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Therapeutical Cues from the Tumor Microenvironment

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1. Introduction

The tumor can be considered a complex organ where transformed cells characterized by high genomic instability and altered gene expression are interconnected and communicate with the surrounding microenvironment. The microenvironment (Fig. 1) is composed of a large variety of stromal cells including inflammatory, stem and progenitor cells, fibroblasts, myofibroblasts, perycites, endothelial and mural cells of the blood vessels which are interconnected with the components of the extracellular matrix (ECM). The crosstalk between these components and cancer cells deeply affects their survival and proliferation. Due to the critical role of these interactions in cancer progression, each cellular or molecular component represents a potential target for the development of useful drugs in cancer treatment. The cells of the microenvironment are more genetically stable compared to the

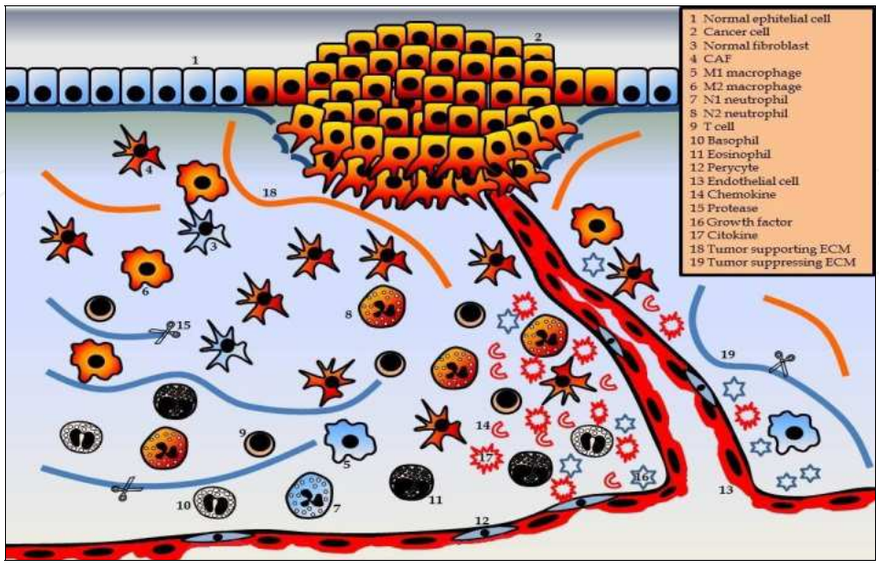

Fig. 1. Schematic representation of the tumor microenvironment; CAF: Cancer Associated Fibroblasts; ECM: extracellular matrix.

tumor cells, and a combinatorial treatment targeting both cancer cells and components of the microenvironment holds the potential for a more successful outcome. In this chapter will list some examples of molecular cues from tumor cells and the surrounding microenvironment that are important for tumor growth and progression and due to their key role in this process are targets for therapeutic drugs under investigation or in clinical use. We will focus on tumor cells and the regulation of proliferation and survival by receptor tyrosine kinases and apoptosis. A number of cellular components of the tumor microenvironment involved in the process of angiogenesis and how ECM affects this process will be taken into account and the effects of some ECM molecules directly affecting tumor cell survival and growth will be also discussed.

1.1 Cell signaling: Receptor tyrosine kinases

Networks of protein-protein and protein-metabolite interactions are commonly found in biological systems where signals must be passed from one location or component within the microenvironment to another. Receptor tyrosine kinases (RTKs) have the critical function of transducing signals derived from extracellular ligands to several intracellular pathways. Regulation of such networks is often achieved by transient, reversible modifications of the components involved. To date, more than 200 different types of post translational modifications (PTMs) have been identified, but by far the best appreciated and among the most important involve phosphorylation of serine, threonine and tyrosine residues. Phosphorylation is one of the most studied PTM because it is vital for a large number of protein functions and characterizes many cellular processes spanning from signal transduction, cell differentiation and development to cell cycle control and metabolism. A primary role of phosphorylation is to act as a switch to turn "on" or "off" a protein activity or a cellular pathway in an acute and reversible manner (Hunter, 1995).

