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REVIEW PAPER

Molecular ultrasound assessment of tumor angiogenesis

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Abstract Angiogenesis, the growth of new blood vessels, plays a critical role in progression of tumor growth and metastasis, making it an attractive target for both cancer imaging and therapy. Several molecular markers, including those that are involved in the angiogenesis signaling pathway and those unique to tumor angiogenic vessels, have been identified and can be used as targets for molecular imaging of cancer. With the introduction of ultrasound contrast agents that can be targeted to those molecular markers, targeted contrast-enhanced ultrasound (molecular ultrasound) imaging has become an attractive imaging modality to non-invasively assess tumor angiogenesis at the molecular level. The advantages of molecular ultrasound imaging such as high temporal and spatial resolution, non-invasiveness, real-time imaging, relatively low cost, lack of ionizing irradiation and wide availability among the imaging community will further expand its roles in cancer imaging and drug development both in preclinical research and future clinical applications.

Keywords Molecular ultrasound · Molecular imaging · Imaging · Targeted contrast-enhanced ultrasound · Tumor · Angiogenesis · Cancer · Anti-angiogenic therapy · VEGF · Integrin · Endoglin

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Introduction to tumor angiogenesis

Angiogenesis is the development of new vasculature from pre-existing blood vessels and/or circulating endothelial stem cells [1]. This process is required for pre and postnatal development and for tissue repair [2, 3]. It is well established that angiogenesis is also one of the key aspects in the growth and metastasis of solid tumors [4–6]. Typically, tumor-associated angiogenesis goes through two phases, an avascular and a vascular phase that are separated by the ‘‘angiogenic switch’’ (Fig. 1a). The avascular phase of tumors corresponds to small and occult lesions that stay dormant and subsist on diffusion of nutrients from the host microvasculature. After reaching a certain size (usually around 1–2 mm [7]), a small subset of dormant tumors enter the vascular phase in which exponential tumor growth ensues. Angiogenesis is a complex multistep process regulated by many factors. At the onset of angiogenesis, a number of pro-angiogenic growth factors (e.g., vascular endothelial growth factors, platelet-derived growth factor, fibroblast growth factors) and proteolytic enzymes (e.g., matrix metalloproteinases, cathepsin cysteine proteases, plasmin) are secreted into the interstitium. This leads to the degradation of basal membrane surrounding the pre-existing vasculature, along with proliferation and migration of smooth muscle and endothelial cells (Fig. 1a). All these events finally lead to the alignment and organization of endothelial cells to form new vessels and a vascular network within the tumor [1].

Advances in knowledge of tumor angiogenesis have resulted in the identification of several molecules involved in tumor angiogenic signaling. These molecules have been exploited for their use as targets for molecular imaging and quantification of tumor angiogenesis. Furthermore, discovery of these molecules has led to realization of the

