2002; Lawson et al. 2002). Indeed, formation of the aorta is impaired in zebrafish embryos mutant for *sonic you* (*syu*), the zebrafish homologue of Shh (Chen et al. 1996; Brown et al. 2000) or after morpholino knock-down of Shh (Lawson et al. 2002). Shh induces the expression in the adjacent somites of VEGF which, in turn, drives arterial differentiation of angioblasts. In the chick, the early extra-embryonic blood islands contain a mixture of subpopulations of cells expressing Nrp-1 and Nrp-2, which subsequently become lineage markers of arteries and veins, respectively (Herzog et al. 2005). This suggests that even early angioblasts may already be committed to either the arterial or venous lineage. Further evidence that VEGF has a role stems from findings that, when released from Schwann cells, it induces arterial specification of vessels, tracking alongside these nerves (Mukouyama et al. 2002) and that Nrp-1, a receptor selective for the VEGF<sup>165</sup> isoform, is expressed in arterial beds (Moyon et al. 2001; Stalmans et al. 2002). VEGF also determines arterial EC specification after birth in the heart and retina, where the matrix-binding VEGF<sup>188</sup> isoform is critical for arterial development (Stalmans et al. 2002; Visconti et al. 2002).

The Notch pathway acts downstream of VEGF in arterial EC specification (Lawson et al. 2002). Notch signalling is initiated when the Notch receptors (Notch 1-4) are activated by their ligands Jagged-1, Jagged-2 and Delta-like-1, -3 and -4 (Alva and Iruela-Arispe 2004). During vascular development, defects in Notch signalling disrupt normal arterial-venous differentiation, resulting in loss of artery-specific markers (e.g. ephrinB2) and ectopic expression of venous markers (e.g. Flt-4) in the aorta (Lawson et al. 2001). Conversely, over-activation of Notch suppresses differentiation of vessels to veins. Furthermore, Hey2, a transcription factor that is induced by Notch signalling, confers features of arterial EC gene expression on vein-derived ECs, up-regulating arterial-specific genes, including *ADHA1* (aldehyde dehydrogenase 1 family, member A1), *EVA1* (epithelial V-like antigen 1) and *keratin 7*, while suppressing vein-specific genes, such as *GDF* (growth and differentiation factor), *lefty-1* and *lefty-2* (Chi et al. 2003). The hairy-related transcription factor gridlock is required for the early assignment of arterial endothelial identity (Zhong et al. 2000). Zebrafish lacking this protein show a disrupted assembly of the aorta in the posterior part of the body (Zhong et al. 2000, 2001), while gridlock over-expression caused a similar disruption of the vein without affecting the artery (Zhong et al. 2001). All these genetic findings appear to refute the hypothesis that physiological cues are responsible for arterio-venous differentiation. However, even after ECs attain a specific arterial or venous phenotype late in embryonic development, this genetic programme still remains remarkably plastic (Moyon et al. 2001).

### 3.5

#### Tissue-Specific Endothelial Cell Differentiation

ECs in different organs acquire highly specialised properties which permit these cells to optimally perform specific functions within each tissue and organ (Ruoslahti and Rajotte 2000). For instance, ECs in the brain are tightly linked to each other and are surrounded by numerous peri-endothelial cells which constitute a barrier that protects brain cells from potentially toxic blood-derived molecules. The development of the blood-brain barrier requires the interactions between astroglial cells that express glial fibrillary acidic protein, pericytes and adequate angiotensinogen levels (Lindahl et al. 1998). The tight junctional complex between ECs consists of numerous integral membrane and cytosolic proteins, belonging to the families of cadherins, occludins, claudins and membrane-associated guanylate kinase homologous proteins (Rubin and Staddon 1999; Tsukita and Furuse 1999). In contrast, vessels in endocrine glands lack these tight junctions. Their endothelium is rather discontinuous and fenestrated, allowing high volume molecular and ion transport as well as hormone trafficking. Overall, the factors that regulate acquisition of specific endothelial properties are largely unknown. However, it appears that the interaction with the host environmental extracellular matrix, in concert with VEGF, plays a major role (Risau 1995; Andries et al. 1998). Besides vascular cell heterogeneity in distinct organs, ECs within the same organ can even be heterogeneous. In the heart, for instance, ECs in distinct locations of the coronary vascular tree differ in their expression of the endothelial constitutive nitric oxide (NO) synthase isoform (Andries et al. 1998), brain-derived neurotrophic factor (Donovan et al. 2000) or adhesion molecules (Derhaag et al. 1996, 1997). Even within a single vessel, ECs may have distinct cell fates. For example, three types of ECs, each with a distinct cell fate, build the inter-segmental vessels in the zebrafish embryo (Childs et al. 2002).

Recently, genetic studies in mice, zebrafish and *Xenopus* have started to unravel the transcriptional code that determines EC fate (Brown et al. 2000; Liao et al. 2000). This code involves basic helix-loop-helix (bHLH) transcriptional activators [hypoxia-inducible factor (HIF)-2 $\alpha$ , stem cell leukaemia factor, Tfeb; Carmeliet 1999] as well as Id repressors as demonstrated by the perturbation of developmental and tumour-associated angiogenesis in mice lacking Id-1/3 (Neufeld et al. 2002). However, there are other mechanisms determining endothelial heterogeneity and organ-specific angiogenesis. For instance, the activity of VEGF and Ang-1 varies in different tissues. Low permeability tumours over-express Ang-1 and/or under-express VEGF or PlGF, whereas tumours with high permeability lack Ang-1 but over-express Ang-2 (Jain and Munn 2000). Another example is the effect of Ang-1, which stimulates angiogenesis in the skin but suppresses vascular growth in the heart (Suri et al. 1998; Visconti et al. 2002).



Moreover, tissue-specific angiogenic factors determine the angiogenic switch restrictedly in particular organs. A striking example of this novel class of cues comprises the endocrine gland-derived VEGF (EG-VEGF) and Bv8, which selectively affect EC growth and differentiation (fenestration) in endocrine glands (LeCouter et al. 2001, 2002, 2004; Ferrara et al. 2004b). Other organ-specific angiogenic factors include Bves and fibulin-2 in the heart (Wada et al. 2001), and glial-derived neurotrophic factor in the brain. That ECs in different tissues are distinct is further suggested by their considerably different response to anti-angiogenic factors. Indeed, ECs in endocrine glands rapidly lose their fenestrations and even become apoptotic in response to VEGF inhibitors, resulting in a 70% loss of the microvasculature in these organs. In contrast, the microvasculature in other organs is much more resistant to such pruning in response to anti-VEGF therapy (Tang et al. 2004a; Kamba et al. 2005). Malignant cells also induce ECs in tumour vessels to acquire a distinct fate and express unique markers ("vascular zip codes") that are absent or barely detectable in quiescent blood vessels of normal tissue (Ruoslahti 2002, 2004). Tumour cells also change the responsiveness of ECs to cues—for instance, epidermal growth factor (EGF) up-regulates its receptors in tumour-associated vessels, thereby making these ECs responsive to the mitogenic activity of EGF—a finding of significant therapeutic relevance.

