

Review Article

Multimodality molecular imaging of CD105 (Endoglin) expression

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Abstract: Since most solid tumor growth depends on angiogenesis, non-invasive imaging of tumor angiogenesis can allow for much earlier diagnosis and better prognosis of cancer, as well as more accurate treatment monitoring, which will eventually lead to personalized molecular medicine. CD105, also known as endoglin, is required for endothelial cell proliferation. The currently accepted standard method for quantifying tumor angiogenesis is to assess microvessel density based on CD105 staining, which has been shown to be an independent prognostic factor for survival in patients of almost all solid tumor types. In this review, we will summarize the progress to date on multimodality molecular imaging of CD105 expression during tumor angiogenesis which includes targeted contrast-enhanced ultrasound, molecular magnetic resonance, near-infrared fluorescence, single-photon emission computed tomography, and positron emission tomography. Although molecular imaging of CD105 expression is surprisingly understudied, non-invasive imaging of CD105 expression has already been achieved with every single molecular imaging modality. In the future, significant research effort should be directed towards non-invasive visualization of CD105 expression, such as quantitative imaging, the use of long-lived isotopes for antibody-based imaging, development of peptide, small molecule, or antibody fragment-based imaging agents, multimodality imaging of CD105 expression with a single agent, the application of nanotechnology, among others.

Keywords: Tumor angiogenesis, CD105 (Endoglin), molecular imaging, positron emission tomography (PET), single-photon emission computed tomography (SPECT), monoclonal antibody (mAb), cancer, anti-angiogenic therapy

Introduction

Cancer is the second leading cause of death in the United States (<http://www.cdc.gov>). In 2010, a total of 1,529,560 new cancer cases and 569,490 deaths from cancer are projected to occur in the United States alone [1]. One of the key requirements during tumor development is angiogenesis, the formation of new blood vessels, without which the tumor cannot grow beyond a few millimeters in diameter [2, 3]. Tumor angiogenesis is regulated by a variety of proteins such as growth factors/growth factor receptors, G-protein-coupled receptors for angiogenesis-modulating proteins, endogenous angiogenesis inhibitors, integrins, among others [3-5]. The fact that tumor progression is de-

pendent on angiogenesis has inspired scientists to search for anti-angiogenic molecules and design anti-angiogenic strategies for cancer treatment and prevention of cancer recurrence/metastasis [6, 7].

Many traditional medical imaging techniques, such as computed tomography (CT), magnetic resonance imaging (MRI), and ultrasound, have been routinely used to monitor the therapeutic effects of cancer intervention [8, 9]. However, with the shift in drug discovery from conventional cytotoxic drugs to novel agents against specific molecular targets, these conventional imaging modalities are usually no longer adequate. Molecular imaging, "the visualization, characterization and measurement of biological

processes at the molecular and cellular levels in humans and other living systems" [10], has evolved dramatically over the last decade and played an increasingly more important role in cancer diagnosis and patient management. In general, molecular imaging modalities include molecular MRI (mMRI), magnetic resonance spectroscopy (MRS), optical bioluminescence, optical fluorescence, targeted contrast-enhanced ultrasound, single photon emission computed tomography (SPECT), and positron emission tomography (PET) [11]. Many hybrid systems that combine two or more of these imaging modalities are also commercially available (both clinically and pre-clinically) and certain others are under active development [12-14].

Non-invasive molecular imaging of tumor angiogenesis can allow for much earlier diagnosis and better prognosis of cancer, as well as more accurate treatment monitoring, which will eventually lead to personalized molecular medicine. Over the last decade, many tumor angiogenesis-related targets have been explored for imaging and therapeutic applications to fight cancer. Among these, two most extensively studied targets are vascular endothelial growth factor receptors (VEGFRs) and integrin $\alpha_v\beta_3$, for which several excellent review articles are available [7, 15-22].