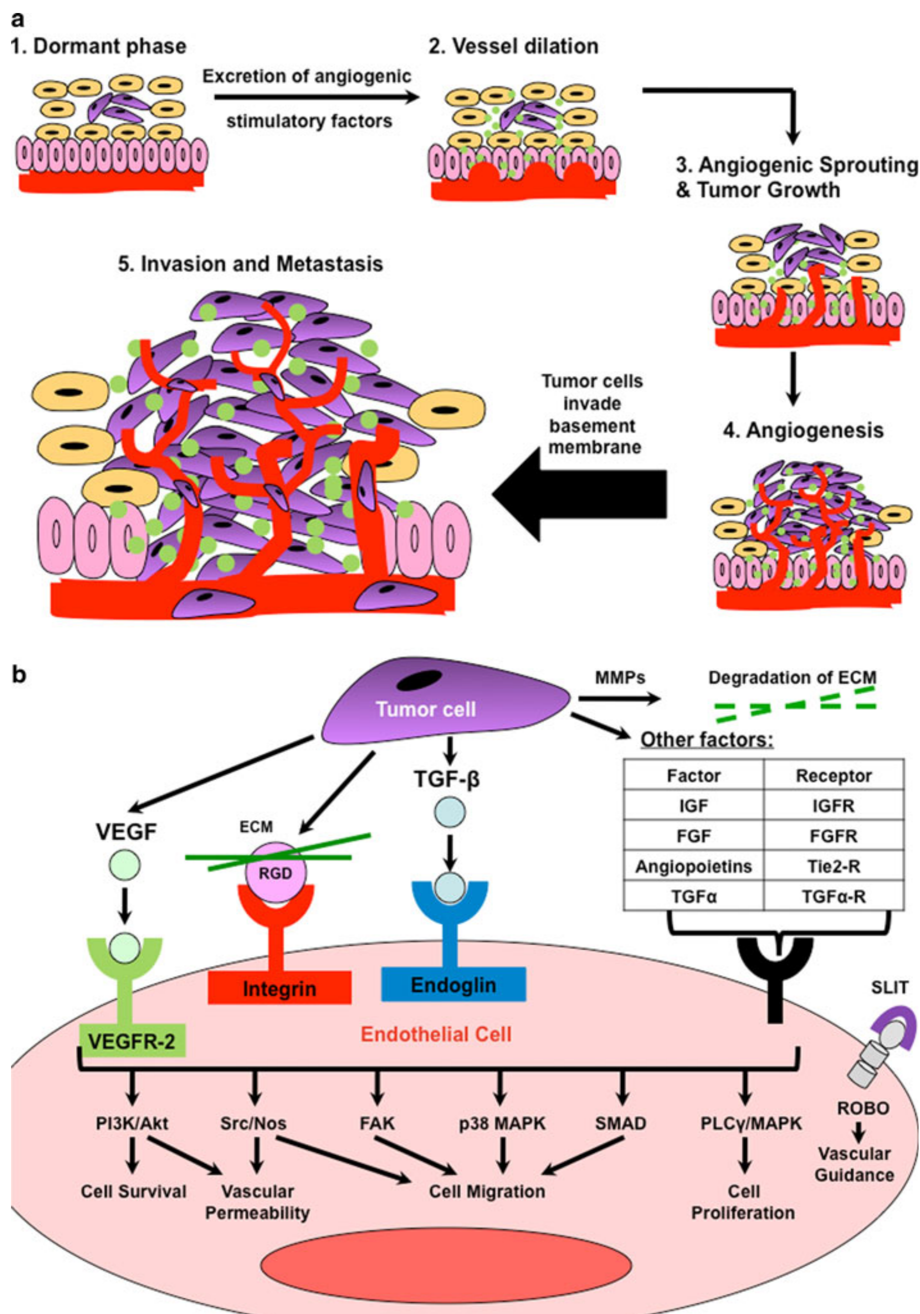


Fig. 1 Tumor angiogenesis is a complex multi-step and multi-signalling process. a When a dormant tumor (step 1) reaches critical size (usually ~1–2 mm) and receives intracellular signals from the tumor microenvironment (e.g., hypoxia), the tumor cells (*purple*; *yellow cells*: normal epithelial cells; *pink vertical cells*: stroma/basement membrane; *red line*: blood vessel) begin to excrete growth factors (step 2), cytokines, and other signaling molecules (*green*). These factors can influence the stromal cells to also produce these factors, which signal (see b) blood vessels to dilate, and grow towards the tumor cells. Microvessels are sprouted (step 3) within the tumor, which survives and proliferates due to the new delivery of nutrients, growth factors, and oxygen. Eventually, a complex vascular network has been created within the tumor (step 4) which promotes (step 5) invasion beyond the basement membrane and metastasis to other tissues. Schematic is adapted from [1]. b Several growth factors are involved in promoting tumor angiogenesis (in a). The most characterized players include ligand-receptor complexes of VEGF-VEGFR-2, RGD (from ECM matrix)-integrin, and TGFβ-endoglin. These, along with other factor-receptor (shown in table) or protein–protein (SLIT and ROBO) binding complexes, result in activation and cross-talk among downstream signaling pathways to promote increased endothelial cell survival, vascular permeability, cell migration, cell proliferation, and vascular guidance. Adapted from Cell Signaling Technologies [90]

concept that tumor vessels can be selectively targeted for therapy. The development of anti-angiogenic therapy (e.g., an antibody or small molecule inhibitor of new vessel formation [6, 8], or anti-vascular therapy e.g., a small molecule inhibitor of new vessel formation as well as destructor of pre-existing tumor microvessels [8]) has been one of the most promising avenues for cancer therapeutics in the last few years.

Molecular markers of tumor angiogenesis

There are numerous proteins/enzymes involved in the angiogenic signal transduction pathway (Fig. 1b) such as vascular endothelial growth factor receptor type 2 (VEGFR-2), integrins, and endoglin (to mention only a few), and, several studies have demonstrated that these molecules are over-expressed on tumor angiogenic vessels compared with normal vessels [9–11]. Among the best-characterized angiogenic signal transduction pathways is the avenue modulated by vascular endothelial growth factor (VEGF) and its receptors (VEGFRs) [12, 13]. The VEGF family is composed of 7 members with a common VEGF homology domain, and, amongst them, VEGF-A plays an important role in tumor angiogenesis. VEGF-A is a homodimeric, disulfide-bound glycoprotein existing in several isoforms with different numbers of amino acid residues. VEGF-A binds to two receptor tyrosine kinases, VEGFR-1 and VEGFR-2 [14], and of these two receptors, VEGFR-2 acts as a direct signal transducer of tumor angiogenesis. Activation of VEGFR-2 triggers multiple signaling networks that result in endothelial cell survival, mitogenesis, migration, differentiation, and vascular permeability (Fig. 1b). Tumor-associated endothelial cells over-express VEGFR-2 and its expression has been associated with tumor progression and poor prognosis in several tumors, including colorectal, gastric, and pancreatic carcinomas; angiosarcoma; breast, prostate and lung cancers, malignant gliomas and melanomas [15, 16]. The expression of VEGFR-2 in tumor vascular endothelial cells in much higher compared with endothelial cells of normal tissue [14] which makes it an attractive molecular target for angiogenic molecular imaging and cancer therapy.

Another signaling pathway involved in the angiogenic response involves the composition of the extracellular matrix (ECM) and a family of transmembrane proteins, the integrins (Fig. 1b). Integrins are expressed both on endothelial cells for modulation of cell migration and survival during angiogenesis, as well as on cancer cells for invasion and movement across blood vessels for metastasis. Integrins consist of two non-covalently bound chains, the α (alpha) and β (beta) subunits. In mammals, 18 α and 8 β subunits assemble into 24 different types of receptors with varying functions. Integrin $\alpha_v\beta_3$ in particular has received a lot of attention as it is highly expressed on tumor-associated endothelium, and almost absent on normal vessels making it very useful as a molecular target of angiogenesis [17, 18]. Integrin $\alpha_v\beta_3$ plays a role in tumor invasion and metastasis by recruiting and activating proteases such as matrix metalloproteinases (MMPs). Expression of MMPs is associated with the removal of the ECM barrier to allow cancer cells and endothelial cells to invade the basement membrane. A number of MMPs are also specifically involved in angiogenesis, including MMPs 1, 2, 3, 9, and 14 [19]. Several ECM molecules, such as fibronectin and tenascin, are also markers of angiogenesis. Spliced variants of fibronectin (the extra B domain, ED-B) [20] and tenascin (tenascin-c) [21, 22] are highly expressed around tumor neovasculature. ED-B of fibronectin, inserted in fibronectin by alternative splicing, is specifically expressed in neovessels of tumors [20]. A spliced variant of tenascin known as tenascin-c which interacts with several ECM molecules, is highly expressed in the tumor neovasculature of lung cancers and modulates cell migration, proliferation and cellular signaling [21, 22].

Identification of other key players in angiogenic signaling is emerging. Transforming growth factor β (TGF-β) signaling plays a role in several biological processes, including embryonic development, carcinogenesis, wound healing and angiogenesis [11]. In normal cells, the TGF-β pathway restricts cell growth, differentiation and cell death. In contrast, in malignant cells, various components of the TGF-β signaling pathway become mutated thereby exploiting the ability of TGFβ to modulate growth promoting processes, including cell invasion and angiogenesis. TGF-β signaling is mediated by TGFβ-binding to TGFβ receptors, of which, there are three classes: Types I and II are heterodimeric receptors whereas type III are homodimeric receptors. Endoglin is a TGFβ-type III receptor which has been shown to participate in signaling angiogenesis. Endoglin is predominantly expressed on proliferating endothelial cells [23], and inhibition of its expression has been shown to restore the growth suppressing signals of the TGFβ signaling pathway [24]. Thus, endoglin is an attractive molecular target of angiogenesis since it is over-expressed on tumor-associated endothelium [11].