## 4

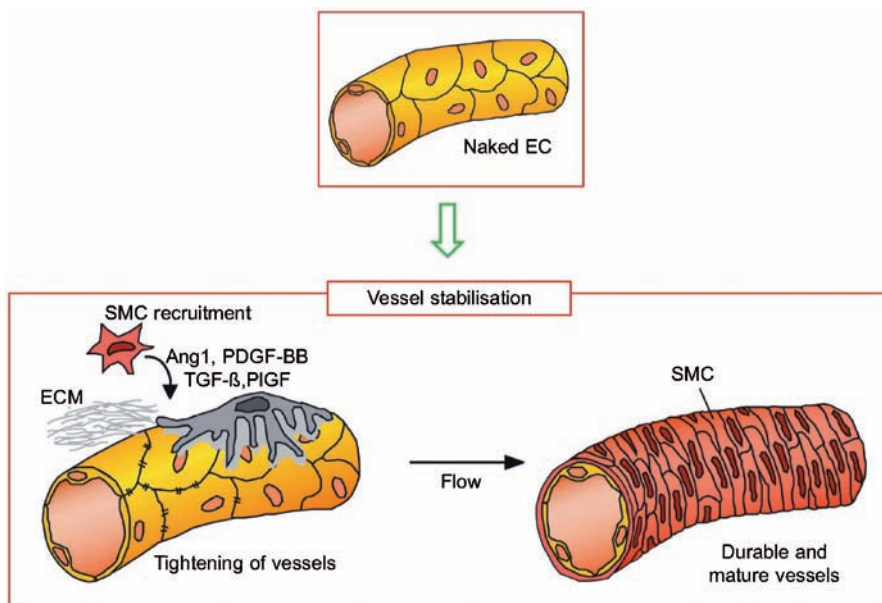
### Angiogenesis

Once a nascent primitive vascular labyrinth has been formed through vasculogenesis, it successively expands and remodels into a more complex, hierarchically and stereotypically organised network of larger vessels, ramifying into smaller vessels. This process, referred to as angiogenesis, involves sprouting, bridging and intussusceptive growth from pre-existing vessels, navigation and guidance, as well as remodelling and pruning. We will now discuss this process by viewing it as a step-wise progression of the following orderly series of events (Fig. 1). In response to angiogenic factors released in nearby hypoxic regions, activated ECs induce extracellular matrix (ECM) degradation, proliferate and navigate towards these angiogenic cues, sprouting into new vessels. Supported by surrounding SMCs and pericytes, nascent vessels consequently stabilise, mature and acquire specified functional properties to accommodate local requirements—namely arteriogenesis (Fig. 3).

#### 4.1

##### Vascular Permeability and Extracellular Matrix Degradation

Water and nutrients move from blood to tissues across the walls of capillaries and venules. The wall of blood vessels is composed of ECs and mural cells,



**Fig. 3** Vessel maturation and stabilisation. When vessels sprout, they initially consist of naked endothelial cell channels. These nascent vessels become stabilised by recruitment of smooth muscle cells and pericytes (a process called arteriogenesis), deposition of extracellular matrix and tightening of cell junctions. Blood flow plays a critical role in making these vessels durable

namely pericytes and SMCs, which are embedded in an ECM. The expression of cell adhesion molecules in quiescent vessels—such as vascular endothelial cadherin (VE-cadherin) in adherent junctions and claudins, occludins and JAM-1 in tight junctions—provides mechanical strength and tightness to the vessel wall and establishes a permeability barrier. Between vascular cells, an interstitial matrix of collagen-I and elastin provides visco-elasticity and strength to the vessel wall. The ECM is responsible for the contacts between ECs and the surrounding tissue, and thus prevents vessels from collapsing. This stable vessel structure must be first destabilised before new vessels can sprout—a process which we will now describe.

The key angiogenic factor VEGF was originally discovered by H. Dvorak as a potent vascular permeability factor. When vascular permeability increases, plasma proteins (such as fibronectin, fibrinogen, etc.) extravasate and provide a provisional matrix for migrating ECs. Vascular leakage results from changes in EC fenestration, a redistribution of intercellular adhesion molecules, such as PECAM-1 and VE-cadherin, as well as from alterations in the cell membrane, a process involving Src kinase (Eliceiri et al. 1999; Gale and Yancopoulos 1999). However, excessive vascular leakage can result in pathological outcomes, such as circulatory collapse, intracranial hypertension, formation of peritoneal ad-



hesions, metastasis or blindness. Moreover, it contributes to the pathogenesis of chronic inflammatory disorders such as psoriasis and myocardial or brain infarction, and leads to increased interstitial fluid pressure in tumours (Jain 2005). Therefore, changes in vascular permeability need to be tightly regulated. Ang-1, a ligand of the endothelial Tie-2 receptor, is a natural inhibitor of vascular permeability. It tightens vessels by affecting the endothelial junctional molecules PECAM, VE-cadherin and occludins (Gamble et al. 2000; Thurston et al. 2000), thus counteracting the VEGF effect and protecting against abnormal plasma leakage.

To emigrate from their resident site, ECs need to loosen their inter-endothelial contacts, relieve peri-endothelial cell support and break down the surrounding ECM. Ang-2, an inhibitor of Tie-2 signalling and antagonist of Ang-1, is involved in the destabilisation of mature vessels by detaching SMCs and loosening the underlying matrix (Maisonpierre et al. 1997; Gale and Yancopoulos 1999). Proteolytic degradation of the ECM is mediated by several proteinase families, i.e. plasminogen activators [such as urokinase plasminogen activator (uPA) and its inhibitor, PAI-1], matrix metalloproteinases [MMPs and tissue inhibitors of metalloproteinases (TIMPs)], chymases, heparanases, tryptases, cathepsins and kallikreins (and their inhibitor kallistatin; Kostoulas et al. 1999; Luttun et al. 2000; Berchem et al. 2002; Jackson 2002; Miao et al. 2002; Yousef and Diamandis 2002). Proteinases also liberate matrix-bound angiogenic growth factors, such as FGF-2, VEGF, insulin-like growth factor-1 (IGF-1), transforming growth factor- $\beta$  (TGF- $\beta$ ) and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), and proteolytically activate angiogenic chemokines such as interleukin-1 $\beta$  (IL-1 $\beta$ ; Coussens et al. 1999; Bergers et al. 2000). In addition, proteinases cleave VEGF into shorter isoforms which differ in their solubility, receptor binding and biological activities (Park et al. 1993). In contrast, proteinases involved in the breakdown of ECM also play a role in the resolution of angiogenesis, as they liberate matrix-bound inhibitors [thrombospondin-1 (TSP-1), canstatin, arrestin, tumstatin, angostatin, endostatin, cleaved anti-thrombin III, platelet factor 4, arresten, endorepellin; Nyberg et al. 2005] and inactivate angiogenic cytokines such as SDF-1 $\alpha$  (Orimo et al. 2005).

When considering the critical role of ECM in vessel growth and maintenance, it is conceivable that proteolytic remodelling of the ECM must occur in a balanced manner. Indeed, excessive breakdown removes critical support and guidance cues for migrating ECs and, consequently, impairs angiogenesis; on the other hand, insufficient degradation prevents vascular cells from leaving their original site. This concept was illustrated by genetic studies of the plasminogen and MMP systems. Indeed, loss of PAI-1 suppressed pathological angiogenesis in tumours, ocular and other disorders, whereas adenoviral PAI-1 gene transfer reverted this phenotype (Bajou et al. 1998; Lambert et al. 2001; Devy et al. 2002). Conversely, plasmin proteolysis is required for angiogenesis, as vascularisation of ischaemic hearts was reduced in uPA-deficient mice (Heymans et al. 1999), while tumour vascularisation was impaired in

plasminogen-deficient mice (Bajou et al. 2001). Similarly, pathological angiogenesis was decreased in mice lacking components of the MMP system, such as MMP-2 and MMP-9, whereas over-expression of membrane-type (MT)-MMP-1 results in highly vascularised tumours (Sounni et al. 2002). A fine-tuned balance between proteinases and their inhibitors is therefore crucial and might explain why the uPA inhibitors PAI-1 and MT-MMP-1 are risk factors for a poor prognosis in several cancers (Bajou et al. 1998; Luttun et al. 2000).