CD105, also known as endoglin, is a member of the TGF- β family of receptors that is required for endothelial cell proliferation [23, 24]. The currently accepted standard method for quantifying tumor angiogenesis is to assess microvessel density (MVD) based on CD105 immunohistochemistry (IHC). Not surprisingly, CD105-based MVD is an independent prognostic factor for survival in patients of almost all solid tumor types [25-27]. One key feature of CD105 is that it is selectively expressed on angiogenic endothelial cells at significantly higher levels (up to 3×10^6 copies per cell) than other angiogenesis-related targets such as the VEGFRs ($< 0.2 \times 10^6$ copies per cell) [28, 29]. Therefore, non-invasive imaging of CD105 expression has the potential to accelerate drug development by providing a reliable measure of angiogenesis in the entire body of an intact system, thereby facilitating individualized treatment monitoring and dose optimization in animal models, clinical trials, and ultimately in the day-to-day management of cancer patients. In this review, we will

summarize the progress to date on multimodality molecular imaging of CD105 expression during tumor angiogenesis.

Targeted contrast-enhanced ultrasound

Ultrasonography is the most commonly used clinical imaging modality due to its safety, low cost, ease of use, and wide availability [30]. Ultrasound contrast agents have been used for a variety of applications such as blood pool enhancement, characterization of liver lesions, and perfusion imaging [31, 32]. These contrast agents are typically small acoustically active particles (e.g. microbubbles) ranging from several hundred nanometers to a few micrometers in diameter [33]. Targeting is achieved either through manipulating the chemical properties of the microbubble shell or through conjugation of target-specific ligands to the microbubble surface [30, 34]. Since microbubbles are too large to extravasate, the disease process must be characterized by molecular changes in the vascular compartment to be imaged, which makes CD105 an ideal target.

In one study, avidin (Av) was incorporated into the shell of perfluorocarbon-exposed sonicated dextrose albumin microbubbles (Av-MBs) to anchor biotinylated monoclonal antibodies (mAbs) [35]. A rat anti-mouse CD105 mAb (MJ7/18) and an isotype-matched control mAb were investigated for biotinylation, microbubble incorporation, and cellular studies. It was found that MJ7/18-conjugated microbubbles bound specifically to endothelial cells but not fibroblasts, while the control mAb-conjugated Av-MBs did not exhibit CD105-specific targeting.

After demonstrating the proof-of-principle, a follow-up study was carried out to follow the vascular response of therapy in mouse models of subcutaneous and orthotopic pancreatic adenocarcinoma [36]. For comprehensive investigation of angiogenesis in tumor-bearing mice treated with anti-VEGF mAbs and/or gemcitabine (a nucleoside analog with known activity against pancreatic adenocarcinoma [37]), the localization of microbubbles targeting CD105, VEGFR-2, or VEGF-activated blood vessels (the VEGF-VEGFR complex) was monitored by ultrasound. In the subcutaneous model, receptor-targeted microbubbles gave significantly better enhancement of tumor vasculature than the non-targeted or control mAb-conjugated micro-

bubbles. In addition, video intensity from targeted microbubbles correlated with the level of target expression (CD105, VEGFR-2, or the VEGF-VEGFR complex), as well as with MVD in tumors under either anti-angiogenic or cytotoxic therapy. Together, these two studies demonstrated that microbubble-based targeted ultrasound represents an attractive non-invasive tool for imaging tumor angiogenesis and monitoring the therapeutic effect of various anti-cancer therapies.

Although exhibiting relatively high spatial resolution (50-500 μm), ultrasound has certain disadvantages such as relatively poor tissue penetration (usually a few centimeters depending on the frequency used) and limited sensitivity [11]. Further development of targeted ultrasound will involve the expansion of targeted disease states, improvements in technology for ligand attachment to microbubbles, better characterization of the acoustic behavior of targeted contrast agents, and development of more sensitive/accurate imaging methods. Acoustic destruction of "payload-bearing" microbubbles has been used to deliver drugs or to augment gene transfection [38]. Therefore, CD105-targeted microbubbles may also have future applications in tumor vasculature-targeted cancer therapy.

