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Imaging aspects of the tumor stroma with therapeutic implications

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Abstract

Cancer cells rely on extensive support from the stroma in order to survive, proliferate and invade. The tumor stroma is thus an important potential target for anti-cancer therapy. Typical changes in the stroma include a shift from the quiescence promoting- antiangiogenic extracellular matrix to a provisional matrix that promotes invasion and angiogenesis. These changes in the extracellular matrix are induced by changes in the secretion of extracellular matrix proteins and glucose amino glycans, extravasation of plasma proteins from hyperpermeable vessels and release of matrix modifying enzymes resulting in cleavage and crosslinking of matrix macromolecules. These in turn alter the rigidity of the matrix and the exposure and release of cytokines. Changes in matrix rigidity and vessel permeability affect drug delivery and mediate resistance to cytotoxic therapy. These stroma changes are brought about not only by the cancer cells, but also through the action of many cell types that are recruited by tumors including immune cells, fibroblasts and endothelial cells. Within the tumor, these normal host cells are activated resulting in loss of inhibitory and induction of cancer promoting activities. Key to the development of stroma targeted therapies, selective biomarkers were developed for specific imaging of key aspects of the tumor stroma.

Keywords

Extracellular matrix; matrix metallo proteinases; angiogenesis; immune cells; cancer associated fibroblasts; tumor microenvironment

1. Introduction

Transformation and clonal expansion of cells has been the primary focus of cancer research and drug development of the last decades. Over the last years this cell autonomous view of the transformed cells culminated in the development of novel therapies that target specific signalling pathways and genetic alterations, which are activated within the cancer cells. However, the limited success of such interventions led to the realization of the importance and unique characteristics of the tumor stroma. Tumor stroma cells, including immune cells, fibroblasts and vascular endothelial cells can constitute a large fraction, often more than half, of the tumor mass. These cells are not innocent bystanders, but rather affect in a

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complex manner the tumor metabolism, proliferation, invasion, metastasis and response to therapy. Although these stroma cells are typically not genetically altered, they do show epigenetic changes and are often 'educated' or activated in the tumor microenvironment to promote tumor progression.

An additional critical component of the tumor stroma is the extracellular matrix (ECM). This matrix includes macromolecular building blocks as well as the extracellular enzymatic activities, both of which are altered in cancer to promote invasion, angiogenesis and hinder drug delivery. Examples include changes in collagen and fibrin, and thus also tissue stiffness, and enzymes that either degrade the matrix, such as MMP's or cross link it such as tissue transglutaminase; hydrophilic glycosaminoglycans such as hyaluronan, are also important constituents of the ECM, affecting its elasticity. Both the synthesis of hyaluronan by hyaluronan synthases and its degradation by hyaluronidases are altered in cancer. The extracellular matrix provides an important reservoir for adhered growth factors that can be released by enzymatic activity to induce signalling thus affecting migration, proliferation and differentiation of cancer and stroma cells.

The robustness of the tumor microenvironment stems from the extensive communication between the tumor and the stroma, which is mediated by changes in the physical microenvironment (interstitial pressure, stiffness etc), by metabolic crosstalk and effects on metabolite and nutrient delivery, and by intercellular cytokine signalling. Due to its impact on tumor progression, the tumor stroma is an important target for cancer imaging and therapy; and multiple tools were developed over the last years for visualization and intervention directed at specific stroma components (Figure 1).

2. Targeting the tumor stroma

2.1 Targeting cancer associated fibroblasts

Fibroblasts are the main cellular component in the microenvironment of many tissues and solid tumors. Normal fibroblasts, in their inactive state, show a suppressive role towards preneoplastic cells (Schauer, Sood, Mok, & Liu, 2011). However, fibroblasts are recruited and activated by tumor cells, as part of tumor desmoplasia. Such activated fibroblasts are termed cancer associated fibroblasts (CAFs). CAFs contribute to tumor progression by altering the extracellular matrix (ECM), secreting growth factors, recruiting and modulating immune response, and by contributing to tumor metabolism (Cirri & Chiarugi, 2012; Hanahan & Weinberg, 2011; J. Zhang & Liu, 2013). The importance of CAFs in cancer response to therapy was highlighted in many studies. Recently, a high throughput screen of chemotherapies, demonstrated a significant effect of fibroblasts in conferring resistance to therapy in multiple cancer cell lines, by affecting signaling pathways (Straussman, et al., 2012).

CAFs originate from different cellular sources, including resident fibroblasts and bone marrow derived mesenchymal stem cells. However, some studies suggested contribution of tumor cells, and endothelial cells through epithelial/endothelial to mesenchymal transition (Hanahan & Weinberg, 2011; J. Zhang & Liu, 2013). Adipocytes, inflammatory cells and pericytes can also contribute to the CAF population. CAFs are therefore a heterogeneous population, mainly identified by cellular morphology and the expression of alpha smooth muscle actin (α -SMA), vimentin and fibroblasts activating protein (FAP) (Hanahan & Weinberg, 2011; J. Zhang & Liu, 2013). CAFs were suggested as a target for anti-tumor treatments as they have an important role in the tumor progression and because of their genetic stability compared with the cancer cells. Targeting these cells can result in indirect effects on tumor angiogenesis and drug delivery.

Although CAF are the most prevalent cell type in the tumor microenvironment, so far they were not a common target for molecular imaging (figure 2). In a recent study, monitoring fibroblasts contribution to tumor stroma was approached with a color coded system of fluorescent expressing nude mice. In this system the non fluorescent tumor cells were injected to the spleen and metastasize to the liver in GFP expressing nude mice. GFP expressing host cells were recruited to the tumor (Suetsugu, et al., 2011).

We have previously reported that exogenously administered fibroblasts are systemically recruited to remote tumor site and this process can be followed by non invasive imaging (Dorit Granot, et al., 2007; D. Granot, Kunz-Schughart, & Neeman, 2005; Vandsburger, et al., 2013). Fluorescence imaging and magnetic resonance imaging (MRI) were used for detection of fibroblasts labeled ex vivo with near infra-red fluorescent dyes, magnetite nanoparticles, biotin-BSA-GdDTPA and with expression of h-ferritin (figure 2). Recruited labeled fibroblasts co-localized with the neovasculature at the tumor rim, consistent with their contribution to tumor angiogenesis (Dorit Granot, et al., 2007). Over-expression of ferritin heavy chain (FHC) as an MRI reporter gene, enabled quantitative tracking of the recruitment of the fibroblasts to ovarian carcinoma tumors. Using this novel method cell densities lower than previously demonstrated are detected. Correlation of fractional blood volume with the R2 maps revealed that the fibroblasts are preferentially recruited to the vascular area within the tumors (Vandsburger, et al., 2013).

Fibroblast activation protein- α (FAP α) is a cell surface glycoprotein selectively expressed by CAF in tumors but rarely in normal fibroblasts. FAP α was suggested as a potential targeting candidate for tumor treatment (Brennen, Rosen, Wang, Isaacs, & Denmeade; Lo, et al., 2008). To specifically inhibit CAFs, researchers used FAP as a targeting molecule for small molecules and antibodies targeting FAP that is specifically expressed in the tumor area (Hofheinz, et al., 2003; Ostermann, et al., 2008; Scott, et al., 2003). Li et al. developed a probe injected IV that is activated by FAP cleavage to generate a near infra-red signal. The signal is generated by uncoupling the fluorescent group Cy5.5 from a quencher group (QSY21), through enzymatic cleavage of a peptide substrate linker (KGPGPNQC) specific for FAP α . This enables the researchers to image FAP specific activity (J. Li, et al., 2012).

To examine the role of CAF in ECM remodeling in collagen deposition, Perentes et al. developed a method to image in vivo collagen deposition by CAF (Perentes, et al., 2009). They used a dorsal skin fold window chamber transplanted in mice expressing GFP under the control of the vascular endothelial growth factor a (*Vegfa*) promoter (VEGF-GFP mice). The labeled cells were shown to be CAFs (Fukumura, et al., 1998). The transparent tumor chamber facilitated the tracking of the same cells and fibers for several days using multiphoton laser scanning microscopy and second harmonic generation (SHG) of fibrillar collagen to visualize the matrix of normal and tumor tissues *in vivo* (Perentes, et al., 2009).

In summary, CAFs are an important component of solid tumors, not only due to their large abundance but also due to their significant contribution to tumor progression, mediated by their effects on the tumor extracellular matrix and the tumor vasculature. The development of novel imaging modalities specific for CAFs would contribute to improving monitoring of therapeutic strategies targeting this stroma component.

2.2 Targeting extra cellular matrix (ECM) components

The ECM is composed of proteoglycans, glycosaminoglycans and fibrillar proteins, in addition to cytokines, growth factors and many secreted enzymes. The interaction of these different ECM components and the balance between ECM deposition and ECM degradation

are important for tissue homeostasis. In oncogenesis this dynamic system is disturbed. Thus, the ECM plays a critical role in tumor development, progression and metastasis (Figure 3).

Many of the ECM components have been implicated in tumor progression, growth, cell migration, invasion and angiogenesis (Egeblad, Nakasone, & Werb, 2010; Gialeli, Theocharis, & Karamanos, 2011; Kaspar, Zardi, & Neri, 2006; Mangala, Fok, Zorrilla-Calancha, Verma, & Mehta, 2007; Paolo P. Provenzano, et al., 2012; Whatcott, Han, Posner, Hostetter, & Von Hoff, 2011). The ECM organization and composition in the tumor microenvironment has specific identifying features, which change during tumor development. For example, the tumor ECM is stiffer due to altered collagen organization and deposition, and there is an increase in MMPs released to the tumor ECM. In addition the tumor ECM composition and organization affect drug delivery to the tumor cells (Egeblad, Nakasone, et al., 2010). As a result, ECM components were identified as targets for tumor therapy and are utilized as tools for specific targeted drug delivery to the tumor.

