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REVIEW

Signaling Pathways in Tumor Vasculogenic Mimicry

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Abstract—Solid tumor growth is dependent on the development of an adequate blood supply. For years, sprouting angiogenesis has been considered as the exclusive mechanism of tumor vascularization. However, in recent years, another mechanism of tumor vascularization has been identified that does not involve endothelial cells, a process called vasculogenic mimicry (VM). VM describes the unique ability of highly aggressive tumor cells to form vessel-like networks by virtue of their high plasticity. VM has been observed in several tumor types, and its occurrence is strongly associated with poor prognosis. This review focuses on signaling molecules and cascades involved in VM. In addition, the clinical significance of VM regardless of anti-angiogenesis treatment modalities is described.

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Key words: highly aggressive tumor, vasculogenic mimicry, signaling pathway, prognosis

Formation of new microvessels on the basis of existing ones, or neoangiogenesis, is a requisite for tumor growth [1]. Abundance of microvessels in a tumor favors its rapid proliferation due to continuous nutrition and oxygen supply and elimination of toxic metabolic products. In recent years, more than 40 antiangiogenic drugs have passed phase II and III of clinical trials, and some proved to be efficient when combined with chemotherapeuticals [2]. Nevertheless, most tumors do not respond to antiangiogenic therapy [3]. In addition to a series of rational explanations, heterogeneity of tumor vessels may be a key factor: classical angiogenesis in a tumor occurs in parallel with formation of mosaic vessels, and vessel cooption is also observed, providing tumor growth along vessels existing in a tissue [4].

It was found recently that cells of highly aggressive metastatic melanomas can form highly structured vascular channels bounded by basal membrane in the absence of endothelial cells (ECs) and fibroblasts [5]. Formation of vascular channels by aggressive tumor cells (TCs) was named vasculogenic mimicry (VM), which emphasizes formation of these channels *de novo*, without implication of ECs, i.e. independently of angiogenesis. Vascular channel formation is a unique feature of highly aggressive phenotype; poorly aggressive TCs cannot form such structures [6]. Formation of a channel network within the tumor is expected to maintain homeostasis and prevent premature necrosis within the tumor. Strong statistical correlation between VM and metastatic rate supports this hypothesis.

Vasculogenic mimicry is observed in various aggressive tumors, such as cancer of breast, prostate, ovary, lung, kidney, and soft tissue sarcoma [7], suggesting that it is a novel characteristic of aggressive tumor. VM has no physiological analogs in either adults or children, so it can be regarded as tumor-specific. The only exception is formation of placental vascular channels by cytotrophoblasts during embryogenesis. This fact opens novel possibilities for blocking tumor growth with minimal effect on normal physiological processes.

The basis of cancer disease is blockage of cell differentiation. Tumor cells undergo some dedifferentiation during tumor progression. First, they lose differentiation proteins whose absence gives a selective advantage for "proliferation self-sufficiency". Moreover, a tumor with repressed function of programmed cell death, being monoclonal in its nature, acquires growing cellular polymorphism. DNA microchip analysis of gene expression profile has shown that highly aggressive melanoma cells compared with poorly aggressive ones express genes characteristic of endothelial, epithelial, hematopoietic, connective, muscular, and stem cells, suggesting partial genetic reversion of aggressive TCs into polypotent embryo-like

Abbreviations: AOs, antioxidants; CLS, capillary-like structures; ECs, endothelial cells; ROS, reactive oxygen species; TCs, tumor cells; TSCs, tumor stem cells; VM, vasculogenic mimicry.

phenotype [8]. Highly aggressive TCs that can form VM channels also express genes implicated in angiogenesis. Despite high expression of VEGF, VEGFR1, VEGFR2, bFGF, bFGFR, COX-2, von Willebrand factor, VE-cadherin, and laminin-5γ2 in these cells, VM does not depend on angiogenesis in the tumor [9]. These cells also express matrix metalloproteinases MMP-1, -2, -9, and -14 modifying the extracellular matrix, which is necessary for classical angiogenesis [10]. Thus, highly aggressive TCs can imitate the behavior of ECs and initiate formation of vascular channels.

This review focuses on the molecular characteristics of VM, signaling pathways involved in VM, and clinical significance of VM in diagnosis of tumors and prognosis of cancer disease outcome.

FUNCTIONAL IMPORTANCE OF VM

Numerous recent studies have shown that the presence of VM channels in tumor material of patients correlates with rapid progression of the tumor, elevation of metastatic, and, as a consequence, short survival of patients [11]. Seftor et al. demonstrated high expression of blood clotting proteins in aggressive melanoma of the eye [12]. It is known that blood coagulation is initiated by tissue factor (TF). Deposition of fibrin in tumor vessels and thrombosis are prevented by TFPI-1 (tissue factor inhibitor 1) and TFPI-2 [13]. Both inhibitors are actively expressed in aggressive melanoma of the eye and are negligible in lowly aggressive melanoma. It is worth noting that in tumor material from patients both TFPI-1 and TFPI-2 are localized along the VM channels. The fact that VM-positive tumor, like ECs, supplies itself with an anticoagulation mechanism implies that VM channels are functionally active and can provide blood circulation within the tumor, particularly in zones of deep hypoxia. Involvement of tumor VM in blood microcirculation was demonstrated on a model of ischemic limbs [14]. Five days after injection of fluorescently labeled metastasizing melanoma cells into the mouse ischemic limb, a formation of "mosaic" vessels composed of ECs and TCs, as well as vascular channels formed by TCs was observed. This study has significantly altered our understanding of the role of VM.

Using dynamic resonance angiography and histological and immunohistochemical analysis of experimental breast tumor, a Japanese research group has shown that the vasculogenic component is predominantly localized in the central area, whereas neoangiogenesis predominantly occurs on the periphery [15]. Moreover, blood flow exists between neoangiogenic and VM loci, because a contrasting fluorescence dye stained both peripheral and central tumor areas. The dye did not accumulate in the central area of tumors lacking VM. Thus, in tumors VM is involved in the integrated system of blood circulation.

MOLECULAR DETERMINANTS OF VM

Ability to form a unique vascular network, which was first found in histological material of patients with uveal melanoma, was later confirmed *in vitro* on so-called 3D-cultures in gel matrixes (Matrigel and collagen gel) [16, 17]. A test for formation of capillary-like structures (CLS) in 3D-culture was developed initially for *in vitro* identification of angiogenesis inhibitors and activators [18]. ECs attached to the gel imitating the extracellular matrix and formed CLS. The cells of metastatic tumor were also capable of forming these structures (Fig. 1a). CLS formation in 3D-cultures is presently regarded as an *in vitro* model of VM [19].