Each RTK is composed of an extracellular N-terminal segment able to bind a specific extracellular ligand, a transmembrane domain and an intracellular C-terminal segment containing the enzymatic domain and a series of tyrosin phosphorylation sites. After ligand binding, the dimerization of the receptor induces a trans-phosphorylation of the intracellular domain followed by the activation of the catalytic subunit and the transduction of the signal at intracellular level. The intracellular domain phosphotyrosines are recognized by downstream signal proteins, generally through Src homology-2 (SH2) or phosphotyrosine-binding (PTB) domains and their binding induces the activation of the intracellular signaling cascade (Hubbard & Miller, 2007). The two main pathways through which RTKs act are the MAPK pathway and the PI3K-AKT, although other pathways can be activated under specific situations. After receptor dimerization an adaptor protein allows its coupling with a G protein that in turn activates a series of intracellular kinases. One of the most studied G proteins is called Ras. Mutations that constitutively activate this protein and induce an aberrant stimulation of the pathway are often observed in several types of cancer. Besides Ras, numerous other proteins involved in the RTKs pathway are mutated in different cancer cells, indicating that these proteins retain a fundamental role in tumor development and progression. Considering the central function it is obvious that these molecules represent a fundamental channel for the development of novel drugs for cancer therapy.

1.2 Apoptosis

A key process profoundly influencing tumor progression is apoptosis. Apoptosis is the process of programmed cell death occurring in multicellular organisms and is characterized

by morphological changes including cell shrinkage, membrane blebbing, cromatine condensation and nuclear fragmentation. Factors such as cellular stress, oncogene activation and dysregulation of cell growth all lead to the activation of p53, which either arrest the cell cycle or activates apoptosis. Apoptosis regulates the proper tissue cell number eliminating damaged or excessive cells and it is also triggered by radiation and chemoterapeutic drugs used for cancer treatment (Sato et al., 1999). Two distinct intercommunicating apoptotic pathways have been described; the intrinsic and the extrinsic. The intrinsic pathway is activated following DNA damage and other severe cellular stresses. The intracellular signals hinge on the mitochondria with the release of cytochrome c which in turn binds to the cytosolic protein Apaf-1 to form the so called apoptosoma decisive for caspase activation. Caspases are a network of proteases that ultimately destroy the cellular structures and induce DNA fragmentation leading to cell death. The extrinsic apoptotic pathway is a receptor-mediated pathway and is activated by extracellular cues upon engagement of the so called death receptors by specific secreted ligands such as Apo2L/TRAIL. Upon activation the receptors for TRAIL activate the caspases specific of the extrinsic apoptotic pathway which ultimately leads to cell death (Jin & El-Deiry, 2005).

1.3 Cells of the tumor microenvironment

None of the cells of the tumor microenvironment except tumor cells are per se malignant; however the interactions with each other and directly or indirectly with the cancer cells may lead to the acquisition of an abnormal phenotype and expression pattern. Numerous studies have reported that the cells of the tumor microenvironment not only respond to and support carcinogenesis, but also can actively contribute to tumor initiation, progression, and metastasis. It has been demonstrated that stromal cells can cause transformation of the adjacent cells through transient signaling that, in some cases, leads to the disruption of homeostatic regulation, including control of tissue architecture, adhesion, cell death, and proliferation (Hu & Polyak, 2008).

Fibroblasts in particular are important members of the stromal microenvironment, as they maintain the adjacent epithelia homeostasis through the secretion of growth factors and direct mesenchymal–epithelial cell interactions. It is becoming increasingly clear that fibroblasts are also prominent modifiers of cancer progression. Knowledge of the role of resting and activated fibroblasts in cancer is still evolving. A subpopulation of fibroblasts, the so-called cancer-associated fibroblasts (CAFs), is increasingly recognized as an important promoter of tumor growth and development. CAFs directly stimulate tumor cell proliferation and angiogenesis through paracrine secretion of growth factors, hormones and cytokines in a context-dependent manner (Rasanen & Vaheri, 2010). Many of the CAF-secreted factors acting in isolation are sufficient to induce transformation of epithelial cells, indicative of a tumor-initiating capability of CAFs (Pietras & Ostman, 2010). Moreover CAFs can affect cancer progression by secreting and organizing altered ECM within the tumor stroma (Kalluri & Zeisberg, 2006).

