
Endothelial Cells in the Tumor Microenvironment

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Abstract

Angiogenesis is a critical process required for tumor progression. Newly formed blood vessels provide nutrition and oxygen to the tumor contributing to its growth and development. However, endothelium also plays other functions that promote tumor metastasis. It is involved in intravasation, which allows invasive cancer cells to translocate into the blood vessel lumen. This phenomenon is an important stage for cancer metastasis. Besides direct association with cancer development, endothelial cells are one of the main sources of cancer-associated fibroblasts (CAFs). The heterogeneous group of CAFs is the main inducer of migration and invasion abilities of cancer cells. Therefore, the endothelium is also indirectly responsible for metastasis. Considering the above, the endothelium is one of the important targets of anticancer therapy. In the chapter, we will present mechanisms regulating endothelial function, dependent on cancer and cancer niche cells. We will focus on possibilities of suppressing pro-metastatic endothelial functions, applied in anti-cancer therapies.

Keywords

Endothelial cells · Cancer development · Tumor endothelial cells · Cancer microenvironment · Cancer niche · Sprouting · Metastasis · CAFs · Microvessels · Tip cells · Tumor angiogenesis · VEGF · Hypoxia · Endothelial-mesenchymal transition · TGF- β

6.1 Introduction

The vascular endothelium is a versatile structure that separates the circulating blood from tissues. Moreover, apart from regulation and maintenance of blood fluidity, it plays multifunctional roles in the delivery of water and nutrient, maintenance of metabolic homeostasis, trafficking of immune cells, activation of innate and acquired immune responses, as well as angiogenesis [30, 73]. The endothelium is a thin monolayer, composed of endothelial cells (ECs) that are able to organize the growth and development of connective tissue cells, forming the surrounding layers of the blood vessel wall. This process is controlled by a paracrine/endocrine network which involves fibrinolytic, pro- and anticoagulants, vasoactive, pro- and anti-inflammatory factors, as well as growth factors produced by ECs [84]. Thus, ECs must be constantly poised to sense and respond to changes within their environment. In tumor and its microenvironment, some agents

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like hypoxia and chronic growth factor stimulation might lead to endothelial dysfunction. There is more and more evidence showing that these abnormalities contribute to cancer progression.

The tumor has been recently described as an aberrant organ not only composed of cancer cells but also of numerous stromal, inflammatory, and vascular cells. Like other organs, in order to develop, the tumor requires a blood supply to provide nutrients and oxygen and waste removal. Initially, cancer cells might adopt tissue-resident vessels. However, the tumor eventually recruits its own vascular supply through the angiogenesis process [123]. The tumor-associated angiogenesis has been defined as sprouting of new vessels from preexisting vessels, which involves endothelial cells [112]. Tumor modulates its microenvironment by releasing numerous cytokines, chemokines, and growth factors to activate normal, quiescent endothelial cells and adapt them to the angiogenic response. Moreover, surrounding stromal cells might also secrete a plethora of factors and cytokines influencing tumorigenesis and metastasis. Within them, TGF- β is considered one of the main factors modulating interactions between cancer and surrounding cells, located within the tumor niche. Among the TGF- β -dependent effects is regulation of cancer cell proliferation, affecting immune response by suppressing immune cells function, conversion of fibroblasts to myofibroblasts and epithelial-mesenchymal transition (EMT). Furthermore, TGF- β promotes the formation of cancer-associated fibroblasts (CAFs), a specialized group of fibroblasts involved in tumor growth and invasion of cancer cells by modulation of the tumor niche [119]. Until now normal fibroblasts (NFs) have been considered the main source of CAFs, but in the last years, endothelial cells have also become an important origin of CAFs. It has been shown that TGF- β is responsible for such EC conversion in a process called endothelial-mesenchymal transition (EndMT) [56]. During EndMT, endothelial cells lose endothelial markers and gain mesenchymal ones, which is followed by increased expression of transcription factors such as Snail and Slug. The changes are accompanied by defaulting of their cellular func-

tion and taking on some characteristics of mesenchymal cells, including loss ability to form capillary tubes and cell-cell junctions, increased cell migration properties, and secretion of extracellular matrix proteins.

In this review, we will focus on the role of endothelial cells in tumor microenvironment particularly on their direct and indirect role in cancer metastasis. While endothelial cells were originally believed to be involved in the direct development of primary tumor due to vascularization, there is more and more evidence suggesting their indirect effect on cancer progression. CAFs are known to play an important role in tumor growth and progression via secretion of various growth factors and chemokines. The contribution of endothelial cells in CAF formation will be discussed. Finally, we will also present current and future therapeutic possibilities targeting at endothelial cells, CAF formation, and chemokines in the context of anti-metastatic treatment.

6.2 Heterogeneity of Normal and Tumor Endothelial Cells

The vascular endothelium is a specific inner cellular lining that separates the circulating blood from the tissues. That thin monolayer plays an important multifunctional property, including the control of vasomotor tone, proliferation/angiogenesis, permeability, hemostasis, humidification, thermoregulation, leukocyte transmigration, sieve function, and scavenging innate and adaptive immunity [2]. This plethora of functions is a consequence of the fact that ECs, being part of the vascular tree, are differentially regulated in space and time. Thus, ECs differ in various organs, but also between distinct segments within or between neighboring of vascular architecture of the same organ. The EC thickness varies across the vascular tree, ranging from less than 0.1 μm in capillaries and veins to 1 μm in the aorta [2]. Endothelial cells are usually flat, but they might be plump or cuboidal occasionally [2]. Endothelium cells in monolayer are held by two main types of junctions: adherent junctions (AJs) and tight junctions (TJs). Their organization

