Advances and Prospects of Vasculogenic Mimicry in Glioma: A Potential New Therapeutic Target?

Heng Cai1–3,* Wenjing Liu4,* Xiaobai Liu1–3 Zhiqing Li1–3 Tianda Feng1–3 Yixue Xue5 Yunhui Liu1–3

1Department of Neurosurgery, Shengjing Hospital of China Medical University, Shenyang 110004, People’s Republic of China; 2Liaoning Clinical Medical Research Center in Nervous System Disease, Shenyang 110004, People’s Republic of China; 3Key Laboratory of Neuro-Oncology in Liaoning Province, Shenyang 110004, People’s Republic of China; 4Department of Geriatrics, First Hospital of China Medical University, Shenyang 110001, People’s Republic of China; 5Department of Neurobiology, College of Basic Medicine, China Medical University, Shenyang 110122, People’s Republic of China

*These authors contributed equally to this work

Abstract: Vasculogenic mimicry (VM) is the formation of a “vessel-like” structure without endothelial cells. VM exists in vascular-dependent solid tumors and is a special blood supply source involved in the highly invasive tumor progression. VM is observed in a variety of human malignant tumors and is closely related to tumor proliferation, invasion, and recurrence. Here, we review the mechanism, related signaling pathways, and molecular regulation of VM in glioma and discuss current research problems and the potential future applications of VM in glioma treatment. This review may provide a new viewpoint for glioma therapy.

Keywords: glioma, vasculogenic mimicry, glioma stem cells, hypoxia, drug delivery systems, noncoding RNAs

Introduction

Gliomas are among the most common central nervous system tumors. At present, comprehensive high-grade gliomas treatment involves surgical intervention combined with postoperative radiotherapy and chemotherapy. However, the median survival time for patients with glioblastoma is less than 14 months, as the tumors are prone to recurrence and the patient mortality rate is very high.1 High-grade gliomas are the typical vascular-dependent solid tumor,2 rich in tumor angiogenesis, and difficulty restricting tumor blood supply is one reason why clinical treatment is problematic.3

Antiangiogenic therapy has been an adjuvant therapy for high-grade gliomas for the past decade. The angiogenesis inhibitor bevacizumab has been used in the treatment of glioma. However, neuro-oncologists have found that angiogenic inhibitors have not achieved the desired therapeutic effect in clinical practice.4–6 It appears that glioma cells (GCs) exhibit “therapy resistance”, suggesting the presence of a blood supply source in gliomas that differs from traditional angiogenesis.

Discovery of Vasculogenic Mimicry (VM)

VM was first discovered by Maniotis et al in highly invasive malignant melanoma.7 VM is a matrix-rich conduit without endothelial cells (ECs), and an EC-independent tumor microcirculation model. Specifically, VM refers to a channel formed by a series of changes, including self-deformation and matrix remodeling of tumor cells to undergo “phenotypic transformation into ECs”.7 The tumor cells that comprise this channel structure show a variety of phenotypic transformations, such as dedifferentiation, where cells show the dual phenotypic characteristics of ECs and tumor cells.
Maniotis et al.\(^7\) first found a grid-like structure formed by interconnections between the stroma in malignant melanoma tissue sections. Using transmission electron microscopy, they observed that the grid structure in the tumor tissue was composed of a special kind of channel. This channel contained hemoglobin and plasma components that pass through it. They further used iodate Schiff staining and found that some of the ducts were strongly positive, suggesting that they were rich in matrix components. However, there was no expression of CD34, an EC marker, in the ducts. This finding suggested that there were no vascular ECs present in these structures.

Furthermore, Maniotis et al.\(^7\) found that the channels were rich in laminin, collagen IV, collagen VI, and heparan sulfate proteoglycan. Phenotypic analysis of tumor cells suggested that they had undergone a phenotypic transformation into ECs. Hemoglobin, red blood cells, platelets, and other blood components were observed in the duct, indicating that this channel was involved in the microcirculation supplied by the tumor vessels.\(^7,8\)

This kind of tumor cell, with an “EC phenotype”, forms the structure of the channels through complex processes of cell deformation, proliferation, migration, and matrix remodeling, to provide the blood supply required for invasive tumor growth. This is also one of the reasons why there is a lack of necrosis in malignant melanoma tissue sections.