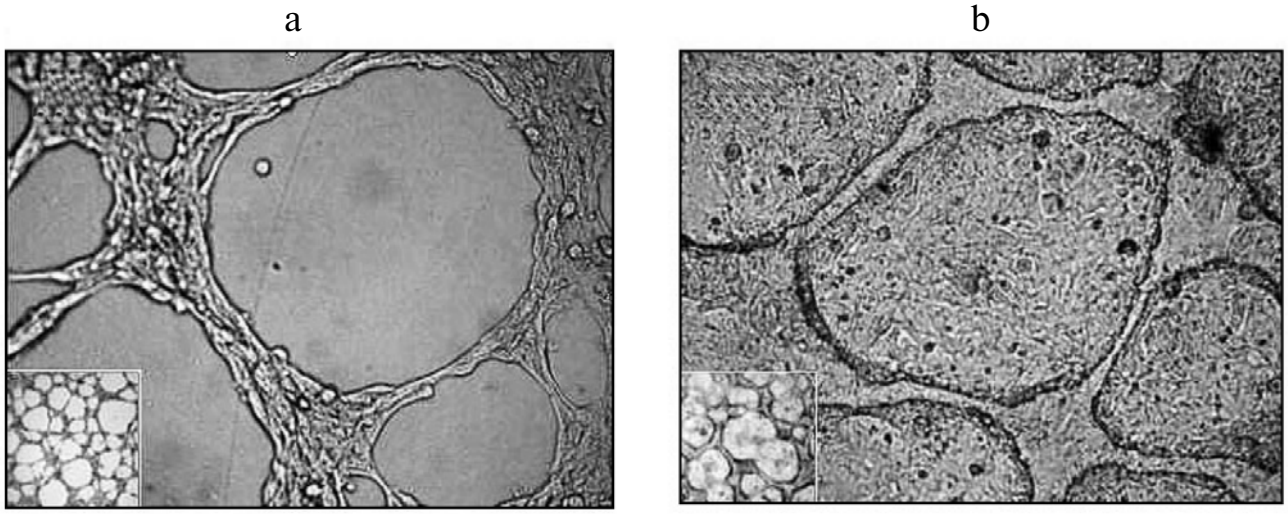

Fig. 1. Formation of vascular channels *in vitro*. a) CLS; b) tubular structures.

Highly aggressive TCs *in vitro* can also form tubular structures. In this case, critical parameter is high density of the cells on Matrigel. Formation of tubular structures begins three days after cell seeding on Matrigel and ends to the end of third week. Microinjection of contrasting dye in these channels demonstrates that at least part of them represents coreless tubular structures that can conduct the dye along the channel [17] (Fig. 1b).

We have optimized the protocol for CLS formation on Matrigel, and some determinants of CLS formation have been revealed. Surprisingly, a reproducible feature of VM was appreciable decrease in cell number at the moment of irreversibility of CLS formation. This change is characteristic of transition of the cell population from proliferation to differentiation. Adhesion to Matrigel seems to activate differentiation signals, allowing the cells to imitate behavior of ECs, and decrease in cell number at the moment of organization of melanoma cells to CLS can be explained by elimination of those cells that are unable to fulfill the endothelium-like function [20]. Our data support the observation of Segura et al., who detected apoptotic cells during vessel formation on Matrigel by human ECs of the HUVEC line [21]. If apoptosis is indeed the driving force for VM establishment, inhibition of caspases should interrupt CLS formation. Supporting this, the pan-caspase inhibitor zVAD-fmk completely cancelled the cooperation of TCs in CLS. DEVD, a specific inhibitor of caspase-3, also blocked CLS formation. These observations were confirmed on five melanoma cell lines. Detailed investigation of this phenomenon has shown that the levels of cytochrome *c* and caspase-3 increased with time of cell growth on Matrigel and decreased at the 16th hour, when CLS formation became irreversible. These data suggest that activation of caspases is one of the limiting stages of proper assembly of melanoma cells into CLS [20]. Thus, we have identified a mechanism that participates in the earliest stages of CLS formation, before organization of cells for CLS formation. This is supported by the fact that addition of the inhibitor at the CLS stage does not counteract further formation of CLS. During differentiation, caspase activity is required for unspecific so-called non-apoptotic function [22-25].

Historically, VE-cadherin was the first identified as VM mediator [26]. VE-cadherin is a transmembrane protein, a member of the cadherin family, which is expressed on the surface of ECs and contributes to homotypic cell–cell interactions. Its role in integration of ECs was confirmed in experiments with VE-cadherin knockout mice; the animals died in the embryonic period [27, 28]. High level of expression of this protein was observed in aggressive melanoma cells capable of CLS formation. In lowly aggressive cells, the level of VE-cadherin was negligible. In 2006, Hess et al. demonstrated that cultivation of melanoma cells in a medium with monoclonal antibodies against VE-cadherin or the use of antisense technology led to complete disability of highly aggressive melanoma cells to form CLS [26]. Thus, vascular channel formation by aggressive TCs follows the same regularity as formation of blood vessels by ECs: recognition of TCs that can enter VM occurs with involvement of VE-cadherin. In connection with this, VE-cadherin is now regarded as a vasculogenic switch.

As mentioned above, TCs that are capable of forming vascular channels are characterized by high expression of the extracellular matrix component laminin-5γ2. Laminins are main components of basal membrane and play an active role in tumor metastasizing: they activate cell migration and are implicated in neoangiogenesis signal transduction [29]. A growing amount of data suggests that the proteolytic cleavage of the laminin-5γ2 chain by matrix proteinase MMP-2 to γ2 and γ2x fragments regulates the integrin-mediated migration of TCs [30]. Antibodies against MMP-2 blocked the cleavage of laminin-5γ2 and interrupted the process of vascular channel formation [31]. The data of numerous recent studies suggest that exactly these three molecules (VE-cadherin, laminin-5γ2, and MMP-2) as well as their ligands and receptors are necessary for formation and maintenance of stability of blood vessels, because the absence of any of these leads to fetal death caused by defects in vascularization [32]. High levels of these proteins are also observed in aggressive tumors; less aggressive ones are characterized by low, if any, production of them. This fact confirms an idea that highly aggressive TCs "recognize" each other by means of VE-cadherin and, like ECs, can modify the extracellular matrix and initiate formation of vascular channels.