Other molecular targets of tumor angiogenesis can be identified either by immunohistology or by differential gene expression analysis on isolated tumor endothelial cells [25]; several of these include prostate-specific membrane antigen (PSMA), ephrin (Eph) ligands and receptors, and magic roundabout-4 (Robo-4), among others [26]. PSMA is a type II transmembrane glycoprotein over-expressed in prostate cancer. It is also expressed on the neovascular endothelium of most solid tumor types [27]. Invasion studies with PSMA-null cells showed that PSMA

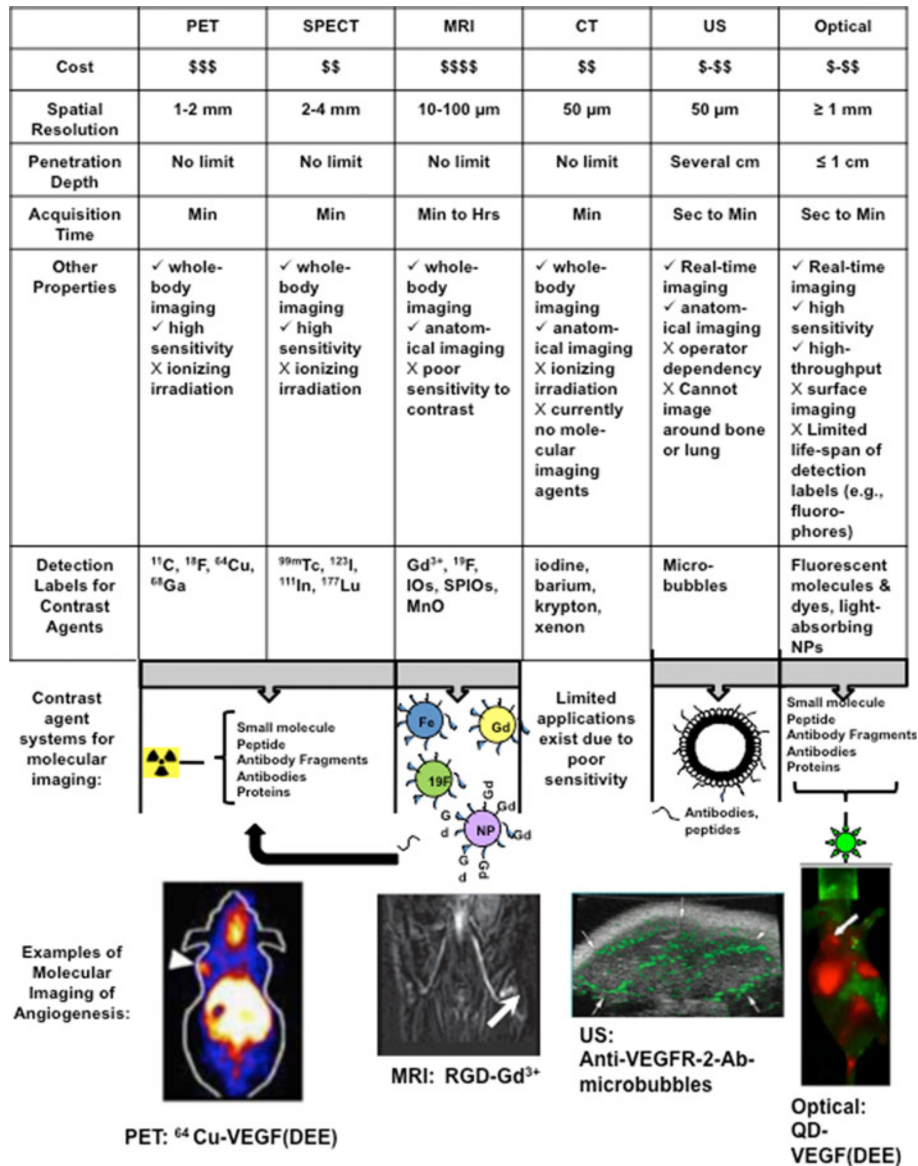
regulates cell invasion by controlling signaling from integrins [28]. Robo-4, a new member of the roundabout receptor family [29], is exclusively expressed in endothelial cells [30]. Transfection of Robo4 into endothelial cells has been shown to increase the number of Þlopodial extention from the cell suggesting its role in endothelial cell migration [31]. The ephrin (Eph) ligands and its receptors are also involved in the process of angiogenesis. The ephrins bind to two families of trans-membrane receptor tyrosine kinases, EphA and EphB. Several ephrins and Eph receptors have been shown to be present on tumor vascular endothelium in ovarian cancer [32]. Amongst them, EphB2 in particular is thought to play an important role in promoting tumor recruitment of blood vessels and tumor cell intravasation into the blood circulation, contributing to both tumor growth and metastasis [33, 34].

The advantage of identifying new proteins that are highly speciÞc for tumor-endothelium is not only that they are attractive targets for drug development but also for distinguishing the process of angiogenesis from inßammation. Angiogenesis also plays a role in inßammation, where the aforementioned molecular targets (i.e., VEGFR-2, integrin $\alpha_V\beta_3$, etc.) can also be over-expressed on angiogenic vessels associated with inßammation; therefore, it is important to have the ability to differentiate inßammation from cancer. This is especially important considering the relationship between cancer and inßammationÑcancer formation has been shown to induce an inßammatory response (i.e., the immune system recognizes the cancer cells as foreign and initiates an immune response [35, 36] and inßammation is associated with an increased risk of cancer (e.g., conditions such as hepatitis or pancreatitis involve production of free radicals that can lead to cellular damage and/or transformation [37]). Since patients with pre-existing inßammatory conditions are at an elevated risk for developing cancer, they may undergo more frequent screening in the future (e.g., via molecular imaging) for cancer; therefore, it is important to be able to distinguish the cancer tissue in a background of inßamed tissue by using tumor-speciÞc contrast agents (i.e., targeted to tumor-speciÞc molecular markers). In depth understanding of the cellular and molecular basis of tumor angiogenesis over the past two decades, and continuing research to identify new molecular targets of angiogenesis has resulted in the identiÞcation of several promising molecules over-expressed on tumor endothelium. These molecules can be used for cancer imaging, staging, therapeutic treatment, and monitoring.

Molecular imaging of tumor angiogenesis

Molecular imagingÑthe in vivo visualization and quantiÞcation of molecular markers involved in biological/cellular processes [38, 39]Ñcan be performed with various imaging modalities including, positron emission tomography (PET), single-photon emission computed tomography (SPECT), magnetic resonance imaging (MRI), magnetic resonance spectroscopy (MRS), ultrasound (US), and optical imaging (Fig. 2). Spectroscopic techniques, including MRS [40] and optical spectroscopy methods such as Raman spectroscopy, [41, 42] infrared spectroscopy, [42] or Fourier Transform infrared spectroscopy [43], among others [44, 45] can measure molecular markers directly by detecting changing energies associated with molecular bonds. These methods involve applying a pulse of energy (e.g., magnetic Þeld or photon) to excite molecules to a different energy state, and measuring the difference in energy states during relaxation following the energy pulse; thus, different energies can be measured for speciÞc molecules. Other modalities require the use of contrast agents (Fig. 2), constructed of a label for readout by an imaging modality (e.g., radiolabel for PET or SPECT imaging) and a ligand that will bind to the targeting molecule of interest. Ligands for molecular targeting can be small molecules, peptides, oligonucleotides, proteins, antibody fragments or antibodies. More simplistic contrast agents can involve direct conjugation or an insertion of a small linking molecule between an imaging label and ligand, while more sophisticated contrast agents can involve multiple labels and/or using a biocompatible nano- or micro-sized particles (e.g., carbon nanotube, liposome, microbubble; see Fig. 2).