Since its initial discovery, VM has been found in other solid tumors, including hepatocellular carcinoma,\(^9-12\) Ewing’s sarcoma,\(^12,13\) acute leukemia,\(^14\) ovarian carcinoma,\(^15,16\) cervical cancer,\(^17\) prostate adenocarcinoma,\(^18\) nasopharyngeal carcinoma,\(^19\) non-small cell lung cancer,\(^20\) lung adenocarcinoma,\(^21\) osteosarcoma,\(^22\) gastric cancer,\(^23,24\) breast cancer,\(^25,26\) and renal clear cell carcinoma.\(^27\)

The in vitro detection of VM involves Periodic Acid-Schiff (PAS)-CD34 double immunohistochemical staining to observe the structure of the lumen in the section. If the endothelial marker CD34 (or CD31) is present, PAS-positive staining indicates tumor vessels. If the endothelial marker CD34 (or CD31) is absent, PAS-positive staining indicates VM. Another detection method has been developed based on the Matrigel three-dimensional (3D) culture model of tumor cells in vitro. Arrangement of tumor cells in a 3D reticulum structure suggests that the tumor cells have undergone phenotypic transformation into ECs. Additionally, the glycoprotein-rich VM channel was also observed in 3D in an in vitro tumor cell culture model by using X-ray tomography for 3D reconstruction.\(^28\) Through time-lapse dynamic magnetic resonance angiography combined with electron microscopy and immunohistochemistry, this VM structure has been confirmed to be involved in tumor microcirculation.\(^29\)

**VM Formation in Glioma**

VM was discovered and reported in glioma by Yue and Chen\(^30\) in 2005. They collected 45 cases of WHO II–IV grade astrocytoma tissues and found PAS (+)/CD34 (-) channels in two high-grade astrocytoma tissues. These channels connected to the CD34 positive glioma microvessels and formed part of the microcirculation, which confirmed the existence of VM in gliomas.\(^30\) In a subsequent study of 101 glioma tissue samples, VM was found in glioma tissue sections from 13 samples. The positive rate of VM positively correlated with the survival time.\(^31\) The positive rate of VM in highly malignant adult glioblastoma specimens was higher than that in lower-grade glioma tissues.\(^32,33\) CD105 (a vascular EC marker) and CD133 double-positive GCs were also found in high-grade gliomas in children, suggesting that VM is not unique to adult high-grade glioma.\(^34,35\) VM formation in glioma is shown in Figure 1.

**Differentiation of Glioma Stem Cells (GSCs)**

Using gene chip technology, researchers analyzed and the expression of specific genes in GCs with VM. Their findings suggest that GCs undergoing VM may regain pluripotent characteristics, exhibit an embryonic phenotype, and undergo “transdifferentiation”.\(^36\) However, VM has been detected in some malignant tumors with bidirectional differentiation. Taken together, these studies have shown that to form VM, GCs must show “transdifferentiation” characteristics and be able to differentiate.\(^37\) Recently, Mei et al.\(^38\) collected 64 glioblastoma tissue samples. Live-cell imaging confirmed that malignant GSCs could differentiate into ECs and produce VM. Among them, CD133 (+) GSCs were considered to have a stronger ability to induce VM formation than CD133 (-) GSCs.\(^39\) Wu et al.\(^40\) found that bevacizumab could induce autophagy in GSCs and activate the vascular endothelial growth factor/vascular endothelial growth factor receptor-2 (VEGF/VEGFR-2) signaling pathway, which also promotes VM. This mechanism may, in part, explain the poor clinical efficacy...
of bevacizumab in the treatment of glioma. An increasing number of studies have shown that GSCs play a vital role in the development of VM in glioma. However, Ke et al found that non-stem-like cells of glioma were more prone to gain VM-related gene expression and phenotype than were stem-like cells of the same origin.

Based on the critical role of GSCs in glioma VM, the researchers developed a variety of drug delivery systems (Table 1), hoping to produce the dual effects of inhibiting glioma stem cell growth and glioma VM at the same time. Some receptors, such as VEGFR-2, neuropilin 1 (NRP1), ephrin A, and epidermal growth factor receptor (EGFR), are highly expressed on GSCs within the VM. Multifunctional targeted drug delivery is feasible when these receptors are efficiently targeted. Liposomes or micelles possess a high binding capacity for receptors and demonstrate superiority in tumor-homing imaging. This type of treatment is expected to become a new direction for the treatment of glioma.