Another protein involved in VM is cyclooxygenase (COX). It catalyzes the first reaction of prostaglandin synthesis in the arachidonic acid cycle [33]. There are two COX isoforms in humans: COX-1 and COX-2. COX-1 is constitutively expressed in normal cells and is involved in tissue homeostasis. COX-2 is not only a mediator of inflammation, but it also mediates a series of other biological functions associated with pathological hyperplastic and neoplastic processes. COX-2 is now regarded as one of the key molecular targets in targeted antitumor therapy and prophylaxis [34]. Recent studies suggest that breast cancer cells expressing COX-2 can form CLS in 3D-culture, while cells characterized by low expression of this enzyme lack this ability [35]. Inhibition of COX-2 with either Celecoxib or specific siRNAs blocks the formation of CLS. Moreover, CLS are disrupted on addition of exogenous PGG2, which is indicative of dependence of CLS formation and maintenance on prostaglandins.

Oxidative stress is known to play an important role in pathogenesis of cancer. The level of reactive oxygen species (ROS) in tumor cells is considerably higher than in normal cells, which is apparently one of the factors determining establishment of the disease [36, 37]. Moreover, ROS are an important mediator in tumor angiogenesis. We supposed that ROS also play an impor-

tant role in vascular channel formation by TCs. Our data have shown that decrease in ROS level by 50-60% caused by antioxidants (AO), such as resveratrol, epigallocatechin gallate, NAC, and Trolox, completely blocked the ability of cells to form CLS. These data were confirmed *in vivo* on an experimental model of melanoma: the density of vascular channels significantly decreased in response to resveratrol. Detailed investigation of this phenomenon has shown that antioxidants inhibit cytochrome *c* release, thus decreasing the level of caspase-3. In our experiments, AOs also decreased expression of VEGF and the receptors VEGFR1 and VEGFR2. We believe that ROS level is a sensitive indicator of the ability of TCs to become involved in VM [38].

We have only discussed several known molecular determinants whose activation or blockage affects VM. There is a reason to hope that adequate understanding the role of key VM markers and identification of novel elements of this phenomenon will reveal agents that can not only effectively block establishment of VM, but also disassemble already formed vessels.

SIGNALING CASCADES INVOLVED IN VM

Hypoxia, HIF-1α, VEGFA/VEGFR1 signaling pathway, and VM. A tumor having a diameter less than 2 mm (dormant tumor) is avascular. It is characterized by a certain balance between proliferation and apoptosis. Switching of the tumor to the angiogenic program depends on the general balance between pro- and antiangiogenic signals, which is more often shifted to tumor proliferation [1]. Almost all tumors secrete VEGF (vascular endothelial growth factor). The binding of VEGF with its receptor stimulates programmed dedifferentiation of ECs into EC precursors, their proliferation, and vessel formation. Tumor angiogenesis is stimulated by hypoxia, the usual state of growing tumor cells having insufficient blood supply [39]. In response to hypoxia, TCs activate synthesis of hypoxia-inducible factors HIF-1α and HIF-2α. HIF-1α plays a specific role in blood supply. Its binding with the enhancer sequence of the *VEGFA* gene activates expression of VEGF, thus stimulating new vessel formation [40]. Once the tumor enters the vascular stage, new vessels continue to form during tumor growth. Recently, it was shown that HIF-1α is localized along the VM channels and is not stained in the CD-31 zone of blood vessels [41]. These results were confirmed by staining with pimonidazole, a marker of hypoxia: severe hypoxia was observed in the tumor zones where VM channels are localized, thus indicating association of VM with c HIF-1α. In 2007, we first demonstrated that VM in melanoma, as well as tumor angiogenesis, are under the control of VEGFA [20]. The next year our data were confirmed by two independent research groups. Su et al. demonstrated that the HIF-1α inhibitor rapamycin blocked VEGF expression, decreased the levels of VE-cadherin, EphA2, and MMP-2, and prevented vascular channel formation in ovarian cancer [42], and the use of VEGFA siRNA confirmed the role of VEGF as trigger of VM in osteosarcoma [43].

When bound, VEGF activates two tyrosine kinase receptors on ECs – VEGFR1 and VEGFR2. Despite high homology between these two receptors, they transmit different signals. VEGFR1 possesses high affinity to VEGF and low kinase activity; the affinity of VEGF to VEGFR2 is lower, but the kinase activity of VEGFR2 is higher than that of VEGFR1. It is now certain that the VEGFA/VEGFR2 signaling pathway is implicated in tumor angiogenesis [44, 45]. VEGFR1 is supposed to play the role of a "trap": the greater amount of the ligand is bound with VEGFR1, the lesser amount of it is bound with VEGFR2. Hiratsuka et al. showed that deletion of the VEGFR1 tyrosine kinase domain had no effect on embryonic and postnatal development of mice [46]. This indicated that the VEGFR1 tyrosine kinase domain is not necessary for EC function. Recently, we obtained experimental confirmation that the VEGFR1 tyrosine kinase domain is required for vascular channel formation [47]. A complete surprise was that a specific inhibitor of VEGFR2, PTKI, does not block CLS formation. On the other hand, siRNA-mediated suppression of VEGFR1 expression made the melanoma cells completely unable to form CLS, which suggests that the VEGFA/VEGFR1 signaling pathway exerts a control over VM and does not depend on tyrosine kinase activity of VEGFR2. It is known that PlGF (placental growth factor) also can bind with VEGFR1 to induce tubulogenesis of ECs [48]. In our experiments, PlGF did not induce CLS formation. PlGF and VEGF, when bound with VEGFR1, apparently activate different signaling pathways, and target genes activated by PlGF cannot trigger VM. We have also shown that VEGFA/VEGFR1 signaling pathway in the cell activates protein kinase Cα (PKCα), and these data were confirmed *in vivo*: Ro32-0432, a specific PKCα inhibitor, decreased the density of VM channels in a dose-dependent way [47]. Contemporary antiangiogenic therapy is directed to decrease EC proliferation or cause their apoptosis, and is not efficient for destruction of vascular channels lined with TCs [9, 49]. However, when the vascular density decreases due to antiangiogenic therapy, which leads to hypoxia, stimulation of VM occurs as a consequence for compensation of oxygen and nutritional deficiency. Thus, antiangiogenic therapy unintentionally activates VM. A recent hypothesis is that first blood vessels in a metastasis are formed from TCs with randomly distributed ECs, so the first blood vessels are either a mosaic or are VM channels [50]. Classical angiogenesis appears at later stages of tumor growth, so a tumor that can form VM channels has more chance to survive, and combining antiangiogenic drugs with VM inhibitors seems to be necessary for elevation of therapeutic effectiveness.