Molecular MRI

MRI is a non-invasive diagnostic technique based on the interaction of protons (or other nuclei) with each other and with surrounding molecules in a tissue of interest [39]. Different tissues have different relaxation times which can result in endogenous contrast for MRI. MRI has good spatial resolution (usually sub-millimeter level) with exquisite soft tissue contrast yet it suffers from inherent low sensitivity, which can be partially compensated for by working at higher magnetic fields (4.7 - 14 T in small animal models), acquiring data for a much longer time period, and/or using exogenous contrast agents. Many exogenous agents can enhance the contrast in MRI by selectively shortening either the T_1 (longitudinal) or T_2 (transverse) relaxation time. Traditionally, gadolinium (Gd) chelates have been used to increase the T_1 contrast [40] while iron oxide nanoparticles have been used to increase the T_2 contrast [41].

Recently, molecular MRI of CD105 expression in

tumor-bearing rats was achieved with Gd-DTPA-containing stabilized liposomes (Gd-SLs) [42]. A series of targeted and non-targeted MRI contrast agents were compared in glioma-bearing rats: Gd-DTPA, Gd-SLs, Gd-SLs conjugated to anti-CD105 mAbs (CD105-Gd-SLs), and Gd-SLs conjugated to control mAbs (IgG-Gd-SLs). Serial T_1 -weighed MRI before and after contrast agent administration revealed that the area with enhanced MRI contrast was restricted for CD105-Gd-SLs but not for the other three groups (i.e. the MRI contrast enhancement were more diffused; **Figure 1**). In addition, the degree of contrast enhancement over time also varied between different groups. For example, Gd-DTPA gave an early contrast enhancement which peaked at 30 min and declined to baseline values at 60 min, while the signal intensity for CD105-Gd-SLs continued to increase over a period of 120 min. The signal intensity of IgG-Gd-SLs and Gd-SLs both peaked at 60 min followed by a decline, yet the rate of decrease was quite different. Ex vivo histology further revealed that the enhancement in the CD105-Gd-SLs group resulted mainly from new microvessels. However, both mature microvessels and new microvasculature were responsible for contrast enhancement in the other three groups.

To date, this is the only report available in the literature for molecular MRI of CD105 expression. Although the proof-of-principle has been demonstrated for mMRI of CD105 expression [42], whether mMRI can significantly improve cancer patient management remains unclear. Since the major disadvantage of MRI is its inherent low sensitivity and Gd-based MRI can only be reliably detected at millimolar concentration, superparamagnetic iron oxide (SPIO) nanoparticles, which can be detected at much lower levels because of its high magnetism [41], deserve to be investigated for mMRI of CD105 in the future.

Near-infrared fluorescence (NIRF) imaging

Optical imaging is a relatively low-cost method suitable primarily for small animal studies. In fluorescence imaging, excitation light illuminates the subject and the emission light is collected at a shifted wavelength [43]. The major types of contrast agents used for fluorescence imaging include organic dyes [44], fluorescent proteins [45], and quantum dots [46]. The main drawback of fluorescence imaging is that it is