Molecular imaging strategies of tumor angiogenesis can be divided into two categories: (1) imaging environments or factors that contribute to angiogenesis (e.g., tumor hypoxia induces VEGF production, which in turn induces angiogenesis; in this example, one can image hypoxia or VEGF); and (2) imaging angiogenic vessels by targeting speciÞc markers on tumor-associated endothelial cells. Several contrast agents for imaging tumor endothelial markers with various imaging modalities (Fig. 2) have been developed preclinically, and the most common targets are the most characterized to dateÑintegrins and VEGFR-2 (Table 1). For integrins, the RGD amino acid sequence (arginine-glycine-aspartic acid) to which integrins bind has been used as the binding ligand for (1) radiolabelled contrast agents for use with PET and SPECT; (2) attachment to Gadolinium-loaded or magnetic nanoparticles for imaging with MRI; (3) for attachment to microbubbles for imaging with ultrasound (discussed further below); and, (4) ßuorescently labeled contrast agents for detection by optical imaging (Table 1). Currently, the only RGD-targeted imaging agent for approved clinical use is the PET radiotracer, ^{18}F-galacto-RGD radiotracer [46]. Contrast agents targeted to VEGF receptors can either be labeled with ligands that bind these receptors or by an antibody or a peptide that directly binds to VEGFR-2 (Table 1; Fig. 2).



	PET	SPECT	MRI	CT	US	Optical
Cost	$$$	$$	$$$$	$$	$-$$	$-$$
Spatial Resolution	1-2 mm	2-4 mm	10-100 μm	50 μm	50 μm	≥ 1 mm
Penetration Depth	No limit	No limit	No limit	No limit	Several cm	≤ 1 cm
Acquisition Time	Min	Min	Min to Hrs	Min	Sec to Min	Sec to Min
Other Properties	✓ whole-body imaging ✓ high sensitivity X ionizing irradiation	✓ whole-body imaging ✓ high sensitivity X ionizing irradiation	✓ whole-body imaging ✓ anatom-ical imaging X poor sensitivity to contrast	✓ whole-body imaging ✓ anatom-ical imaging X ionizing irradiation X currently no mole-cular imaging agents	✓ Real-time imaging ✓ anatom-ical imaging X operator dependency X Cannot image around bone or lung	✓ Real-time imaging ✓ high sensitivity ✓ high-throughput X surface imaging X Limited life-span of detection labels (e.g., fluoro-phores)
Detection Labels for Contrast Agents	^{11}C, ^{18}F, ^{64}Cu, ^{68}Ga	^{99m}Tc, ^{123}I, ^{111}In, ^{177}Lu	Gd^{3+}, ^{19}F, IOs, SPIOs, MnO	iodine, barium, krypton, xenon	Micro-bubbles	Fluorescent molecules & dyes, light-absorbing NPs

Fig. 2 Advantages and disadvantages of various molecular imaging modalities, including positron emission tomography (*PET*), single photon emission computed tomography (*SPECT*), magnetic resonance imaging (*MRI*), computed tomography (*CT*), ultrasound (*US*), and optical imaging (adapted from Willmann et al. [91]). Values are listed for small animal imaging systems. Basic contrast agent design includes a binding ligand such as a small molecule, peptide, antibody, antibody fragment, or protein to bind to the protein target of interest, as well as a label for readout by the different imaging modalities. Readout labels are shown as (1) radiolabels for PET or SPECT (radioisotopes listed separately); (2) Gadolinium (Gd^{3+})-loaded nanoparticles (nanoparticles are ~10–200 nm; see review Nune et al. [92]) or liposomes (~200 nm lipid-shelled vesicle; see review: Blanco et al. [93]), iron oxide (*IO*) nanoparticles (*NPs*), ^{19}F-loaded nanoparticles for MRI, or not shown: superparamagnetic iron oxide (*SPIO*) NPs, or monocrystalline oxide (*MnO*; see review on molecular MRI contrast agents: [94]) for use with MRI; (3) microbubbles (e.g., ~1–4 μm phospholipid-shelled bubbles filled with gas core; see Fig. 3) for use with US imaging; or, (4) fluorescent dyes or molecules (e.g., green fluorescent protein (*GFP*)) or optical-absorbing nanoparticles (e.g., quantum dots (QDs) are 1–10 nm nanocrystals; see Bentolila et al. [95]) for use with optical imaging. Examples of molecular imaging of angiogenesis in small-animals are provided for each modality: (1) PET imaging of VEGFR-2 expression in 4T1 breast subcutaneous tumors (shoulder, *arrow*) with a ^{64}Cu-labelled VEGF peptide sequence binding specifically to VEGFR-2 (VEGF(DEE); reprinted with permission from Wang et al. [96]); (2) T1-weighted MRI imaging of integrin $\alpha_v\beta_3$ expression in M21 melanoma (*white arrow*) with Gadolinium-loaded dendrimers targeted with cyclic RGD (reprinted with permission from Barrett and Choyke [97]); (3) VEGFR-2-targeted microbubbles (conjugation of biotinylated anti-VEGFR-2 antibody (*Ab*) to streptavidin-coated microbubbles (see Fig. 3)) and molecular ultrasound in C6 rat glioma subcutaneous xenografts (*arrows*: tumor borders) in mice. Green targeted contrast signal was overlaid on *gray* Brightness-mode (B-mode) image (reprinted with permission: Willmann et al. [82]); and, (4) near-infrared optical imaging of quantum dots conjugated to VEGF(DEE; same peptide as in PET image; reprinted with permission from Chen et al. [84]) in U87MG glioma subcutaneous tumors in mice (shoulder, *arrow*)

Table 1 Examples of molecular imaging modalities and contrast agents targeted to the angiogenic markers integrins and VEGFR2 (adapted from [72, 73])

Molecular target/event	PET	SPECT	MRI/MRS	US	Optical
Integrins (tumor angiogenesis)	^{64}Cu-RGD (SWNT); ^{64}Cu-RGD (QD); ^{64}Cu-RGD (SPIO); ^{64}Cu-knottin peptides [72, 74]	^{111}In-perßuorocarbon NP-RGD [75]	RGD peptide Gd containing paramagnetic and ßuorescent liposomes; RGD peptide-SPIOs [76]	Knottin-RGD conjugated MBs; RGD MBs; Anti-β_3 Ab-MB; [63] Echistatin-coated MBs [77]; β_3-targeted perßuoro-carbon NP	RGD-QD705; RGD-Rhodamine/PE-liposomes; Cy5.5-knottin peptides; Raman: SWNT-RGD [72, 78]
Vascular endothelial growth factor (VEGF) receptor (VEGFR; Tumor angiogenesis)	^{89}Zr-Avastin; ^{64}Cu-DOTA-VEGF; ^{64}Cu-DOTA-VEGF (peptide) [79, 80]	^{111}In-Avastin; ^{125}I-VEGF$_{165}$ (^{125}I or ^{99}Tc)-VEGF$_{121}$, ^{111}In-hnTf-VEGF [81]		Anti-VEGFR2 Ab-MB; [82] KDR peptide-conjugated MBs [62]	VEGF-Cy5.5; VEGF-QD [83, 84]