In vivo imaging of the ECM is critical to better understand the roles that different components play in the tumor and to better target and utilize the components for tumor therapy.

2.2.1 Fibrillar proteins proteoglycans and glycosaminoglycans—ECM building blocks may be imaged in vivo by MRI, positron emission tomography (PET), bioluminescence imaging (BLI) and Intravital microscopy. Tumor specific targeting may be accomplished by active targeting of an ECM component, which is specific to tumor ECM or by passive targeting utilizing the tumor enhanced permeability response. ECM components may be targeted for imaging by antibodies or peptides, which bind to a specific ECM component. The antibody or peptide will be labeled with the relevant probe for each imaging modality.

2.2.1.1 Collagen: Collagen is the major component of the ECM, having both a structural and functional role. Many collagens and collagen degradation products play active roles in cell signaling (Ricard-Blum, 2011). In the tumor, collagen structure and architecture deviates from that of normal tissue. Deposition of certain collagen subtypes arise and collagen fibrils become linearized, as a result the tissue becomes stiffer; linearized collagen has been suggested to promote cell invasion (P. P. Provenzano, et al., 2006). Moreover proliferation, differentiation and apoptosis may be effected by tumor associated collagen (Egeblad, Rasch, & Weaver, 2010). Correlation between collagen architecture with type and tumor stage has been reported (Nadiarnykh, LaComb, Brewer, & Campagnola, 2010; P. P. Provenzano, et al., 2006). In addition, tumor collagen was shown to induce chemoresistance and affect drug delivery to the tumor cells (Egeblad, Rasch, et al., 2010).

Second harmonic generation (SHG) is one of the most popular methods to image collagen both in vitro and in vivo. This technique has a great advantage over many others since no labeling of the collagen and no fixation or sectioning is necessary. SHG enables in vivo imaging of endogenous collagen with no manipulation of the tissue. It is used in many studies to visualize a variety of processes such as dynamic changes in collagen deposition, degradation and structure during tumor progression, and the changes in collagen in response to treatment such as chemotherapy.

In vivo imaging of the dynamic processes of collagen homeostasis and collagen reaction in response to treatment are essential for better understanding tumor progression and drug delivery to the tumor cells. Brown et al (2003) used SHG to image collagen dynamics in vivo (E. Brown, et al., 2003). Human sarcoma cells were inoculated into a dorsal skinfold chamber in immune deficient mice. The mice were treated with the hormone relaxin known

to upregulate MMPs. Tumor collagen was imaged for several days by SHG. Collagen length and SHG signal of pre-existing fibers decreased significantly in relaxin treated mice compared to controls (E. Brown, et al., 2003).

SHG enables the study of interactions between the tumor cells and their surrounding microenvironment in vivo, by combining SHG to view the collagen and multiphoton microscopy to image fluorescently labeled tumor cells. The behavior of non-metastatic and metastatic mammary tumor cells in the context of their collagen microenvironment was compared in vivo. GFP Labeled mammary tumor cells were injected into mice and tumor-collagen interactions were followed. Non-metastatic tumor cells behaved differently from metastatic tumor cells. On one hand, the non-metastatic cells moved with no contact with the collagen fibers; on the other hand, the metastatic cells interacted with the collagen fibers, spread along them and showed a linear motion along the fibers. In addition, the tumors from the metastatic cell line had lower collagen density (W. Wang, et al., 2002). Differentially labeling two cancer cell lines, one with low metastatic ability and the other with high metastatic ability enabled imaging of the behavior of the two cell lines in vivo within the same microenvironment. Cancer cells from both cell lines were inoculated in the same location in one animal, showing differences in single cell behavior in the same microenvironment (Sahai, et al., 2005). The distribution of collagen in the tumor is not uniform. Using SGH and fluorescence microscopy, Kakkad et al followed collagen deposition and hypoxia, by expressing EGFP under the control of hypoxia response element (Kakkad, et al., 2010). Dense collagen was detected in normoxic regions of the tumor while it was excluded from hypoxic regions of the tumor.

In addition to SHG there are a few examples of collagen imaging by near infra-red fluorescence (NIRF). Yasunaga et al conjugated a collagen 4 antibody to an infra-red dye and demonstrated specific antibody accumulation in tumors in vivo by NIRF imaging. However the specificity of the antibody was evident only 7 days post antibody injection. (Yasunaga, Manabe, Tarin, & Matsumura, 2011).

NIRF was applied also for imaging degraded collagen and thus MMP activity. Collagen mimetic peptides (CMP) that fold into triple helix were found to bind to degraded collagen. CMPs conjugated to an infra-red fluorophore were injected intravenously to mice with prostate tumor xenografts allowing detection after 4 days. In addition a general MMP activatable probe was administered to the mice, and both CMP and MMP activatable probe colocalized in the tumor. Hence CMPs bind collagen degraded by MMPs (Y. Li, et al., 2012).

An MR method for collagen imaging is MR elastography. Briefly, changes stiffness are detected by MRI from the propagation of displacement within the tissue in response to harmonic mechanical vibration. Using this method the shear modulus of the tissue can be mapped. In tumors, deposition of collagen content and the associated rigidity can be followed (Murphy, et al., 2013). This approach is used for clinical mapping of rigidity in tumors and thus can be an important approach for monitoring response to stroma-targeted intervention.

Further imaging methods are still necessary, since it is not always possible to decipher between collagen subtypes based on SHG imaging. In addition, degradation fragments of different collagen subtypes are involved in many signaling pathways active in tumor growth. Imaging of these fragments is important to better understand the tumor microenvironment. A second possibility is to image the enzymatic activity of collagen degrading enzymes; this will be discussed in a later section.

2.2.1.2 Fibronectin: Fibronectin (FN) is a glycoprotein abundant in the ECM. It has many binding sites to cell surface integrins and to different ECM components such as collagen and fibrin (Pankov & Yamada, 2002). FN has both a structural and a functional role, effecting tissue morphology as well as cellular processes. There are a variety of FN isoforms originating from alternative splicing. The isoforms fibronectin extra domain A and fibronectin extra domain B are prevalent in embryonic development and in the adult in tissue remodeling during wound healing and tumorigenesis. Fibronectin extra domain B is abundant in tumor neovasculature and its expression correlates with a negative prognosis in cancer patients (Kaspar, et al., 2006). As a result FN isoforms are an attractive target for tumor therapy. Peptides were designed to target and alter the morphological structure of FN. In addition, FN isoforms were targeted by antibodies which may be useful for specific drug delivery to the tumor (Kaspar, et al., 2006). Utilizing labeled antibodies or peptides for imaging enabled live real-time assessment of tumor FN.

Thus, several extra domain B specific antibodies were conjugated to fluorescent labels for in vivo imaging showing perivascular accumulation in tumors (Birchler, et al., 1999; Neri, et al., 1997). The probe accumulated selectively in the tumors as opposed to a control antibody and was retained for over 24 hours (Birchler, et al., 1999). Clinical evaluation of the extra domain B specific antibodies was demonstrated by PET and single photon emission computerized tomography (SPECT) on 20 patients with brain lung or colorectal cancer (Santimaria, et al., 2003).

To allow detection by MRI, a peptide that binds specifically to fibronectin extra domain B was conjugated to superparamagnetic iron oxide nanoparticles (SPIO). Mice were inoculated with Lewis lung carcinoma cells, and after tumor establishment, the conjugated contrast agent was injected intravenously. Contrast agent accumulated in the tumor, and was detectable 24hr post injection (Park, et al., 2012).

Further MRI imaging of tumor associated FN used CLT1-GdDTPA, a cyclic decapeptide, which binds specifically to fibrin- FN clots in tumors (Pilch, et al., 2006). CLT1-Gd-DTPA/ DOTA successfully enhanced tumor contrast in vivo, in tumor bearing mice for at least 60 min, while the unconjugated contrast agent was rapidly cleared (Tan, et al., 2012; Tan, Wu, Jeong, Chen, & Lu, 2010; Ye, et al., 2008).

2.2.1.3 Glucose amino glycans: Hyaluronan (HA) is a high molecular weight negatively charged linear polysaccharide implicated in many cellular functions. HA is involved in signaling pathways through specific interaction with cell surface receptors such as CD44 and RHAMM. In many cancers HA is elevated, leading to increased interstitial fluid pressure in the tumor (Paolo P. Provenzano, et al., 2012). In addition, HA is involved in several cellular processes such as cancer cell adhesion and migration and angiogenesis. Targeting HA levels in cancer therapy is investigated by targeting HA synthesis and degradation (Whatcott, et al., 2011). HA conjugated to fluorescent probes or MR contrast agents was applied for detection of CD44 positive tumors by NIRF or MR imaging respectively (Cho, et al., 2012; Lee, et al., 2013; Yoon, et al., 2012).

Visualization of endogenous HA is difficult due to the high negative charge resulting in non-specific binding. Given the importance of HA in different pathways involved in tumor progression, an imaging method for direct visualization of endogenous HA would be of great benefit.