EphA2, FAK, and VE-cadherin. In 2006, Hess et al. demonstrated that in sites of cell–cell contacts VE-cadherin is colocalized with epithelial kinase EphA2, a receptor tyrosine kinase, which is constitutively expressed on melanoma cells with metastatic phenotype, and cooperation of these two molecules is necessary for VM channel formation [51]. They proposed a possible signal transduction pathway establishing VM. According to their model, the homotypic binding of VE-cadherin molecules on adjacent TCs leads to translocation of EphA2 onto the plasmatic membrane, where it can bind to the ligand, the transmembrane protein ephrin A1, thus leading to (auto?)phosphorylation of EphA2. The phosphorylated EphA2 induces conversion of inactive GDP-bound Rac1 (small GTPase; membrane GTPase) form and Cdc42 in their GTP-bound active forms. This is consistent with the fact that in highly aggressive melanoma cells, the levels of active Rac1 and Cdc42 are considerably higher than in melanoma cells that do not form CLS on Matrigel, and inhibition of Rac1 and Cdc42 synthesis in ECs using RNA technologies completely blocked the melanoma cell assembly into CLS [52, 53]. Activated Rac and Cdc42 are involved both in formation of lamellipodia and filopodia required for cell motion [54-56] and in translocation of FAK (focal adhesion kinase, also known as protein tyrosine kinase 2, PTK2) to the newly forming focal adhesion sites [57]. When localized on a membrane, FAK can phosphorylate EphA2 and, possibly, integrins, which in turn facilitate cell migration via activation of paxillin [58]. Although expression of the *PTK2* gene (encoding FAK) is nearly equal in both highly and poorly aggressive melanoma cells, the activity of FAK is considerably higher in the highly aggressive cells. Blockage of FAK phosphorylation at tyrosines 397 and 576 makes the enzyme inactive, and this completely eliminates the CLS forming ability of highly aggressive melanoma cells. FAK is now regarded as the VM trigger (Fig. 2).

Signaling pathways triggered by VE-cadherin and EphA2 can also activate phosphatidylinositol 3-kinase (PI3K), which participates in branched reaction cascades implicated in programs of cell survival and proliferation

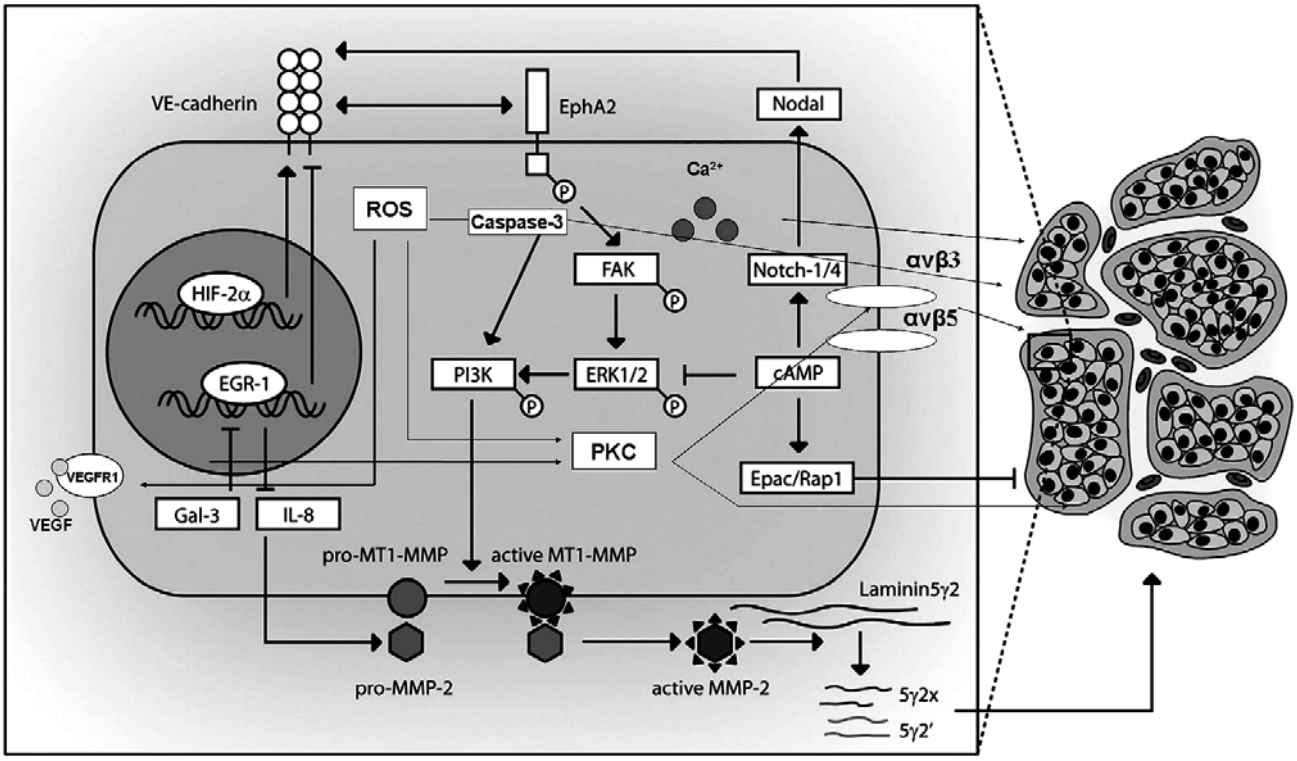

Fig. 2. Hypothetical model of signaling pathways involved in VM. Homotypic binding of VE-cadherin molecules of adjacent TCs leads to translocation of EphA2 onto the plasmatic membrane, where it binds with the transmembrane protein ephrin A1, thus leading to (auto?)phosphorylation of EphA2. Transduction of the signal activated by VE-cadherin and EphA2 can be realized in two ways – via activation of FAK or PI3K. PI3K facilitates the transition of pro-MMP to the active conformation MMP-2, which cleaves laminin-5γ2 to the fragments γ2 and γ2x. Accumulation of these fragments in the extracellular medium facilitates cell migration and VM channel formation. Inhibition of COX-2 counteracts the vascular channel formation. cAMP inhibits VM via activation of Epac/Rap1. Downregulation of BMP-4 activity leads to decrease in VE-cadherin and EphA2 expression. Activation of Nodal in TCs maintains VM via elevation of VE-cadherin expression. Hypoxia elevates expression of the *VEGF* gene that controls VM. Inhibition of TFPI-2 suppresses VM via inhibition of MMP-2 activation, and TFPI-1 fulfills an anticoagulation function in the vascular channels.