Strategies for choosing the speciÞc angiogenic marker to target and the imaging modality are dependent upon the contrast agent type and properties (e.g., biodistribution, kinetics), as well as the application. For example, the molecular marker PSMA, a marker of prostate cancer, has been shown to be expressed on both prostate cancer cells and prostate cancer-associated endothelial cells [47]. Therefore, this target is not optimal for imaging with a small molecule or antibody that can leak out of tumor microvessels and also bind to prostate cancer cells; in this case, the quantiÞed signal would not accurately represent a measure of tumor angiogenesis, but rather, the tumor as a whole. However, if a larger particle, such as a microbubble (i.e., lipid-shelled, gas-Þlled bubbles that are 1Ð4 µm in diameter and used as a contrast agent with ultrasound imaging), that remains in the vasculature and only binds to endothelial-speciÞc PSMA, the associated quantiÞed signal of bound contrast agent could directly measure PSMA expression levels in prostate cancer endothelium. Application-speciÞc choices of imaging modalities and contrast agents are strongly driven by the advantages and disadvantages of the modality (Fig. 2). In addition to the routine use of molecular imaging for diagnosing and staging of cancer (currently performed mostly with PET and SPECT), the in vivo identiÞcation and quantiÞcation of tumor molecular proÞles, including angiogenic markers, with molecular imaging can be used to decide on target-speciÞc chemotherapy on a patient-speciÞc basis (personalized medicine), and for monitoring the direct effect of therapy on the targeted molecule. The direct monitoring of therapeutic treatmentÑfor example, monitoring VEGFR-2 expression following a therapy targeted to VEGFR-2 and resulting in downregulation of VEGFR-2 expressionÑ often occur much sooner than other changes, such as tumor cell death or changes in tumor size. Therefore, measurement of these therapeutic effects before overt morphological changes can provide an earlier assessment, and may avoid exposing non-responding patients to unnecessary possible side effects from a therapy they may not beneÞt from.

In summary, molecular imaging has many applications, and can involve frequent imaging of a patient, which can dictate the choice of the molecular imaging strategy. PET and SPECT imaging both involve the use of ionizing irradiation, which is impractical for frequent imaging (e.g., in a screening setting) as the cumulative irradiation dose may harm the patient. MRI does not involve ionizing irradiation exposure; however, this modality is expensive, and sensitive quantiÞcation of molecularly targeted contrast agents often involves high-doses that can be toxic [48]. Compared to these modalities, optical imaging is inexpensive and highly quantitative; however, it has limited applications due to its lack of depth penetration in tissue (i.e., limited to surface imagingÑfor example, skin cancer, esophagus or colon with optical endoscopy, or bladder cancer imaging with optical cystoscopy [49Ð51], and availability to clinicians. Ultrasound imaging with molecularly targeted contrast microbubbles is of particular interest from this point of view, since this modality is relatively inexpensive, offers real-time contrast imaging, allows relatively deep tissue penetration [52], and does not involve ionizing irradiation, and is widely available and portable (Fig. 2). These advantages make molecular ultrasound imaging ideal for protocols involving frequent imaging such as early detection strategies through screening (e.g., for cancer in high risk patients) and therapeutic monitoring. In the following section we focus on use of molecular ultrasound for imaging and quantiÞcation of angiogenic markers and monitoring anti-cancer therapies.

Principles of molecular ultrasound imaging

Contrast-enhanced ultrasound imaging is based on the reception, analysis and display of acoustic signals produced by reflection or backscatter of sound (echo) with use of contrast agents. The most commonly used contrast agent are microbubbles that are gas-liquid emulsions consisting of a gaseous core (e.g., perfluorocarbon, sulfur hexafluoride, or nitrogen) that is enclosed by a shell composed of biocompatible materials (albumin, galactose, lipids, polymers; Fig. 3). The gaseous core of the microbubbles causes a very high echogenic response following insonification with ultrasound, resulting in a high contrast-to-tissue background ratio. Owing to their micron size (usually ranging in size between 1 and 4 μm diameter), these microbubbles stay within the vascular compartment, and do not leak out into the extra-vascular space. Thus, microbubbles are highly suitable for imaging angiogenic markers that are overexpressed on tumor vascular endothelial cells [53].

Microbubbles have been engineered for safe clinical applications (e.g., identification and characterization of focal liver lesions [54, 55]. Following intravenous administration, microbubbles do not coalesce to form emboli, but dissolve leaving remnants that are easily metabolized or excreted. Further, biodistribution studies revealed that microbubbles have low circulation residence times as they are rapidly removed by the reticuloendothelial system (RES) [48, 56, 57]. To increase the circulation time of the microbubbles in serum, additional coatings, such as polyethylene glycol (PEG) polymer arms are added onto the microbubble shell. Additionally these coatings help stabilize the microbubble by providing additional steric protection, preventing aggregation and help escape immune surveillance by the body [58] (Fig. 3).

To make ultrasound a molecular imaging tool, contrast microbubbles can be functionalized with ligands such as antibodies or peptides that bind molecular marker of interest with high affinity [59]. These binding ligands can either be coupled to the microbubble using non-covalent attachment (e.g., biotin-streptavidin [60]), or chemical conjugation [60] or they can be integrated into the microbubble shell directly during the production process or after manufacturing (Fig. 3) [61, 62]. Furthermore, the attachment of ligands can be on the PEG arm which acts as a spacer between lipid shell and the site of binding in the tissue [53]. The nature of the binding chemistry is important for potential clinical translation of targeted contrast microbubbles. For example, streptavidin can cause immunogenic and allergic reactions in patients and, therefore, cannot be used in clinically translatable microbubbles. Alternative binding strategies of ligands onto the microbubbles shell (e.g., maleimide/thioether, amine (NH_2)/amide attachment) or direct insertion of a