## 4.2

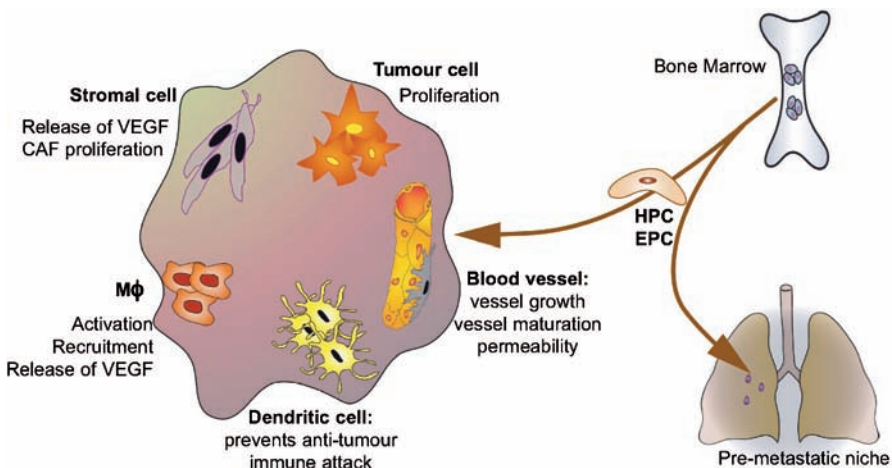
### Endothelial Budding and Sprouting

Once the physical barriers are dissolved, proliferating ECs migrate to distant sites. This is a complex, tightly regulated process, requiring the involvement of numerous stimulators and inhibitors. For reasons of brevity, we will only review some key signals. The most important signalling of all involves VEGF, which via binding its receptor VEGFR-2, regulates embryonic, neonatal and pathological angiogenesis in a strict dose-dependent manner. The latter phenomenon is exemplified by genetic studies. Indeed, loss of a single VEGF allele results in lethality due to early embryonic vascular defects (Carmeliet et al. 1996; Ferrara et al. 1996), while reduction of VEGF levels by only 25% impairs spinal cord perfusion and causes motor neuron degeneration, reminiscent of amyotrophic lateral sclerosis (Oosthuyse et al. 2001). Several additional gene-manipulating studies in mice, zebrafish and *Xenopus* have documented the principal role of VEGF in vascular development and illustrated its potential to stimulate new vessel growth (Cleaver and Krieg 1998; Nasevicius et al. 2000; Liang et al. 2001; Stalmans et al. 2003). Conditional inactivation of VEGF after birth or expression of particular VEGF isoforms in knock-in mice revealed that VEGF is requisite for vascular expansion during post-natal growth in various organs (e.g. kidney, bone, heart and retina) and, when insufficiently available, causes tissue ischaemia, impaired growth and organ failure (Carmeliet et al. 1999b; Haigh et al. 2000; Maes et al. 2002; Mattot et al. 2002; Stalmans et al. 2002; Eremina et al. 2003). On the other hand, over-expression of VEGF, for instance in the skin of transgenic mice, stimulates abundant cutaneous capillary growth and an inflammatory skin condition resembling psoriasis (Xia et al. 2003). Numerous studies also established VEGF as a key angiogenic player in cancer (Ferrara 2002). Because of its predominant role, VEGF is currently being evaluated for both pro- and anti-angiogenic therapy (Ferrara 2000a, b).

Gene targeting studies in mice have elucidated the functional role of PlGF, a homologue of VEGF, which binds to VEGFR-1. Loss of PlGF-while not causing any vascular defects during embryonic development, reproduction or normal adult life-impaired angiogenesis and plasma extravasation in pathological conditions, including ischaemia, inflammation and cancer (Carmeliet et al. 2001). The important role of PlGF in pathological angiogenesis is further evidenced by findings that PlGF stimulates angiogenesis and collateral growth (see below)

in the ischaemic heart and limb of wild-type mice (Luttun et al. 2002) and, in combination with VEGF, in the ischaemic heart of a mouse model resistant to the VEGF treatment alone (Autiero et al. 2003). PlGF contributes to the angiogenic switch in pathological conditions by affecting, directly and indirectly, multiple cell types (Fig. 4).

First, PlGF has direct effects on ECs by inducing its own signalling via VEGFR-1 and by amplifying VEGF-driven angiogenesis (Carmeliet et al. 2001; Autiero et al. 2003). Second, PlGF-by directly stimulating SMCs and fibroblasts, which express VEGFR-1-recruits SMC around nascent vessels and thus promotes vessel maturation and stabilisation (Green et al. 2001; Ishida et al. 2001; Luttun et al. 2002; see Sect. 5). Third, PlGF stimulates the mobilisation of VEGFR-1-positive HSC/HPCs from the bone marrow (Carmeliet et al. 2001; Lyden et al. 2001) and, indirectly via up-regulation of VEGF expression, recruitment of VEGFR-2-positive EPCs to the site of neovascularisation (Hattori et al. 2001, Luttun et al. 2002). At such sites they promote new vessel growth by directly incorporating into the vessel wall and/or by creating a pro-angiogenic microenvironment through the release of angiogenic molecules (Rehman et al. 2003). Furthermore, PlGF can also recruit HPCs to distant sites to form pre-metastatic niches. Fourth, PlGF is chemo-attractive for monocytes and macrophages, which express VEGFR-1 (Sawano et al. 2001; Luttun



**Fig. 4** PlGF is a master switch of pathological angiogenesis and stimulates tumour vascularisation and growth by affecting multiple cell types. Within the tumour environment, PlGF stimulates either vascular cells (endothelial and smooth muscle cells) or non-vascular cells (monocytes/macrophages, stromal cells and dendritic cells). PlGF may also affect VEGFR-1-expressing tumour cells directly. In addition, PlGF stimulates the mobilisation and recruitment of VEGFR-1-positive HSC/HPCs from the bone marrow to the primary tumour and pre-metastatic niches. It remains to be determined whether PlGF also directly affects EPC recruitment by interacting with its receptor VEGFR-1 on EPCs

et al. 2002)-activated macrophages are a rich source of a variety of angiogenic molecules (Autiero et al. 2003) and also produce PlGF, thereby providing a positive feedback.

The role of PlGF and VEGFR-1 in both endothelial and haematopoietic lineages explains why blocking VEGFR-1 more efficiently suppresses inflammatory angiogenic disorders (atherosclerosis, arthritis) than blocking VEGFR-2 (Luttun et al. 2002). Similar effects would thus be expected when VEGFR-1 activation is prevented by PlGF inhibitors or antibodies. VEGF-B is another homologue of VEGF, but its angiogenic activities remain to be determined.

Another angiogenic signalling system involved in vessel growth and stabilisation comprises the Tie-2 receptor, which binds the angiopoietins (Ang-1 and Ang-2). Ang-1, via phosphorylation of Tie-2, is chemotactic for ECs, induces vascular sprouting, stimulates EC survival, mobilises EPCs and HSC/HPCs, and stabilises networks initiated by VEGF, presumably by stimulating the interaction between endothelial and peri-endothelial cells (Suri et al. 1996, 1998; Gale and Yancopoulos 1999; Hattori et al. 2001). All these activities may explain why Ang-1 stimulates vessel growth in skin, ischaemic limbs, gastric ulcers and in some tumours (Suri et al. 1998; Shim et al. 2002; Plank et al. 2004). However, Ang-1 also suppresses angiogenesis in other tumours and the heart (Ahmad et al. 2001; Visconti et al. 2002). In fact, Ang-1 may restrain vessel sprouting by tightening vessels via effects on junctional molecules (Thurston et al. 2000), by recruiting pericytes and by promoting endothelial-mural cell interactions as an adhesive protein (Carlson et al. 2001). Ang-2 in concert with VEGF is also angiogenic and has been proposed to stimulate the growth of immature (SMC-poor) tumour vessels by loosening the endothelial-peri-endothelial cell interactions and degrading the ECM via up-regulation of proteinases, thereby counteracting the activity of Ang-1 (Ahmad et al. 2001; Etoh et al. 2001; Gale et al. 2002). However, the angiogenic activity of Ang-2 seems to be contextual as well since, in the absence of VEGF, Ang-2 causes EC death and induces vessel regression (Maisonpierre et al. 1997).

Several additional factors regulate the proliferation of ECs. Integrins are heterodimeric cell surface receptors of specific ECM molecules which, by bidirectionally transmitting signals between the outside and inside of vascular cells, assist vascular cells to build new vessels in co-ordination with their surroundings (Hood and Cheresch 2002; Hynes 2002). The  $\alpha v\beta 3$  and  $\alpha v\beta 5$  integrins have long been considered to regulate the angiogenic switch positively (Lee and Juliano 2004), because their pharmacological antagonists which are currently being evaluated in clinical trials suppress pathological (i.e. tumour) angiogenesis (McNeel et al. 2005). Furthermore, a combination of antibodies against  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$  integrins reduces tumour vascularisation (Senger et al. 2002). However, genetic deletion studies suggest that vascular integrins inhibit, rather than stimulate, tumour angiogenesis (Reynolds et al. 2002). This inhibitory activity may be attributable to the ability of these integrins to suppress VEGFR-2-mediated EC survival, trans-dominantly block other inte-

grins, or mediate the anti-angiogenic activity of angiogenesis inhibitors such as tumstatin, endostatin, and canstatin (Carmeliet 2002; Reynolds et al. 2002; Hamano et al. 2003; Sudhakar et al. 2003, 2005; Lee and Juliano 2004, Magnon et al. 2005). Thus, while these genetic insights do not invalidate the promising (pre)clinical results obtained with integrin antagonists for cancer treatment, a better understanding of whether and in which conditions integrins play positive or negative roles in tumour angiogenesis is desirable.

