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Optical Imaging and Tumor Angiogenesis

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Abstract Tumor angiogenesis is essential for tumor growth and progression. Therefore, targeting tumor blood vessels is a promising approach for cancer therapy. Angiogenesis, the formation of blood vessels, is a multistep process, and strongly influenced by the microenvironment. There are no in vitro assays that can resemble this dynamic process in vivo. For this reason, animal models and imaging technologies are critical for studying tumor angiogenesis, identifying therapeutic targets as well as validating the targets. Non-invasive molecular imaging in animal models presents an unprecedented opportunity and ability for us to perform repetitive observations and analysis of the biological processes underlying tumor angiogenesis and tumor progression in living animals in real time. As we gain a better understanding of the fundamental molecular nature of cancer, these techniques will be an important adjunct in translating the knowledge into clinical practice. This important information may elucidate how the tumor blood vessels behave and respond to certain treatments and therapies. J. Cell. Biochem. 90: 484–491, 2003.

Key words: imaging; tumor angiogenesis; VEGF

NON-INVASIVE CANCER IMAGING

Biological discovery has moved at an accelerated pace in recent years, with a considerable focus on the transition from in vitro to in vivo models. As a result, there has been a significant increase in the need to adapt and develop novel non-invasive, high resolution in vivo imaging approaches for studying cancer development and quantitatively determining molecular and cellular events in vivo [Weissleder and Mahmood, 2001; Weissleder, 2002]. Non-invasive imaging methods allow continuous monitoring of tumor development in vivo. Real time spatiotemporal analysis of tumor growth can reveal the dynamics of cancer progression. Furthermore, the effects of therapy on individual populations of cells, or even specific molecules can be evaluated non-invasively in living experimental animals. These approaches offer the ability to perform repetitive observations and interventions of the biological processes underlying cancer growth and development. If these techniques prove effective in mice, they may be translated into the clinic in the future, where they can be used to non-invasively detect tumors in situ and monitor treatment of human cancers.

Non-invasive molecular imaging is particularly critical for angiogenesis studies [Folkman and Beckner, 2000]. Blood vessel formation is a complex process that includes cell proliferation, migration, vascular tubule formation, and remodeling. Moreover, endothelium is heterogeneous and local microenvironment and tumor–host interaction affects endothelium behavior [Li et al., 2000; Geng et al., 2001; Trepel et al., 2002]. There is no in vitro assay that can replicate this complicated dynamic process in vivo, thus imaging and animal models are essential. Traditionally, we have relied on surgery and tissue biopsies. This only gives a snap shot of a very dynamic process, and important information is lost during the process. Non-invasive molecular imaging will overcome these limitations. These powerful technologies allow us to extract information about the angiogenesis

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process and tumor–host interaction and its molecular mechanism in living animals in real time. These methods are also critical in evaluating therapeutic responses of antiangiogenic therapies and they will be an important adjunct in translating the knowledge into clinical practice. Notably, antiangiogenic therapy is intended to target the angiogenic vessels only. It causes fewer side effects since normal vessels are typically quiescent. Traditional clinical trial designs, looking for the maximum toxicity dose, would not apply to this new type of therapy [Cristofanilli et al., 2002]. Instead, identifying surrogate markers for antiangiogenic therapy is imminent. Non-invasive imaging may offer the ability to optimize therapy for each treatment and for each individual.

The ability to visualize the dynamic biological process by in vivo imaging revolutionizes many areas in biology. Recent advances in the application of fluorescent proteins have permitted microscopy to move from static images to dynamic recording in living cells and living animals [Phair and Misteli, 2001]. Tissue visualization with light is probably the most common imaging in medicine and medical research. Optical imaging is inexpensive, high-resolution and allows real-time monitoring. It has the ability to monitor a single cell's behavior in animal models and it holds promise for the detection and elucidation of disease and pathogenesis at the microscopic level, potentially even in situ [Weissleder, 2002]. Optical imaging provides a powerful approach to study tumor angiogenesis and tumor development [Brown et al., 2001; Jain et al., 2002]. In addition, optical imaging complements other imaging technologies. The compatibility of this technology with other modalities would allow the creation of combined modalities for simultaneous detection that yields a superior feature set.