[59]. This enzyme is involved in the establishment of VM [60]. The active PI3K induces expression of MT1-MMP, which converts pro-MMP-2 into its active form. In numerous recent works, the role of MT1-MMP and MMP-2 in cleavage of laminin into two fragments, γ2 and γ2x (Fig. 2), is regarded as crucial. Elevation of the concentration of these fragments in the extracellular medium contributes to migration and proliferation of ECs and to vascularization. As mentioned above, γ2 and γ2x are molecular messengers triggering VM. Specific PI3K inhibitors decreased both activity of metalloproteinases and accumulation of γ2 and γ2x, eventually resulting in complete blockage of vascular network formation by TCs. Expression of VE-cadherin and EphA2 by highly aggressive TCs and triggering signaling pathways involving FAK and PI3K by these proteins are consistent with the hypothesis of the ability of highly aggressive TCs to imitate the behavior of ECs.

Tumor galectins and VM. Galectin-3 (Gal-3) was recently identified as a protein required for VM channel formation. Gal-3 is a β-galactosyl-binding lectin that contributes to cell adhesion, cell cycle regulation, tumor growth, and angiogenesis [61]. Gal-3 is not expressed in normal cells and most benign tumors, whereas it is almost always found in highly aggressive tumors, thus serving as their marker. During neoplastic progression, Gal-3 accumulates in the TC cytoplasm, induces transition of the tumor into more aggressive type, and stimulates angiogenesis and formation of distant metastases [62, 63]. Elevation of Gal-3 level is indicative of oncogenicity of many tumor types. Galectins can act both outside the cell via interaction with the extracellular matrix and within the cell by modulating some intracellular signaling pathways (see review [64]).

The shRNA-induced decrease in Gal-3 expression completely blocked CLS formation in 3D-culture of aggressive melanoma cell line C8161. Activation of RAF kinase and PI3K was observed in cells with a low level of Gal-3. DNA microarray analysis demonstrated a drastic decrease in VE-cadherin, IL-8, and MMP-2 expression in such cells [65, 66]. IL-8 activates expression of MMP-2, and both VE-cadherin and MMP-2 are VM mediators. Thus, expression of Gal-3 is a determinant of CLS formation (Fig. 2). The authors explain the stimulating effect of Gal-3 on VE-cadherin and IL-8 expression as an influence of EGR-1 (early growth response protein), a transcription factor implicated in transduction of an extracellular cell differentiating signal (Fig. 2). The EGR-1-induced repression of VE-cadherin and IL-8 is supposed to be important for maintaining the differentiation status in normal tissue. In malignant neoplasms, expression of Gal-3 counteracts the EGR-1 binding to a gene promotor, thus activating transcription of proliferation initiating genes. Summarizing the above, a novel, previously undescribed pathway is identified that is induced by Gal-3 and implicated in VM.

The cAMP signaling pathway. Perhaps the very important role of cAMP is activation of cAMP-dependent protein kinases [67]. The most studied is a serine–threonine protein kinase A (PKA). Being activated, PKA uses ATP to phosphorylate one or another of many biologically active cell proteins, such as enzymes, receptor and channel proteins, nuclear histones, transcription factors, etc. As a rule, serine, threonine, or tyrosine residues undergo phosphorylation. It is worth noting that besides PKA-mediated cAMP signaling, a PKA-independent cAMP signaling pathway also exists. Activation of the latter pathway of signal transduction involves the protein messengers Epac1 and Epac2 [68, 69]. An *in vitro* study has shown that the cAMP signaling pathway is implicated in CLS formation by melanoma cells (MUM-28 and C816) growing on Matrigel [70]. The adenylate cyclase activator forskolin increased the cAMP level and reversibly inhibited CLS formation (Fig. 2). This effect was mediated by activation of Epac/Rac-1 and did not depend on PKA. Forskolin also inhibited the PI3K-dependent phosphorylation of Akt (Fig. 2). On the other hand, accumulation of cAMP in melanoma cells led to inhibition of phosphorylation of ERK1/2, the pharmacological inhibitors of which counteract VM channel formation. It is worth noting that the role of cAMP in tumor angiogenesis is not clearly presented in the literature. It is supposed that cAMP is implicated in differentiation of arterial ECs rather than venous ones, and this effect involves cooperation with the Notch signaling pathway [71]. Besides implication in EC differentiation, the cAMP signaling pathway also controls localization of VE-cadherin in ECs. It was shown on HUVEC cells that forskolin and prostacyclin, agents that can elevate cAMP level, cause accumulation of VE-cadherin in sites of cell–cell junction [72]. Elevated expression of VE-cadherin elevates EC adhesion to the matrix. A somewhat different picture emerges on melanoma cells: forskolin decreases expression of VE-cadherin. These data are difficult to interpret. One can suppose that, although a cAMP signaling pathway is involved in vascular channel formation by TCs, this process is likely under the control of several signaling pathways whose balance depends on cell type and, possibly, on the nature of the signal.

Ca^{2+} signaling pathway and integrins. CLS formation in 3D-culture passes through a series of sequential events: cell migration, formation of cell–cell contacts, and cell elongation. The cells stop proliferation, recognize each other by forming contacts via VE-cadherin, elongate, and attach to the extracellular matrix. Cell–cell interaction is known to occur via formation of focal adhesion sites. This process is Ca^{2+}-dependent, because removal of Ca^{2+} from the extracellular medium leads to disintegration of focal contacts. Ca^{2+}-binding sites were identified on extracellular domains of cadherins, F-actin, tubulin, and integrins, which implies the need for Ca^{2+} not only for homotypic

recognition of cells, but also for formation of stable junctions [73]. These data brought us to a more detailed study of the role of Ca^{2+} in CLS formation. Our experiments confirmed the crucial role of both extra- and intracellular Ca^{2+} in CLS formation. They also confirmed the need for cellular actin cytoskeleton reorganization for proper TC assembly into CLS (Fig. 2). A fact of great interest, which was not previously discussed in literature, is that intact tubulin is required for CLS formation [74].