Formation of Glioma VM in Hypoxic Environments

The rapid proliferation of GCs causes a relative lag in tumor angiogenesis, which then leads to the formation of a hypoxic microenvironment in localized tumor regions. In this hypoxic microenvironment, GCs are arranged autonomously into channels. These GC-arranged VM channels are the key to maintaining the malignant biological characteristics of tumors and have some EC functions and phenotypes. The hypoxic microenvironment also activates some related signaling pathways, molecules, and the prolyl hydroxylase activity decreases, allowing the hypoxia-inducible factor (HIF) subunit alpha subunit to escape von Hippel-Lindau degradation. HIF alpha subunits accumulate in the cytoplasm, where they combine with HIF beta to form heterodimers and then translocate to the nucleus to activate target gene transcription. Additionally, hypoxia inhibits HIF degradation, allowing HIF-1α or HIF-2α to be in the nucleus and bind to the hypoxia response element of the target gene. Activation of VEGF, cytokines, stem cell characteristic maintenance-related genes, and epithelial-mesenchymal transition (EMT) inducers could also lead to VM.

Rapid cell proliferation and unorganized perfusion in glioma microcirculation can cause an increase in interstitial fluid pressure, inducing ECs to cross the blood-tumor barrier and form a hypoxic environment in the center of the glioma.
| No | Drug Carrier | Types of Drugs | Name of Drug | Special Conjugate | Other Conjugate | The Transport and Identification Mechanism | The Aim of Treatment | Ref |
|----|--------------|----------------|--------------|-------------------|----------------|-------------------------------------------|---------------------|-----|
| 1  | Liposomes    | Single         | Combretastatin A4 | Peptide containing the Asn-Gly-Arg | NA | Endocytosis, CPP | Anti-VM | 43 |
| 2  | Liposomes    | Single         | PTX           | Multifunctional tandem peptide R8-c(RGD) | NA | Endocytosis, CPP | Anti-VM and anti-BCSCs | 44 |
| 3  | Liposomes    | Single         | DOX           | Hyaluronic acid ion-pairing nanoparticle | NA | Endocytosis, CPP | Anti-VM and anti-BCSCs | 45 |
| 4  | Liposomes    | Single         | PTX           | Tandem peptide R8-dGR | Integrin αvβ3 and NRP1 receptors recognizing peptide | Endocytosis, CPP | Anti-VM and anti-BCSCs | 46 |
| 5  | Liposomes    | Dual           | PTX; artemether | Mannose-vitamin E derivative; dequalinium-lipid derivative | NA | Glucose transporters; adsorptive-mediated endocytosis | Anti-VM; induction of apoptosis in brain cancer cells and BCSCs | 47 |
| 6  | Liposomes    | Single         | PTX           | TR peptide | Integrin αvβ3-specific vector | Endocytosis, CPP | Anti-VM and anti-BCSCs | 48 |
| 7  | Liposomes    | Single         | PTX           | SHH targeting peptide; VEGFR 2 targeting peptide | CK peptide; GYG linker | PEG-PLA | Anti-VM and anti-BCSCs | 49 |
| 8  | Liposomes    | Single         | DOX           | d-peptide of nicotine acetylcholine receptors | VEGFR 2 and NRP1 recognizing peptide | Endocytosis, CPP | anti-VM | 50 |
| 9  | Micelles     | Single         | PTX           | Tumor-homing peptides | GRP78 | Endocytosis, CPP | Anti-VM and anti-BCSCs | 51 |
| 10 | Micelles     | Single         | PTX           | EGFR/EGFvIII Dual-Targeting Peptide | NA | Endocytosis, CPP | Anti-VM and anti-BCSCs | 52 |
| 11 | Liposomes    | Dual           | Lycobetaine, OCT | nRGD | NA | Endocytosis, CPP | Anti-VM, anti-BCSCs and anti-tumor-associated macrophages | 53 |
| 12 | Micelles     | Single         | PTX           | Peptide ligand RAP12 of LRP1 | NA | PEG-PLA | Anti-VM and anti-BCSCs | 54 |
| 13 | Liposomes    | Single         | DOX           | Myristic Acid-Modified DA7R Peptide | NA | Endocytosis, CPP | Anti-VM and anti-BCSCs | 55 |

(Continued)
the glioma. This hypoxia causes GCs with “stem cell characteristics” to form VM channels, which are then connected to endothelial-dependent blood vessels to form early VM structures. This structure is a mixed structure in which glioma microvessels and VM coexist. The spatial and temporal correlations between the VM networks and GCs with “stem cell characteristics” suggest that these cells are the early driving forces of VM.