BIOLOGICAL AND CLINICAL SIGNIFICANCES OF TUMOR BLOOD VESSELS

Mounting evidence has demonstrated that tumor growth and progression depend on tumor angiogenesis. This fundamental principle states that tumor growth beyond a certain size is strictly dependent on tumor angiogenesis [Folkman, 2001]. By extrapolation, the same is true for tumor metastasis [Folkman, 2002]. Consistent with this notion, studies indicate that tumors with a luxuriant vasculature have a higher fraction of dividing cells and lower necrosis rates than tumors with a poorly developed vasculature [Folkman, 1990]. Moreover, clinical studies have shown a direct correlation between the density of tumor vessels and an adverse prognosis in patients with a variety of solid tumors [Bosari et al., 1992; Papamichael, 2001; Rioux-Leclercq et al., 2001]. Taken together, these studies suggest that the ability of a tumor to induce neovascularization determines its rate of growth and its likelihood of metastasis. Considering the importance of vascular growth in tumor progression, therapeutic approaches targeting the tumor endothelium should provide long term, effective control of the disease. The success of recent clinical trials of antiangiogenic cancer therapy further confirms the promise of this approach.

In addition to tumor angiogenesis that enables tumors to grow, tumor vascular survival is also a critical issue for cancer therapy, because tumor vascular survival keeps tumor cells alive. When cancer patients are admitted to the clinic, the tumors and tumor blood vessels are already well established. Simply targeting tumor angiogenesis is clearly not sufficient to reduce the tumor burden. Identification of therapeutic targets to inhibit tumor vascular survival and induce vascular regression is essential for the success of effective antiangiogenic cancer therapy [Garcia-Barros et al., 2003]. Peptide growth factors and their receptors, particularly endothelium-specific receptor tyrosine kinases, regulate vascular formation and vascular survival [Yancopoulos et al., 2000]. In addition, the tumor microenvironment not only regulates tumor angiogenesis, it also affects vascular survival and vascular response to therapy [Jung et al., 2000; Geng et al., 2001]. For example, blocking VEGF, a potent vascular survival factor [Ferrara, 2000], enhances tumor vascular response to therapy [Geng et al., 2001; Kozin et al., 2001].

OPTICAL IMAGING OF HOST ENDOTHELIUM-TUMOR CELL INTERACTION AND ANGIOGENESIS INITIATION

It has long been speculated that tumor host interaction is a critical component in tumor development. On one hand, the local microenvironment affects tumor cell behavior. On the other hand, tumor cells changes the local environment. There exists a very close and

intimate relationship between these two compartments. However, without appropriate methods it is almost impossible to know what type of interactions exist between them and what exactly happens when tumor cells are primed to initiate angiogenesis. These questions are particularly relevant to tumor angiogenesis initiation, tumor progression, and metastasis, since the angiogenic trigger is presumably present in these cases.

Optical imaging offers single cell resolution and real time imaging. Optical imaging has been used to study gene expression, tumor angiogenesis, physiological function of tumors, and tumor metastasis [Yang et al., 2000; Brown et al., 2001; Hoffman et al., 2002]. In a previous study, we used green fluorescent protein (GFP)-labeled tumor cells and mouse dorsal skinfold window chamber models to study tumor host interaction and tumor angiogenesis initiation. The window chamber was placed on the dorsal skinfold of a mouse. One side of the epidermis was removed and a facial plane with associated vasculature remains. A murine mammary tumor line transfected with GFP (4T1-GFP) was implanted onto the fascial plane and the chambers were sealed with a glass cover slip. The chamber with tissue is semi-transparent and ideal for optical imaging. Using this approach, we were able to visualize tumor formation from a single tumor cell [Li et al., 2000] (Fig. 1).

Several intriguing observations were obtained from this study. (1) Tumor cells alter the host "normal vasculature" morphology. Implantation of only 50 tumor cells in the window chamber caused dramatic vascular morphologic changes in just a few days (Fig. 1B–C). The surrounding host "normal" vessels became dilated and torturous. Interestingly, it has been known that tumor vessels are structurally and functionally abnormal. Here we show that surrounding host vessels become abnormal under the influence of a few tumor cells. However, it is not clear what cause this change and what is the benefit for tumor development. (2) Host environment alters tumor cell behavior. 4T1 is an epithelial tumor line, which has a typical cobble stone-like morphology when cultured in vitro (Fig. 1H). However, a few days after implantation in vivo, the cells became enlongated or polarized. The tumor cells underwent epithelial-to-mesenchymal transformation (EMT), and they migrated towards nearby blood vessels (Fig. 1E–G). When the cells reached to the blood vessels, they grew around the vessel and formed a cuff, and then grew along the vessels. The data suggest a chemotaxis-like movement of the tumor cells toward the host blood vessels prior to any evidence of tumor angiogenesis. It suggests that the host endothelium is secreting a signal or signals that recruit the tumor cells. It is unclear at this point what these signals are.