CD105 Imaging

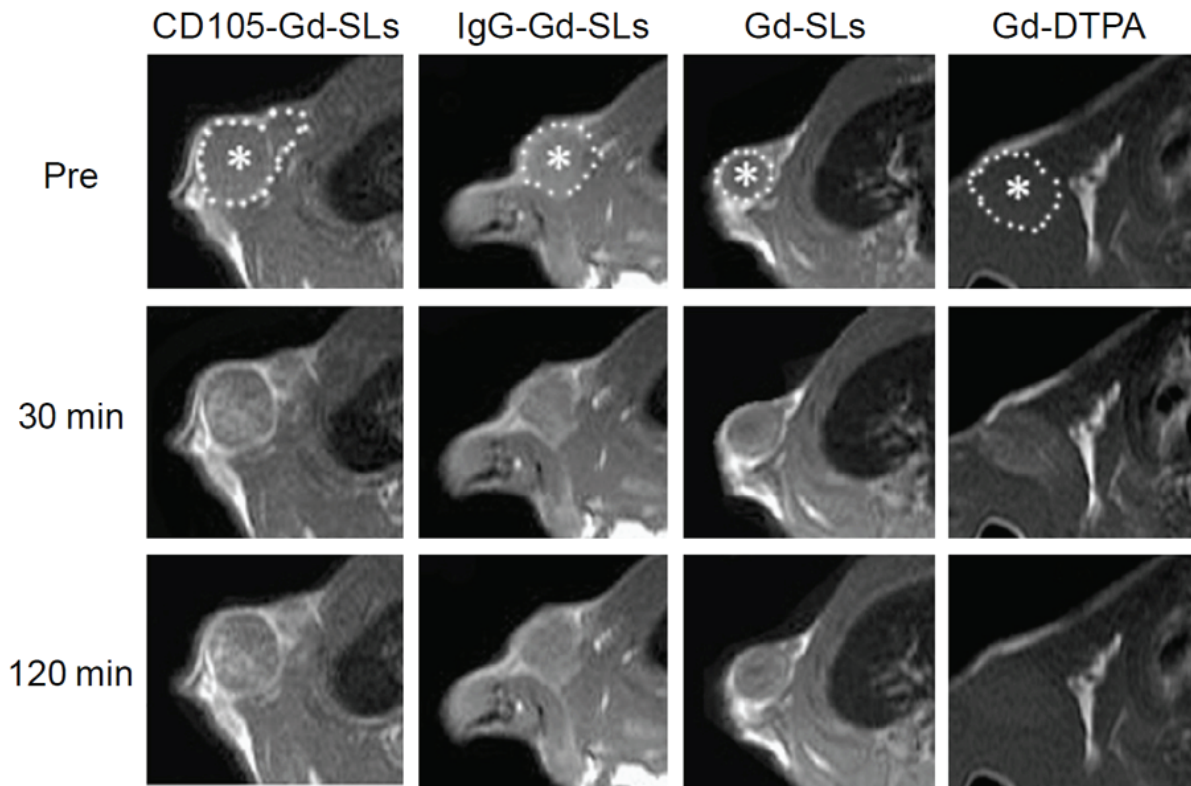


Figure 1. Molecular MRI of CD105 expression. T₁-weighted images at different time points post-injection of various contrasts agents are shown. *: subcutaneous tumor. Adapted from reference [42].

typically not quantitative and the image information is surface-weighted due to tissue absorption. In most cases, significant background signal is also observed because of tissue autofluorescence. For in vivo applications, imaging in the near-infrared region (NIR; 700 - 900 nm), where the absorbance spectra for all biomolecules reach minima thus providing a clear optical window [47], can provide better opportunities for visualizing tumor angiogenesis in both small animal models and various clinical scenarios.

One study investigated the applicability of a human umbilical vein endothelial cell (HUVEC)-based in vitro model to mimic physiological and angiogenic vasculature [48]. High fluorescence signal was observed in proliferating HUVECs due to CD105 expression. We recently investigated tumor angiogenesis in a mouse tumor model through NIRF imaging of CD105 expression [49]. TRC105, a human/murine chimeric mAb which binds to both human and murine CD105,

was conjugated to a NIRF dye (800CW). After confirming the CD105-binding affinity/specificity of 800CW-TRC105 in vitro, in vivo/ex vivo NIRF imaging, blocking studies, and ex vivo histology were performed on 4T1 murine breast tumor-bearing mice to evaluate the ability of 800CW-TRC105 to target tumor angiogenesis. Serial NIRF imaging after intravenous injection of 800CW-TRC105 revealed that the 4T1 tumor could be clearly visualized as early as 30 minutes post-injection (**Figure 2**). Tumor uptake of 800CW-TRC105 plateaued at about 24 h post-injection with excellent tumor contrast. In vivo target specificity of 800CW-TRC105 was further confirmed with several control studies (e.g. blocking studies and imaging with unconjugated 800CW dye).