Integrins comprise a large family of transmembrane linker proteins localized on the cell surface that mainly promote stable interactions between cells and the extracellular matrix [75]. The ligand-binding capability of integrins depends on bivalent cations. Cells are attached to the extracellular matrix by small sites of their surface called focal contacts. These sites are the loci of integrin receptors that specifically bind various matrix components, such as collagen, fibronectin, laminin, and vitronectin. The intracellular domain of integrin receptor binds with actin filaments of cytoskeleton via a chain of cytoplasmic proteins. The integrin–extracellular matrix association is not only structural, but also functional: adhesion of the cell to various components of the extracellular matrix "turns on" a chain of signal transmission from focal contacts into the cell. In mammals, 24 integrins have been characterized to date, including 18 α- and 8 β-subunits. In our studies, we focused on the role of αvβ3 and αvβ5, using integrin β1 as a control. Expression of integrin αvβ3 correlates with metastatic potential of melanoma cells [76], and both integrins, αvβ3 and αvβ5, activate PKCα [77], a mediator of VM. Integrin β1 is responsible for cell adhesion and motility. Our data suggest that integrins αvβ3 and αvβ5 both participate in CLS formation, whereas integrin β1 has no essential role in this process (Fig. 2). CLS formation depends on cell migration and formation of cell–cell and cell–extracellular matrix junctions. At this stage, such junctions should be flexible, so the contribution of integrin β1 that "glues" the cell to the matrix [78] is not critical. Our data are confirmed by a recent report on the ability of integrin αvβ3 to activate expression of VEGF and VEGFR1, which are the triggers of VM [79].

It is known that the ECM–integrins/Rab-Src-FAK–stress fibers–ERK signaling cascade regulates cell invasion and motility. It was shown in multiple myeloma that activation of PKCα is under the control of VEGF-mediated and ECM–integrin β1 signaling pathways [80]. In this model, VEGF induces activation of PI3K, and integrin β1 mediates cell junction with fibronectin of the extracellular matrix, which stimulates PKCα translocation into the membrane and activation of this kinase. On the other hand, the PKCα inducer 12-O-tetradecanoylphorbol-13-acetate (TPA) also activates integrins αvβ3 and β1 via a so-called "inside-out" signaling pathway [81]. Cooperation of "outside-in" and "inside-out" signaling pathways occurs when one integrin is activated by the binding with ligand on the extracellular matrix and, via PKCα, activates another integrin within the cell by means of an "inside-out" signaling pathway and, possibly, participates in regulation of vascular channel formation by TCs. Summarizing these data, establishment of VM depends on Ca^{2+}-sensitive reorganization of actin cytoskeleton, including changes in cell shape, as well as creation of adhesion foci required for cell motility and elongation and formation of cell–cell junctions. The role of each of these signaling pathways clearly requires further study. However, the fact that a Ca^{2+}/integrin signaling pathway exerts control over VM suggests the therapeutic importance of decrease in intracellular Ca^{2+} level.

Notch signaling pathway. During vascularization of an embryo, the EC precursor angioblasts differentiate to form a primitive honeycomb-like network. This network pattern is also observed in VM. The ability of highly aggressive TCs to express genes characteristic of many cell types is supposed to be a consequence of reactivation of embryonic signaling cascades. It was recently shown that expression of Nodal, an embryonic morphogen from the TGFβ family, is elevated in metastatic melanoma cells [82]. Inhibition of Nodal leads to reversion of melanoma cells to melanocytes that cannot form CLS. Moreover, decrease in expression of Nodal is accompanied by decrease in VE-cadherin level, thus indicating that expression of Nodal is necessary for maintenance of de-differentiated status of TCs.

Quite recently it was demonstrated that Nodal expression is under the control of the Notch signaling pathway [83]. This highly conserved intracellular signaling pathway is activated on interaction of transmembrane ligands, members of the families Jagged (Jagged 1 and 2) and Delta (Delta-like 1, 3, and 4), with Notch receptors (Notch1-4) [84]. Following the binding with ligand, activation of Notch receptor occurs due to proteolytic action of γ-secretase by the release of the intracellular domain, which migrates into the nucleus and, forming a complex with DNA, regulates transcription of genes *Hes1* and *Hes5*, as well as *Hey1* and *Hey2*. During embryogenesis, the Notch signaling pathway controls establishment of bilateral symmetry [85]. Much large clinical material is accumulated suggesting that dysfunction of the Notch signaling pathway is implicated in development of glioma, breast cancer, pancreatic cancer, colorectal cancer, and various hematopoietic neoplasms and serves as a trigger for tumor progression to more aggressive phenotype [86], which is a prerequisite for vascular channel formation by TCs. On the other hand, expression of Notch and its ligands is also observed in ECs, and activation of this signaling pathway in ECs terminates proliferation and allows differentiation of the cells. Blockage of the Notch signaling pathway in endothelium was shown to permit unproductive angiogenesis and decelerate tumor growth [87]. These two independent observations prompted the study on the role of the Notch signaling pathway in VM. As

mentioned above, activation of the Notch pathway directly depends on the functional state of γ-secretase. Inhibition of this enzyme by DAPT or BZ stabilized CLS formed by melanoma cells on Matrigel: CLS retained stability for 32-36 h, while spontaneous CLS decay was observed in 20-24 h incubation of the cells on Matrigel. These data were confirmed by blocking the ligand–receptor linkage. Anti-Jagged1 neutralizing antibodies also stabilized CLS. It becomes obvious that attenuation of the Notch signaling pathway is necessary for maintenance of CLS integrity. It is worth noting that we did not study the contribution of individual Notch receptor/ligand signaling pathways, but used substances that block activity of γ-secretase, which uniformly cleaves the intracellular domain of all four Notch receptors.

Analysis of xenografts has shown that vascular channels formed by TCs become larger in diameter and more branched in response to DAPT. A fact of particular interest is that necrosis was absent in those tumor areas in which high densities of VM channels were observed. This fact assuredly confirms the functional activity of VM and its ability to maintain tumor growth. Our data also confirm the recently described phenomenon that antiangiogenic therapy, although it initially leads to decrease in tumor size, results in more aggressive tumor growth characterized by elevated invasion and metastasizing. We have shown that expression of known VM mediators MMP-2 and VEGFR1 increased in mouse xenografts in response to anti-Notch therapy. Thus, anti-Notch therapy impels highly aggressive TCs to acquire endothelium-like features (VM), and VM allows a tumor to survive by supplying the tumor areas characterized by pronounced hypoxia with nutrition and oxygen. Our data suggest that under suppression of angiogenesis, a tumor uses an alternative, endothelium-independent vascularization, and the contribution of VM to the total blood circulation in the tumor should be taken into account in antiangiogenic therapy. Clinical trials of antiangiogenic effect of γ-secretase inhibitor are now in the third stage. Final conclusions will be made on completion of these trials.