Inflammatory cells can also deeply affect cancer cells behavior. Tumor associated macrophages (TAMs), which represent the major inflammatory component of the stroma of many tumors, are strongly associated with tumor progression and metastasis. In contrast to the reactive macrophages which are characterized by a so-called M1 phenotype, TAMs are alternatively activated and acquire an M2 phenotype, thus promoting tumor growth, angiogenesis, metastasis, as well as suppressing potential anti-tumor immune activities. TAMs affect vascular density and ECM by secreting a vast array of pro-angiogenetic factors

and proteolytic enzymes including VEGF, FGF, PDGF, TNF-α, MMPs, plasmin, urokinase-type plasminogen activator (uPA) and the uPA receptor (uPAR) (Guruvayoorappan, 2008; Allavena et al., 2008). One key role for the microenvironment that has gained prominent recognition is neoangiogenesis. Tumors simply cannot progress without concomitant growth and organization of the stromal endothelial cells (EC) and pericyes. Malformed "leaky" tumor vessels contribute to tumor hypoxia, acidosis, and increased interstitial fluid pressure. The tumor in turn responds with a unique repertoire of gene expression, which in turn acts to alter cell growth, invasion, and ultimately metastasis (Li et al., 2007).

1.4 Tumor angiogenesis and ECM

Investigations into the roles of the tumor stroma have established that the ECM plays an important role in tumor vascularization (Wernert, 1997). Due to the accumulation of several gene mutations, cancer cells have the ability to be self-sufficient in growth signaling, insensitive to antigrowth signals, unresponsive to apoptotic events and capable of limitless replication. Although all of these neoplastic properties are necessary for tumor development, they are not sufficient to become clinically relevant malignancies, unless the tumor is able to recruit its own blood supply. In most tumors new blood vessels are formed through a process called angiogenesis, in which new blood vessels sprout from the preexisting vasculature (Folkman & Klagsbrun, 1987). Following EC proliferation newly formed vessels differentiate into arterioles and venules, necessary to provide blood supply to the growing tumors. In fact, solid tumors will not grow beyond the size of 1-2 mm, unless they induce the formation of new blood vessels to provide the oxygen and the nutrients necessary for their relentless growth and expansion (Campbell et al., 2010). During the different stages of angiogenesis, temporal and spatial regulation of ECM remodeling leads to local changes in net matrix deposition or degradation, which in turn contribute to control of EC growth, migration and differentiation. ECM remodeling is regulated by pro- and anti-angiogenic factors, matrix-degrading proteases and cell-ECM interactions (Sottile, 2004). In this context discovering the roles played by novel ECM proteins in vascularization is a crucial step in the process of creating new treatments for pathological angiogenesis.

1.5 ECM and tumor development

The ECM composition and the stroma's mechanical properties are other important characteristics that profoundly affect tumor cell behavior. The ECM tensile strength or stiffness can regulate cell growth, differentiation and migration, as well as the recruitment of other types of tumor-associated cells. Moreover, the stiffness and the increased interstitial pressure of the tumor stroma influences drug diffusion through the tumor and also the intrinsic drug sensitivity of malignant cells (Augsten et al., 2010).

On the other end, tumor invasion and metastasis are accomplished through ECM breakdown mainly due to matrix metalloproteinases (MMPs) activation. The ECM disruption promotes abnormal inter- and intra- cellular signaling leading to dysregulation of cell proliferation, growth and cytoskeleton reorganization. In turn, the ECM degradation gives rise to an increase of growth factors and ECM-derived molecules that modulate the properties of the different tumor-resident cell types.

An important event in tumor progression is the acquisition by cancer cells of the capability to dissociate from their original site and invade adjacent and distant tissues. Invasion involves the breaching of several barriers, including the basement membrane and the

stromal matrix. Tumor cell invasion and metastasis is a compelling issue of cancer treatment accounting for ~ 90% of cancer related deaths, hence the targeting of tumor invasion is increasingly recognized as a fundamental therapeutic approach. Other key elements of the tumor microenvironment are the single ECM components. The ECM is mainly composed by an intricate interlocking mesh of fibrillar and non-fibrillar collagens, elastic fibers, non-collagenous glycoproteins such as proteoglycans (PGs) and glycosaminoglycans as hyaluronan (HA). Within these structural elements, the stroma is made also of matricellular proteins, a group of extracellular proteins that do not contribute directly to the formation of structural elements but serve to modulate cell-matrix interactions and cell function (Steeg, 2006; Brooks et al., 2010). Tumors often display an altered ECM composition due to the factors produced by tumor and stromal cells. Most solid tumors exhibit a profoundly different ECM-protein profile compared to their normal counterparts. Many of these proteins, though the integrins or other cell surface molecules, affect tumor cells behavior in terms of proliferation, apoptotic rate and migration.