The abovementioned study represents the first successful example of NIRF imaging of CD105 expression in vivo. Due to the poor tissue penetration and intense scattering of light, optical imaging (even in the NIRF region) will only be

CD105 Imaging

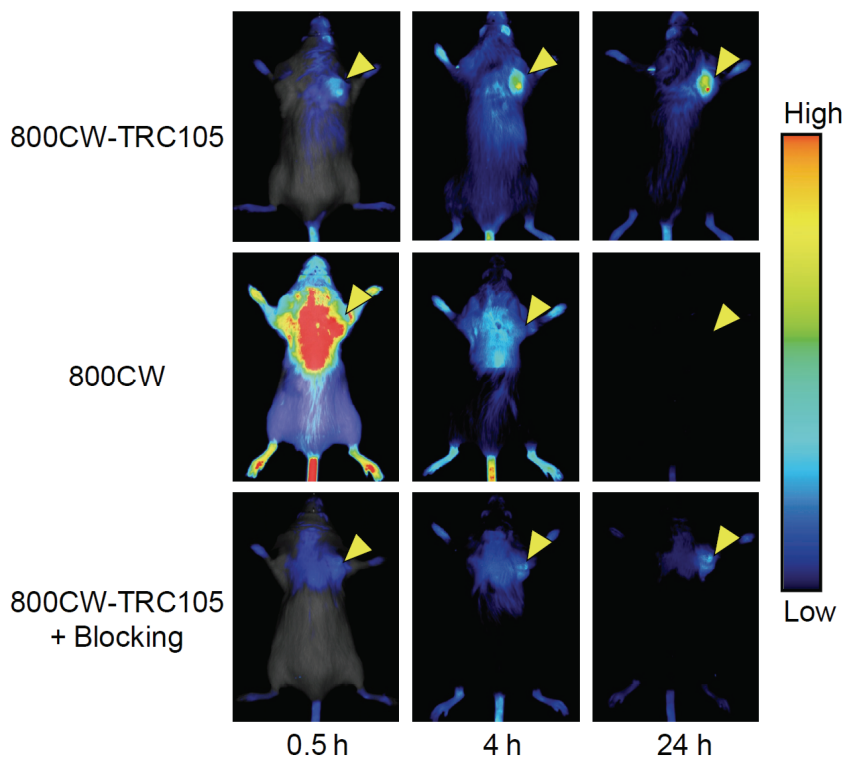


Figure 2. Optical imaging of CD105 expression. The 4T1 murine breast cancer tumors (arrowheads) in Balb/c mice can be clearly visualized in a small animal optical scanner after intravenous injection of 800CW-TRC105. The CD105 specificity was confirmed by the much lower tumor uptake of 800CW or 800CW-TRC105 when co-injected with TRC105 (denoted as “800CW-TRC105 + Blocking”).

possible in the clinical setting in limited sites such as the tissues and lesions close to the skin surface, tissues accessible by endoscopy, and during intra-operative visualization. In future studies, spectral imaging techniques (where fluorescence signals can be separated based on the emission spectra of different fluorophores [50]) and fluorescence-mediated tomography [51, 52] may also facilitate accurate interpretation of the fluorescence imaging data.

Single-photon emission computed tomography

Radionuclide-based imaging techniques (i.e. SPECT and PET) have been routinely used in the clinic over the last decade. Because of the wider availability of gamma cameras and SPECT scanners in the past, CD105 imaging was achieved with SPECT earlier than with PET. Due to the use of collimators to define the angle of incidence of emitted gamma rays [53], SPECT imaging has a very low detection efficiency ($< 10^{-4}$). Common radioisotopes used for SPECT imaging are ^{99m}Tc ($t_{1/2}$: 6.0 h), ^{111}In ($t_{1/2}$: 2.8 d), ^{123}I ($t_{1/2}$: 13.2 h), and ^{131}I ($t_{1/2}$: 8.0 d).