2.2.2 ECM modifying Enzymes—Extracellular enzymatic remodeling of the ECM affects its biological and biophysical properties. Both the presence of the enzyme itself, as well as its catalytic activity provide important targets for imaging. Tools for in vivo imaging

of enzymatic activity were developed and are implemented for many ECM modifying enzymes. The concept of imaging enzymatic activity is usually carried out by conjugating a substrate analog to a contrast agent, in MRI or fluorescent probe for NIRF. Switchable 'smart' contrast agents are sensors in which the imaging signal is quenched for the conjugated probe. Once enzymatic degradation occurs the relevant signal is enhanced, and enzymatic activity can be imaged.

2.2.3.1 Matrix metalloproteinase: Matrix metalloproteinases (MMP) comprise a family of over 20 protein degrading enzymes (Malemud, 2006; Noel, Jost, & Maquoi, 2008). Many MMPs are over expressed in tumors, affecting progression, growth, invasion and metastasis in a complex manner, either promoting progression or providing a protective influence against tumor development (Gialeli, et al., 2011; Noel, et al., 2008). Under the premise that MMPs are an important target for tumor therapy, many MMP inhibitors have been developed. However so far these inhibitors failed in clinical trials, due to lack of MMP specificity as well as treatment at the wrong time phase of tumor progression (Gialeli, et al., 2011). In vivo imaging may help us better understand the exact expression and activity profile of specific MMPs as well as the exact location of activity within the tumor.

PET tracers developed to image MMP distribution using radiolabeled MMP inhibitors showed poor tumor uptake and non-specific accumulation (Medina, et al., 2005; Scherer, McIntyre, & Matrisian, 2008; Wagner, et al., 2007; Zheng, et al., 2003; Zheng, et al., 2002).

For detection of MMP activity by PET, a MMP2 substrate was conjugated to Polyethylene glycol and to a hydrophobic tetramethylrhodamine (TMR) domain and labeled with ^{18}F . MMP2 cleavage releases the hydrophobic TMR- ^{18}F . In vivo TMR- ^{18}F accumulated in MMP2 expressing tumor xenografts but not in control tumors (Chuang, et al., 2012).

Watkins et al developed a SPECT probe to follow MMP14 activity. This probe is conjugated to a labeled cell penetrating peptide, which is internalized into the cells after MMP14 cleavage. Signal concentration in the cells is correlated with MMP activity. This probe showed promising results in cancer cells in vitro, but has yet to be studied in vivo (Watkins, et al., 2009).

A number of different methods were developed utilizing NIRF for MMP activity imaging in vivo. Bremer et al synthesized an MMP2 cleavable peptide conjugated to infra-red fluorophores. The fluorescent signal is quenched until the probe is cleaved by MMP2. In addition, they created another probe with the scrambled sequence of the MMP-2 substrate probe. They demonstrate the activity and specificity of the probe by using two tumor cell lines, fibrosarcoma positive for MMP2 expression and breast tumor cells negative for MMP2 expression. In-vivo, in fibrosarcoma tumors but not in breast tumors, signal intensity rose after intravenous injection of the probe, but not the control probe. These results indicate the probe was activated specifically by MMP2 (Bremer, Bredow, Mahmood, Weissleder, & Tung, 2001).

Huang et al added radiolabeling to a similar infra-red labeled probe which is activated after cleavage by several MMPs (MMP 7, 9, 12, 13). PET imaging was used to detect the distribution of the probe, allowing the quantification of the enzymatic activity detected by NIRF. Probe specific fluorescence was demonstrated after MMP cleavage in a tumor xenograft model, which could be suppressed using an MMP inhibitor (C. W. Huang, Li, & Conti, 2012).

In a different approach McIntyre et al created a proteolytic beacon, which fluoresces in the green and red spectrum. Using a peptide sequence that is cleaved specifically by MMP7 the

green signal is enhanced. Thus, this probe can be detected in both its cleaved and uncleaved forms, and the two can be distinguished by green/red fluorescence ratio. The probe proved efficient *in vivo*, in mice injected with MMP7 expressing and non-expressing tumor cells (McIntyre, et al., 2004).

Zhao et al. generated a probe with a MMP2 and MT1-MMP cleavage site for NIRF imaging. The probe is constitutively fluorescent but upon cleavage the fluorescent tag is inserted into the cell membrane, thus the probe tags the tumor cells in the vicinity of MMP activity. This approach led to the tagging of the invasive front of tumors *in vivo*. When the probe is first administered, fluorescent signal is dispersed throughout the mouse body, but after 24 hours the probe is retained only in the tumor. This approach prevents the signal of extracellular MMP activity from leaking to adjacent tissue (Zhao, et al., 2010).

In addition a switchable probe compatible to fluorescent imaging and MRI was developed. This probe was labeled with cy5, gadolinium or both, and consists of dendrimeric nanoparticles coated with activatable cell penetrating peptides. The activatable cell penetrating peptides are polycations attached to polyanions by a MMP2 and MMP9 cleavable peptide. Upon cleavage the polycation is free to adhere and be taken up by cells in the vicinity. The fluorescent or MRI label is linked to the polycations. These agents were tested *in vivo* and demonstrated specific signal enhancement in both fluorescent and MRI signal in a number of mouse xenograft models. The activatable cell penetrating peptides were even able to detect residual tumor after debulking surgery, and metastasis (Olson, et al., 2010). Fluorescent detection of residual tumor is of interesting clinical potential for guidance of surgical procedures. Lepage et al used a different concept to image MMP7 activity *in vivo*, by MRI. They developed a proteinase modulated contrast agent. Proteolytic cleavage of the proteinase modulated contrast agent changes their solubility and therefore enhances their retention in the specific tissue. This contrast agent is specifically cleaved by MMP7. In nude mice bearing MMP7 positive and negative tumors the contrast agent was retained for longer in the MMP7 positive tumors (Lepage, et al., 2007). A similar concept was used to detect MMP2 activity *in vivo* (Lebel, et al., 2008).

The use of a substrate conjugated to a MRI contrast agent as opposed to a fluorescent probe gives the added value of anatomical context. Many probes were developed to image MMP activity *in vivo*. The specificity of these probes towards a single MMP is important since different MMPs are active at different tumor stages and sometimes promote opposite effects. An important step forward would be to develop a method to quantify MMP type specific enzymatic activity based on the imaging results.

2.2.3.2 Hyaluronidase: Hyaluronidases (Hyal) are the enzymes responsible for HA catabolism. Several clinical trials investigated the effects of addition of Hyal to chemotherapy (Whatcott, et al., 2011). Preclinical studies demonstrated that administration of hyal reduces the tumor HA content and improve drug delivery and response to chemotherapy (Alexandrakis, et al., 2004).

Hyal activity was imaged using MRI contrast agent conjugated to HA. When conjugated, this complex showed low relaxivity, while after degradation by hyal the relaxivity increased resulting in enhanced contrast (Shiftan, et al., 2005; Shiftan & Neeman, 2006). HA was conjugated to Gd-DTPA on agarose beads. *In vitro* HA-Gd-DTPA-beads were significantly activated upon degradation by Hyal. *In vivo*, signal significantly elevated after Hyal digestion, in the tumors injected with HA-Gd-DTPA-beads, compared to HA-Gd-DTPA-beads with no tumor (Shiftan, et al., 2005). Analysis based on *in vitro* studies was used to quantify the activity of Hyal in the tumor (Shiftan & Neeman, 2006).

2.2.3.3 Transglutaminase: Transglutaminases are enzymes which form large protein aggregates by catalyzing covalent protein cross-linking (Gaudry, Verderio, Jones, Smith, & Griffin, 1999). They affect the stiffness of tumor ECM, due to changes in fiber organization and cross-linking (Levental, et al., 2009). Tissue transglutaminase is highly expressed in certain cancers such as breast cancer and plays a role in cancer cell invasion, metastasis, survival and chemoresistance (Herman, Mangala, & Mehta, 2006; Mangala, et al., 2007). MR contrast agent conjugated to enzyme substrate analogs was applied for molecular imaging of tissue transglutaminase activity (Mazooz, et al., 2005; Tei, et al., 2010). Briefly, low molecular weight tissue transglutaminase substrate analogs, were conjugated to Gd-DTPA and to fluorescent dyes. Upon enzymatic activity the contrast media were covalently linked to the extracellular matrix, thus enabling the imaging of transglutaminase activity in breast cancer cell spheroids and in tumor xenografts.

The unique cancer ECM takes part in many tumorigenic processes, in many different ways. These ECM components provide important potential targets for therapy. In addition they may affect the level of success or failure of existing cancer therapies. Thus, dynamic imaging of the ECM is of clinical significance. MMPs, for example, play different roles in cancer progression depending on MMP and on the stage in tumor development; the MMP may be either cancer promoting or inhibiting. Better non invasive in-vivo imaging of MMPs will help in elucidating MMPs role at different stages of tumor development. The many pre-clinical and clinical trials for tumor therapy involving ECM components demand well established in vivo imaging biomarkers of the ECM, so that therapeutic success can be monitored.

2.3 Targeting tumor associated immune cells

The immune system plays a substantial role in tumor development. During the early stages of tumor growth it is believed that the immune system can suppress growth of neoplastic tissue by eliminating tumor cells. Tumor progression and spread is conceptualized by the immunoediting hypothesis in which the tumor environment is selected for escape from the immune system (Schreiber, Old, & Smyth, 2011; Vesely, Kershaw, Schreiber, & Smyth, 2011). Inflammation constitutes a double-edged sword that may provide both anti-tumor and tumor-promoting properties (Grivennikov, Greten, & Karin, 2010). It is the plasticity of the immune cells that allows the intensification of the antitumor response during therapy (Coussens, Zitvogel, & Palucka, 2013).