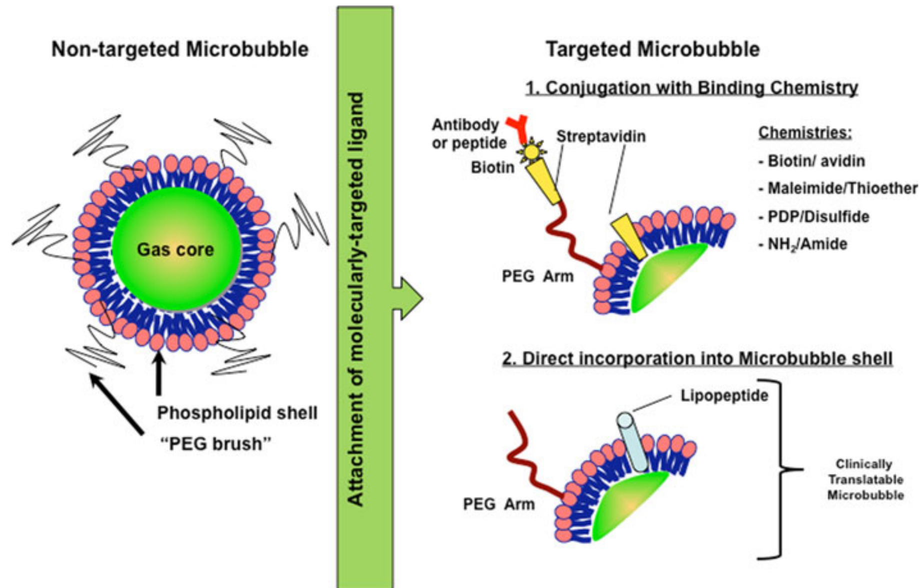


Fig. 3 Design considerations for molecularly-targeted microbubbles (adapted from [53]). Most non-targeted microbubbles (*left*) are 1–4 μm microspheres consisting of a phospholipid monolayer shell and filled with a heavy perfluorinated gas (e.g. perfluorobutane or perfluorocarbon gas). Additional coatings of biocompatible polymers (e.g., polyethylene glycol (*PEG*)) or proteins (e.g., albumin) are added for physical stability, to escape immune surveillance, to prevent microbubble aggregation, to provide spacing between lipid shell and binding ligand, and as platform for attaching binding ligands to molecular targets. Ligands (such as antibodies or peptides) for binding proteins expressed on endothelial cell surfaces can be attached by several approaches, including (1) non-covalent attachment using biotin/streptavidin, biotin/avidin, maleimide/thioether, 2-(Pridylthio)propionyl (PDP)/disulfide, or amine (NH_2)/amide attachment systems via the PEG arm or lipid shell components; or, (2) direct incorporation of peptides conjugated to lipids (to form lipopeptides) during the manufacturing process, which is needed for clinical translation of molecularly-targeted microbubbles (see text)

lipid-associated molecule (Fig. 3) are being explored as potential alternative strategies for designing clinically translatable microbubbles [61, 62].

With the advent of microbubbles, ultrasound technology has undergone technical advances as traditional ultrasound imaging techniques were inadequate to selectively isolate imaging signal from microbubbles. New imaging techniques exploit the unique behavior of a microbubble in an ultrasonic Þeld for the purposes of detection as well as quantiÞcation of signal. Upon insoniÞcation with low ultrasound transmit frequencies, microbubbles gives rise to nonlinear echoes. These echoes are readily distinguishable from the surrounding issue. Today, most contrast ultrasound systems are equipped for detection of these nonlinear echoes. Modern ultrasound imaging approaches for enhancing microbubble-enhanced ultrasound imaging signal and for quantiÞcation of microbubble-enhanced imaging signal are reviewed elsewhere [53].

Assessment of tumor angiogenesis with molecular ultrasound imaging

Several preclinical studies have validated the use of targeted microbubbles for detection of tumor angiogenesis in mouse models of human cancers (Tables 1 and 2). Molecular ultrasound performed using microbubbles targeting makers such as VEGFR-2, integrin $\alpha_v\beta_3$, and endoglin (Table 2) showed high accumulation in tumor tissue which translated to higher signals compared to non targeted microbubbles. Further, results from these studies correlated with data obtained by ex vivo analysis such as immunohistochemistry and immunoblotting. Earlier studies generated targeted microbubbles primarily by attaching monoclonal antibodies against markers of angiogenesis using streptavidinÐbiotin conjugation chemistry. Subsequently, several research studies worked towards enhancing the ultrasound signal due to microbubbles and making it feasible for clinical translation by several modiÞcations. One such modiÞcation is to use small peptide sequences that bind the molecular marker with high afÞnities to target microbubbles. This idea was demonstrated by Willmann et al. [63], showing that an $\alpha_v\beta_3$ integrin-targeted microbubble (constructed by attaching a high-afÞnity binding small knottin peptide onto the surface of contrast microbubbles small [64]) resulted in twofold higher in vivo signal than an anti-integrin $\alpha_v\beta_3$ antibody-targeting microbubble in human ovarian tumor xenografts in mice. An important consideration for designing clinically translatable microbubbles conjugated with peptides for binding

Table 2 Summary of studies on the use of targeted microbubbles for molecular ultrasound imaging of tumor angiogenesis

Animal model	Molecular target	Binding ligand	Ligand-microbubble conjugation system	Reference
Subcutaneous human colon cancer xenografts in mice	Human KDR/VEGFR2	Human KDR/VEGFR2 peptide	Heterodimeric KDR-targeted peptide	Pysz et al. [62]
Orthotopic rat breast cancer model in rats	Human KDR/VEGFR2	Human KDR/VEGFR2 peptide	Heterodimeric KDR-targeted peptide	Pochon et al. [61]
Subcutaneous human ovarian cancer xenografts in mice	$\alpha_v\beta_3$ integrin	Biotinylated knottin peptide	StreptavidinÐbiotin	Willmann et al. [63]
Subcutaneous human ovarian cancer xenografts in mice	VEGFR2 and $\alpha_v\beta_3$ integrin	Biotinylated anti-VEGFR2 and anti-$\alpha_v\beta_3$ integrin antibodies	StreptavidinÐbiotin	Willmann et al. [67]
Subcutaneous mouse angiosarcoma and rat glioma xenografts in mice	VEGFR2	Biotinylated anti-VEGFR2 antibody	StreptavidinÐbiotin	Willmann et al. [82]
Subcutaneous murine breast cancer tumors in mice	VEGFR2	Biotinylated anti-VEGFR2 antibody	StreptavidinÐbiotin	Lee et al. [85]
Subcutaneous human melanoma xenografts in mice	VEGFR2	Biotinylated anti-VEGFR2 antibody	StreptavidinÐbiotin	Rychak et al. [86]
Subcutaneous murine breast cancer xenografts in mice	VEGFR2	Biotinylated anti-VEGFR2 antibody	StreptavidinÐbiotin	Lyshchik et al. [87]
Subcutaneous human prostate cancer xenografts and murine clone C tumors in mice	Tumor endothelial cell (target was not identiÞed)	Biotinylated RRL containing peptide	StreptavidinÐbiotin	Weller et al. [88]
Orthotopic human glioma model in rats	$\alpha_v\beta_3$ integrin	Biotinylated Echistatin peptide	StreptavidinÐbiotin	Ellegala et al. [77]