One pioneering study investigated an ^{111}In -

labeled anti-CD105 mAb (MJ7/18) and compared its neovascular binding, tumor accumulation, and in vivo behavior to an isotype-matched control mAb [54]. In a B16 melanoma model, the tumors in animals receiving ^{111}In -labeled MJ7/18 were more easily identified than animals receiving the radiolabeled control mAb. However, the tumor contrast was only modest. Ex vivo autoradiography and histology experiments of the tumor sections corroborated the different patterns of in vivo tumoral accumulation for the two antibodies. MJ7/18 exhibited intense activity in the peripheral region of the tumor, where the highest concentration of vessels was found, and much lower activity in the tumor centre. On the other hand, little accumulation of activity could be found in the tumors of mice that had been injected with ^{111}In -labeled control mAb. It was suggested that imaging of abundantly expressed endothelial targets could circumvent delivery barriers normally associated with other tumor targeting strategies and could potentially be used to quantitate molecular angiogenic markers.

At about the same time, a ^{125}I -labeled anti-CD105 mAb (MAEND3) was tested in a canine

mammary carcinoma model [55]. After demonstrating differential expression of CD105 on human breast cancer and endothelial cells, two dogs with spontaneous mammary tumors were intravenously injected with ^{125}I -labeled MAEND3 and imaged eight hours later. Rapid and intense uptake of the radiolabeled mAb with excellent tumor-to-background ratio was observed in the tumor areas of both dogs, which were confirmed as ductal mammary adenocarcinomas after surgical excision ten days later. Another study also explored the use of a ^{125}I -labeled anti-CD105 mAb for radioimmunotherapy applications in mouse tumor models [56]. Significant growth suppression of the tumors was observed while a ^{125}I -labeled control mAb did not show any significant anti-tumor efficacy.

A few years after these studies in preclinical models (mice and dogs respectively), a $^{99\text{m}}\text{Tc}$ -labeled anti-CD105 mAb (E9) was investigated in freshly excised kidneys from renal carcinoma patients [57]. Since E9 does not cross-react with animal tissues, this strategy can serve as clinically relevant ex vivo model. After perfusion of $^{99\text{m}}\text{Tc}$ -E9 through the renal artery, immunoscintigraphy revealed the presence of well-defined radioactive hot spots, which matched the positions of the tumors as identified by pre-surgery MRI and subsequent histology. Gamma-counting revealed that the median values of radioactivity uptake per gram of wet weight were > 10 fold higher in the tumors than in normal kidney tissues. Not only was CD105 specificity of the tracer confirmed by blocking studies, immunoscintigraphy with $^{99\text{m}}\text{Tc}$ -E9 was also able to identify tumors that were not detected during pre-surgery MRI. These findings warranted future investigation of the in vivo pharmacokinetics of $^{99\text{m}}\text{Tc}$ -E9 (preferably after humanization) in renal cancer patients.

Although the major advantage of SPECT imaging is that it can be used for simultaneous imaging of multiple radionuclides, since the gamma rays emitted from different radioisotopes can be differentiated based on the energy [58], thereby allowing simultaneous detection of multiple biological events with multiple isotopes, such strategy has rarely been adopted. Another imaging modality, PET, offers many advantages over SPECT (e.g. much higher sensitivity) and the increasing popularity of clinical PET and PET/CT scanners can facilitate clinical translation of promising new PET tracers.