FGFs stimulate EC growth directly and, by recruiting pro-angiogenic mesenchymal and inflammatory cells, also indirectly (Carmeliet 2000a). Though PDGF-BB has been documented to stimulate microvascular sprouting of ECs, its main activity is to recruit pericytes and SMCs around nascent vessel sprouts, thereby stimulating vessel maturation and stabilisation, and increasing vessel perfusion (Lindahl et al. 1998, 1999; see Sect. 5). Molecules such as TGF- $\beta$ 1, activin-A and TNF- $\alpha$  stimulate or inhibit EC growth, depending on the context (Pepper 1997; Gohongi et al. 1999; Guo et al. 2000).

Chemokines are another interesting class of molecules, capable of stimulating or inhibiting EC growth, depending on the type of receptor they activate. Chemokines binding CXCR2 and CXCR4 are angiogenic (e.g. GRO- $\alpha$ , GRO- $\gamma$ , ENA-78, GCP-2, IL-8, SDF-1 $\alpha$ , 9E3, eotaxin, I-309, MCP-1, fractalkine), while chemokines binding CXCR3 (e.g. PF-4, MIG, IP-10, ITAC, BCA-1, SLC/6CKine) have angiostatic activity (Bernardini et al. 2003). At least two of those have received increasing recognition. IL-8 is expressed in several tumours and inflammatory conditions, and is even up-regulated in tumours after anti-VEGF therapy, while anti-IL-8 antibodies block tumour growth (Mizukami et al. 2005). Furthermore, emerging evidence indicates that SDF-1 $\alpha$  stimulates angiogenesis via direct effects on ECs, as well as via recruitment of bone marrow-derived EPCs and HPCs both in ischaemic and malignant tissues (Ceradini et al. 2004; Butler et al. 2005); antagonists of SDF-1 $\alpha$  block tumour growth (Guleng et al. 2005; see above).

EGF is a mitogen for epithelial cells and is over-expressed in various tumours. While it does not regulate vascular development, it has been implicated in tumour angiogenesis. Indeed, EGF induces the expression of its own receptors in ECs and is mitogenic for EGFR-positive ECs. In addition, EGF indirectly stimulates tumour angiogenesis by inducing the release of VEGF and the expression of VEGF receptors in tumour vessels (van Cruijssen et al. 2005). Another growth factor, hepatocyte growth factor (HGF), stimulates angiogenesis when exogenously administered (Jiang et al. 2005). Other molecules are capable of stimulating EC growth *in vitro* or angiogenesis in experimental models, but their endogenous role in angiogenesis during development or disease often remains incompletely determined. Some examples include erythropoietin, IGF-1, neuropeptide-Y, leptin, Thy-1, tissue factor, interleukins and others (Carmeliet 2003a).

Angiogenic sprouting is a complex process, requiring a finely tuned balance between activators and inhibitors. Some of the endogenous angiogenesis

inhibitors that are currently being evaluated for clinical use include angio-statin, endostatin, anti-thrombin III, interferon- $\beta$ , leukaemia inhibitory factor and platelet factor 4, tumstatin, C-terminal hemopexin-like domain of MMP-2 (PEX) and vasostatin (O'Reilly et al. 1994, 1997; Carmeliet 2000b, 2003a; Nyberg et al. 2005).

### 4.3

#### Vascular Lumen Formation

Sprouting ECs assemble into solid cords which then undergo tubulogenesis to form vessels with a central lumen. Little is known about how lumen formation is regulated *in vivo*. Gene targeting studies revealed that VEGF co-ordinatedly regulates vessel size and guidance. VEGF exists in different isoforms with distinct affinities for the ECM. Thus, VEGF<sup>121</sup> is diffusible, VEGF<sup>189</sup> binds to the matrix, whereas VEGF<sup>165</sup> has an intermediate profile (in mice, all VEGF isoforms are shorter by one residue). By virtue of their distinct affinities, the isoforms produce a gradient with VEGF<sup>120</sup> acting over a long-range and VEGF<sup>188</sup> over a short range (Ruhrberg et al. 2002; Gerhardt et al. 2003). In the mouse retina, a gradient of matrix-bound VEGF produced by astrocytes guides endothelial tip cells and regulates lumen formation of nascent vessels. Indeed, an alteration of the gradient by loss-of-function manipulation led to a reduction in vessel branching and a concomitant increase in vessel lumen size, while a gain-of-function induced the opposite vascular changes (Gerhardt et al. 2003). Further evidence for a role of VEGF gradients in tip cell guidance and vessel lumen regulation was deduced from analysis of three mouse lines (the VEGF<sup>120</sup>, VEGF<sup>164</sup> and VEGF<sup>188</sup> lines), each engineered to express a single VEGF isoform. VEGF<sup>164</sup> mice are normal, but VEGF<sup>120</sup> or VEGF<sup>188</sup> mice exhibit serious vascular remodelling defects (Carmeliet et al. 1999b; Stalmans et al. 2002). Vessels in VEGF<sup>120</sup> mutants are enlarged, stunted and exhibit reduced branching. Their tip cell filopodia extend chaotically in all directions, which is thought to cause lumen enlargement at the expense of directed branch formation and elongation. These defects presumably result from replacement of the normal VEGF gradient by a non-directional deposition of the highly diffusible VEGF<sup>120</sup>. In VEGF<sup>188</sup> mice, a shortage of diffusible VEGF causes the opposite phenotype, i.e. supernumerary branches at the expense of luminal enlargement.

In combination with VEGF, Ang-1 also augments lumen diameter (Suri et al. 1998). Egfl7, a recently identified endothelial-derived secreted factor, is expressed at high levels in the developing embryo. Knock-down of Egfl7 in zebrafish embryos revealed that Egfl7 specifically blocks vascular tubulogenesis (Parker et al. 2004). Other molecules involved in the control of lumen formation are the integrins  $\alpha 5$ ,  $\beta 1$  and  $\alpha v \beta 3$  (Bayless et al. 2000), probably because of their interaction with the surrounding ECM. Finally, thrombospondin-1 (TSP-1) and tubedown-1 (tdn-1) suppress vascular lumen formation (Gendron et al. 2000).