Note: Vascular endothelial growth factor receptor 2 (VEGFR2), RGD peptide (arginine-glycine-aspartic acid), RRL peptide (arginine, arginine, leucine)

molecular targets is the microbubble and peptide stability. For example, the knottin peptide conÞguration provides stability against protease degradation compared with the short, linear RGD sequence [64, 65]. In addition to peptide stability, increased microbubble stability can enhance signal by providing an increased efÞciency of binding. For example, Jun et al. [66] demonstrated that microbubbles conjugated with a cyclic form RGD peptide (arginine-glycine-aspartate-D-tyrosine-lysine) was more stable in circulation (circulation time was greater than 1 h) compared to control microbubbles targeted with AGD peptide (biotin-alanine-glycine-apartate). Increased circulation time can provide sufÞcient time for more microbubbles to attach the target, thus, providing high targeted signal and improved signal-to-background ratio.

Another approach to increase microbubble-enhanced ultrasound imaging signal for detection of tumor angiogenesis is the concept of dual- or multi-targeting microbubbles. Here, two or more ligands that bind to different angiogenic markers are attached to the surface of microbubbles which may increase the likelihood of microbubbles to actually attach to angiogenic vessels. This concept was shown recently [67] exploring the use of dual-targeted microbubbles carrying antibodies targeted to both VEGFR2 and integrin $\alpha_v\beta_3$. In this study, dual-targeted microbubbles accumulated more to tumor vessels of human ovarian cancer xenografts in mice than single-targeted microbubbles targeting either VEGFR2 or integrin $\alpha_v\beta_3$ [67]. This increased imaging signal at sites of tumor angiogenesis produced by using dual-targeted microbubbles may be useful in cases such as the early detection of cancer when tumors are too small to cause detectable morphologic changes but large enough to induce tumor angiogenesis [67].

Regarding moving targeted contrast microbubbles into the clinic, several steps are necessary in order to formulate a clinically translatable microbubble and rigorously test its use prior to clinical translation. An exemplary study [62] demonstrates these steps for the use of a novel, clinically translatable microbubble targeted to human kinase insert domain receptor (KDR is the human protein analogous of murine VEGFR-2): The Þrst step in designing a clinically translatable microbubble was to identify peptides that bind to the target of interest with high afÞnity, and then to conjugate it to the microbubbles in a manner that avoids immunogenic chemistries (see above). Two peptides were identiÞed by phage display and were found to bind to human KDR with high afÞnity [68]. These two individual peptides were then linked by a hydrophilic spacer to form a heteropeptide (further increasing the binding afÞnity of that hetropeptide to KDR to be 0.2Ð0.5 nM [69]. The heteropeptide was then connected to a lipid (separated by PEG to provide steric separation from the microbubble shell) to form a heterolipopeptide, which could be incorporated directly into the microbubble shell during manufacturing (BR-55 microbubbles; Bracco Research, Geneva) [61, 62].

The next step for translating peptide-targeted microbubbles involves testing the microbubbles in a clinically relevant animal model. However, this can be challenging if the binding peptide binds to the human target with high afÞnity but may not recognize the respective counterpart in the animal model. Testing cross-reactivity of a novel binding peptide between e.g. human and mouse receptors can be performed in cell culture experiments by testing binding afÞnity to isolated mouse VEGFR-2 protein [61] or to cells expressing VEGFR-2 [62]. Pysz et al. [62] demonstrated cross-reactivity of KDR-targeted microbubbles to adhere to mouse VEGFR-2-positive and human KDR-positive vascular endothelial cells under shear ßow conditions in cell culture. Non-targeted microbubbles did not bind to any cell type, thus, demonstrating one level of target speciÞcity. In addition to positive conÞrmation of binding, another level of speciÞcity to the VEGFR-2 was further tested by pre-treating the cells with an anti-mouse VEGFR-2 antibody to block the VEGFR-2 receptor from binding the KDR-microbubbles. This experiments showed that mouse VEGFR-2-positive cells failed to bind human KDR-targeted microbubble when blocked with anti-mouse VEGFR2 antibody indicating that KDR-microbubbles can cross-react with and bind speciÞcally to mouse VEGFR-2 [62].

Following proof of binding peptide cross-reactivity between human and e.g. mouse receptors, in vivo imaging studies in animal models of tumor angiogenesis can be performed. Pysz et al. [62] tested KDR-targeted microbubbles in a mouse subcutaneous tumor model for human colon cancer with a dedicated small-animal ultrasound imaging system. Similar to the cell culture studies, the speciÞc binding of KDR-targeted microbubble was demonstrated Þrst by comparison to non-targeted microbubbles, and second, by pre-administration of a mouse anti-VEGFR-2 antibody to block the receptor from binding to KDR-targeted microbubbles. These experiments demonstrated higher in vivo imaging signals with KDR-targeted microbubbles compared to non-targeted microbubbles and showed that the in vivo imaging signal using KDR-targeted microbubbles could be blocked following intravenous administration of blocking anti-VEGFR2 antibodies [62], conÞrming in vivo binding speciÞcity of human KDR-targeted microbubbles for murine VEGFR-2.

According to the aforementioned example, the path towards clinical translation of molecular ultrasound and molecularly targeted microbubbles involves (1) designing a safe microbubble that is targeted to a relevant target for human cancer and speciÞc for the human protein of interest; (2) testing its binding ability to bind to both the human

target and a similar, homologous rodent target with high specificity in vitro or in cell culture; and (3) to test its binding ability to the rodent target with high specificity in a human relevant application such as cancer imaging. Additionally, ex vivo quantification of molecular levels with immunoblotting and/or immunostaining could confirm and correlate ultrasound signal representing target expression to actual molecular marker expression on vessels.

Prior to full clinical use of the peptide-targeted microbubbles, its safety and toxicity needs to be tested. Several non-targeted microbubbles have been approved for clinical use in cardiology and radiology applications [56]; thus, the initial building blocks to clinical translation are already established. Additional testing of molecularly targeted microbubbles needs to include biodistribution analyses. Biodistribution studies on lipid shell perfluorocabon-filled microbubbles targeted to VEGFR2 was analyzed in vivo in living mice by using dynamic micro-PET [48]. Anti-VEGFR-2 antibodies were radiolabelled by conjugating radiofluorination agent N-succinimidyl-4-[^{18}F]fluorobenzoate to the antibodies as a tracer for in vivo assessment of targeted microbubble biodistribution using micro-PET imaging. Imaging revealed accumulation in the liver and spleen indicating that the microbubbles are cleared by the reticuloendothelial system and that 50% of targeted microbubbles cleared from the blood pool after ~3.5 min and ~95% were cleared from the blood circulation after 30 min [48]. Toxicity studies in several animal models and first in human dose escalation studies are the final steps for further moving a clinically translatable targeted contrast microbubble into clinical applications.