Cancer immunotherapies aim to direct immune response towards tumor cells “labeled” with tumor-associated antigens (TAAs), and to remove immunosuppressive microenvironment around tumors, that hampers anti-tumor response. The first approach includes tumor vaccines in the form of peptides/proteins, recombinant vectors, tumor cells as well as dendritic cells (DCs) and other antigen presenting cells. The second is based on immunomodulatory molecules like cytokines, antibody-cytokine fusions, toll-like receptors, adjuvants and inhibitors of immunosuppressive entities. Efficiency of a treatment can be boosted by chemotherapy, radiation, small-molecule targeted therapeutics, and hormonal therapy. Combination of different approaches proves to be more effective than monotherapy (Schlom, 2012).

Effector cells that shape the immune response in the tumor stroma include tumor-promoting regulatory DCs, M2 macrophages, N2 neutrophils, myeloid DCs, regulatory T-cells ; as well as anti-tumor classic DCsM1 macrophages, N1 neutrophils, cytotoxic T-cells (CTLs) and natural killer cells (Shurin, Ouellette, & Shurin, 2012). All of these cell types elicit complex responses that change over time. Biopsy and *ex vivo* analysis does not give full spatiotemporal information about their function and fate in a living tissue (Akins & Dubey, 2008). Complete, multidimensional data is essential both for monitoring the progress of the

therapy and for conducting basic pre-clinical research aimed at designing novel treatments characterized by stronger clinical responses.

Molecular imaging and intravital microscopy are used to study antitumor immune responses (Figure 4). PET, SPECT and MRI are utilized for repeated, non-invasive observation of tumor growth, metastasis, regression and immune cells trafficking into the tumor stroma. While nuclear imaging methods have higher sensitivity, MRI provides higher spatial resolution. Development of non-toxic, stable contrast agents for MRI enables *in-vivo* imaging of adoptively transferred lymphocytes, as well as tumor associated macrophages and dendritic cells.

Those methods help to link directly the outcome of pre-clinical research with potential clinical applications by providing the similar means of imaging and quantification both for humans and for animals. They are not feasible to study interactions between single elements of the tumor stroma on the cellular and subcellular level. Intravital optical methods, and particularly 2P microscopy allow imaging of intercellular interactions in superficial layers of the body cavities and organs with single-cell resolution.

2.3.1 imaging and targeted therapy for tumor T cells—Throughout the last decade multiple studies have been published on multimodal imaging of T-cell – tumor interactions in living animals. This reflects a need for new tools and translatable models that could boost introduction of new therapies. Utilization of T-cells transduced with reporter genes allows repeated follow up of the adoptive transfer after administration of the contrast agent. Efficient tumor recognition and subsequent cytotoxic activity depends on antigen specificity, MHC (or HLA for human) matching and T-cell activation status. Prior immunodepletion generates a niche for T-cell expansion; yet this condition does not reflect a usual clinical setting.

Antigen specificity is essential for T-cell mediated tumor elimination. It was shown that adoptively transferred T-cells, immune against Moloney murine sarcoma virus/Moloney murine leukemia virus-induced tumors, but not naive T-cells are able to localize into these tumors in immunodeficient mice. Time-dependent migration and expansion of herpes simplex virus thymidine kinase (*HSV-tk*) transduced immune T-cells was detected in the virus-induced, but not control tumors. Detection of T-cells was performed with microPET after injection of ^{18}F -FHBG, a radioactive substrate for HSV-TK (Dubey, et al., 2003). However, throughout a real adoptive transfer therapy in human, HLA matching is required for effective antitumor T-cell action. There is a need for development of more complex pre-clinical models that would reflect more precisely the clinical setting. This problem was addressed by some groups that showed accumulation of Epstein-Barr (EBV) specific T-cells in EBV+ tumors expressing the T cells' restricting HLA allele (Koehne, et al., 2003) and HLA-dependent killing of Wilms tumor protein (WT1) expressing leukemias as well as solid tumors by T-cells sensitized to WT1 (Dobrovina, et al., 2004). Imaging of primary immune response constitutes another approach to obtain biological information more relevant to the clinic. Primary immune response against Moloney murine sarcoma virus/Moloney murine leukemia virus-induced tumor was observed in mice reconstituted with hematopoietic stem and progenitor cells transfected with trifusion reporter gene (renilla luciferase, enhanced green fluorescent protein, *HSV-tk*). This method allowed combining PET imaging with BLI. The authors were able to show localization of T-cells to the tumor and lymph nodes, as well as T-cell depletion from the lymph nodes after treatment with immunosuppressive drug dexamethasone (Shu, et al., 2005). BLI provides only 2D information about localization of the cells and thus is rarely used as an independent modality to monitor antitumor responses. However, it has been reported that cytotoxic T-cell activity

can be visualized after adoptive transfer thanks to coupling enhanced Granzyme B promoter to firefly luciferase reporter gene (Patel, et al., 2010).

Memory T-cells have greater tumoricidal potential than the naive subsets. Proper activation status determines their ability to eliminate tumor cells. PET tracking was used to compare the ability of ovalbumin-specific naive and memory T-cells to eradicate OVA-expressing tumors. Both cell subsets were able to eliminate tumors, however memory T-cells accumulated and proliferated faster than naive T-cells, which, due to slower expansion rate needed to be transferred in higher numbers to achieve similar effect. PET was utilized here to track T-cells in the tumors and the lymph nodes, and also was proven to be a feasible tool for noninvasive quantification of cells in regions of interest (Su, Chang, Gambhir, & Braun, 2006).

Application of novel reporter genes for simultaneous imaging of multiple cell subsets can dramatically increase the amount of information that can be obtained from each experiment. This makes pre-clinical research more resource efficient and helps to scrutinize the full complexity of the cellular behavior. The possibility of using human norepinephrine transporter as a reporter for simultaneous imaging with SPECT and PET, after introduction of norepinephrine analogs [^{123}I]MIBG and [^{124}I]MIBG, was demonstrated. In this manner, EBV-specific T-cells were successfully tracked to EBV+ tumors. Transfecting two T-cell subsets (CD4+ and CD8+) with two distinct reporter genes (human norepinephrine transporter and HSV-TK) allowed independent follow up of cells in the same animal (Dobrovinn, et al., 2007). Multimodal approach was also used for tracking hemagglutinin - specific T-cells labeled by FDA-approved non-immunogenic ^{111}In -oxine. Simultaneous monitoring of the tumor volume and T-cells migration was possible thanks to SPECT-CT fusion system. By this means the study demonstrated improved antitumor cytotoxic activity of T-cells adoptively transferred to lympho-depleted mice (Pittet, et al., 2007).

Ex-vivo expansion and selection of antigen-specific T-cells is time consuming, which is impractical in the clinic. The alternative is to use T-cell receptor-engineered lymphocytes, transduced with chimeric antigen receptors (CARs), which are capable of eliciting objective tumor responses (Morgan, et al., 2006). *In-vivo*, however, their tumoricidal activity is compromised compared to the expanded and selected T-cells.

Feasibility of introducing therapeutic and nuclear reporter genes into human T-cells and subsequently monitoring their spatial and temporal distribution in a prostate systemic cancer model with PET, BLI and CT was demonstrated (Dobrenkov, et al., 2008). In another study splenocytes from fully immunocompetent mice, engineered to express murine/human chimeric T-cell receptor specific for tyrosinase were transferred to lymphopenic, preconditioned mice bearing HLA-matched tyrosinase-expressing and control tumors. PET CT allowed to visualize 3D systemic distribution of antigen-specific T-cells in the anatomical context, their homing to the tumor, and its subsequent eradication (Koya, et al., 2010).

Finally, in the clinical setting PET was successfully used to track adoptively transferred autologous T-cells, in a patient suffering of III–IV grade glioblastoma multiformae. The patient's T-cells were electroporated with a plasmid construct encoding IL-13 zetakine for tumor targeting and HSV-TK gene for PET tracking and frozen. 9 months after resection, the tumor relapsed and the therapeutic cells were administered to the patient throughout a 5 week cycle of intralesional infusions. PET, FDA approved contrast agent ^{18}F -FHBG was detected at the tumor resection site and in the organs involved in the contrast clearance (Yaghoubi, et al., 2009).

Introduction of cross-linked iron oxide nanoparticle to the OVA-specific CD8⁺ T-cells allowed tracking them into OVA-expressing melanoma tumors. Thanks to high-resolution MRI imaging it was possible to observe spatio-temporal heterogeneity of T-cell recruitment to the tumor as well as the changes of its volume over time (Kircher, et al., 2003). Another study showed that OVA-specific T-cells, efficiently labeled with anionic γ -Fe₂O₃ SPIO uncoated nanoparticles, homed first to the spleen before they were recruited to the tumor (Smirnov, et al., 2006).

In addition to imaging the stroma, MRI is clearly valuable for monitoring tumor burden and for detection of tumor regression with therapy, including stroma targeted therapy. Thus for example, MRI was proven effective in correlating initial changes with the final outcome of glioblastoma rejection, induced by engineered T-cells expressing membrane tethered IL-13 zetakine chimeric T-cell antigen receptor, which were directly administered to the tumor bed (Lazovic, et al., 2008).