varies along the vascular tree [12]. For instance, the large artery is rich in TJs, whereas venules display less organized TJs. Similarly, in the brain, where protection of the nervous system is required, junctions are well developed and rich in TJs [31]. In contrast, post-capillary venules have a poorly organized TJs due to the dynamic trafficking of circulating cells and proteins suspended in plasma [31]. Another feature of endothelium diversity is its continuity. Continuous endothelium might be fenestrated or non-fenestrated. Fenestrated continuous endothelium is found in the places where increased filtration or increased transendothelial transport is needed, like capillaries of exocrine and endocrine glands. Non-fenestrated continuous endothelium is found in capillaries, veins, and arteries. Discontinuous endothelium occurs in some sinusoidal vascular beds, first of all in the liver [2]. It has been proposed that angiogenesis, being one of the main processes engaging endothelial cells, requires at least a few cells of discontinuous. ECs, called tip cells, are directly engaged in vessel sprouting. Highly proliferative stalk cells follow tip cells, and phalanx cells that are involved in improving the perfusion and oxygenation of newly formed blood vessels [51].

Mentioned ECs heterogeneity is provided mainly by one of two distinct mechanisms based on microenvironment pressure or epigenetic modulation [3]. Endothelium is not only a specific inner cellular lining separating the circulating blood from the tissues, but it is exposed to a great variety of factors, secreted by tissue microenvironments. Moreover, to properly perform its functions across the vascular tree, ECs have to detect and respond to environmental stimuli, which is guaranteed by endothelial cells heterogeneity. This mechanism is reversible when ECs are removed from their microenvironment and grow in tissue culture. The second mechanism involved posttranscriptional modification that seemed to be epigenetically programmed and independent of extracellular signals. Although it is widely accepted that microenvironment stimulation is responsible for triggering epigenetic modifications, they may remain during the removal of the signals and be transmitted during mitosis [3].

It should be noted that EC heterogeneity also translates into the heterogeneity of tumor endothelium. In line with Folkman's hypothesis, tumor growth strictly depends on blood vessels [41]. At the same time, tumor blood vessels are formed by ECs recruited from surrounding tissue transformed to tumor endothelial cells (TECs). The tumor vasculature, in contrast to well-differentiated normal vessels, it is composed of a chaotic mixture of abnormal, disorganized artery–capillary–vein hierarchy vessels [109]. Unlike normal blood vessels, tumor vessels are more dilated and tortuous. They branch irregularly, have chaotic flow patterns, and increased permeability to macromolecules [75]. Due to an imbalance between pro- and antiangiogenic factors and with a predominance of stimulators (angiogenic switch), a classic hierarchical branching pattern system of arterioles, veins, and capillaries is disturbed. The layout of neoplastic capillaries is morphologically immature: chaotic, strongly twisted, with variable vessel diameter and irregular edge [29]. In line to the unsettle tumor vasculature, endothelial cells, forming tumor vessels, are structurally abnormal. TECs have a disturbed redistribution of phospholipids, a discontinuous or absent basement membrane, increased fenestrations and extended intercellular junctions, and a high proliferative rate compared to normal ECs and tend to grow one on top of the other and invade into the vessel lumen [3]. Phenotypic changes, accompanied by changes at the molecular levels, have been identified comparing normal ECs to TECs, isolated from normal and tumor tissues. In 2000, St. Croix et al. performed a comparative analysis of gene expression profiles between tumor endothelial cells and normal endothelial cells and identified the specific genes for TEC called tumor endothelial markers (TEMs) [95]. Since then, several studies have been published on molecular differences between TECs and NECs [15, 66, 77] e.g tumor endothelial markers (TEMs), endoglin (CD105), or endothelial protein-disulfide isomerase EndoPDI [50] has been also demonstrated that TECs can secrete several factors that affect their survival in an autocrine manner [17, 18, 74, 101].

Increased permeability of the walls, hemorrhage, and plasma leakage result from a reduced number of pericytes and increased proteolytic activity within the vessel formation zone. TECs are characterized not only by an increased size, but they also presented aneuploidy, abnormal centrosomes, and high activation of the MAPK pathway, promoting cell survival [5, 43]. TECs exhibit several differences which contribute to their proangiogenic phenotype, including changed responsiveness to growth factors such as EGF, adrenomedullin, and VEGF. VEGF stimulates the migration of TECs and enhances their survival in an autocrine manner, which leads to the antiapoptotic phenotype of TECs [51]. TECs show upregulated aldehyde dehydrogenase (ALDH) expression which is manifested by a formation of increased tube number even under starvation conditions [80].

It is suggested that the persisting hypoxia together with the secretion of cytokines promotes tumor angiogenesis by inducing the mobilization of bone marrow-derived endothelial progenitor cells to cancer [45]. Glioblastoma cells and lymphoma ones are examples of tumor cells that are capable of differentiating into TECs [98, 106]. Interaction between tumor cells and the microenvironment leads to alteration of ECs into TECs that express high levels of biglycan through epigenetic modifications, which stimulates tumor cells to metastasize through activation of different signaling pathways [67]. Furthermore, it was reported that endothelial progenitor cells release microvesicles with gene fragments that can activate endothelial cell angiogenic properties [33]. Due to the mechanisms mentioned above, TECs become cytogenetically abnormal and unstable in the tumor microenvironment.