2. Potential therapeutic targets

2.1 Receptor tyrosine kinases

RTKs are specifically activated by several extracellular molecules including growth factors, cytokines, and hormones. Most of the targeted inhibitors under development or already in clinical use are molecules with high affinity for growth factors RTKs, such as the Fibroblast Growth Factor Receptor (FGFR), the Vascular Endothelial Growth Factor Receptor (VEGFR), the Platelet-Derived Growth Factor Receptor (PDGFR), the tyrosine-protein kinase Kit receptor (c-Kit) and the Epidermal Growth Factor Receptor (EGFR). The FGF family contains 22 related peptides able to bind four different FGF receptors (FGFR 1-4). FGFs have been implicated in both physiological and pathological angiogenesis, vasculogenesis and blood vessel remodeling, influencing proliferation and differentiation of different cell types. FGF2 (aka bFGF) is a potent mitogen and chemotactic factor for endothelial and smooth muscle cells and stimulate pericyte formation. It also stimulates the production of the urokinase-type plasminogen activator (uPA) and MMP expression inducing vessel destabilization and ECM breakdown. Receptor dimerization and the presence of heparin or heparan sulfate proteoglycans are necessary for its activation as well as internalization of the ligand to achieve full activation. FGFR1 is the best characterized FGF receptor and it was found to be amplified in 8-10% of breast cancers (Elbauomy et al., 2007). FGFR1 is over-expressed and occasionally amplified also in oral and esophageal squamous carcinomas (Ishizuka et al., 2002)), prostate cancer (Sahadevan et al., 2007), and in a number of bladder carcinomas (Simon et al., 2001). Amplifications have also been observed in ovarian tumors (Gorringe et al., 2007). The EGFR/ERBB family was one of the first to be linked to cancer development and progression. EGFRs regulate cell proliferation, migration and differentiation. Moreover, their signaling is a key driver of the Epithelial-Mesenchimal Transition (EMT) which is a major event involved in the metastatic dissemination of tumor cells (Wilkins-Port & Higgins, 2007). The EGFR family comprises four different receptors, EGFR/HER1 (ERBB1), HER2 (ERBB2), HER3 (ERBB3) and HER4 (ERBB4) and all of them have been implicated in the development of several tumor types. The EGFR downstream signaling cascade includes the Ras-MAP kinase pathway (Herbst, 2004). Over-expression of EGFRs or mutations in their downstream pathways has been linked to poor response to

chemotherapy whereas its correlation with prognosis has not been demonstrated due to contrasting results.

VEGF is an essential growth and survival factor for ECs promoting their survival, proliferation, migration and morphogenesis into functional tubular structures. Moreover, its activity influences vascular permeability (Roskoski, Jr., 2007). The critical role of VEGF in the formation of a new vasculature in growing tumors with the recruitment of circulating ECs and progenitor cells to the sites of neovascularization is well known and plays an important role in tumor progression (Folkman, 1971). In several tumor types including colorectal cancer, VEGF expression has been linked with tumor aggressiveness, poor disease-free and overall survival, distant metastatic spread and decreased response to preoperative radiotherapy (Giatromanolaki et al., 2006; Giralt et al., 2006; Nozue et al., 2001; Zlobec et al., 2005). There are five human VEGF family members and three VEGF receptors. Each VEGF molecule displays a characteristic receptor binding pattern; VEGF-A binds to both VEGFR1 and VEGFR2; VEGF-B and PlGF (Placental Growth Factor) bind to VEGF1, instead VEGF-C and VEGF-D bind to VEGFR3. The activation of VEGFR2 though the binding VEGF-A plays a pivotal role in the regulation of EC function and angiogenesis and for this reason VEGFR2 is one of the most studied receptors of this family. Another important cytokine is the PDGF which is involved in the control of the proliferation, survival and motility of several mesenchymal cell types.

In addition, it is important for the maintenance of the perivascular structures that surround and support tumor blood vessels. For this reason PDGF receptors are under investigation as targets for anti-angiogenic cancer therapy. Two isoforms of the PDGF receptors are known, PDGFRα and PDGFRβ. Both can form homo- or heterodimers and stimulate cell proliferation, but only PDGFRββ and PDGFRαβ dimers can mediate smooth muscle cell and fibroblast chemotaxis. After their activation PDGF receptors activate the Ras/MAPK pathway modulating cell proliferation, migration and differentiation and the PI3K/AKT pathway promoting cell survival. Another member of the PDGFR family is KIT, which was shown to be over-expressed in many tumors, including gastrointestinal stromal tumors (GISTs), mast cell tumors, melanomas, endometrial and ovarian carcinomas and small cell carcinomas of the lung. The binding of stem cell factor to KIT induces receptor dimerization and activation of the intracellular signaling mediated by the PI3K, Ras/MAPK, JAK/STAT pathways to stimulate cell survival (Sattler & Salgia, 2004).