T-cells behavior inside the tumor stroma can be directly observed *in-vivo* with 2P microscopy. It was shown in tumor explants model that tumor infiltrating lymphocytes (TILs) of the transgenic DPE-GFP mice, expressing GFP in all T-cells exhibit random migratory pattern, crawling along ECM fibers, visualized thanks to second harmonic generation signal they emit. T-cells revealed polarized morphology with the lamellopodia formed at the leading edge. Dynamic analysis of 3D reconstructions revealed long- and short- term interactions of TILs with tumor cells stably expressing enhanced cyan fluorescent protein (CFP). Upon contact with TILs tumor cells were shown to change their morphology from spindle-shaped to round with condensed body and to shed the membrane blebs, which is an indication of apoptotic cell death. After adoptive transfer OVA-specific CD8⁺ DPE-GFP effector CTLs exhibited higher migratory velocities and displacement within tumors expressing their cognate OVA antigen, comparing to the control tumors. As expected long-term interactions engaged by TILs as well as tumor regression and apoptosis occurred exclusively in the antigen-specific manner. TILs were also shown to be involved in long-lasting interactions with the cells containing autofluorescent material, further determined as macrophages. In-vivo imaging of T-cell behaviour in subcutaneously implanted tumors, confirmed findings from the explant model (Mrass, et al., 2006).

Time-lapse 2P microscopy of skin flap model, where OVA-specific GFP-expressing T-cells cells were adoptively transferred into the mice bearing OVA-expressing and control thymomas, revealed that CTLs transiently stop moving within antigen-expressing tumors in the early phase of rejection and resume their movement in the later phase when tumor size decreases. Initial arrest of CTLs motility was caused by the contact mediated killing of antigen-expressing GFP tumor cells, that disappeared over time as a result. Direct contacts between CFP⁺ CTLs and GFP⁺ tumors were visualized at early stage of rejection. Later, CTLs resumed migration in the areas where the tumors cells had been found dead. Cells that resumed motility were found to migrate along the ECM fibers imaged thanks to second harmonic generation signal GFP-expressing CTLs, migrating along blood vessels visualized by injection of rhodamine-labeled dextran, exhibited more elongated shape than the cells found in the other areas. 2P imaging of frozen section revealed that antigen specificity is required to facilitate CTLs migration deep inside the tumor. However, polyclonal, in-vitro activated T-cells co-transferred with OVA-specific CTLs were able to accumulate and infiltrate deep into tumors as well. This suggests that CTL-mediated killing created permissive microenvironment for T-cell migration. All of this findings were reproduced in fibrosarcoma tumor model (Boissonnas, Fetler, Zeelenberg, Hugues, & Amigorena, 2007).

2.3.2 imaging and targeted therapy for tumor associated macrophages and dendritic cells—Tumor associated macrophages (TAMs) constitute another component of

the immune tumor stroma involved in cancer progression. They were shown to induce angiogenesis, facilitate intravasation of tumor cells and modulate T-cell immunity. Their accumulation can be non-invasively imaged with MRI. It was possible to locate intratumoral TAMs in soft tissue sarcoma model, by labeling them with AMTA 680 magneto-fluorescent dye and subsequently detecting with MRI and fluorescence molecular tomography (FMT) (Leimgruber, et al., 2009).

Dendritic cells (DCs) can trigger T-cell maturation and proliferation needed to exert efficient cytotoxic effect on tumor cells. In DC therapy, cells can be injected to the subjects via intravenous, intradermal and subcutaneous route, or directly to the lymph nodes. Accurate monitoring of the administration and trafficking of DCs is necessary to evaluate the optimal route for their administration and to assess their distribution. Un-accurate delivery of DCs was suggested as one of the frequent reasons for failure of DCs-mediated immune induction. A clinical study performed on stage-III melanoma patients demonstrated that SPIO- and ^{111}In -labeled DCs can be tracked by MRI to neighboring lymph nodes upon ultrasound-guided intranodal injection. MRI imaging revealed more DC positive lymph nodes in comparison to routinely used scintigraphy. High resolution anatomical information provided by MRI allowed to confirm the exact intranodal localization of DCs, scintigraphy failed to resolve that; in some cases DCs were delivered into perinodular fat instead of the lymph node itself. Therefore MRI was concluded as a method more accurate than scintigraphy in verification of the DCs delivery to the lymph nodes in the patients (de Vries, et al., 2005).

2P and confocal imaging was also utilized to visualize AMTA 680 fluorescently labeled TAMs in-vivo, inside the stroma of GFP-expressing tumor, in dorsal skinfold chamber. TAMs were found to be immotile, engaging interactions with tumor cells by sending cytoplasmic protrusions (Leimgruber, et al., 2009). Another study utilized multiple methods of macrophage labeling based on phagocytosis of injected Texas red-dextran and GFP expression under *c-fms* and *lys* promoters. It was shown that macrophages localize in the close association with the fluorescent dextran labeled blood vessels around the GFP-expressing tumors. Macrophage density was shown to be much higher in the tumor margins than deep inside the tumor stroma. The frequency of tumor motility was associated with the presence of perivascular macrophages. Tumor cells migrated towards and intravasated in close vicinity of them, within one cell diameter. It was calculated that tumor cell intravasation is related to the number of macrophages, not the blood vessels density. Additionally, the existence of paracrine loop between tumor cells and macrophages, involving epidermal growth factor (EGF) and colony-stimulating factor (CSF-1), was confirmed (Wyckoff, et al., 2007).

2.4 Targeting the tumor vasculature

Formation of new blood vessels is a critical step in tumor progression beyond a few millimeters in size, due to diffusion limits of oxygen and nutrients (Carmeliet & Jain, 2000; Folkman, 1971). Induction of angiogenesis in tumors, namely the “angiogenic switch”, was hypothesized to be the critical switch enabling tumors to exit from dormancy and grow beyond the diffusion limits (Bergers & Benjamin, 2003; Gilad, et al., 2005; Hanahan & Folkman, 1996). To enable the continuous growth, tumor cells and supporting stroma cells, secrete pro-angiogenic factors that stimulate sprouting of new vessels. These factors include growth factors and enzymes (like the proteolytic MMPs) that activate the endothelial cells. VEGF-A is the main inducer for angiogenesis through its receptor VEGFR2 and is an established target both for molecular imaging and for anti-angiogenic treatment (Chung & Ferrara, 2011; Eklund, Bry, & Alitalo, 2013).

The newly formed vessels are composed of the tube forming endothelial cells and their supporting perivascular pericytes (Armulik, Abramsson, & Betsholtz, 2005). The expansion of the tumor vasculature includes growth of the endothelial sprouts from preexisting vessels and the growth and remodeling of the primitive network into a complex network (Carmeliet, 2000). The tumor vessels are usually disorganized and chaotic in comparison to normal vascular beds (Carmeliet & Jain, 2000). Targeting and imaging tumor angiogenesis has been the focus of many studies (Missbach-Guentner, et al., 2008; Neeman, Gilad, Dafni, & Cohen, 2007). Several mouse models were suggested for studying tumor angiogenesis (Eklund, et al.; Mriouah, et al.).

Bevacizumab, a humanized monoclonal antibody against VEGF, was the first antiangiogenic drug to be approved for clinical use in humans (Ferrara, 2004). Clinical and pre-clinical monitoring of anti angiogenic treatment requires the development of functional and molecular imaging biomarkers (Figure 5).

2.4.1 Functional imaging of tumor angiogenesis—In the tumor area the major contribution of the angiogenic process is perfusion, oxygenation and nutrient supply to the surrounding tissue/tumor. Parameters such as perfusion, microvascular density and vessel permeability are important for evaluating the angiogenic process and response to anti-angiogenic treatment. Measuring the angiogenic response to treatment poses an imaging challenge, particularly considering the diverse roles of angiogenic growth factors and calls for development and implementation of multiple imaging modalities (Boult, Terkelsen, Walker-Samuel, Bradley, & Robinson, 2013). Various imaging techniques are used to provide functional information on the tumor angiogenesis including dynamic contrast enhanced MRI (DCE-MRI), ultrasound, PET (especially with water O-15) and dynamic contrast enhanced CT.

Blood flow and perfusion changes can be monitored by Dynamic contrast enhanced MRI (DCE-MRI) (Goh & Padhani, 2006), and by arterial spin labeling (Ansiaux, et al., 2005). DCE-MRI tracks the pharmacokinetic behavior of injected low molecular weight contrast agent as it distributes in the tumor vasculature and leaks across permeable vessels. Model based analysis of DCE-MRI data was used for mapping multiple parameters including blood volume, vascular permeability, blood flow, vascular surface area, and interstitial pressure. DCE-MRI is used to measure vasculature parameters in many preclinical models and also in the clinic for initial diagnosis and tumor staging (Ocak, et al., 2007). Arterial spin labeling requires no administration of exogenous contrast media and reports on the exchange of water molecules between blood and the tumor. MRI is often used for assessment of the efficacy of anti angiogenic treatment in the clinic and in pre-clinical studies.

Blood vessels can also be visualized by CT by administration of contrast media, typically low molecular weight iodinated agents and acquisition of serial images over time. CT allows measurements of blood flow and blood volume in the tumor. However, CT has a major disadvantage of radiation exposure in patients. In animal research microcomputed tomography (μ CT) is associated with exposure to relatively high radiation dose making it more suitable for samples or in post mortem studies (Maehara, 2003; Savai, et al., 2009; Wessels, et al., 2007). Flat-panel volume computed tomography (fpVCT) enables imaging large volume in short time with higher resolution and lower radiation dose (compared with regular CT) allowing the researcher to scan a whole live animal (Greschus, et al., 2005; Kiessling, et al., 2004).