6.3 Angiogenesis in Tumor Development

Efficient functioning of the circulatory system, responsible for gas exchange, transport of nutrients, and metabolic products, is the basic condition for appropriate development during ontogeny. In embryo development, de novo formation of

the vascular plexus from angioblasts (EPCs; endothelial precursor cells) is one of the earliest organogenesis processes, called vasculogenesis [1]. Next, the existing vascular network undergoes proliferation, reorganization, and maturation in the process of angiogenesis (neovascularization) [11]. A new capillary mesh network is created by sprouting of endothelial cells. The last stage is the maturation of the vessel through the migration of pericytes and vascular smooth muscle cells (VSMCs) on a newly formed basal membrane (BM). Under physiological conditions, neovascularization occurs during embryo implantation, the women's monthly cycle, and wound healing, and in the muscles [16]. In pathological conditions, when the activity between pro-angiogenic factors and antiangiogenic ones is disturbed, it occurs during chronic inflammation and hypoxia and in asthma, rheumatoid arthritis, psoriasis, Crohn's disease, diabetic retinopathy, as well as endometriosis and obesity. However, angiogenesis plays the most significant role in the process of neoplasia [65].

In the initial stage, in order to survive and proliferate, tumor takes oxygen and nutrients by diffusion. The environment in which it develops undergoes hypoxia and acidification as a result of excess metabolic products. When its volume exceeds 1–2 mm³, the tumor must become angiogenic and recruit their vasculature to grow. Cancer cells, together with host/niche cells, stimulate the development of their blood vessels, using various mechanisms of tumor angiogenesis [36]. The most common one and best described is vessel sprouting (Fig. 6.1). In the classical model, the vasodilatation of the mother vessel occurs, which contributes to reduced BM density. It leads to partial degradation of BM and protrusion of endothelial cells in that place. As the ECs do not lose intracellular connection with each other and they migrate parallelly, the polarity of the cells is preserved. At the same time, the new lumen is formed by polarized ECs. They release proteins which rebuild the basal membrane along which pericytes migrate. This phenomenon stabilizes the capillary and contributes to its maturation [83]. The last step of vessels maturation described above is impaired during cancer angiogenesis.

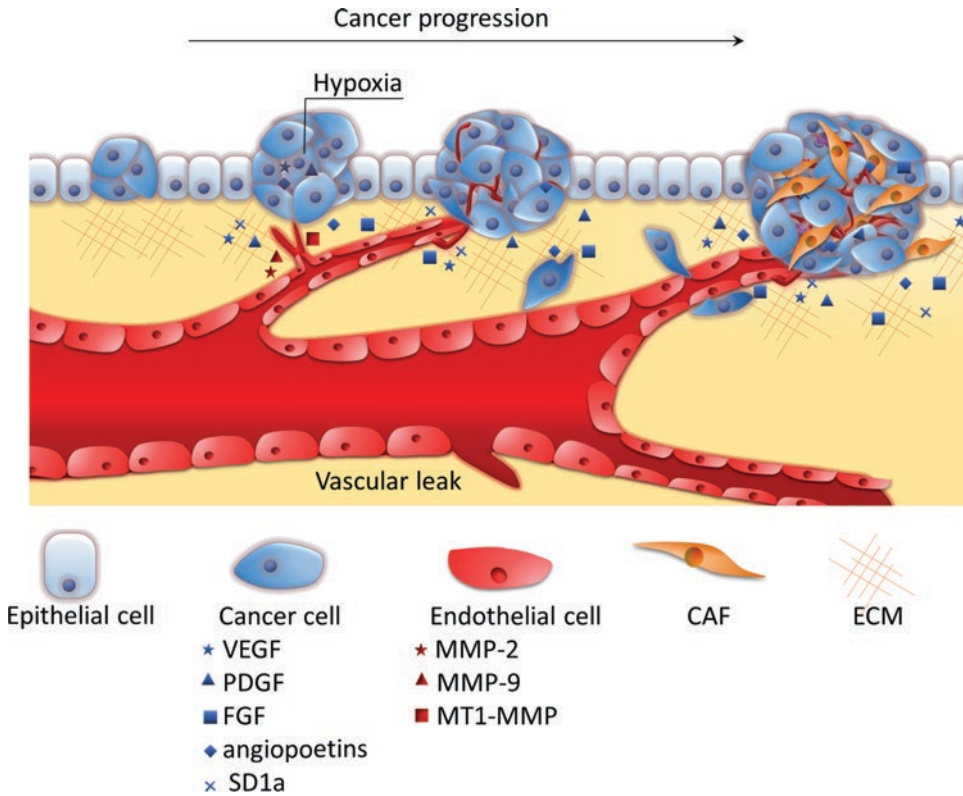


Fig. 6.1 Mechanisms of tumor vascularization. At the point when developing cancer reaches its size 1–2 mm, hypoxia and nutrient deprivation result in release of tumor cell-soluble growth factors, chemokines, and cytokines (VEGF (blue star), PDGF (triangle), FGF (square), angiopoietins (diamond), and SD1a (cross)). The factors induce the sprouting and proliferation of endothelial cells on nearby blood microvessels. The created tumor blood vessels are leaky and tortuous with partially exposed basal

lamina where vascular leaks are observed. Additionally, the vascular remodeling is also enhanced by factor secreted by cancer-associated fibroblasts (CAFs) that are recruited to the tumor niche. CAFs cause the rearrangement the profile of extracellular matrix protein and release matrix metalloproteinases (MMPs: MMP-2 (red star), MMP-9 (triangle), and MT1-MMP (square)) that cleave and remodel ECM therefore activating the endogenous angiogenesis inhibitors such as tumstatin and endostatin

Hypoxia-induced factor-1 α (HIF-1 α) is the main factor that initiates sprouting [69]. It induces secretion by ECs proangiogenic factors such as platelet-derived growth factor, type B (PDGF-B), hepatocyte growth factor (HGF), angiopoietins, epidermal growth factor (EGF), placental growth factor (PIGF) [29, 65] and is the main stimulator of angiogenesis vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) [94]. VEGF, which works in an auto- and paracrine manner, contributes to extravasations of plasma proteins, e.g., fibrinogen, which initiates integrin-dependent migration of ECs, a release of metalloproteinases, and activation of the mitogen-activated protein kinase (MAPK) pathway.