Positron emission tomography

PET was first developed in the mid-1970s [59]. The most widely used PET isotopes include ^{11}C ($t_{1/2}$: 20 min), ^{18}F ($t_{1/2}$: 110 min), and ^{64}Cu ($t_{1/2}$: 12.8 h). Over the last several years, PET imaging with ^{64}Cu has become increasingly more popular and significant research effort has been devoted to the development of ligands that can stably chelate ^{64}Cu [60]. Since the targeting ligands used for CD105 imaging to date are exclusively mAbs which have relatively long circulations half-lives (hours to days), long-lived PET isotopes (e.g. ^{64}Cu) are desirable for CD105-targeted radioimmunopET imaging.

The abovementioned TRC105, with a very high avidity for human CD105 (K_D : 2 ng/mL), is currently in a multicenter Phase 1 first-in-human dose-escalation trial at multiple centers in the United States [61]. Multiple Phase 2 therapy trials are planned or underway in patients with various solid cancer types. To investigate the in vivo pharmacokinetics and tumor targeting efficacy, we recently labeled TRC105 with ^{64}Cu for PET imaging of CD105 expression [62]. In vitro, in vivo, and ex vivo studies were performed on 4T1 murine breast tumor-bearing mice to evaluate the ability of ^{64}Cu -DOTA-TRC105 (DOTA denotes 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) to target tumor angiogenesis. In vitro, FACS analysis and fluorescence microscopy revealed no difference in CD105 binding affinity between TRC105 and DOTA-TRC105. In vivo, serial PET imaging revealed that the 4T1 tumor uptake of the tracer was fast, prominent, persistent, and CD105-specific (**Figure 3**), which was further validated by several control experiments (e.g. with ^{64}Cu -labeled cetuximab, an isotype matched mAb which binds to the human epidermal growth factor receptor [63]). At late time points, the tumor uptake was higher than most organs which provided excellent tumor contrast.

This study represents the first successful example of PET imaging of CD105 expression. Since TRC105 is already in clinical investigation and therapeutic efficacy has been shown in various animal tumor models [28, 64, 65] and certain cancer patients, this study identifies a new perspective for tumor angiogenesis-related research and warrants future clinical translation of ^{64}Cu -DOTA-TRC105, where it may be used to evaluate the pharmacokinetics, tumor targeting

CD105 Imaging

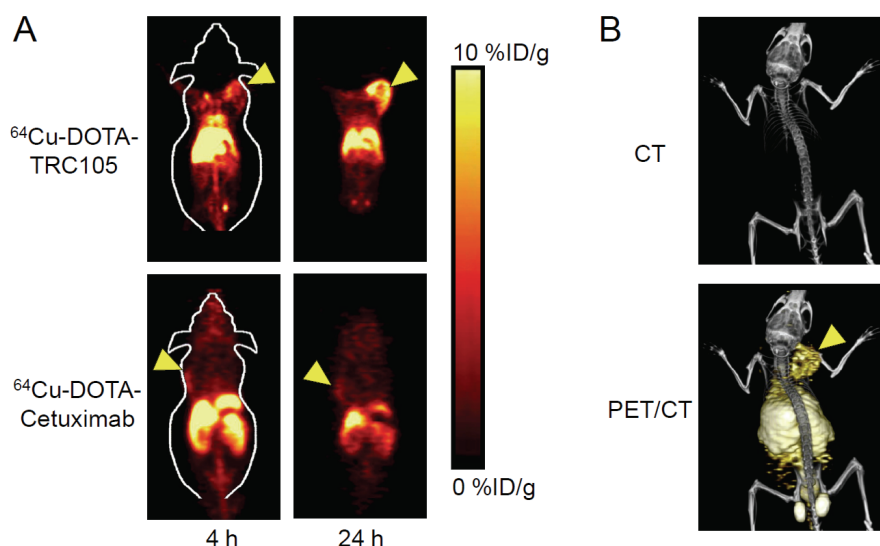


Figure 3. PET imaging of CD105 expression. **A.** Coronal PET images of 4T1 tumor-bearing mice at 4 and 24 h post-injection of ^{64}Cu -DOTA-TRC105 or ^{64}Cu -DOTA-cetuximab. **B.** CT and PET/CT images of ^{64}Cu -DOTA-TRC105 in 4T1 tumor-bearing mice at 24 h post-injection. Arrowheads indicate the 4T1 tumors.