Using fpVCT Kiessling et al. were able to detect vessels in 40–50 μ m in diameter by injecting high dose of iodine/barium contrast agents (Kiessling, et al., 2004). The method also allows for monitoring changes in blood vessel diameter, recruitment of blood vessels,

changes in blood vessels density in the tumor at multiple time points and monitoring changes related with necrosis of the tumor (Missbach-Guentner, et al., 2008). Changes in blood vessels in bone metastasis from breast cancer origin could be monitored following treatment (Bauerle, et al., 2008). Tumor progression as well as the development of tumor vasculature was followed *in vivo* in a transgenic mouse model for mammary carcinogenesis (WAP-T mice), using iodine containing contrast agent (Isovist 30^{TM}) and a blood pool agent (eXia 160^{TM}). The use of blood pool agent enhanced the spatial resolution due to prolonged half life in the blood and reduced extravasation from blood vessels (Gerstel, et al.; Jannasch, et al., 2009).

PET-perfusion measurements are performed using labeled water O-15, to measure blood flow or labeled carbon monoxide (C-15) that immediately binds to red blood cells *in vivo* for assessment of blood volume (Dimitrakopoulou-Strauss, Strauss, & Burger, 2001; Mullani, et al., 2000). Labeling red blood cells or albumin with a radioisotope was also reported. In tumors, leakage of tracer such as 68Ga-DOTA-albumin allows the calculation of vessel permeability, when dynamic imaging is performed (Hoffend, et al., 2005). Radiotracers are often used for functional vasculature imaging in the clinic, however the short half-life of C-15 and O-15 are a drawback, and require on-site radiochemistry.

Combined use of gray scale and Doppler measurements makes ultrasound imaging a cheap and safe measurement tool for blood flow dynamics and resistance of large vessels in tumors in 2D and 3D both clinically and in laboratory animals (Alcazar, 2006; Fleischer, et al., 1999). Clinically, Doppler is used to follow blood flow and its velocity for monitoring and staging different types of cancer (Alcazar, 2006; Yang, Tse, Lam, Metreweli, & Chang, 2002). High-frequency power Doppler imaging allows the noninvasive characterization of the vasculatures in primary and recurrent tumors of mice by measuring perfusion and vascular density (J.-J. Chen, Fu, Chiang, Hong, & Yeh, 2013; J. J. Chen, Chiang, Hong, & Yeh).

Microbubbles are gas filled vesicles with a diameter between 1–5 μ m that behave hemodynamically like red blood cells and can be detected at high sensitivity (Lindner, 2004). As microvessels are below the spatial resolution of normal ultrasound, using microbubbles allows for detection of smaller vessels (Horie, Chen, Li, Mori, & Kodama, 2013). Microbubbles were also applied for monitoring response to anti angiogenic treatments in several tumor models (Deshpande, Pysz, & Willmann, 2010; Postema & Gilja, 2011).

Ultrasound can also be used for guided delivery of therapy. For example, anti angiogenic therapy can be delivered by using microbubbles as carriers of the anti angiogenic agents, coupled with ultrasound-targeted microbubble destruction. In this method a distractive pulse causes the bubbles to explode and cause local damage and local release of the microbubble therapeutic payload. The specificity can be enhanced by conjugating targeting ligands to the surface of the bubbles (Fujii, et al.; Goossen, de la Rosette, Hulsbergen-van de Kaa, van Leenders, & Wijkstra, 2003; Kiessling, Huppert, & Palmowski, 2009; Klivanov, 2006). Conjugation of shRNA for VEGFR2 to microbubbles as was demonstrated by Fujii et al. resulted in reduction in tumor size and perfused areas and lower tumor microvascular blood volume, as a direct result from gene knockdown (Fujii, et al., 2013).

FMT is a non-invasive optical technique that was used to quantitatively measure *in vivo* the blood vessel density in response to anti-tumor treatment (Montet, et al., 2007; Q. Zhang, et al., 2011). Zhang et al. used FMT with a vascular pool agent (AngioSense⁷⁵⁰) to visualize functional vessel normalization and to follow changes in the same vessels before and after anti VEGF or rapamycin treatment.

Optical coherence tomography (OCT) provides non-invasive and label-free imaging of living tissues and organisms. Microstructural OCT allows for detection of vessels larger than 100µm. Angiographic imaging relies on the measurement of scattering dynamics which is altered by blood flow (Vakoc, et al.; L. V. Wang, 2009). The wide-field imaging of OCT reveals the morphological nature of tumor vascular networks. This was used to highlight the important role of the microenvironment in tumor vasculature (Vakoc, et al., 2009).

Combination of architectural and vascular OCT images acquired simultaneously allows distinguishing between intra tumor and peri tumor vessels, for monitoring the different anti vascular response at these sites (Winkler, et al.).

Using window chamber in mice to image tumor growth and angiogenesis was reported many years ago (Clark & Clark, 1932). However, novel imaging techniques, fluorescent proteins and probes made this model a useful tool for high resolution of tumor vasculature (E. B. Brown, et al., 2001; W. Cai & Chen, 2007; Q. Huang, et al., 1999; Palmer, et al., 2011). Imaging tumor vasculature using a window chamber is a powerful tool enabling researchers to monitor changes in vasculature in high resolution at different time points in order to learn about vasculature development in early vs. late tumor stages, vascular changes in response to treatment and combination of vascular imaging with other tumor or stroma components. Combination of spectral and fluorescent imaging were used for imaging of blood supply and oxygenation at high spatial resolution (Alcazar, et al., 2006).

Photoacoustic imaging can be used for imaging exogenously administered contrast media with high absorption, and also for detection of endogenous light absorbing molecules. Deoxyhemoglobin, oxyhemoglobin and melanin account for most of the optical absorption in mammalian tissue in the visible spectrum (Hu & Wang, 2010). Consequently, fPAM, is a sensitive blood detector with high contrast and specificity. It is suitable for imaging the volumetric morphology of the subcutaneous microvasculature, and for mapping blood oxygenation in healthy and tumor tissues (H. F. Zhang, Maslov, Stoica, & Wang, 2006).

2.4.2 VEGF/VEGFR axis—The vascular endothelial growth factor (VEGF) family consists of 7 isoforms, and is a key pathway regulating tumor angiogenesis. VEGF-A and its main receptor VEGFR2 are therefore targets for molecular imaging and for therapy (W. Cai & Chen, 2007; Turkbey, Kobayashi, Ogawa, Bernardo, & Choyke, 2009). The VEGF promoter was used to induce expression of GFP (Izzo, Mackanos, Beckham, & Jansen, 2001) or Luciferase (Y. Wang, et al., 2006) as a way to track tumor induction of angiogenesis. Expression of luciferase under the VEGFR-2 promoter provides a way to track angiogenic response in endothelial cells during tumor growth (N. Zhang, Fang, Contag, Purchio, & West, 2004).

While these reporter gene approaches can be used only for preclinical research, targeted delivery of labeled VEGF or VEGF targeted antibodies can be used for mapping the distribution of VEGF and its receptors also in human. Conjugation of VEGF to DOTA - ⁶⁴Cu for PET imaging revealed low VEGFR-1 expression and good correlation to VEGFR-2 as measured by western blot (K. Chen, Cai, Li, Wang, & Chen, 2009). In addition, radiolabeled bevacizumab was used for PET imaging (Nagengast, et al., 2007).

Targeted imaging of endothelial cells was done by conjugating VEGFR2 antibody to microbubbles for imaging tumor angiogenesis by ultrasound (Pillai, et al.; Willmann, et al., 2008), and using Tie2 GFP mice for intravital imaging of endothelial cells in a window chamber (Hillen, et al., 2008; Motoike, et al., 2000).

2.4.3 $\alpha v \beta 3$ integrin—Endothelial cells express integrins that modulate their migration and survival during angiogenesis. The $\alpha v \beta 3$ integrin binds to Arginine-Glycine-Aspartic acid (RGD) in vitronectin, fibronectin and thrombospondin and it is highly expressed on endothelial cells of newly formed tumor vasculature (Ramjaun & Hodivala-Dilke, 2009).

Multiple probes were generated for detection of $\alpha v \beta 3$ by different imaging modalities. Based on the binding of RGD peptide to $\alpha v \beta 3$ tracers were developed for imaging vasculature by conjugating RGD to ^{125}I (R. Haubner, et al., 1999) ^{18}F (Roland Haubner, et al., 2001; Liu, et al.) and ^{64}Cu (Yapp, et al.) using PET. Similarly RGD labeled with fluorescent markers (e.g. quantum dots) were reported for fluorescence imaging (Weibo Cai, et al., 2006). A contrast agent conjugated to a monoclonal antibody was reported for detection of $\alpha v \beta 3$ integrin with MRI (Sipkins, et al., 1998). Conjugation of biomarkers to microbubbles for ultrasound imaging, allows to visualize the expression of $\alpha v \beta 3$ integrin in tumors and in response to anti angiogenic therapy. Palmowski et al. used molecular ultrasound imaging to track changes in vasculature and elevation of ICAM and $\alpha v \beta 3$ after carbon ion irradiation by conjugating RGD peptide or ICAM antibodies to microbubbles (Palmowski, et al., 2009).