TUMOR STEM CELL AND VM

Until recently, the idea prevailed that all tumor cells possess equal proliferative potential and are equally responsible for the development of oncological disease. However, in many cancer types only a small portion of cells have this feature. The commonality of properties characteristic of stem cells (SCs) and many tumor cells suggests the conception of tumor stem cells (TSCs) [88]. These cells seem to appear because of a failure in regulatory systems of damaged SCs or their direct progeny. Behavior of SCs is under strict genetic control and occurs in accordance with signals from their close surrounding (niche). Appearance of mutations in SCs, their inheritance by mother cells, and inadequate SC response to outer signals can lead to transformation of healthy cells into tumor cells. These tumor cells are characterized by unlimited lifespan and (like physiological SCs) capability of differentiating into other cell types. Aggressive tumor cells, which are capable of vascular channel formation, also express genes required for maintenance of polypotent phenotype [89]. Moreover, aggressive TCs actively express signaling molecules such as Nodal, Notch, and Wnt, which are implicated in maintenance and differentiation of embryonic stem cells. Interestingly, these signaling molecules not only maintain SC polypotency, but also are necessary for establishment of VM.

To date, we know very little about signaling pathways in SCs, which maintain polypotency. Non-differentiated SCs are characterized by high expression of Nodal, whereas a low level of this signaling molecule is associated with activation of differentiation [90]. Another signaling pathway, which is constitutively active in non-differentiated cells, is the TGFβ/activin pathway [91]. Activation of BMP-4 (bone morphogenic protein), a protein implicated in early embryonic differentiation (gastrulation and mesoderm formation), is also controlled by the TGFβ/activin pathway. Implication of this protein in tumor progression was discovered quite recently, when BMP-4 was identified as an important mediator of invasion and metastasizing [92]. Recently, expression of BMP-4 in aggressive TCs was shown to be necessary for the formation of tubular structures: cells with low BMP-4 activity were unable to form a vascular network [93]. Moreover, decrease in BMP-4 expression correlated with decrease in expression of EphA2 and VE-cadherin genes, which, as shown above, are triggers of VM.

Stem cells can be in the polypotent state for a long time because of niches. The SC niches create a microenvironment and control local processes of SC proliferation and differentiation by integrating signals from adjacent stroma cells, as well as from the organism and outer environment. Niches create a system of signals and address specificity of SC differentiation in the locus of a lesion or natural cell loss. It is the locus where the SCs migrate and undergo activation, proliferation, and differentiation into the cells of the given tissue. Resemblance between TSCs and aggressive TCs that can be involved in VM in markers and signaling pathways suggests that the microenvironment of a malignant tumor acquires some embryonic features. For example, aggressive melanoma cells of line C8161, when grown on Matrigel in a medium conditioned by human fetal cells, form spheroid aggregates imitating the behavior of SCs [94]. Analysis of the protein expression pattern of these cells has shown that expression of VE-cadherin significantly decreases, and its level only returns to normal seven days after removal of the conditioned medium. As mentioned above, highly aggressive TCs can also modulate the extracellular matrix by secreting MMP and laminin 5γ2. These experiments sug-

gest that aggressive TCs can manipulate the microenvironment without implicating cell–cell junctions.

Hypoxia, one important parameter of the niche, is necessary for maintenance of undifferentiated status of SCs. The phenomenon of hypoxia-induced maintenance of undifferentiated status of SCs has been described for neuroblastoma, breast cancer, and prostate cancer [95-97]. Staining with pimonidazole (a marker of hypoxia) has shown that the microenvironment of VM channels in the tumor is also characterized by deep hypoxia [98], which is indicative of the need for deep hypoxia around VM channels for maintaining the polypotent phenotype of highly aggressive TCs.

Of certain interest is the origination of aggressive TCs that can form vascular channels. Two possible scenarios have been proposed. The tumor – for survival and progression – can induce differentiation of TCs into the endothelium-like phenotype allowing formation of vascular channels, which are required for nutrition supply and, possibly, metastasizing. This hypothesis is supported by the data of Monzani et al. [99]. They found a TSC subpopulation in tumor material containing VM channels. Another variant is not excluded, namely, that TSCs are the TCs that lost tissue-specific functions during the dedifferentiation process and turned back into polypotent cells expressing the markers of both TSCs and ECs. Since such cells are required for tumor life support, they persist in the tumor. To date, we have no data allowing the choice of one scenario or the other. It is possible that both pathways producing cells capable of VM can occur, and the choice depends on the TC type.

CLINICAL SIGNIFICANCE OF VM

VM is presented on histological sections as channels between TCs containing red blood cells and plasma. These channels are PAS-positive structures (PAS, periodic acid Schiff) and represent microcirculation channels in the tumor. Several PAS-positive structures can be distinguished in tumors: parallel structures, loops, networks, and dermal packages [100]. The pattern of these structures is crucial for blood supply and, hence, for the prognosis of the disease.

Analysis of tumor samples from 234 patients with eye melanoma demonstrated 91-92% survival for 10-years in those patients who had no PAS-positive loops, networks, or parallel structures, whereas only 55-57% survival was found in those patients who had these structures. These signs were independent prognostic factors [101]. The prognostic value of VM has been demonstrated in breast cancer, ovarian cancer, mesothelial sarcoma, rhabdomyosarcoma, hepatocellular sarcoma, and renal cell carcinoma (Fig. 3) [102-105]. Clinical data enable testing the validity of VM pattern determination in tumor as a diagnostic marker. Additional clinical studies are obviously required, such as revealing the synergistic relations between VM and number of microvessels in various types of tumors, before VM status determination becomes a routine prognostic factor.

As we noted earlier, aggressive melanoma cells, as well as some cell lines of breast or ovarian cancer, are capable of CLS formation when grow in 3D-culture. Based on these observations, a group of American scientists studied in detail the *in vitro* VM modulating effect of TNP-470 and endostatin, two inhibitors of tumor angiogenesis widely used in clinics. Their experiments demonstrated that neither TNP nor endostatin influenced the formation of the vascular network by melanoma cells [9]. These experiments are crucial for studying the VM phenomenon in clinics and allow opening a new page in the evaluation of physiological significance of neoangiogenesis and consideration of vasculogenic component in the blood microcirculation in the tumor.