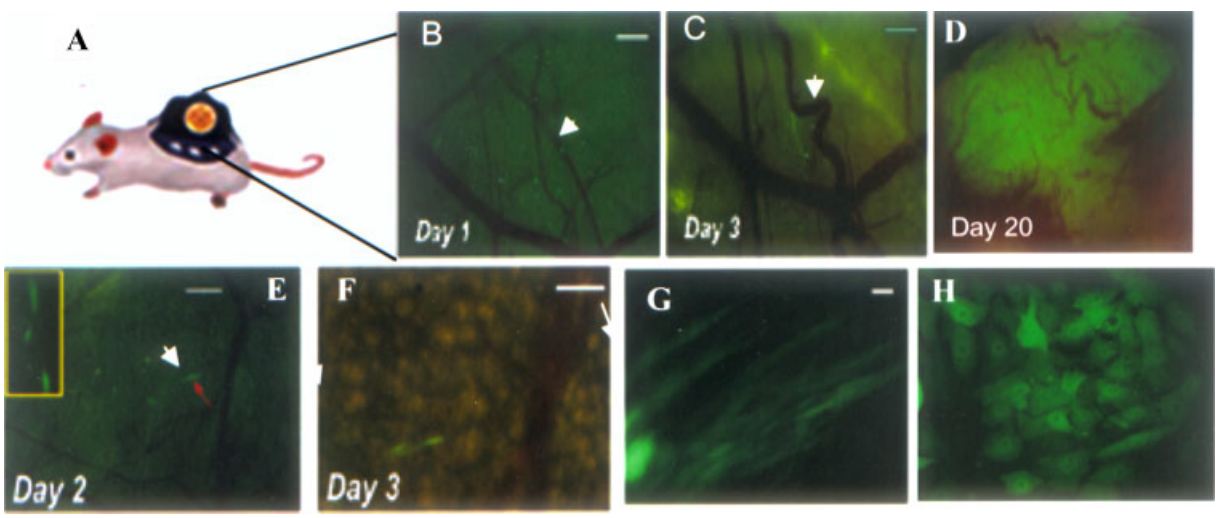

Fig. 1. Imaging tumor–host interaction in living animals. Rodent skinfold window chamber model is ideal for optical imaging (**A**). Implantation of a few 4T1-GFP cells (green dot in **B**) in the window chamber changes host vessel morphology in 3 days (**C** as indicated by arrows). A vascularized tumor forms in 20 days from a single tumor cell (**D**). Conversely, host environment changes tumor cell behavior. 4T1-GFP cells in vivo polarized/elongated and migrated towards surrounding blood vessels (**E–G**). The morphology of 4T1 cells in vivo (E–G) is completely different from the one seen in cultured dish (**H**), typical epithelial cell morphology. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Potential candidates include oxygen or other nutrient gradients, growth factors, or other cytokines. This observation is consistent with the "two-compartment" theory proposed by Folkman that tumor and endothelial cells secrete chemotactic signals that attract each other. (3) A paracrine regulation exists between tumor cells and host endothelium and the interaction is critical for tumor cell behavior and survival. 4T1 cells neither have VEGF receptor nor respond to VEGF treatment in vitro [Li et al., 2000]. However, injection of a soluble VEGF receptor (ExFlk) to block VEGF signaling in vivo leads to tumor cell apoptosis and tumor regression within 5 days before the appearance of neovascular sprouts (Fig. 2). The results indicate that there exist a two-way paracrine exchange of growth factors and survival factors between tumor cells and neighboring vascular endothelial cells. (4) Tumor angiogenesis starts at a much earlier time than textbooks have suggested. The traditional view is that tumor angiogenesis does not start until a tumor reaches a few cubic millimeters in size. However, we observed the earliest tumor angiogenesis started when there were only 100–300 cells (Fig. 1). A possible explanation for the discrepancy is the resolution of the experimental systems. Specifically, GFP-labeled tumor cells in combination with the dorsal skinfold window chamber provides high clarity that allows the observation of tumor growth from individual tumor cells, while earlier studies depended mostly on immunohistochemistry of well-established tumors that does not allow similar observations of earlier angiogenic activities at equivalent spatial and temporal resolutions.