#### 4.4

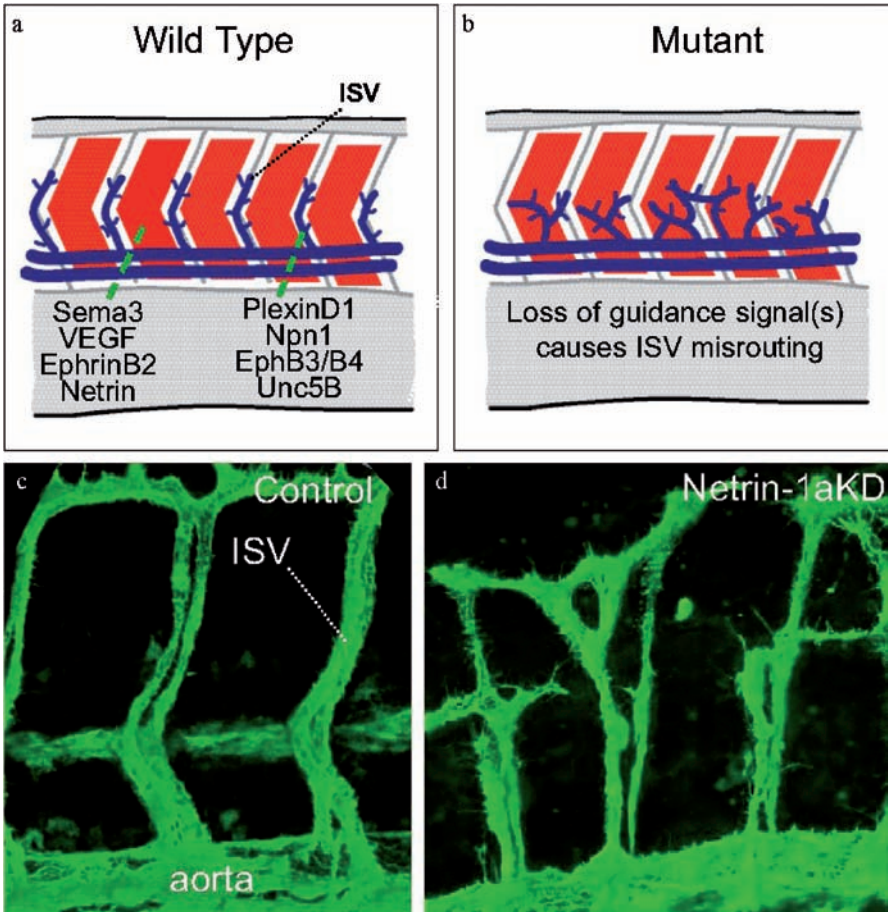
#### Guided Navigation of Vessels

During evolution, organisms have come to perform more specialised tasks, requiring an increased supply of nutrients by blood vessels. Wiring of blood vessels into functional circuits is therefore of utmost importance. The complexity of this task is underscored by the high degree of orderly patterning of the vascular networks. Five centuries ago, Andreas Vesalius illustrated the parallels in the stereotyped branching patterns of vessels and nerves. Today, evidence is emerging that vessels, which arose later in evolution than nerves, co-opted several of the organisational principles and molecular mechanisms that evolved to wire up the nervous system. The choreographed morphogenesis of both networks suggests that they are directed by genetically programmed mechanisms. Specialised endothelial “tip” cells are present at the forefront of navigating vessels and share many similarities with axonal growth cones (Gerhardt et al. 2003). They extend and retract numerous filopodia in saltatory fashion to explore their environment, suggesting that they direct the extension of vessel sprouts. The key function of the tip cells appears to be to “pave the path” for the subjacent “stalk” ECs. Tip cells proliferate minimally, whereas stalk cells proliferate extensively while migrating in the wake of the tip cell, thus permitting extension of the nascent vessel.

Guidance of embryonic vessels requires local guidance cues that instruct them to navigate along specific paths to reach their correct targets. Nerves and vessels face similar challenges in finding their trajectories, which are staked out with multiple checkpoints that divide navigation over a long trajectory into a series of shorter decision-making events (Autiero et al. 2005). Axons and vessels often take advantage of one another to follow the same path. In some cases, vessels produce signals (such as artemin and neurotrophin 3) that attract axons to track alongside the pioneer vessel (Honma et al. 2002; Kuruvilla et al. 2004). Conversely, nerves may also produce signals such as VEGF to guide blood vessels (Mukouyama et al. 2002). Very recent evidence reveals that the same cues that control axon guidance also function to pattern blood vessels. Four families of axon guidance cues, acting over a short-range (cell- or matrix-associated signals) or long-range (secreted diffusible signals), have been identified, namely netrins and their deleted in colorectal cancer (DCC) and Unc5 receptors, semaphorins and their neuropilin and plexin receptors, Slits and their Robos receptors and ephrins and their Eph receptors (reviewed in Dickson 2002; Carmeliet 2003b; Huber et al. 2003). A role for Unc5b and netrin1a in vessel guidance was identified by analysis of the developing inter-segmental vessels (ISV) in zebrafish embryos. Pathfinding of these vessels is stereotyped and believed to be genetically programmed by an interaction of attractive and repulsive cues. In control embryos, ISVs sprout from the dorsal aorta to the dorsolateral roof of the neural tube. After knock-down of Unc5b or netrin1a, ISVs exhibit supernumerary, often ectopically located filopodial

extensions (Lu et al. 2004), and the dorsal trajectory of most ISVs is irregular, with numerous extra branches deviating from the normal stereotyped path (Fig. 5). These findings suggest a tight control of ISV navigation by netrin family members.

Cross-talk between semaphorins and their receptors (e.g. neuropilins and plexins) is also necessary for ISVs to select the appropriate branching site along the dorsal aorta and to follow the pathway along the somite boundaries (Miao et al. 1999; Serini et al. 2003; Shoji et al. 2003; Torres-Vazquez et al. 2004; Gu



**Fig. 5a–d** Role of guidance signals in intersomitic vessel guidance. **a** Schematic representation of the zebrafish embryo trunk, showing the somites (red) producing the indicated guidance cues and intersomitic vessels (ISV) (blue). **b** In the absence of these guidance cues, the ISVs are misrouted. **c** This diagram shows the stereotyped pattern of ISVs in zebrafish embryos [the endothelial cells express an enhanced green fluorescent protein (eGFP) transgene]. **d** After knock-down (KD) of *netrin1a*, the ISVs are misguided



et al. 2005). A vascular-specific Robo homologue, Robo4, has been identified (Park et al. 2003). In vitro, Slit2 is able to repel ECs and Robo4 may mediate this effect (Park et al. 2003). A Robo4 knock-down study in zebrafish showed that some Robo4-expressing ISVs failed to sprout from the dorsal aorta or arrested midway through their dorsal migration path, whereas others deviated from their normal dorsal trajectory (Bedell et al. 2005). Repulsive ephrin-Eph signals provide short-range guidance cues for vessels to navigate through tissue boundaries. For instance, ephrinB2 repels EphB3/EphB4-expressing ISVs from entering somites (Wang et al. 1998; Adams et al. 1999; Oike et al. 2002). Understanding this process in the primitive zebrafish embryo may have therapeutic implications, as many of these guidance signals are excessively expressed in tumours, which characteristically develop a chaotic, misguided vasculature (Autiero et al. 2005; Carmeliet and Tessier-Lavigne 2005).

## 4.5

### Vessel Maintenance

When vessels sprout, they initially consist of naked EC channels. Once assembled in new vessels, these ECs become quiescent and survive for years. The importance of endothelial survival is demonstrated by the finding that diminished endothelial survival causes vascular dysfunction and regression in the embryo as well as in the adult (Carmeliet et al. 1999a; Gerber et al. 1999; Baffert et al. 2005). The molecular mechanisms enabling a confluent endothelium to maintain its physiological function in various vascular beds for long periods of time is still unclear. However, some insights have been obtained from *in vivo* and *in vitro* studies. For instance, Ang-1 promotes endothelial survival, while in the absence of angiogenic stimuli Ang-2 suppresses endothelial survival, thus contributing to the regression of tumour vessels (Suri et al. 1998; Gale and Yancopoulos 1999; Holash et al. 1999). Haemodynamic forces are also essential for the maintenance of the vascular integrity in different vascular beds, as physiological shear stress reduces endothelial turnover and abrogates TNF- $\alpha$ -mediated endothelial apoptosis (Dimmeler et al. 1996). Flow is critical too for maintaining vessel branches, as hypoperfused sprouts often regress.

But perhaps the most critical survival factor for quiescent ECs in the adult is VEGF. Thus, when VEGF levels are reduced, for instance by exposure of premature babies to hyperoxia, retinal vessels regress (Alon et al. 1995; Meeson et al. 1999). The recent clinical experience with VEGF inhibitors has revealed that these anti-angiogenic agents may cause rare but important adverse effects such as thrombosis, hypertension, bleeding and renal dysfunction (Hurwitz et al. 2004). Some of these adverse effects of anti-VEGF therapy can be explained by the requirement of threshold levels of VEGF for the survival and maintenance of quiescent vessels in healthy organs. For instance, the thrombotic risk may be related to the reduced release of fibrinolytic components (Pepper et al. 2001), the increased release of fibrinolytic inhibitors and pro-coagulants (Ma

et al. 2005), the reduced release of NO (an inhibitor of platelet aggregation and vasospasms, Yang et al. 1996), and EC dysfunction resulting from deprivation of VEGF vessel maintenance signals (Gerber et al. 1999; Kamba et al. 2005). The hypertension is probably attributable to reduced vasodilatation by NO, and possibly to pruning of normal vessels (Sane et al. 2004), while proteinuria and glomerulonephritis may be related to the maintenance role of VEGF in podocyte functioning (Eremina et al. 2003). Bleeding in centrally located cavitory necrotic lung tumours is likely to be due to vessel disintegration. As mentioned above, VEGF inhibitors cause the microvasculature to regress by 70% in endocrine organs, further highlighting the importance of VEGF as a maintenance cue for quiescent vessels in healthy organs (Tang et al. 2004a; Kamba et al. 2005).