Clinical trials of antiangiogenic drugs (more than 40 pharmaceuticals are presently at stages II and III of clin-

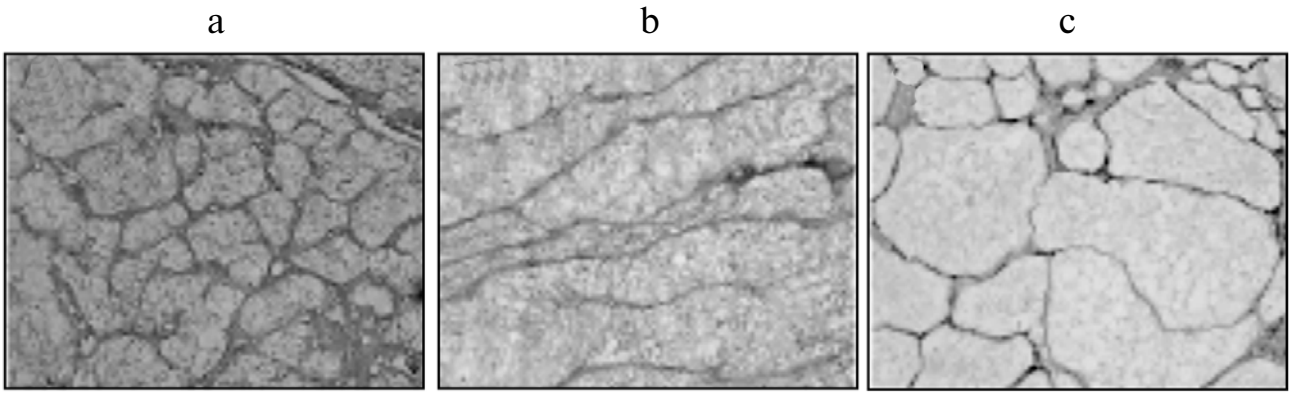

Fig. 3. PAS-positive structures in histological material of clear cell renal cell carcinoma. a) VM channels are a vascular network; b) VM channels are parallel; c) double staining of the sections with antibodies against CD31 and PAS reagent. The integral system of tumor blood supply is seen.

ical trials) have demonstrated that not all tumors are sensitive to this therapy. Moreover, although the tumor decreases to some extent after the first line therapy, the second and third line therapies are ineffective. The *in vivo* experiments have demonstrated that prolonged treatment with angiostatin, a natural inhibitor of angiogenesis, induced resistance to the treatment in some mice [106]. Histological analysis of samples insensitive to the drug indicated the presence of VM, unlike samples that were sensitive to the treatment. Thus, the insensitivity of many tumor types to antiangiogenic therapy can be explained, at least partially, by the appearance of a vascular network formed by TCs.

VM is not generally accepted as a supplemental tumor blood supply. Although the fact is not denied that channels formed by TCs are present in malignant tumors, the contribution of such a network of channels to the tumor blood supply is questioned. But all investigators agree that the appearance of VM in a tumor correlates with elevated risk of metastasizing and short survival.

The first mention of VM occurred in 1999. Over the years, experimental material has been accumulated supporting a hypothesis on trans-differentiation of aggressive TCs into cells that are capable of vascular channel formation. On one hand, the aggressive TCs demonstrate high expression level of genes encoding VEGF receptor–ligand system proteins, Tie-1, Tie-2, EphA2, VE-cadherin, and MMP-2, which are necessary for TCs acquiring the endothelium-like characteristics, and on the other hand, Notch and its target gene *Nodal* expression is observed, enabling the aggressive TCs to simulate the behavior of embryonic cells. As the tumor progresses to a more aggressive phenotype, a constant selection of cells occurs, which favors those able to fit to a rapidly changing microenvironment: only those TCs survive that favor the tumor progression. The ability for trans-differentiation combined with a highly invasive, and, hence, metastatic potential in a number of cells allows them to form a channel network within the tumor, which can partially compensate insufficiently rapid development of the microcirculation network of blood vessels and create conditions for survival and proliferation of cells by preventing necrosis within the tumor. As a result, a small fraction of cells appears and develops in the tumor that is capable of invasive growth and, due to this feature, possesses elevated metastatic ability.

Interestingly, VM is not equally presented in different tumor types: in melanoma, 60-62% of blood supply occurs via VM channels; in soft tissue sarcoma 35-37%; in renal cell carcinoma 30-32%; in ovarian and breast cancers 15-18%; and in colorectal cancer 10-12% (our unpublished data). It is worth noting that the vascular system of the tumor is strikingly different from that of healthy tissue: in tumor the vessels are either greatly widened or barely visible. These vessels are not always connected with each other and are poorly stabilized by pericytes, which elevates the risk of bleeding. Their chaotic structure also decelerates the bloodstream [107]. Against the background of the extreme imperfection of the blood circulation in the tumor, 10-12% of VM channels are unlikely to significantly alter the situation, but 60-62% clearly can be significant. Those researchers who operate under small portions of VM do not consider VM as an additional system of tumor blood supply. In part they are right. In our experiments, we selectively inhibited VM in experimental melanoma model in three ways: antioxidants, Ca^{2+} chelators, and PKC inhibitor. In all three cases neither the volume nor the weight of the tumor was significantly changed, while the VM channel density significantly decreased. However, the number of metastases in lung significantly decreased. We find it difficult to interpret these data in any way. Nobody doubts today that the cancer patient does not die from a large tumor, but rather of metastases. It is the presence of metastases that makes extremely difficult a full recovery from cancer. Identification of markers that are tightly associated with the metastatic potential of each individual tumor is necessary not only for rational therapy of cancer patients, but also for the individualization of the treatment ("the right drug for right patient").

Establishment of VM is a complex biological process that involves several signaling pathways. Its contribution to the total blood circulation in the tumor is not yet determined. But beyond doubt, VM can be invaluable for solid tumors that grow as massive nodes with a small amount of vascular stroma. Studies on molecular mechanisms of VM establishment will allow not only better understanding the interaction between metastatic cells, its environment, and switching to the aggressive tumor form, but also will offer a novel approach to diagnosis and treatment of malignant diseases.

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