Digestion by MMPs of extracellular matrix (ECM) releases the tumor growth factor (TGF- β), basic fibroblast growth factor (FGF-2), and insulin-like growth factor-1 (IGF-1), i.e., anti-apoptotic factors, activating the survival signal transduction pathway [27, 47, 52]. The second group consists of tissue-resident cells, including normal tissue epithelial cells, vascular cells (endothelium and pericytes), normal fibroblasts, adipocytes, and leukocytes (mast cells and macrophages) [20, 22, 34, 92]. It has been confirmed that leukocytes as well as ECs are important sources of VEGF-A, which is able to accelerate tumor angiogenesis [35]. But TASCs might increase vascular density in human tumors

through secretion of other numerous chemokines and growth factors (Fig. 6.1).

Cancer stem cells (CSCs) can differentiate to endothelial cells and, as a consequence, induce new vessels via a phenomenon known as vascular mimicry. However, that ability does not lead to the form of mature and proper blood vessels which would counteract hypoxia. During progression, cancer recruits numerous types of cells to the cancer niche, which can modulate tumor vascularization. The cells located in the tumor microenvironment, called tumor-associated stromal cells (TASCs), can be divided into two main groups. Leukocytes (lymphocytes, neutrophils, monocytes, and macrophages) infiltrating tumor constitute the first group delivered from the bone marrow via systemic circulation. Macrophages that are recruited to the tumor environments, called TAMs (tumor-associated macrophages), have been described as a source of non-thrombogenic EC-like surfaces, constituting a potential scaffolding for tumor vascularization through mimicry vasculare [89]. However, the mechanism of that process is still unknown.

Tumor cells play a crucial role in initiation and regulation of cancer angiogenesis. It must be noted that, other cells, located in the tumor niche, also secrete numerous signaling molecules and induce pathways that influence the angiogenic response. Apart from sprouting new vessels in response to VEGF stimulation, blood vessels might also originate from cells of the bone marrow or tumor stem cells dedifferentiated to ECs (vascular mimicry). A wide diversity of molecular pathways which are able to induce tumor vascularization can make antiangiogenic therapies ineffective [96].

Tumor endothelial cells may undergo endothelial to mesenchymal transition (EndMT) and become carcinoma-associated fibroblasts, CAFs. It was demonstrated that stromal-derived factor-1 (SDF-1) in CAFs recruits EPCs promoting angiogenesis. Overexpression of MMP-2 by CAFs stimulates epithelial hyperplasia and abnormal branching in the mammary gland. It was shown that high level of MMP-2 production in stromal cells is required to support pathological neoan-

giogenesis of gliomas. Neovascularization is promoted also by induction of IL-8 secretion by CAFs, isolated from metastatic colon cancer patients [117]. CAFs express a membrane-bound serine protease, called fibroblast activation protein (FAP), which is associated with poor prognosis in several cancer types.

Significant associations were found between tumor angiogenesis and miRNAs in activated endothelial cells. miRNAs have opposing effects on cancer and endothelial cells. Their overexpression inhibits angiogenesis and enhances proliferation of cancer cells. MicroRNA-126 (miR-126) is an endothelial-specific miRNA that regulates angiogenic signaling and vascular integrity as a negative regulator of VEGF-A. However, it was observed that overexpression of miR-126 in endothelial cells enhances VEGF-A activity and promotes vessel formation by repressing the expression of sprouty-related protein-1 (Sprd-1) [105]. In oral squamous cell carcinoma, a low miR-126 expression is correlated with tumor progression through the activation of angiogenesis and lymphangiogenesis via VEGF-A pathway [91]. miR-126 is involved in cancer cell–stromal cell crosstalk. CAFs induces downregulation of miR-126 in adjacent human umbilical endothelial cells (HUVEC). The lowered miR-126 confers increased tube formation in the early invasive stage of cervical cancer [53]. VEGFR2 can be targeted by miR-221 and miR-222 [55].

6.4 Intravasation of Cancer Cells

Metastasis is a multi-step process, divided into two main phases: (1) translocation of cancer cells from the primary tumor to distant tissues and (2) colonization of these cancer cells at the secondary site [48]. Here we focused on the role of the endothelium in the first phase. The tumor metastatic potential is dependent on its rapid extravasation into the vascular system [13, 61, 82]. That process is composed of several steps: adhesion of invading cancer cells to ECs, changes in the endothelial barrier and intravasation,

dissemination into the bloodstream as migrated and proliferated circulating tumor cells (CTCs), and finally, after extravasation, colonization of other organs [6, 59, 61, 97]. Transendothelial migration (TEM) of invasive cancer is a critical phenomenon in the intra- and extravasation. During that phenomenon, tumor cells migrate between two endothelial cells [61, 93]. In vitro studies suggest that tumor cells might also pass through individual endothelial cells, in a process called transcellular migration [58, 99]. An interaction between transmigrated cancer cells and ECs induces contraction and disruption of their cell-cell contacts as well as secretion of pro-inflammatory factors by the latter [103]. Metastatic microenvironment is also characterized by platelet aggregation and formed microthrombi which promote ECs activation through induced inflammation [100]. As described above, blood vessels arising during cancer progression [49] are usually immature without proper junctional contact between ECs. The blood vessels are leaky and vulnerable due to abnormal pericyte coverage. Those injuries enable cancer cells to intravasate through the blood barrier [37, 116]. According to a favorable theory, both intravasation and extravasation are active processes, regulated by several factors such as TGF- β [7], VEGF [38, 60, 86], angiopoietin-2 (Angpt2) [88], stromal-derived factor-1 α (SDF-1 α) [118], or TNF [121]. A notable difference between intravasation and extravasation is found in the fact that intravasation mostly involves abnormal tumor vasculature whereas extravasation targets at normal blood vessels. It has been observed that the interaction between tumor cells and ECs is modulated by VEGF or TNF favor intravasation. The process is modulated when the number of blood microvessels increases and disruption of the blood barrier occurs [Fig. 6.2]. Additionally, presence of macrophages seems to be necessary for this process. However, macrophage-secreted TNF increases endothelial permeability, but its depletion does not reduce intravasation. The authors suggested the importance of other macrophage-secreted factors (probably IL-6) or juxtacrine interactions in induction of intravasation.