Collectively, our study reveals the importance of host endothelium–tumor cell interaction and the initiation of tumor angiogenesis (angiogenic switch). The timing of the initiation of angiogenesis for disseminated tumor cells is a very important issue, since it will lead to a better understanding of the role the process of angiogenesis plays in tumor metastasis. Angiogenesis initiation in metastatic tumor cells may be very different from that in primary tumors. Optical imaging may provide a means to study the differences.

OPTICAL IMAGING OF TUMOR VASCULAR SURVIVAL AND VASCULAR RESPONSE TO THERAPY

Peptide growth factors regulate endothelial cell survival [Yancopoulos et al., 1998], and tumor vascular survival affects tumor vascular response to therapy. Using the skin flap window model, we observed a heterogeneous response to irradiation exists among different types of tumors grown in the same host (C57/BL mice) [Geng et al., 2001]. Three different tumors were implanted into the tumor windows. Vascularized tumors developed in one week, at which time they were treated with local irradiation. Irradiation induced a dose and time-dependent injury to tumor blood vessels within the window. Radiation-sensitive tumor vessels in melanoma B16F0 showed rapid and marked regression following low dose (2 Gy) of irradia-

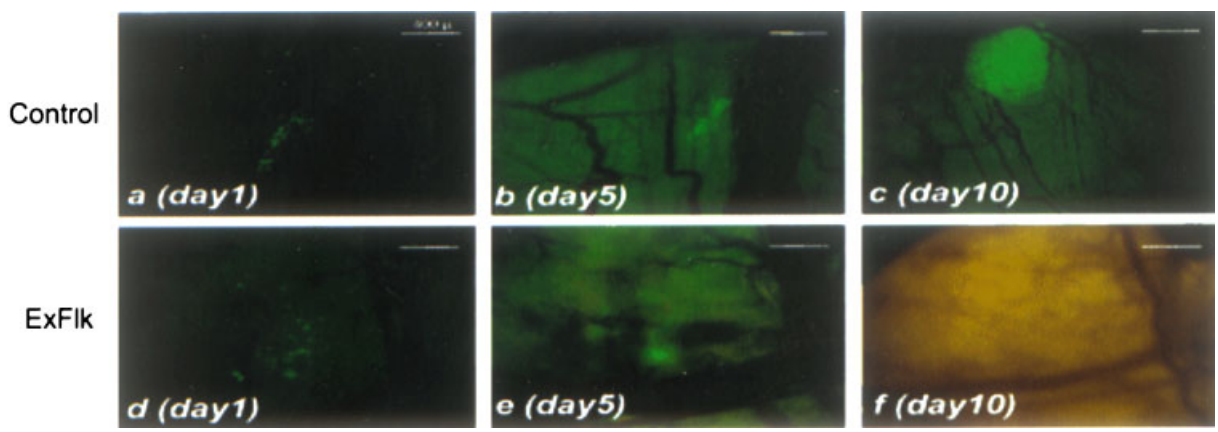

Fig. 2. A paracrine regulation exists between tumor cells and host endothelium. 4T1-GFP cells were injected into the window chamber and treated with ExFlk. Blocking VEGF signaling (**bottom panel**) inhibited tumor cell migration (day1 and 5) and tumor cells died in 5 to 10 days, compared to the control-treated group in which tumor cells migrated toward vessels and formed a tumor in 10 days. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

tion. Intermediate tumor vessels in Lewis Lung Carcinoma showed limited response to 2 Gy of irradiation, but responded well to 6 Gy of irradiation. Radiation resistant tumor vessels in glioma GL261 showed little response even at 6 Gy (Fig. 3). Blocking VEGF function enhanced tumor vascular response to irradiation therapy [Geng et al., 2001]. The study shows the usefulness of optical imaging in examining tumor vascular survival and tumor vascular response to therapy.