Monitoring anti-angiogenic therapy with molecular ultrasound imaging

Expression levels of molecular markers targeted by anti-angiogenic therapy can be directly monitored using molecular ultrasound. Moreover, changes in angiogenic marker expression from conventional cancer therapies can also be measured with molecular ultrasound. A few preclinical studies have tested this idea in small animal models of human cancers (Table 3). Korpanty et al. [70] used VEGFR-2- or endoglin-targeted microbubbles to measure therapeutic response of subcutaneous or orthotopic pancreatic cancer tumor-bearing mice that were treated with anti-vascular endothelial growth factor (VEGF) monoclonal antibodies (100 μg twice weekly of bevacizumab; Genentech) and/or gemcitabine (chemotherapeutic agent that inhibits DNA replication; 2 mg twice weekly; Eli Lilly). They detected decreasing accumulation of microbubbles labeled with either anti-VEGF-VEGFR-2 complex, VEGFR-2 or endoglin antibodies compared to control microbubbles (IgG labeled) after tumor-suppressive therapy. This result correlated with ex vivo expression analysis of the marker expression levels. Pysz et al. [62] reported the ability of KDR-targeted microbubbles to monitor anti-VEGF therapy (5 mg/kg B20-4.1.1.1, an anti-VEGF antibody targeting both human and mouse VEGF; Genentech) in human LS174T colon cancer xenografts in mice with molecular ultrasound (Fig. 4). Comparison of molecular ultrasound imaging signal with KDR-targeted microbubbles in mice receiving anti-angiogenic treatment or placebo (saline) showed a significant decrease (~40%) in treated mice (compared to pre-treatment baseline signal) as early as 24 h after initiation of anti-angiogenic therapy (Fig. 4). In contrast, no difference in molecular ultrasound KDR-targeted imaging signal was observed in non-treated mice. Furthermore, KDR-associated molecular ultrasound signal was observed prior to any changes in tumor size; thus, demonstrating the advantage of early assessment of anti-angiogenic therapy prior to overt morphological-anatomical changes become visible in tumors.

Future outlook of molecular ultrasound imaging

The potential of molecular ultrasound imaging with various angiogenic molecular targets has been clearly established with preclinical research applications in the areas of cancer

Table 3 Summary of studies on the use molecular ultrasound imaging for monitoring anti-angiogenic therapy

Anti cancer therapy	Type of cancer	Ligand	Ligand-microbubble conjugation system	Reference
Anti-VEGF antibody	Subcutaneous human colon cancer xenografts in mice	Human KDR/VEGFR2	Heterodimeric KDR-targeted peptide	Pysz et al. [62]
MMP inhibitor AG3340	Subcutaneous human squamous cell carcinoma xenografts in mice	Biotinylated anti-VEGFR2 antibody and biotinylated RGD peptide	Streptavidin–biotin	Palmowski et al. [89]
Anti-VEGF antibody and/or gemcitabine	Subcutaneous and orthotopic human pancreatic cancer xenografts in mice	Biotinylated anti-Endoglin, anti-VEGFR2, anti-VEGF-VEGFR complex antibodies	Streptavidin–biotin	Korpanty et al. [70].

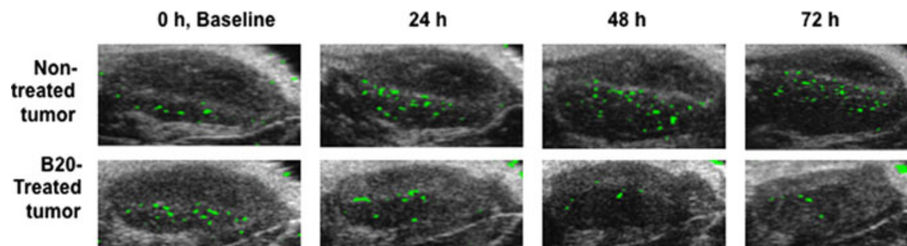


Fig. 4 Molecular ultrasound allows longitudinal monitoring of anti-angiogenic therapy. Ultrasound images represent subcutaneous human colon cancer xenografts in nude mice imaged with a novel, human KDR-targeted contrast microbubble [62]. Imaging signal from microbubbles attached to VEGFR2 is shown as *green signal* overlaid on B-mode image. *Note*, that the in vivo molecular imaging signal measured in tumor of non-treated mouse increases over time; whereas imaging signal in mouse treated with an anti-angiogenic therapy (B20 anti-VEGF-antibody) deceases as early as 24 h after initiation of therapy. Of note is also, that the molecular ultrasound imaging signal decreases before overt morphological changes in treated tumor become visible (for more details please refer to [62])

detection and therapeutic monitoring. However, clinical translation of molecular ultrasound imaging requires several improvements including design of biocompatible molecularly targeted microbubbles, improvements in targeted microbubble quantiÞcation, and improvements in instrumentation for sensitive and enhanced detection. First, microbubbles must be conjugated to molecular targeting moieties without the use of strept(avidin)/biotin conjugation chemistries, since those chemistries are immunogenic [71]. Several alternate strategies such as chemical coupling of ligands or direct insertion into microbubble shell during manufacturing (e.g., KDR-binding lipopeptide [61, 62]) have to be explored.

Second, improvements and standardization of targeted microbubble quantiÞcation needs to be performed for clinical translation. Imaging techniques that selectively detect only the adhered microbubbles as opposed to circulating microbubbles could aid in better quantiÞcation of signal. Finally, ultrasound devices currently use a two-dimensional imaging approach allowing only a limited assessment of the diseased tissue under consideration. New device technology has resulted in transducer access to a variety of tissues, including endoscopic ultrasound, intravascular ultrasound, transvaginal ultrasound, and transcranial ultrasound, among others. Current on-going advancements in three-dimensional ultrasound imaging techniques will also enable to extent the limited Þeld-of-view of current two-dimensional transducers and allow a more accurate quantiÞcation of molecular marker expression without relying on Þnding e.g., the same two-dimensional imaging plane during longitudinal monitoring of therapy. Three-dimensional ultrasound imaging is also important for reliable tumor detection and assessment of angiogenic treatment in a heterogeneous tissue, such as a cancer.

In conclusion, with rapid advances being made using molecular ultrasound in preclinical research, its translation into clinics is imminent. Once established in a clinical setting, molecular ultrasound may serve as a powerful tool for screening, diagnosing and treatment monitoring of cancer.

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