Matrix Remodeling Is a Critical Step in VM
In the identification of VM, the PAS-positive matrix layer was found to cover the inner surface of the VM structure. At present, the known matrix components include laminin, collagen, mucopolysaccharide, and F tissue factor and its inhibitors. The first several components are also components of the vascular basement membrane, which promotes connection and penetration between the VM structure and glioma microvessels. The balance between F tissue factor and its inhibitors is the key regulatory mechanism controlling anticoagulant function and maintaining VM blood flow.

Immune Cell Infiltration
GCs can recruit tumor-associated immune cells, especially M2 tumor-associated macrophages (TAMs) that express CD68 and CD206.67 GCs secrete IL-4 to activate TAMs and upregulate the expression of CD68, Arg-1, and CD204. Activated TAMs are widely recruited to, and infiltrate, VM-positive areas where they activate and upregulate cyclooxygenase-2. This further activate prostaglandin E and prostaglandin E receptor 1 through the protein kinase C pathway, and promotes VM in glioma.58,69

Regulation of VM in Glioma
Many molecules and signaling pathways are involved in the regulation and development of VM in glioma.

Hypoxia-Related Signaling Pathways
Hypoxia can induce VM. Under hypoxic conditions, leucine-rich repeats and immunoglobulin-like domains 1 (LRIG1) inhibit the EGFR mediated phosphoinositol 3-kinase (PI3K)/AKT pathway and repress the EMT.70 The inhibition of hypoxia-induced VM in gliomas has been studied.70 Under hypoxia, the rapamycin target protein is involved in VM formation in glioma through HIF-1α.63 In the in vitro hypoxia glioma model, B-cell lymphoma 2 (Bel-2) inhibits VM formation in gliomas by inhibiting the activation of the HIF-1α-MMP-2-MMP-14 signaling pathway.71 Silencing Beclin-1 can also significantly reduce hypoxia-induced VM formation.72 Additionally, under hypoxia, some GSCs express vascular endothelial (VE)-cadherin; VE-cadherin and HIF-2α directly interact to contribute to GSC VM formation.73

The VEGF family is a group of regulatory molecules critical for angiogenesis in glioma, and it is also involved in VM regulation in gliomas.41 For example, GSCs express VEGFR-2, which is activated by VEGF and promotes tubule formation. During autophagy in GSCs, phosphorylation of VEGFR-2 is activated by the PI3K-AKT pathway, which promotes the formation of VM in GSCs.40 The role of VEGF in VM in gliomas was also detected by the dynamic 3D culture model.74

| No | Drug Carrier | Types of Drugs | Name of Drug | Special Conjugate | Other Conjugate | The Transport and Identification Mechanism | The Aim of Treatment | Ref |
|----|--------------|----------------|--------------|-------------------|----------------|---------------------------------------------|----------------------|-----|
| 14 | hMSCs        | Single         | Bispecific immunotoxins | VEGF165; ephrin A | PE3KDEL | Injection of engineered hMSCs | Anti-VM and inhibiting tumor growth | 56 |
| 15 | Liposomes    | Single         | DOX          | Heptapeptide A7R | VEGFR 2 and NRPI recognizing peptide | Endocytosis, CPP | Anti-VM and anti-BCSCs | 57 |

Abbreviations: VM, vasculogenic mimicry; PTX, paclitaxel; DOX, doxorubicin; CTT, octreotide; hMSCs, human mesenchymal stem cells; BCSCs, brain cancer stem cells; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2; NRPI, neuropilin-1; RGD, arginine-glycine-aspartic acid; SHH, human sonic hedgehog targeting peptide; LRP1, LDL receptor related protein 1; PEG-PLA, poly(ethylene oxide)- poly(lactic acid); CPP, cell penetrating peptides. NA, not applicable.
VE-cadherin, a member of the cadherin superfamily, is closely related to hypoxia-related signaling molecules. Under hypoxic conditions, VE-cadherin is upregulated in a HIF-1α- and HIF-2α-dependent manner and contributes to hypoxia-induced VM. Abnormal expression of VE-cadherin specifically by ECs was also found in VM glioma-like stem cells, suggesting that VE-cadherin is involved in VM.