Table 1. CD105 expression has been imaged with every single molecular imaging modality

Modality	mAb	Image Label	Model	References
Ultrasound	MJ7/18	microbubble	pancreatic cancer	[35, 36]
MRI	not identified	Gd-DTPA	glioma	[42]
Fluorescence	TRC105	IRDye 800CW	breast cancer	[48, 49]
SPECT	MJ7/18, E9, MAEND3	^{111}In , ^{125}I , $^{99\text{m}}\text{Tc}$	melanoma, canine mammary carcinoma, renal carcinoma	[54, 55, 57]
PET	TRC105	^{64}Cu	breast cancer	[62]

efficacy, dose optimization, and dose interval of TRC105 and TRC105-based anti-cancer agents in the clinic.

Conclusion and future perspectives

Given the pivotal role CD105 plays during tumor angiogenesis, molecular imaging of CD105 expression is surprisingly understudied. Nonetheless, non-invasive imaging of CD105 has been achieved with every single molecular imaging modality (Table 1). Non-invasive imaging of CD105 expression during tumor angiogenesis has clinical applications in many aspects: lesion detection, patient stratification, new drug development/validation, treatment monitoring, and dose optimization. Quantitative correlation of tracer uptake with CD105 expression level would be highly desirable for future treatment monitoring applications, as it would be ideal to non-invasively measure the changes of CD105 expression quantitatively, rather than qualita-

tively, in each individual patient upon anti-angiogenic therapies. Much further effort should be directed towards the development of clinically translatable CD105-targeted imaging agents, which can be widely used in anti-cancer clinical trials thereby paving the road to personalized medicine.

Due to excellent sensitivity and tissue penetration, radionuclide-based imaging techniques (i.e. SPECT and PET) possess significantly higher potential than non-radionuclide-based techniques. To date, most of the CD105-targeted agents are based on mAbs. To provide more insight about the long-term behavior of TRC105 in vivo, other longer lived isotopes (e.g. ^{89}Zr , ^{74}As , etc.) may be explored in future studies. The advantages of antibody-based tracers are that they are quite antigen-specific and have high binding affinity and absolute tumor uptake, which makes them suitable for internal radiotherapy applications (e.g. after labeling with ^{90}Y

or ^{177}Lu [66]) and/or targeted delivery of drugs.

The major limitations of antibody-based imaging are slow tumor accumulation and high background signal in the reticuloendothelial system, which may be overcome by peptide, small molecule, or antibody fragment-based tracers since they typically exhibit fast blood clearance thereby can be labeled with ^{11}C or ^{18}F for PET to confer higher throughput. High-affinity CD105-binding peptides could be generated from various high-throughput screening strategies such as phage display. Anti-CD105 antibody fragments, both human and murine [67, 68], as well as certain bi-specific antibodies [69, 70], may also be investigated in the future for imaging and potential therapeutic applications.

Multimodality imaging of CD105 expression, where the same probe can be simultaneously detected by two or more imaging modalities, should be developed in the future. By combining the advantages of various imaging modalities, quantitative and more accurate information can be obtained which no single modality alone can offer. Dualmodality probes that combine radionuclide-based imaging (very sensitive and highly quantitative) and non-radionuclide based approaches, for example optical imaging which can significantly facilitate ex vivo validation of the in vivo data and MRI probe which can provide high resolution anatomical information, are of particular interest.

Nanotechnology may also have many applications in CD105-targeted imaging and therapy in the future. Since many nanoparticles suffer from poor extravasation [22, 71, 72], CD105 is an ideal target for cancer nanomedicine as extravasation is not required to achieve tumor contrast or uptake. Lastly, the anti-cancer agents developed for CD105 targeting can also have broad applications in many other angiogenesis-related diseases [73].

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