They also prove the importance of remodeling of the endothelial barrier, induced by tumor-endothelial interaction for translocation of tumor cells via the blood barrier [121].

Some data suggested that endothelial-mesenchymal transition, leading to disruption of the cell-cell junction between ECs and disruption of blood barrier, also contributes and facilitates cancer cell intravasation.

6.5 CAF Formation

Endothelial cells forming a single-cell layer lining the inner surface of the blood vessels [55] are characterized by wide plasticity [23]. During cancer progression, the endothelium that undergoes endothelial-mesenchymal transition (EndMT) is becoming, besides normal fibroblasts (NFs), one of the main sources of cancer-associated fibroblasts (CAFs). It has been postulated that about 40% of CAFs are formed from endothelial cells [56].

During EndMT, cells lose cell-cell connections, detach from the cell layer, and elongate. Additionally, their adhesion ability is decreased, and migration properties increased (Fig. 6.3). Those behavioral modulations are accompanied by decreased endothelial marker levels, such as CD31 (platelet endothelial cell adhesion molecule-1 (PECAM-1)) or claudin, and gain of mesenchymal markers, such as fibroblast-specific protein 1 (FSP1; S100A4) or α -smooth muscle actin (α SMA) [9, 10, 76, 85, 120]. CAFs are also characterized by increased expression of contraction proteins like caldesmon and tropomyosin [26, 124].

The best-known inducers of EndMT belong to the transforming growth factor superfamily (TGF- β) which includes TGF- β 1, TGF- β 2, and bone morphogenetic protein (BMP). Activation of receptors for these factors leads to an induction of Smad-dependent and Smad-independent pathways [40, 102, 71]. In the canonical pathway, TGF- β 1 or TGF- β 2 binds to constitutively activate II TGF- β receptor (TGF- β RII) and then to recruit and activate I TGF- β receptor (TGF- β RI)