Matrix Metalloproteinases (MMPs)
MMPs play an essential role in VM formation and are essential protein targets and effectors in the VM regulatory network. It has been reported that both MMP-14 and MMP-2 degrade the gamma 2 laminin subunit into gamma 2′ and gamma 2x fragments and then stimulate glioma cell invasion and VM. In malignant glioma, MMP-14 expression and activation transform MMP-2 precursors into active MMP-2 and affect matrix remodeling, which affects VM formation in glioma. Histone deacetylase activates MMP proteins through the PI3K-ERK signaling pathway and promotes VM formation by regulating the expression of laminin subunit gamma 2 (LAMC2), a mimicry-related molecule in gliomas.

Cytokine Family
Epidermal growth factor (EGF) is increased in GCs, and binding to EGFR activates its downstream pathways, including PI3K-AKT, ultimately activating LAMC2 and cyclooxygenase-2 and promoting VM. The inhibitory effect of LRIG1 on VM in glioma is also mediated by the EGFR signaling pathway. EMT plays an important role in glioma progression. Transforming growth factor beta (TGF-β) induces the development of VM, while the TGF-β1 inhibitor galunisertib inhibits astrocyte-induced VM in glioma. Additionally, the expression of insulin-like growth factor-binding protein 2 (IGFBP2) is positively correlated with VM in patients with glioma. IGFBP2 interacts with the integrin alpha5beta1 subunits and enhances CD144 expression in a FAK-ERK pathway-dependent manner, IGFBP2 can also activate CD144 and MMP2 through transcription factor SP1 activation, enhancing VM in gliomas. Aquaporin-1 may play a role in VM in glioblastoma, and it can be used as a new diagnostic biomarker and a potential therapeutic target. In oligodendroglioma, downregulation of galec tin-1 gene expression, a significant decrease in brain expressed X-linked 2 expression, and inhibition of VM may present new therapeutic strategies for reducing chemotherapy resistance. A histone deacetylase inhibitor has also been identified as a promising candidate for VM inhibition in glioblastoma. Finally, suppression of Axin1 and curA have also been shown to affect VM.
Noncoding RNAs
In recent years, the regulatory roles of noncoding RNAs (ncRNAs) in glioma occurrence, metastasis, invasive growth, and angiogenesis have become the focus of glioma research. ncRNAs include long ncRNAs (lncRNAs), microRNAs (miRNAs), and PIWI-interacting RNAs.

lncRNAs are a newly discovered class of ncRNAs with lengths of more than 200 nucleotides. lncRNAHOXA-AS2 is upregulated in glioma tissues and is positively correlated with the positive rate of VM.78 HOXA-AS2 knockout attenuates the GC viability and represses VM, which may occur through VE-cadherin inhibition. Moreover, HOXA-AS2 knockout inhibits the activity of MMP-2 and MMP-9.78 In addition, LINC00339 expression in glioma positively correlates with VM formation. LINC00339 inhibits miR-539-5p expression, resulting in increased expression of twist family bHLH transcription factor 1 (TWIST1). TWIST1 upregulates MMP-2 and MMP-14 promoter activities and expression.89 The USF1 transcription factor promotes VM in glioma by regulating lncRNA-SNHG16 and lnc00667. Silencing of USF1 can inhibit VM occurrence, which may be regulated by a competitive endogenous RNA mechanism.90 lncRNA SNHG20 also plays a vital role in regulating the formation of VM in glioma.

miRNAs are also essential regulators of VM in glioma. Xue et al.92 found that miR-Let-7f reduces the occurrence of VM in gliomas by inhibiting periostin-induced GC migration. Li et al.93 confirmed that miR-141 expression in primary gliomas is downregulated. miR-141 regulates GC proliferation, migration, and invasion by controlling EphA2 expression, which then affects VM in gliomas. miR-584-3p plays a role in glioma inhibition by inhibiting