## 5

### Arteriogenesis

Establishment of a functional vascular network requires that nascent vessels formed by vasculogenesis and angiogenesis mature into durable, stable, non-leaky and functional vessels (Fig. 3). This stabilisation requires recruitment of SMCs, generation of an ECM and specialisation of the vessel wall for structural support and regulation of vessel function—the process of arteriogenesis (Jain 2003). Endothelial channels are covered by multiple layers of SMCs in the proximal part of larger vessels, and by single pericytes in smaller distal vessels. The coverage of vessels by mural cells (pericytes and SMCs) not only regulates EC proliferation, survival, differentiation and haemostatic control, but also assists ECs in acquiring specialised functions to accommodate various needs in different vascular beds. Moreover, interstitial matrix components, generated by mural cells, interconnect ECs and provide blood vessels with visco-elastic properties. SMCs provide structural integrity of the vessel wall. These multi-functional cells contract spontaneously or in response to agonists, maintaining intravascular pressure and tissue perfusion.

#### 5.1

##### Smooth Muscle Progenitor Cells

A striking feature of SMC biology is the considerable heterogeneity in SMC origin, both during embryonic development and in the adult vasculature (Fig. 2). They can differentiate from ECs, from mesenchymal cells or from bone marrow progenitors and macrophages. For instance, the first SMCs in the dorsal aorta and the SMC-like myofibroblasts in the prospective cardiac valves transdifferentiate from the endothelium (Nakajima et al. 1997; Gittenberger-de Groot et al. 1999). Cardiac neural crest cells are the source of SMC of the large thoracic blood vessels and the proximal coronary arteries (Creazzo et al. 1998;

Gittenberger-de Groot et al. 1999). Mural cells from the distal coronary arteries are recruited from the epicardial layer (Gittenberger-de Groot et al. 1999), whereas coronary vein SMCs are derived from the atrial myocardium (Dettman et al. 1998).

In the adult, the recruitment of SMCs is accomplished by both the division of pre-existing SMCs and the differentiation of bone marrow-derived SMC progenitors, as exemplified in heterotypic cardiac and aortic transplantation in mice (Hillebrands et al. 2001; Saiura et al. 2001). In addition, fibroblasts can differentiate into myofibroblasts, which in turn differentiate into vascular SMCs in response to biochemical or mechanical cues. In humans, SMC progenitors could be identified in the mononuclear fraction of the peripheral blood (Simper et al. 2002). However, the numeric contribution of bone marrow-derived SMCs to vessel growth or maturation of vessels is still controversial. In some animal studies, bone marrow-derived SMCs contributed substantially (10%–50%) to neointima formation and re-endothelialisation in the context of transplant atherosclerosis, balloon injury and primary atherosclerosis (Religa et al. 2002; Sata et al. 2002; Caplice et al. 2003). Other reports, however, demonstrated a more modest (1%–10%) contribution of bone marrow-derived SMCs during these events (Li et al. 2001; Hillebrands et al. 2002). Moreover, SMCs were shown to transdifferentiate from circulating EPCs and mature ECs (Frid et al. 2002; Simper et al. 2002).

## 5.2

### Smooth Muscle Cell Recruitment, Growth and Differentiation

Recruitment of mural cells to nascent vessels is achieved by the involvement of several regulatory pathways (Fig. 3). The PDGF family comprises four family members (i.e. PDGF-A to D) which bind, with distinct selectivity, the receptor tyrosine kinases PDGFR- $\alpha$  and - $\beta$ , expressed on ECs and SMCs. PDGF-BB and its receptor PDGFR- $\beta$  play essential roles in the stabilisation of nascent blood vessels. By releasing PDGF-BB, ECs stimulate growth and differentiation of PDGFR- $\beta$ -positive mesenchymal progenitors and recruit them around nascent vessels (Betsholtz 2004). Absence or insufficient recruitment of periendothelial cells in mouse embryos lacking PDGF-B or PDGFR- $\beta$  increases endothelial growth and permeability, enlarges vessel size and enhances fragility, resulting in bleeding, impaired perfusion and hypoxia (Lindahl et al. 1998; Hellstrom et al. 1999). The subsequent increase in VEGF levels further aggravates vascular permeability and oedema, and promotes haemangioma formation. Similar neovascularisation occurs in the retina of diabetic subjects, when their pericytes are killed by toxic metabolites.

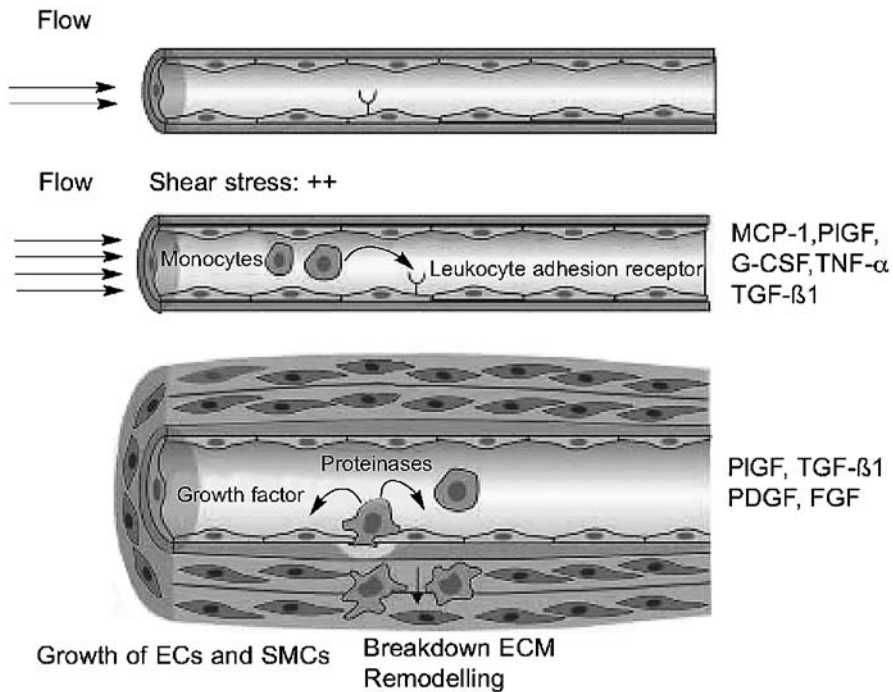
Vessels in tumours are covered by fewer pericytes than in normal tissues (Eberhard et al. 2000; Morikawa et al. 2002; Ostman 2004). These mural cells differentiate from pools of c-Kit<sup>+</sup>Sca-1<sup>+</sup>VEGFR1<sup>+</sup> perivascular progenitor cells, which are mobilised from the bone marrow in response to PDGF-B (Song

et al. 2005). When PDGFs are over-expressed, tumour vessels are covered by more mural cells and tumour growth is accelerated (Ostman 2004). Conversely, when PDGFR- $\beta$  signalling is inhibited, fewer pericytes are recruited, tumour vessels are dilated and EC apoptosis is increased. Combined administration of receptor tyrosine kinase inhibitors (RTKIs), targeting VEGFRs and PDGFR- $\beta$ , increases the anti-angiogenic effect, even in the often-intractable late stage of solid tumours (Bergers et al. 2003). PDGFR- $\beta$  inhibitors also destabilise the larger SMC-covered vessels, which supply bulk flow to tumours, and thereby render them more susceptible to EC-specific inhibitors.