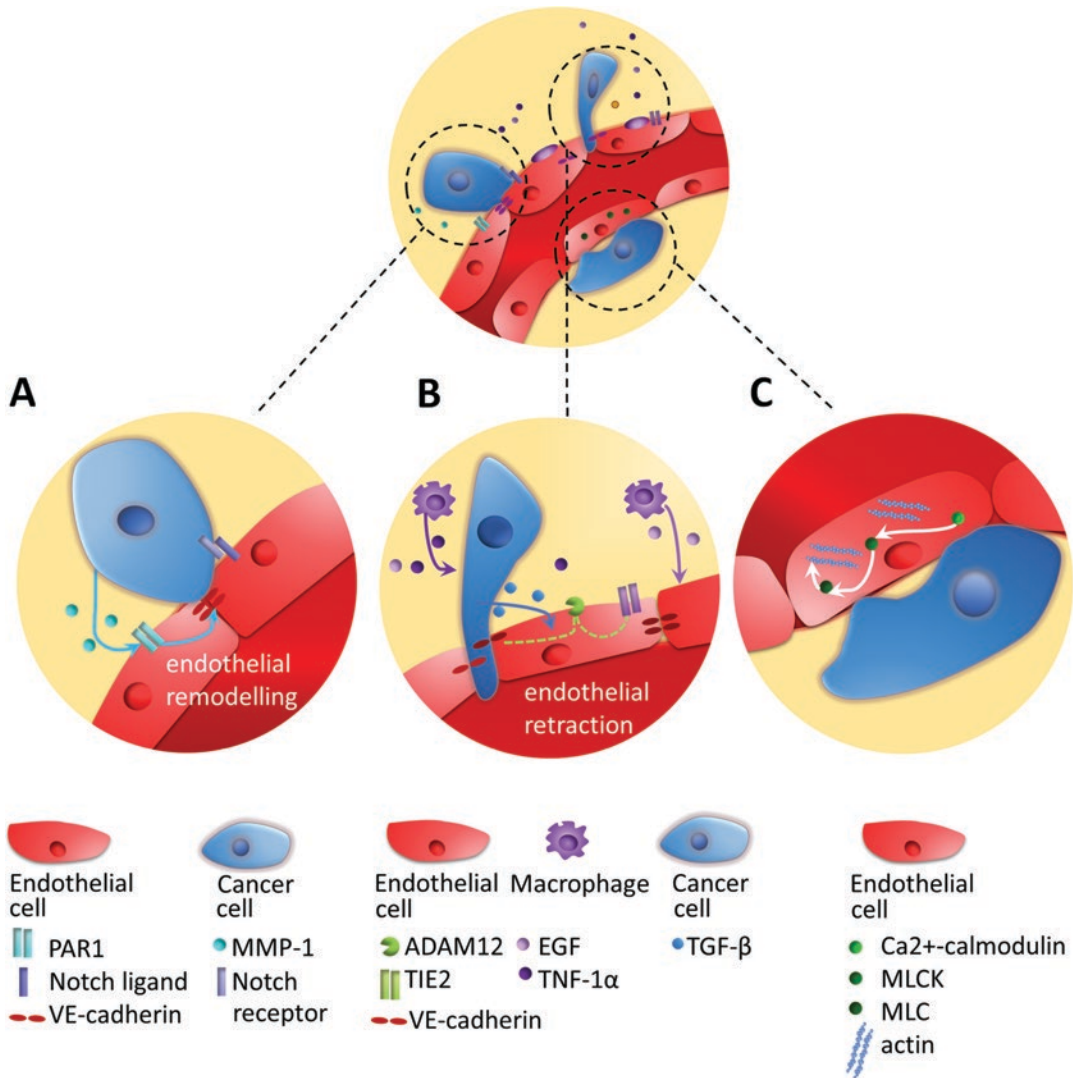


Fig. 6.2 Molecular pathways that regulate the intravasation. Cancer cells enter the circulation by transmigrating either paracellularly through the endothelial cell (EC) junctions or transcellularly through the EC body. Matrix metalloproteinase 1 (MMP-1) is crucial for paracellular intravasation in regions where protease-activated receptor 1 (PAR1) on ECs mediates the remodeling of endothelial junctions (a). Cancer cells can use Notch receptors to bind to Notch ligands on ECs and thereby transmigrate through the endothelial junctions (a). Alternatively, a vascular endothelial cadherin (VE-cadherin) and angiopoietin-1 receptor (TIE2) are cleavage by metalloproteinase-12 (ADAM12), which leads to disruption of endothelial junctions (b). Cancer cells moving to blood vessels are also

promoted by tumor-associated macrophages (b) by secreting epidermal growth factor (EGF). Retraction of endothelial junctions, that facilitate cancer cell transendothelial migration (TEM), might be induced by transforming growth factor β 1 (TGF β 1) secreted by cancer cells (c). That process can be stimulated by macrophage-secreted tumor necrosis factor 1 α (TNF1 α) as well. Transcellular intravasation is observed in sites of cancer cell attachment. There complexes of Ca²⁺–calmodulin induce phosphorylation of myosin light chain (MLC) by myosin light chain kinase (MLCK) causing actomyosin contraction. Finally, that pathway results in creation of transitory pore-like structure enable cancer cell to cross the EC barrier

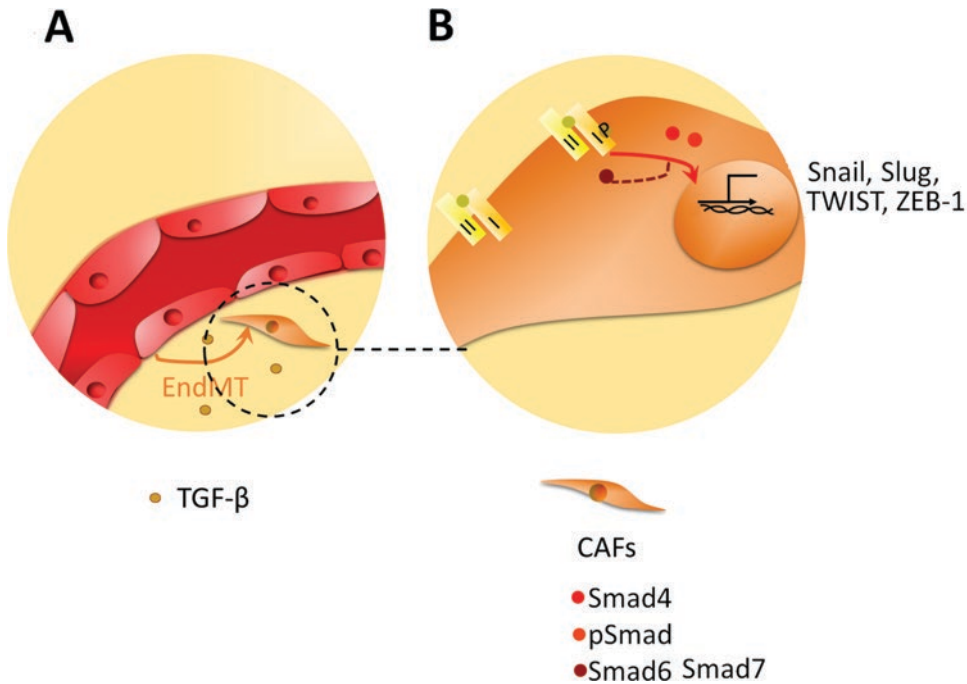


Fig. 6.3 Endothelial-mesenchymal transition. During the tumor progression, the tumor cells secrete TGF- β (a), which affects EndMT. TGF- β by stimulation the located in cell membrane TGF β RI (I) and constitutively active TGF β RII (II) leads to the activation of Smad proteins, which translocate to the nucleus (b). The Smad proteins

induce expression of Snail and Slug, TWIST, and ZEB-1 transcription regulators. These transcription modulators cause an increase in the expression of mesenchymal markers and numerous cytoskeletal proteins, leading to the elongation of the endothelial cell and induced its invasive character (as described in the text)

via its phosphorylation [90]. The last of the receptors binds and phosphorylates Smad2/3 which made a complex with Smad4. The created transcription complex moves to the nucleus and triggers the expression of numerous genes which are specific for EndMT [40, 70] such as NOTCH1, TWIST1, and SNAI1/2 [102]. The *in vitro* observation was confirmed during *in vivo* analysis on mouse models. The knockdown and knockout of several TGF- β signaling-related genes, such as SMAD2, SMAD3, and TGFBR2, prevented EndMT [28, 115].

TGF- β signaling might be induced indirectly by caveolin-1 (CAV1), Wnt pathway, and endothelin-1 (ET-1). CAV1, located in caveolae, is involved in the internalization of TGF- β receptors [32]. It has been shown that its expression is upregulated during cancer progression. *In vivo* studies demonstrated that lack of CAV1 induced

spontaneous EndMT in mice model. Additionally, TGF- β might accelerate the process [62]. Wnt proteins are involved in EndMT by Smad-dependent TGF- β signaling. They can modulate the phenomenon through canonical (i.e., involving β -catenin) and non-canonical Wnt signaling pathways [4, 63, 107]. Although numerous studies demonstrated that Notch signaling work together with TGF- β pathway [21, 42, 79, 107], it has been shown they act independently in development of Kaposi's sarcoma-associated herpes virus [46]. It has been recently revealed that ET-1, which is an endogenous vasoconstrictor polypeptide, might alone or together with TGF- β cause EndMT in human ECs [25, 113, 114].