| No. | The Types of Noncoding RNAs | The Name of Noncoding RNAs | Function | The Target Molecules |
|-----|-----------------------------|-----------------------------|----------|---------------------|
| 1   | lncRNA                      | HOXA-AS2                    | Promotion| Inhibit VE-cadherin expression, and inhibit the expression and activity of MMP-2 and MMP-9, PI3K-AKT signaling pathway |
| 2   | miRNA                       | miR-373                     | Inhibition| Inhibit VE-cadherin expression, and inhibit the expression and activity of MMP-2 and MMP-9, PI3K-AKT signaling pathway |
| 3   | lncRNA                      | LINC00339                   | Promotion| Increase in the expression of TWIST1. TWIST1 upregulates the promoter activities of MMP-2 and MMP-14, and increases the expression and activity of MMP-2 and MMP-14 |
| 4   | miRNA                       | miR-539-5p                  | Inhibition| Increase in the expression of TWIST1. TWIST1 upregulates the promoter activities of MMP-2 and MMP-14, and increases the expression and activity |
| 5   | lncRNA                      | SNHG16                      | Promotion| Increase the expression of ALDH1A1 |
| 6   | lncRNA                      | linc00667                   | Promotion| Increase the expression of ALDH1A1 |
| 7   | miRNA                       | miR-212-3p                  | Inhibition| Inhibit the expression of ALDH1A1 |
| 8   | miRNA                       | miR-429                     | Inhibition| Inhibit the expression of ALDH1A1 |
| 9   | lncRNA                      | SNHG20                      | Promotion| Upgradation of FOXL1 mRNA by SMD pathway |
| 10  | miRNA                       | miR-Let-7f                  | Inhibition| Disturbing periostin induced migration |
| 11  | miRNA                       | miR-584-3p                  | Inhibition| Disturbing hypoxia-induced stress fiber formation and migration of glioma cells |
| 12  | lncRNA                      | lincRNA-SNHG16              | Promotion| Disturbing hypoxia-induced stress fiber formation and migration of glioma cells |
| 13  | miRNA                       | miR-9                       | Inhibition| Disturbing hypoxia-induced stress fiber formation and migration of glioma cells |
| 14  | miRNA                       | microRNA-26b               | Inhibition| Disturbing hypoxia-induced stress fiber formation and migration of glioma cells |

Abbreviations: lncRNA, long noncoding RNA; miRNA, microRNA; TWIST1, transcription factor twist family bHLH transcription factor 1; VE-cadherin, vascular endothelial-cadherin; HOXA-AS2, HOXA cluster antisense RNA 2; ALDH1A1, aldehyde dehydrogenase 1 family member A1; SMD, Staufen1-mediated mRNA decay; EphA2, EPH receptor A2; STMN1, stathmin 1.
VM formation in GCs by antagonizing hypoxia-induced ROCK1-dependent stress fiber formation. miR-9 and miR-26b can also be used as potential anti-VM molecules in GCs.

These results suggest that ncRNAs are critical VM regulatory molecules in glioma. Looking for a noncoding RNA molecule may be a potential target for glioma therapy (Figure 2 and Table 2).

Conclusion
The in-depth study of VM in gliomas has shown that VM can be used as a new entry point for the basic research of gliomas, and as a new direction in glioma growth inhibition. Moreover, VM has become the focus of many researchers to solve antiangiogenesis-targeted drug resistance in the treatment of gliomas.

The main issues remaining to be addressed in VM research in glioma are: (1) the glioma microenvironment and its complexity, in which the relationships among various regulatory factors, specific regulatory mechanisms, and glioma VM are not clear; (2) the relationship between GSCs and VM in glioma is not clear; and (3) at present, glioma VM research is mainly supplemental to glioma angiogenesis research, and the relationship between VM and angiogenesis and their interaction with the malignant progression of glioma have not been reported. Nevertheless, the study of the role of VM in gliomas may still provide a new direction for glioma treatment.

Abbreviations
VM, vasculogenic mimicry; ECs, endothelial cells; GCs, glioma cells; PAS, Periodic Acid-Schiff; 3D, three-dimensional; GSCs, glioma stem cells; VEGF, vascular endothelial growth factor; VEGFR-2, vascular endothelial growth factor receptor-2; NRP1, neuropilin 1; EGFR, epidermal growth factor receptor; HIF, hypoxia-inducible factor; EMT, epithelial-mesenchymal transition; TAMS, tumor-associated macrophages; LRIG1, leucine-rich repeat sequences and immunoglobulin-like domain 1; PI3K, phosphoinositide 3-kinase; Bcl-2, B-cell lymphoma; MMPs, matrix metalloproteinases; VE-cadherin, vascular endothelial-cadherin; LAMC2, laminin subunit gamma 2; TGF-β, Transforming growth factor beta; IGFBP2, insulin-like growth factor-binding protein 2; ncRNAs, noncoding RNAs; lncRNAs, long noncoding RNAs; miRNAs, microRNAs; TWIST1, transcription factor twist family bHLH transcription factor 1; IFP, interstitial fluid pressure.

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Disclosure
The authors report no conflicts of interest in this work.

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