2.4.4 Perivascular mural cells—The importance of pericytes on tumor progression was studied using transgenic mice expressing thymidine kinase in pericytes (using the NG2 promoter). In these mice, pericytes could be depleted by administration of Ganciclovir (GCV) (Cooke, et al., 2012). Although depletion of pericytes attenuated growth of the primary tumor, it enhanced the progression of metastases. Such therapeutic effect could potentially be complemented with non invasive imaging of vasoreactivity by BOLD contrast MRI in response to hypercapnia, a process which depends on vascular maturation and activity of the perivascular contractile pericytes (Abramovitch, Dafni, Smouha, Benjamin, & Neeman, 1999; Neeman, Dafni, Bukhari, Braun, & Dewhirst, 2001).

2.4.5 Lymphatic endothelial cells, lymphangiogenesis and lymphatic function—Although for many years it was believed that tumors are devoid of functional lymphatics, it has become clear that peritumor lymphatics and tumor-induced lymphangiogenesis augment tumor progression and metastasis. Lymphatics also contribute to tumor evasion of immune response and decreasing interstitial pressure in the tumor. Intratumoral lymphatics are generally considered non-functional, whereas peritumoral lymphatics carry the burden of lymphatic drainage from the tumor (Shields, 2013). Lymphangiogenesis has been suggested as a target for tumor therapy, through suppression of the VEGF-C/VEGF-D/VEGFR3 pathway.

CT and MRI were applied for lymph node (LN) imaging and distinction between large and normal LNs, where large nodes may indicate metastatic spread. In addition there are many emerging methods for the imaging of sentinel lymph nodes in all modalities including MRI, CT, PET and ultrasound. In-vivo imaging methods for lymphatic vessels and lymphatic function are now being developed. Molecular probes or contrast agents are used to image lymphatic vessels and flow. These are administered either intravenously or intradermally in the vicinity of the tumors. The molecular weight of the probe is of great significance, it determines whether the probe will enter the lymphatics, accumulate in the LN, enter the vasculature and the rate of these processes.

For example, intradermal injections of TRITC-dextran were used for intravital imaging of melanoma associated lymphatic vessels in a dorsal skin fold chamber (Gaustad, Simonsen, Leinaas, & Rofstad, 2012). Several other studies used multiphoton laser-scanning microscope to image lymphatic vessels in a tumor xenograft model in the mouse tail, ear or hind limb (He, et al., 2005; Hoshida, et al., 2006; Isaka, Padera, Hagendoorn, Fukumura, &

Jain, 2004; Padera, et al., 2002). Padera et al (2002) compared the lymphatic vessels in two VEGFC overexpressing tumor models, melanoma and fibrosarcoma in the hind limb. TRITC-dextran was injected into the tumor area in addition to FITC into the blood vessels. No lymphatic vessels could be detected within the tumor but the peripheral lymphatic vessels in the VEGFC expressing tumors were dilated compared to control tumors (Padera, et al., 2002).

Quantum dots were also applied for fluorescence imaging of lymphatic function. These may be intradermally injected and are drained by lymphatic vessels. Utilizing quantum dots Harrell et al (2007) characterized an increase in lymphatic flow in tumor draining lymphatics compared to non-tumor draining lymphatics in melanoma tumors inoculated into the mouse footpad (Harrell, Iritani, & Ruddell, 2007). Imaging of lymphatic drainage using quantum dots was established with quantum dots of up to 5 different emission wavelengths simultaneously (Kobayashi, et al., 2007). Utilizing macro-zoom fluorescence microscopy Kosaka et al (2012) were able to image microscopic lymphatic flow after quantum dots administration. This too offers great potential for tumor lymphatic imaging (Kosaka, Mitsunaga, Choyke, & Kobayashi, 2013).

NIRF non-invasive imaging of the lymphatic vessel function is widely used. Dynamic imaging of lymphatic vessels is possible by intradermal administration of indocyanine green (ICG). This method was used to image lymphatic vessel function in a tail and hind limb tumor model. Lymphatic vessel drainage and integrity was altered in tumor bearing mice compared to controls (K. Y. Choi, et al., 2011).

To overcome the instability of ICG in solution, a liposomal formulation of indocyanine green (LP-ICG) was developed, which has greater stability and optical properties than ICG. LP-ICG was used to quantify lymphatic flow in tumor bearing mice. Mice were injected with melanoma cells expressing luciferase and LP-ICG was injected in the peritumor area intradermally. This enabled bioluminescence coupled with NIRF imaging to follow both tumor volume and metastasis and lymphatic flow. Lymphatic flow was found to be elevated in tumor bearing mice compared to mice with no tumor, while heavy tumor lymph metastatic burden correlates with lower LP-ICG clearance from these lymph nodes (S. T. Proulx, et al., 2010). Proulx et al (2013) developed a NIRF dye, IRDye conjugated to polyethylene glycol. In 2 different tumor models, murine melanoma and mammary carcinoma they followed lymphatic vessel functionality by following lymphatic drainage and vessel pulse rate. They found that lymphatic vessels were dilated in the tumor periphery and as tumor progressed, lymphatic vessel pulse rate decreased. In addition LN metastasis, visualized by bioluminescence or fluorescence (NIRF) was associated with lymphatic vessel dysfunction and rerouting of lymphatic drainage (Steven T. Proulx, et al., 2013).

Lymphatic function can also be studied by contrast – enhanced MRI using early versus delayed enhancement and using in vivo avidin chase (Dafni, Israely, Bhujwalla, Benjamin, & Neeman, 2002; Dafni, Landsman, Schechter, Kohen, & Neeman, 2002; Pathak, Artemov, Neeman, & Bhujwalla, 2006; Pathak, Bhujwalla, & Pepper, 2004; Saban, et al., 2007; Vandoorne, Addadi, & Neeman, 2010). We introduced the use of intravenously administered biotin-BSA-Gd-DTPA and analysis of the kinetics of vascular leakage and later lymphatic drainage of the contrast material by MRI, for mapping lymphatic drainage from sites of blood vessels hyper-permeability. To segregate between vascular leakage and lymphatic drainage, rapid clearance of the contrast agent from the blood vessels was induced by intravenous administration of avidin (in vivo avidin chase). Increased lymphatic drainage was measured in VEGF overexpressing tumors inoculated into the mouse hind limb (Dafni, Israely, et al., 2002). Pooling and draining voxels were analyzed similarly by the early and late dynamics of contrast enhancement after administration of Gd-DTPA macromolecular

contrast media, showing correlation with the tumor metastatic potential (Pathak, et al., 2006; Pathak, et al., 2004). Later Saban et al (2007) studied lymphatic drainage by analyzing the kinetics of the contrast agents movement from the blood vessels to the interstitial space and drainage by lymphatic vessels. They utilized a biotin-BSA-Gd-DTPA-Cy5.5 for both NIRF and DCE-MRI imaging of lymphatic vessel function. They analyzed the functionality of lymphatic vessels in the tumor and in the tumor periphery, and concluded that the intratumor lymphatics had decreased function (Saban, et al., 2007).

Ruddell et al (2008) used low-molecular weight gadolinium to image lymphatic drainage with DCE-MRI. Gd-DTPA was s.c injected into the dorsal toe of the rear foot in mice bearing melanoma tumors in the hind leg footpad. The contrast agent was taken up specifically by lymph vessels and did not leak into the blood vessels until it reached the heart. The tumor induced lymph drainage was significantly increased compared to normal control mice and to injection of the contrast agent in the contralateral side. 9 min after contrast agent injection in the tumor side it already labeled major lymph vessels and LN and reached the heart. In control mice after 9 min signal in lymph vessels adjacent to injection site was still faint (Ruddell, et al., 2008).

An additional dendrimer-based nano-size paramagnetic molecule was developed by Kobayashi et al (2004) for micro-MR mammo-lymphangiography (Kobayashi, et al., 2004). The contrast agent was injected peritumorally in spontaneous breast cancer mouse models and in murine breast cancer xenografts. The contrast agent was drained by the lymph vessels and signal was enhanced in the lymph vessels and LN. It did not leak into vasculature. In addition a lack of signal enhancement in areas of the LN indicated to the presence of metastasis, which was later validated by histology. This method allows for lymphatic drainage imaging of both normal and tumor associated lymphatics and for the detection of LN metastasis (Kobayashi, et al., 2004).

In the studies described thus far lymphangiogenesis and lymphatic function was imaged by the molecular agent taken up by the lymphatic vessels. These imaging methods are relatively transient. Signal is detected for the short period of time necessary for agent clearance by the lymphatic vessels. To prolong the imaging time frame probes which label lymphatic markers were developed. These include LYVE1 and VEGFR3 targeting peptides.

McElroy et al (2009) utilized a LYVE1 antibody conjugated to a green fluorophore, to track the movement of human pancreatic cancer cells through lymph vessels in vivo. The conjugated LYVE1 antibody was injected near the inguinal lymph node. Signal was maintained for up to 48hr post injection as opposed to control mice with either FITC-dextran which disappeared after 4hr or IgG control which was weaker than the LYVE1 conjugated marker at 4hr and lasted only up to 12hr post injection. They injected RFP expressing cancer cells in the vicinity of the inguinal lymph node 4hr after lymphatic marker injection, and were able to follow cells in the lymphatic vessels (McElroy, et al., 2009).