Cellular elongation and acquisition of migration ability observed during EndMT correlate with cytoskeleton remodeling. The alterations concern to all types of cellular filaments such as

microfilaments, microtubules, and intermediate filaments. EndMT is characterized by a gain of vimentin expression, which was described as a marker of mesenchymal cells. The regulation of actin cytoskeleton is controlled by proteins belonging to the Rho GTPase family (RhoA, RhoB, Rac-1, cdc42) whose activity is regulated by TGF- β signaling. Activation of small G-proteins causes incorporation of globular actin proteins (G-actin) into filaments of F-actin. This process is critical to forming the stress fibers and results in an increased contraction ability of CAFs [87]. The G-actin pool is released from cytosolic complexes with MRTFs (MRTF-A and MRTF-B) which in “actin-free stage” translocate to the nucleus. MRTFs are the well-described coactivators of serum response factor (SRF) which regulate expression of cytoskeleton regulators and focal adhesion protein such as FAK, vinculin, and α -SMA, necessary for shaping the mesenchymal and contractile nature of CAFs [81]. In our studies, we found that activation of MRTFs are dependent on RhoA and Rac-1/MMP-9 and finally induce ILK and vinculin expression [26]. That axis regulates generation and maturation of focal adhesion, which is characterized by accelerated cell movement, typical for EndMT.

Microtubules, the largest cytoskeleton fibers are involved in the translocation of newly expressed mesenchymal markers, one of which is N-cadherin [68]. It has been proposed that the alteration of β -tubulin subunit expression modulates microtubule dynamics [44]. We revealed that upregulation of tubulins β -3 and β -4 levels, during EndMT, is critical for faster CAFs movement [110, 111].

6.6 Perspective: Endothelium as the Therapeutic Agent in Anticancer Therapy

6.6.1 Antiangiogenesis [AA] Therapies

A variety of signaling molecules such as VEGF-VEGFRs, ephrin-Eph receptors, angiopoietin-Tie, and the Delta-Notch play important roles in angiogenesis. These vascular endothelial growth

factors and their receptors regulate both vasculogenesis and pathological angiogenesis. The VEGF family members, i.e., VEGF-A/VEGF-B/VEGF-E and PlGF, regulate angiogenesis and vascular permeability by activating receptors VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk1 in mice). VEGF-C/VEGF-D and their receptor VEGFR-3 (Flt-4) are mainly observed in lymphangiogenesis. VEGFR-2 is a major signal transducer for neovascularization by the activation of the MAPK signaling pathway. VEGF-A, which demonstrates a variety of functions, including proangiogenic and vascular permeability activity, is the main player. Due to this fact, the VEGF-VEGFR system is an important target for antiangiogenic therapy in cancer progression [94, 104]. Currently, there are four main approaches targeting at cancer angiogenesis tested in clinical trials and approved for clinical practice: (1) neutralizing monoclonal antibody that binds circulating VEGF; (2) recombinant protein (decoy receptor or VEGF-Trap) that binds more than one proangiogenic growth factor; (3) small-molecule tyrosine kinase inhibitors that block tyrosine kinase activity of VEGFRs; and (4) therapeutic monoclonal antibodies targeting VEGFR-2 [72, 78].

One of the first antiangiogenic therapies was a therapy with a humanized monoclonal antibody, neutralizing circulating VEGF-A, i.e., Bevacizumab (Avastin®, Roche/Genentech). The first phase III trial results showed that Bevacizumab combined with chemotherapy in metastatic colorectal cancer (MCRC) improved progression-free survival (PFS) (10.6 vs. 6.2 months) and overall survival (OS) (23 vs. 15.3 months) compared to chemotherapy [54]. Aflibercept (Zaltrap®, Sanofi Genzyme) is a human recombinant fusion protein that acts as a decoy receptor of VEGF-A, VEGF-B, and PlGF. Aflibercept treatment was approved in MCRC with infusional fluorouracil, leucovorin, and irinotecan [24]. Tyrosine kinase inhibitors (TKIs) are small-molecular-weight drugs that inhibit the kinase activity of different receptors and their downstream signaling. Sorafenib (Nexavar®, Bayer/Onyx) or Sunitinib (Sutent®, Pfizer) target not only at VEGFR but other kinases such as PDGFR and FGFR [78]. Ramucirumab (Cyramza® Eli Lilly) is a human monoclonal anti-

body that inhibits angiogenesis by blocking binding VEGF to the extracellular domain of VEGFR2. It is recommended in combination with FOLFIRI (folinic acid, 5'-fluorouracil and irinotecan) in MCRC patients if the disease progresses after therapy with Bevacizumab, oxaliplatin, and fluoropyrimidine [8].

Direct suppression of tumor angiogenesis and vascular normalization results in suppression of tumor growth. However, after a long-term therapy, tumor cells acquire a resistant phenotype as a result of hypoxia and low nutrition stress. Overall, the survival benefits of antiangiogenic (AA) drugs have not been impressive and surprisingly most cancer patients stop responding or do not respond to the AA therapy at all. What is more, recently it was shown that AA drugs cause a switch to vasoinvasion of tumor cells, leading to increased metastasis and shortened life in mice [39]. The tumor resistance to AA agents can partly be a consequence of non-sprouting mechanisms of vessel recruitment. In intussusceptive microvascular growth, new vessels are generated by creating columns from connective tissue within the lumen of existing vessels. Glomeruloid angiogenesis is characterized by tight nests of vessels that resemble renal glomerulus. In vessel co-option, tumor cells incorporate host vessels in the normal surrounding tissue, and vasculogenic mimicry tumor cells directly from perfused channels bind to the host vasculature. In turn, in the case of looping angiogenesis, contractile myofibroblasts pull host vessels into the cancer tissue [78].