The Tie-2/Ang and PlGF/VEGFR-1 signalling systems (see Sect. 4.2) are also involved in vessel stabilisation and maintenance. Ang-1, by binding the Tie-2 receptor and counteracting Ang-2 activity, recruits pericytes and promotes the interaction between nascent endothelial channels and mural cells by serving as an adhesive ECM-associated and  $\alpha 5$ -integrin binding protein (Carlson et al. 2001; Xu and Yu 2001). A precise balance of Tie-2 signals thus seems critical, as a hereditary dysfunction of Tie-2 in humans induces vascular malformations, characterised by enlarged vessels with reduced SMC coverage (Vikkula et al. 1996). PlGF, via binding to VEGFR-1, directly affects SMCs and fibroblasts which express VEGFR-1, but may also indirectly influence SMC proliferation and migration via cytokine release from activated ECs (Luttun et al. 2002). Through these effects, PlGF recruits SMCs around nascent vessels, thereby stabilising them into mature, durable and non-leaky vessels (Vikkula et al. 1996; Green et al. 2001; Ishida et al. 2001; Luttun et al. 2002).

The multifunctional cytokine TGF- $\beta 1$  promotes vessel maturation by stimulating ECM production and by inducing differentiation of mesenchymal cells to mural cells (Pepper 1997; Chambers et al. 2003). It is expressed in a number of different cell types, including endothelial and peri-endothelial cells and, depending on the context and concentration, both pro- and anti-angiogenic properties have been ascribed to TGF- $\beta 1$  (Gohongi et al. 1999). Gene targeting studies in mice underscore the importance of TGF- $\beta 1$ , its receptors (RI, RII and endoglin) and the downstream signalling molecules activin receptor-like kinase (ALK)-1 and ALK-5 in the initial phases of resolution and maturation of angiogenesis (Pepper 1997; Weinstein et al. 2000). Hereditary haemorrhagic telangiectasia (HHT), which is characterised by telangiectasia and arteriovenous malformations, has been associated with loss-of-function mutations of endoglin (HHT-1) and ALK-1 (HHT-2, Begbie et al. 2003). However, the precise underlying mechanisms of vascular defects in HHT lesions remain unresolved, as the respective roles of ALK-1 (with Smad1 and Smad5) and ALK-5 (with Smad2 and Smad3) in vessel maturation are debated (Goumans et al. 2002; Lamouille et al. 2002; van den Driesche et al. 2003).

A special type of arteriogenesis represents the enlargement of pre-existing collateral arterioles upon occlusion of a supply artery in the myocardium and peripheral limbs (Fig. 6). This process has been termed “adaptive arteriogenesis” or “collateral vessel growth” and differs significantly from the mecha-



**Fig. 6** Mechanism of collateral vessel growth. Upon occlusion of a supply feeder, the flow through of pre-existing collateral vessel increases, which activates the endothelium. As a result, endothelial cells secrete chemoattractants for monocytes and express leukocyte adhesion receptors, onto which leukocytes bind. After extravasation, monocytes produce endothelial and smooth muscle cell mitogens and proteinases to remodel the vessel wall, resulting in enlargement of the collateral vessel. A number of molecules known to stimulate this process are indicated

nisms of angiogenesis (Helisch and Schaper 2003). As a result of increased collateral blood flow and shear stress, activated ECs express monokines, including monocyte chemotactic protein 1 (MCP-1), and monocyte adhesion molecules, such as intracellular adhesion molecule 1 (ICAM-1). By producing growth factors and proteinases (uPA and MMPs), the recruited monocytes infiltrate and proteolytically remodel the vessel wall, enabling SMCs to migrate and divide. The importance of inflammatory cells in collateral vessel growth is underscored by studies in which depletion of monocytes impairs, whereas delivery of monocytes enhances, vessel enlargement (Heil et al. 2002; Kamihata et al. 2002). Cytokines attracting monocytes or prolonging their life span (such as MCP-1, granulocyte-macrophage colony-stimulating factor, TGF- $\beta$ 1 and TNF- $\alpha$ ) enhance collateral growth, whereas anti-inflammatory cytokines (such as IL-10) act as inhibitory molecules (Buschmann et al. 2001; Hoefer et al. 2002; van Royen et al. 2002; Voskuil et al. 2003). PlGF also enhances

collateral growth, not only by recruiting monocytes but also by stimulating EC and SMC growth (Luttun et al. 2002; Pipp et al. 2003; see Sect. 4.2). Delivery of FGF, FGF-4 or basic FGF stimulates collateral growth, in part via up-regulating PDGFR-expression (Cao et al. 2003; Pipp et al. 2003). However, VEGF alone appears to affect capillary angiogenesis more efficiently than collateral growth, explaining, at least in part, why results of clinical trials have not been more positive (Isner 2002; Helisch and Schaper 2003). Co-administration of VEGF with additional molecules such as PDGF, PlGF or Ang-1 may therefore enhance its therapeutic potential for the treatment of ischaemic heart and limb disease (Chae et al. 2000).

## 6

### Therapeutic Implications

Over the last decade, intensive efforts have been undertaken to develop therapeutic strategies to promote revascularisation of ischaemic tissues or to inhibit angiogenesis in cancer, ocular, joint or skin disorders. Over 500 million people worldwide have been estimated to benefit from either pro- or anti-angiogenic therapy. Unfortunately, clinical trials testing the pro-angiogenic potential of VEGF or FGF have not met the expected results (Isner 2002; Simons 2005). While some of this failure is attributable to suboptimal delivery strategies, stimulation of durable and functional vessel growth is a more formidable challenge than previously anticipated. Novel strategies involving transplantation of bone marrow-derived cells or the delivery of molecules capable of stimulating the growth of not only distal capillaries but also of proximal collateral conduit vessels will be required in the future (Chae et al. 2000; Luttun et al. 2002; Cao et al. 2003; Pipp et al. 2003; Ferrara and Kerbel 2005; Simons 2005).

Most efforts to date have been focussed on developing anti-angiogenic agents, blocking the activity of VEGF (Table 3). The first two VEGF antagonists—the anti-VEGF antibody bevacizumab (Avastin, Genentech; Ferrara et al. 2004a, 2005) and a VEGF<sup>165</sup> aptamer (Macugen, Eyetech; Cunningham et al. 2005)—have recently been FDA-approved for the treatment of malignant and ocular disease, respectively. Bevacizumab provides an overall survival benefit in colorectal, breast and lung cancer patients when combined with conventional chemotherapy. Meanwhile, monotherapy with the multi-targeted receptor tyrosine kinase inhibitors (RTKIs) sorafenib (Bayer and Onyx) or sunitinib (Pfizer), which target ECs as well as cancer, mural, stromal and haematopoietic cells, demonstrates clinical benefit in certain cancers (Branca 2005; Marx 2005; Rini et al. 2005). However, as angiogenesis is a tightly regulated process dependent on the complex interplay of numerous molecules, identifying the key targets for drug development remains challenging, and thus a number of outstanding questions still remain to be addressed. How do we clinically assess the efficacy of anti-angiogenic therapies? Is administration of a single angiogenic

**Table 3** Anti-angiogenic agents

Drug	Target	Class	Development
Bevacizumab	VEGF	MAb	FDA-approved
Anti-PlGF	PlGF	MAb	Preclinical
Anti-VEGFR-1	VEGFR-1	MAb	development
IMC-C1121b	VEGFR-2	MAb	Phase I
VEGF-trap	VEGF, PlGF, VEGF-B, -C (?), -D (?)	Protein	Phase I-II
AEE788	VEGFR-2, EGFR	RTKI	
AG-013736	VEGFR, PDGFR, c-kit	RTKI	
AMG 706	VEGFR-1,-2, PDGFR, c-kit	RTKI	
AZD2171	VEGFR-1,-2,-3	RTKI	
CEP-7055	VEGFR-1,-2	RTKI	
CHIR258	VEGFR-1,-2, FGFR-1	RTKI	
CP-547632	VEGFR-2	RTKI	
GW786034	VEGFR-2	RTKI	
KRN-951	VEGFR-1,-2,-3, PDGFR, c-kit	RTKI	
OSI-930	VEGFR-1,-2,-3, c-kit	RTKI	
XL999	FGFR, VEGFR-1,-2,-3, PDGFR	RTKI	
ZK-CDK	VEGFR-1,-2,-3; CDKs	RTKI	
ZD6474	VEGFR-2, EGFR	RTKI	
PTK787/ZK 2258	VEGFR-1,-2,-3, PDGFR- $\beta$ , c-kit	RTKI	Phase III
SU11248	VEGFR-2,-3, PDGFR- $\beta$ , c-kit	RTKI	
BAY (43-9006)	VEGFR-1,-2,-3, PDGFR, c-kit, Raf	RTKI	

MAb, monoclonal antibody; RTKI, receptor tyrosine kinase inhibitor

molecule sufficient, or do tumours easily find escape routes to switch on alternative angiogenic programmes? Will long-term anti-angiogenic treatment, and in particular combinatorial anti-angiogenic treatment, cause toxicity?