Specific lymphatic marker targeting was utilized also for PET imaging. A LYVE1 antibody was radiolabeled for in-vivo lymphatic imaging by PET. Melanoma tumors expressing luciferase and VEGFC were inoculated into the footpad of mice. The tumor draining LN was clearly visible after i.v injection of ^{124}I -anti-LYVE1-antibody, but the control contralateral LN was not visible, and the IgG control was found mainly in the blood. Immunostaining revealed that the tumor draining LN had more LYVE-1 expressing lymphatic vessels compared to controls (Mumprecht, et al., 2010). In addition to utilizing lymphatic drainage for contrast agent labeling of lymphatics, several mouse models have recently been developed for specific imaging of the lymphatic vessels development. These include transgenic mice expressing fluorescent proteins (GFP, mOrange2, tomato) under the

Prox-1 promoter (I. Choi, et al., 2011; Hagerling, Pollmann, Kremer, Andresen, & Kiefer, 2011; Truman, et al., 2012). Prox-1 is a marker specific to lymphatic endothelial cells both in embryonic development and in the adult. These models may be further developed for studying lymphangiogenesis in cancer progression. An additional mouse model was created in which an EGFP-luciferase fusion protein is expressed under the VEGFR3 endogenous control. Utilizing this model they were able to image lymphangiogenesis in the periphery of induced papillomas. In addition they injected s.c. mouse melanoma tumors of a highly metastatic cell line. Here they visualized lymphangiogenesis in the periphery of the tumors and in lymph nodes of these mice (Martinez-Corral, et al., 2012).

2.5 Perineural invasion

Tumors use multiple invasion routes, following not only the vasculature but also neurons (Friedl, Locker, Sahai, & Segall, 2012). The prognostic significance of perineural invasion was highlighted for head and neck cancer, prostate cancer and pancreatic cancer (Cozzi, et al., 2013; Miller, et al., 2012; Mossner, 2010). Perineural invasion is frequently diagnosed late, through its motor or sensory clinical symptoms. Due to its significance on patient outcome, it provides an important biomarker for treatment selection, and also an interesting target for intervention. Early, imaging based detection of perineural invasion could aid in surgical planning. MRI at 3T showed structural changes in facial nerves for cutaneous squamous cell carcinoma invading the parotid gland (Penn, Abemayor, Nabili, Bhuta, & Kirsch, 2010). In pancreatic cancer, perineural invasion is a major source of positive resection margins. Volumetric rendering of CT data were used for visualization of perineural plexuses (Deshmukh, Willmann, & Jeffrey, 2010). Extrapaneuratic invasion was shown to follow neurovascular bundles. Radiation therapy can affect perineural invasion not only through its direct effect on the cancer cells, but remarkably also through its effect on the paracrine communication between the nerve microenvironment and the tumor cells mediated by glial-derived neurotrophic factor (Bakst, et al., 2012). Thus, in vivo MRI studies in mice, demonstrated impaired perineural invasion for mice with irradiated sciatic nerve administered with non-irradiated cancer cells.

3. Summary and Perspectives

The tumor stroma, including its diverse cellular and non-cellular components, affects cancer progression and tumor response to therapy in multiple ways. Classical therapies of cancer focused on the transformed clonally expanded cell as the primary target for tumor detection and targeted intervention. Over the last years it became clear that the tumor microenvironment, including the multiple types of cells that are recruited to and educated in the tumor offers additional targets for detection and therapy. Tailoring such treatment to the patient requires effective means for visualization of such targets and for detection of the efficacy of intervention. Novel tools developed over the last years for imaging the key cellular and molecular stroma components, offer hope for such guidance of treatment in cancer. In particular, significant progress was made in imaging the ECM and the activity of some of the enzymes that modify it, the tumor blood and lymphatic vessels, and the recruited accessory cells including immune cells and cancer associated fibroblasts. Most of these tools are available now for preclinical basic research and drug development, some of which could potentially also be translated to clinical use. Although validation of such imaging based biomarkers will require considerable investment and effort, its value would be immeasurable in tailoring personalized therapy. However, it is clear that we have just begun to reveal the rich and rapidly changing landscape of the tumor stroma, and thus many challenges remain. Systemic effects of cancer could build the niche to be used for tumor metastasis, and systemic cell recruitment could alter distant organs. Targeting such changes will require further innovative tools for their visualization.

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Abbreviations

2P	two photon
aSMA	alpha smooth muscle actin
BLI	bioluminescence imaging
CAFs	cancer associated fibroblasts
CARs	chimeric antigen receptors
CMP	collagen mimetic peptides
CFP	cyan fluorescent protein
CT	computerized tomography
CTL	cytotoxic T lymphocytes
DCs	dendritic cells
DCE-MRI	dynamic contrast-enhanced MRI
ECM	extracellular matrix
FAP	fibroblast activation protein
FAPα	fibroblast activation protein-alpha
FMT	fluorescence molecular tomography
FN	fibronectin
fpVCT	flat-panel volume computed tomography
HA	hyaluronan
HLA	human leukocyte antigen
HSV-tk	herpes simplex virus thymidine kinase
Hyal	hyaluronidase
ICG	Indocyanine green
IL-13	interleukin 13
LN	lymph node
LP-ICG	liposomal formulation of indocyanine green
MHC	major histocompatibility complex
MMPs	matrix metallo proteinases
MRI	magnetic resonance imaging
NIRF	near infra-red fluorescence
OCT	optical coherence tomography

OVA	ovalbumin
PDGF	platelet derived growth factor
PET	positron emission tomography
SHG	second harmonic generation
SPECT	single photon emission computerized tomography
SPIO	superparamagnetic iron oxide
TAM	tumor associated macrophages
TILs	tumor infiltrating lymphocytes
TMR	hydrophobic tetramethylrhodamine
VEGF	vascular endothelial growth factor

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Figure 1. Tumor-Stroma communication

Growth of solid tumors depends on interaction with multiple stroma components including blood vessels, the immune system, the extracellular matrix and stroma fibroblasts. All these stroma components are altered by in the tumor environment and were suggested as targets for therapy. Non invasive imaging of the unique tumor stroma emerges as a powerful tool for detection, monitoring and guidance of therapy.

Figure 2. Image guided targeted delivery of fibroblasts in ovarian carcinoma

A) Exogenously labeled fibroblasts are actively and systemically recruited to ovarian carcinoma xenografts, where they assume perivascular position and are activated to form cancer associated activated myofibroblasts. B) Histological staining of the recruited fibroblasts. C) Optical imaging of recruitment of fibroblasts expressing the MRI reporter gene h-ferritin. D) Analysis of the volume fraction occupied by the exogenously delivered ferritin expressing fibroblasts. Reproduced with permission from Radiology (Vandsburger, et al., 2013).

Figure 3. Imaging the extracellular matrix in cancer

A) The tumor extracellular matrix (ECM) provides multiple targets for non invasive imaging, including macromolecular building blocks and extracellular ECM modifying enzymes.

B) Optical imaging of MMP activity in tumors. Top) administration of the MMP probe. Bottom) control probe. Reproduced with permission from Journal of Controlled Release (Zhao, et al., 2010).

C) Intravital imaging of collagen using second harmonic generation showing the effects of treatment with relaxin. Reproduced with permission from Nature Medicine (E. Brown, et al., 2003).

D) MRI of hyaluronidase activity detected using a smart sensor in which relaxivity is enhanced by enzymatic cleavage. Reproduced with permission from Contrast Media and Molecular Imaging (Shiftan & Neeman, 2006).

Figure 4. Imaging the tumor-immune communication

A) Multiple immune cells are actively recruited to the tumors where they undergo reprogramming. Novel approach for cellular imaging enables tracking these cells in vivo. B) MRI can be used for guidance of administration of SPIO labeled dendritic cells to melanoma patients. (left) MRI scan showing the inguinal lymph node to which the dendritic cells should be administered (black arrow). (right) MRI shows mis-administration of the dendritic cells into the subcutaneous fat (white arrow). Reproduced with permission from Nature Biotechnology (de Vries, et al., 2005).

C) Imaging of T cell recruitment was demonstrated using PET-CT (A) thoracic tumors were adoptively transferred with tyrosinase expressing T cells (B) Scheme of the tumor model. Reproduced with permission from the Proceedings of the National Academy of Sciences of the United States of America (Koya, et al., 2010).

Figure 5. Imaging the tumor vasculature

A. The tumor vasculature provides multiple targets for structural, functional, molecular and cellular imaging. Such multi-parametric view is essential for visualization of the complex process leading to remodeling of functional blood and lymphatic vessels.

B. Structural imaging of the tumor vasculature. Optical frequency domain imaging (OFDI) was applied for acquisition of exquisite images of the tumor blood vessels. Left) The incident light beam is combined with a reference beam, generating an interference signal in which depth is manifested by amplitude and phase. Right) Human glioblastoma xenograft in a mouse reveals the three dimensional architecture of the vasculature (yellow-superficial, red 2 mm deep). Scale bar, 0.5 mm. Reproduced with permission from Nature Medicine (Vakoc, et al., 2009).

C. Volumetric CT provides whole body view of the systemic reorganization of blood vessels induced by a growing tumor. Reproduced with permission from Nature Medicine (Kiessling, et al., 2004).

D. Imaging tumor lymphatics drain by MRI lymphography. Left) hind foot lymphatics. Right) Maximal intensity projection acquired 30 minutes after intradermal administration of Gd-DTPA into the dorsal toe. Reproduced with permission from Neoplasia (Ruddell, et al., 2008).