Tumor vascular permeability, vessel size, and blood flow are important functional indexes of tumor blood vessels. Optical imaging has the ability to determine these parameters in vivo. Optical imaging offers great clarity for imaging blood vessels, which was elegantly demonstrated by using fluorescent dye or quantum dots [Larson et al., 2003]. Tumor vessels are generally leaky, with a heterogeneous permeability that depends on the tumor site. Multiphoton microscopy in combination with fluorescence labeled molecules can be used to quantify the permeability of individual tumor blood vessels non-invasively deep inside living animals [Brown et al., 2001]. In addition, i.v. injection of fluorescent labeled cells allows quantitation of blood flow and cell blood vessel interaction in vivo [Brown et al., 2001]. Functional vascular indexes are important parameters for antiangiogenic therapy. Optical imaging has the ability to measure these important parameters as an indicator of tumor vascular responses to therapy in vivo.

OPTICAL IMAGING IN ANTIANGIOGENIC CANCER THERAPY

The success of antiangiogenic therapy depends on reliable monitoring systems. The traditional clinical development of chemotherapeutic agents is based on the following measurements: first, the dose-dependent toxicity associated with the agent; second, the dose-limiting toxicity of the agent (DLT); third, the maximum-tolerated dose (MTD) that has a higher probability of reducing tumor burden and prolonging the survival. However, antiangiogenic therapy causes none or very limited toxicity since angiogenesis is quiescent in normal adult. Targeting tumor angiogenesis produces limited side effects on the normal vessels. Therefore, traditional clinical trial design looking for the maximum toxicity dose would not apply to this type of therapy [Ellis et al., 2001; Cristofanilli et al., 2002]. In addition, studies have shown that higher doses of antiangiogenic agent do not translate into better outcomes. Contrarily, lower doses with continuous administration seem to work better in certain types of tumors. Furthermore, tumor development is not a single disease. There exists a huge heterogeneity among tumors and tumor blood vessels. This makes identification of surrogate markers for antiangiogenic therapy critical.

Functional characterization of the tumor vasculature by non-invasive imaging has great potentials in antiangiogenic therapy. Imaging of tumor blood vessels should provide a means to

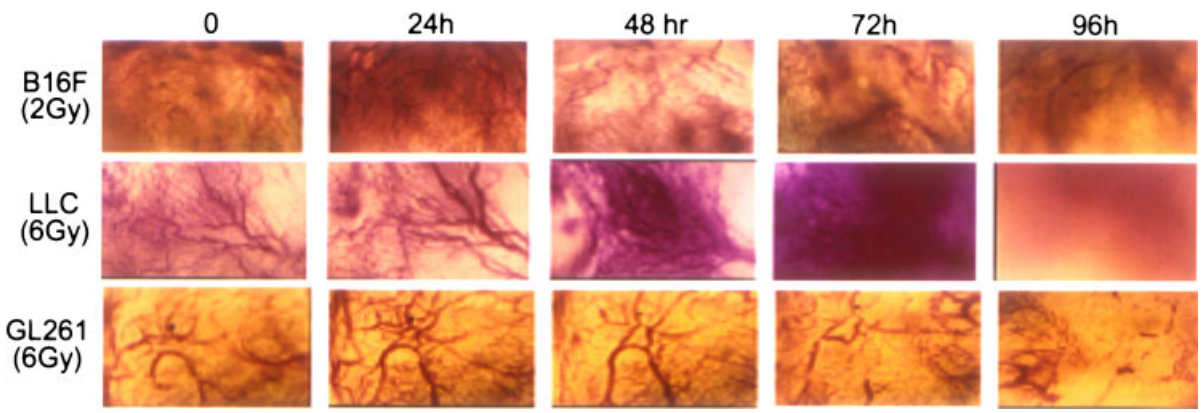

Fig. 3. Tumor vascular response to irradiation is heterogeneous. We implanted three different types of tumor cells (melanoma (B16F0), Lewis lung carcinoma (LLC), and glioma (GL261) in the window chambers that were established in the same strain of mice (C57/BL). Upon the formation of vascularized tumors, the mice received local irradiation. Tumor vascular response was recorded under a microscope at various time points after irradiation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

monitor vascular response to therapy. It may offer the ability to optimize therapy for each treatment and for each individual. Optical imaging and other imaging methods may be able to determine the following parameters for antiangiogenic therapy [Padhani and Neeman, 2001]:

(1) Diagnosis and prognosis of cancer. Early detection of tumor lesions will lead to effective elimination of the cancer. Since angiogenesis in tumors is active, contrast agents directly targeted the angiogenic vessels, such as integrin $\alpha_v\beta_3$ [Hood et al., 2002], may provide a means to detect the tumor lesion. In addition, tumor vascularity is associated with tumor malignance and tumor response to treatment. Imaging technologies may provide a measurement for cancer prognosis.
(2) Selection of the optimal treatment regime. Tumors are heterogeneous, so is the tumor vasculature. Since antiangiogenic inhibitors are often designed to target a specific angiogenic process or a specific signaling molecule, the response of tumors to the inhibitors may vary among tumors. Examination of the tumor vascular functions (e.g., blood flow, perfusion, and permeability) may therefore allow rational selection of patients for specific treatment.
(3) Dose optimization. Tumor vascular response to therapy is heterogeneous. Selection of the optimal dose of inhibitors for each type of tumor and each individual is critical for the effective treatment of the disease. Molecular imaging, including optical imaging, should be able to monitor and quantitate tumor vascular response to therapy, and will aid in dose selection.
(4) Detection of the antiangiogenic response. Since tumor cells are genetically unstable, they may develop tolerance to low oxygen and low nutrient levels after antiangiogenic treatment [Yu et al., 2002]. Antiangiogenic treatment may not lead to a significant reduction of tumor size in a short period of time. Traditional measurement of tumor burdens does not reflect the direct response of the tumors to the treatment. Many antiangiogenic compounds have failed in clinical trials based on the tumor burden measurement [Ellis et al., 2001; Rothenberg et al., 2003]. However, it is not clear whether the compounds fail to inhibit angiogenesis or the approach used is not suitable for the tumor treatment. Noninvasive imaging has the ability to measure various tumor vascular indexes, such as blood flow, perfusion, and vascular permeability, which reflects a direct response to the treatment.
(5) Monitor tumor response. Antiangiogenic treatment is intended to induce tumor blood vessel regression that may lead to tumor regression. Since this approach does not directly target tumor cell, it often results in a slow reduction of tumor burden. A precise monitoring system is essential to monitor the tumor response. In addition, antiangiogenic treatment causes tumor cell apoptosis or tumor necrosis, but it may not instantly translate into the reduction of tumor size. Imaging approaches are able to measure the live tumor cells [Contag et al., 2000] that would be ideal to monitor tumor response. Furthermore, antiangiogenic therapy most likely will be a long-term treatment. Inexpensive, non-invasive imaging is highly desirable.

LIMITATIONS OF OPTICAL IMAGING

The major limitation of optical imaging is tissue light scattering and absorption that affect both image resolution and depth of light penetration of tissues [Ntziachristos and Chance, 2001]. In the ultraviolet and visible regions, tissue scattering and absorption of light is high, which limits its tissue penetration. Thus, optical imaging in these regions is conventionally used to evaluate lesions on the surface, endoscopic-accessible and surgically exposed deep tissues. At the near infrared region (NIR) between 700–900 nm, absorption is low and allows light to penetrate much deeper into tissues, a depth that may be sufficient to practically image small animals and certain human cancers. Endoscopic NIR optical imaging should make it possible to image certain internal organs in patients. In addition, development of multiphoton microscopy significantly advances optical imaging [Piston, 1999]. It has a much better signal-to-noise ratio, greater imaging depth, and longer sample lifetimes. It

provides a powerful approach to measure tumor angiogenesis and functional indexes of tumors [Brown et al., 2001].

SUMMARY

Advances in the biomedical sciences have been accelerated by the development and utilization of new imaging technologies. With animal models widely used in the biological science, finding ways to conduct in vivo experiments more accurately and efficiently becomes a key factor in the success of medical research. Optical imaging is inexpensive, high resolution (up to a single cell level or even sub cellular level), and allows real time imaging. It not only provides powerful tools to study the molecular mechanisms of tumor angiogenesis, identify therapeutic targets as well as to validate the targets in animal models, but will also be a valuable measurement for monitoring drug response in patients.

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