## 6.1

### Potential Biomarkers for Anti-angiogenic Therapy

Reliable biomarkers need to be established to validate the efficacy of anti-angiogenic therapy, to identify responsive patients and optimal doses, to predict efficacy of regimens that include anti-angiogenic agents, and to detect and prevent tumour escape. Traditional chemotherapy drugs are used at the maximum tolerated dose, which is determined by toxicity, and typically results in a reduction of the cross-sectional diameter of a tumour when measured on serial computed tomography scans (Simon et al. 1997). It is tempting to consider



tumour size as a marker of response to anti-angiogenic treatment. However, major functional, structural, cellular and molecular changes can occur in tumours in response to VEGF blockade without a significant reduction of tumour volume (Tong et al. 2004; Willet et al. 2004, 2005; Winkler et al. 2004). Thus, in the absence of an overt cytotoxic effect of anti-angiogenic treatment, other surrogate markers that do not rely on tumour regression must be identified. Some of the candidate markers include classic diagnostic or prognostic biomarkers, as well as newly developed, target- and mechanism-based biomarkers (Park et al. 2004).

Biopsy of tumour tissue is routinely carried out, but is invasive and not readily feasible for some tumours. It represents a prognostic method offering great potential to identify valuable markers for therapeutic efficacy, including the evaluation of protein expression, microvascular density, perivascular cell coverage of tumour vessels, cell proliferation and apoptosis, as well as genomic analysis during anti-angiogenic treatment (Willet et al. 2004, 2005; Ince et al. 2005). Measurements of interstitial fluid pressure (IFP) and tissue oxygenation are parameters that reflect vascular function and delivery of therapeutics. Changes in IFP and tissue oxygen levels might represent valuable surrogate markers of efficacy and vessel normalisation during anti-angiogenic treatment (Willet et al. 2004, 2005).

Less invasive methods include determining changes in growth factor protein concentrations in bodily fluids. For instance, plasma levels of VEGF have been found at higher levels in many tumours and in metastatic disease, and have long been regarded as a potential surrogate marker of cancer growth and anti-angiogenic drug efficacy (Bocci et al. 2004). However, many studies of its use as a surrogate marker have not proved its reliability (Brower 2003). Meantime, new research on tumour growth markers is shifting away from measuring levels of VEGF to measuring its effects, such as the recruitment of progenitor cells from the bone marrow to the tumour where they contribute to neovascularisation. Indeed, recent studies indicate that circulating progenitor cells might represent a reliable surrogate marker of anti-angiogenic therapy (Rafii et al. 2002; Willet et al. 2004, 2005; Beaudry et al. 2005; Blann et al. 2005; Shaked et al. 2005). However, their population is heterogeneous and their concentration in whole blood in humans is very low (Shaked et al. 2005). Measuring, therefore, the number of viable and apoptotic CECs might be more feasible (see Sect. 3.2).

Non-invasive techniques including dynamic contrast-enhanced magnetic resonance imaging, spectral imaging and positron emission tomography have the potential to measure functional parameters and offer surrogate markers regardless of tumour type and localisation (Jennens et al. 2004; Collins 2005; Liu et al. 2005; Miller et al. 2005a). The improvement of these techniques for monitoring the spatial and temporal changes in tumour blood flow, vascular structure, permeability and tumour metabolism (Cohen et al. 1994; Wu et al. 1994; Pham et al. 1998; Herbst et al. 2002; Pahernik et al. 2002; Morgan et al.



2003; Willet et al. 2004; Xie et al. 2004) might allow us to assess more precisely the efficacy of anti-angiogenic treatment.

## 6.2

### Escape from Anti-angiogenic Therapy

Despite promising successes, cancer patients receiving a single class of angiogenesis inhibitors still die. Does that suggest the strategy is insufficient, or does it evoke resistance; if so, how can we avoid resistance? Emerging evidence indicates that treatment with a single anti-angiogenic molecule is more challenging than anticipated, and may not suffice to combat the wide array of angiogenic factors produced by cancer cells and the growing tumour. Redundancy of alternative angiogenic factors in anti-angiogenic monotherapies, up-regulation of angiogenic or anti-apoptotic factors, and heterogeneity of vascular dependency among tumour subpopulations are possible mechanisms that may contribute to the development of acquired drug resistance during anti-angiogenic treatment (Kerbel et al. 2001, Broxterman et al. 2003; Miller et al. 2005b). Indeed, inhibition of a single target leads to up-regulation of additional angiogenic factors. For instance, PlGF is up-regulated after anti-VEGF therapy (Willett et al. 2005), VEGF after anti-VEGFR-2 or anti-EGFR administration (Bocci et al. 2004; Vioria-Petit and Kerbel 2004; Bianco et al. 2005), and IL-8 after HIF-1 deletion (Mizukami et al. 2005). Mutant tumour-cell clones, such as those lacking p53, are able to survive in hypoxic conditions, and their reduced vascular dependence impairs the anti-angiogenic response (Yu et al. 2002). Tumour stromal cells, upon prolonged anti-VEGF treatment, switch to other pro-angiogenic factors (PDGF-B, ephrinB), allowing regrowth of the tumour vasculature (Huang et al. 2004). Furthermore, the PDGFR- $\alpha$  signalling pathway has recently been identified as an alternative mechanism for stromal fibroblast recruitment when tumour cells are deficient in VEGF production (Dong et al. 2004). End-stage tumours might contain more pericyte-coated vessels, explaining why EC-targeted therapies fail to induce regression of established tumours, while targeting pericytes in addition to ECs was more effective (Bergers and Hanahan 2002; Bergers et al. 2003).

Thus, combined treatment of anti-angiogenic agents with distinct complementary mechanisms of action, targeting other angiogenic molecules and targeting not only ECs but also other pro-angiogenic cell types may offer advantages of increased efficacy—at least if toxicity is not a concern (see the following section). Another advantage is that such combinations may give the tumour less chance to escape from anti-angiogenic treatment. Exploring strategies to delay, minimise or even avoid resistance to anti-angiogenic agents might further increase the benefit of anti-angiogenic treatments.

### 6.3

#### Adverse Effects of Anti-angiogenic Therapy

As anti-angiogenic agents will hopefully be delivered earlier and earlier to more and more patients for less advanced and life-threatening disease, probably in combination with additional medications, the safety of anti-angiogenic treatment is a topic of emerging importance. Apart from side-effects experienced in clinical trials (Hurwitz et al. 2004), pharmacological and genetic studies in mice revealed that inhibition of VEGF-driven angiogenesis might be expected to cause many more adverse effects (Marx et al. 2002). Fortunately, such toxicity has not yet been observed in humans, but it may emerge in conditions where genetic or iatrogenic predisposition increase the risk. Some of the unwanted toxicity of VEGF/VEGFR-2 targeting agents can be explained by the requirement of threshold levels of VEGF for the survival and maintenance of quiescent vessels in healthy organs (see Sect. 4.5; Gerber et al. 1999; Eremina et al. 2003; Tang et al. 2004a; Baffert et al. 2005; Kamba et al. 2005). A better understanding of why the PlGF/VEGFR-1 pathway is effective only in pathological but not in physiological angiogenesis (Carmeliet et al. 2001; Luttun et al. 2002; Autiero et al. 2003) should therefore be relevant for the design of safe therapeutic strategies. The challenge now involves developing these novel anti-angiogenic strategies and optimising combinatorial treatment regimens to fully exploit the therapeutic potential of angiogenesis inhibition.

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