Several phase I and II studies targeting at fibroblast activated protein (FAP) with a humanized monoclonal antibody (Sibrotuzumab) failed to produce clinical benefits in the colon and non-small-cell lung cancer alone or in combination with docetaxel. The latest proposed strategy is based on a specific location of FAP which can be used for precise administration of cytotoxic prodrugs. This strategy is expected to enhance efficacy of the drug delivered to the tumor microenvironment [14].

VEGFR-2 is known as a target for Sunitinib which is a receptor tyrosine kinase inhibitor. ECs transfected with miR-221/miR-222 and treated with Sunitinib showed a reduction in total tube

length, and enhancement of cellular proliferation was observed. Sunitinib was not able to abolish the effect of miR-221/222 at pharmacologically relevant concentrations. Such resistance to treatment with Sunitinib may develop when the targeted protein is not accessible for the drug binding. In therapeutic implications, inhibition of miR-221 and miR-222 might improve the patient's survival if administered as an adjuvant therapy in combination with Sunitinib [57].

6.6.2 Inhibition of CAF Formation

Currently, tumor immunomodulation is the main focus of anti-cancer therapies [64]. CAFs, being an important element, regulate cancer invasiveness and characterize by a wide range of cross-talk with other cells located in tumor microenvironments, are a target of anti-cancer therapies. In contrast to preinvasive stages of cancers, the cross-talk processes are mainly observed in invasive cancer stages [64]. That interaction seems to be the main source of chemoresistance. CAFs highly express chemoresistance receptors like retinoic acid receptor β , which improves therapeutic responses of the cells. Cell surface molecules CD10 and GPR77 expressed on CAFs, also contribute to chemoresistance through supporting cancer stemness. Hence, the effectiveness of anticancer therapies in preinvasive stages may not be disturbed but the treatment may additionally intensify tumor growth in invasive stages. Therefore, complexed therapies should be applied.

TGF- β s are the main EndMT inducers contributing to formation of CAFs. Thus, the inhibition of TGF- β pathway seems to be the most promising strategy to decrease the population of CAFs. Theoretically, three levels of inhibition are possessed: (i) ligand inhibition which prevents TGF- β synthesis, (ii) ligand-receptor interaction blocking, and (iii) restriction of signal transduction. Despite the numerous tested inhibitors, studies on their functions mainly focused on modulation of cancer cells. Only one study demonstrated the role of the inhibitor on the cancer niche. TGF β -activated microenvironment increases the metastasis ability of colon cancer

cell into lungs and liver. Zhang et al. [122] revealed that LY2109761 significantly reduces liver metastases and prolongs survival (by about 25%) in a mouse model. Galunisertib treatment resulted in a blocked formation of subcutaneous tumors by primary colorectal cancer stem cells [19]. Results of these studies demonstrated that STAT3 signaling enhances liver and lung metastasis through TGF- β and IL-11-dependent pathways. The authors prove that targeting at TGF- β signaling can alter cancer cells via cells located in the tumor microenvironment.

Finding effective therapies which would inhibit CAF formation or block their effect on cancer progression appears to be quite difficult.

As described above, CAFs are a heterogeneous group of cells, formed from several sources under the influence of different immunomodulators. Therefore, inhibition of only one pathway is not strong enough to counteract an occurrence of CAFs. Secondly, particular CAF subpopulations demonstrated different functions. It has been recently shown that depending on the location in the cancer niche, CAFs can regulate cancer cells contraction (CAFs located in the close area or within the tumor) or affect the tumor through secreted immunomodulators. Additionally, it has been suggested that different levels of the α -SMA marker, demonstrated by CAFs, depend on the origin of these fibroblasts [70]. Expression of particular markers is another problem that should be considered while searching for anti-CAF therapy. Numerous studies revealed that their expression is dependent on the CAF source. Difficulty is that the presence of particular markers is not specific to CAFs, but it can be observed in other cells of the tumor niche, especially macrophages or lymphocytes [108].

6.7 Conclusion

The endothelium plays a critical role in cancer progression. Inhibition of cancer vascularization, intravasation of cancer cells, and CAF formation are the main reasons for creating effective anti-cancer therapies and, above all, inhibition of metastasis.

Nevertheless, many strategies limiting the growth of blood vessels proved to be ineffective. The reasons for that should be explained by diversified vasculature of the types of recruited inducing cells or those localized in the tumor area as well as possibilities of their interaction. It should be emphasized that the above-mentioned processes can be induced by tumor cells and tumor niche cells in response to the applied treatment. These elements should be included in the search for new effective therapies that inhibit tumor vascularization. Intravasation of cancer cells could be limited in three ways: by preventing cancer vascularization; by blocking an invasion of tumor cells into the vessel, i.e., by maintaining cell-cell connections; and finally, by inhibiting tumor cells invasiveness. Unfortunately, the last two possibilities are practically not used in anti-cancer therapies. CAFs may serve as the last mechanism that may constitute a source of anti-cancer strategies. It is known that these cells significantly contribute to a development of the tumor, either by induction of proliferation in pre-invasive cancer stages or through the acceleration of EMT and metastatic capacity in invasive tumor stages. EndMT inhibition, which accounts for approximately 40% of CAFs, could prevent these adverse effects of CAF formations. However, so far, the role of mechanisms conditioning the transformation and functions of individual CAF subpopulations have not been clearly clarified.

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