2.2 Apoptotic molecules

The life and death decision at the cellular level is controlled by environmental cues including the presence or absence of growth factors and physical stimuli such as mechanical stress or radiation. Moreover, cells sense their three dimensional location through specific interactions with the ECM as well as with neighboring cells and these contacts are essential for cell survival. In particular, the lack of appropriate cells/ECM interactions leads to a peculiar form of cell death named anoikis (Gilmore, 2005). The life and death decision is also profoundly influenced by the components of the ECM, which can change dynamically during differentiation, development and other tissue-remodeling events. As mentioned above, cells' anchorage is essential for survival (Meredith, Jr. et al., 1993) which is maintained through the activation PI3K Kinase-AKT pathway granted by integrin engagement; should this interaction be disrupted cells undergo apoptosis (Frisch & Screaton, 2001). For example the use of competitors for integrin binding such as Arginin,

Glicine, Aspartate-based (RGD-based) peptides can induce apoptosis by directly activating caspases (Adderley & Fitzgerald, 2000). ECs are highly susceptible to survival anchorage dependent stimuli (Scatena et al., 1998). As mentioned, also the loss of cell-cell contacts leads to the activation of a default death programmed in a variety of cell types. For instance, deficiency or truncation of VE-cadherin which mediates adhesion between ECs (Hermiston & Gordon, 1995) induces ECs' apoptosis and abolishes transmission of the survival signal by VEGF-A to AKT Kinase and Bcl2 (Carmeliet et al., 1999) and this profoundly affects the angiogeneic potential. Given the importance of adhesion in maintaining cell survival, altered expression of glycoproteins in the tumor microenvironment leads to an impairment of cell survival. For example, PGs expression is markedly altered during malignant transformation and tumor progression and is influenced by cancer cells-derived secreted factors and this may either facilitate or counteract the growth of solid tumors. PGs, such as versican, perlecan and small leucine-rich PGs affect cancer cell signaling, growth and survival, as well as cell adhesion, migration and angiogenesis. Furthermore, expression of cell-surface-associated PGs, such as syndecans and glypicans, is also modulated in both tumor and stromal cells. Cell-surface-associated PGs bind various factors that are involved in cell signaling, thereby affecting cell proliferation, adhesion and motility. An important mechanism of action is offered by a proteolytic processing of cell-surface PGs known as ectodomain shedding of syndecans; this facilitates cancer and EC motility, protects matrix proteases and provides a chemotactic gradient of mitogens. However, syndecans on stromal cells may be important for stromal cell/cancer cell interplay and may promote stromal cell proliferation, migration and angiogenesis. The abnormal PG expression by cancer and stromal cells may be exploited to evaluate tumor progression and patient survival (Theocharis et al., 2010). It is known that heparan sulphate proteoglycans (HSPGs), represent an important reservoir of chemochines, cytochines and enzymes (Bishop et al., 2007; Sasisekharan et al., 2002). Heparanase, can cleave HS side chains from HSPGs and release a multitude of bioactive molecules including bFGF and Hepatocyte Growth Factor (HGF) (Ogasawara et al., 1996). bFGF enhances EC and tumor cell proliferation and this was demonstrated to contribute to hepatocellular carcinoma progression (Pang & Poon, 2006). Heparanase expression is closely related with apoptosis of several tumors cells, including HCC (Ikeguchi et al., 2003b; Ginath et al., 2001) and there is a significant positive correlation between the mRNA expression levels of heparanase and the percentages of apoptotic hepatocytes in liver tissues (Ikeguchi et al., 2003a). Besides affecting EC proliferation bFGF is also responsible for a direct effect on tumor cells protecting them from apoptosis. Cell surface HSPGs act as co-receptors for formation of high-affinity bFGF-receptor complexes, and in this context the HS chains play a leading role (Chua et al., 2004). In this context, the heparanase-induced alteration of cell surface HSPGs, might down-regulate bFGF pro-survival effects resulting in tumor cell apoptosis. In addition, there is also a group of ECM molecules that have been shown to directly impair cell viability. Among these proteins CCN1, a secreted ECM-associated heparin binding protein, can either induce or suppress apoptosis: it can promote EC survival but also induce fibroblast apoptosis mediated by integrin $\alpha_6\beta_1$ and the heparan sulfate proteoglycan sydecan 4. The mechanism involves the activation of Bax which leads to cytochrome-c release and activation of caspase-9 and -3 activation (Todorovicc et al., 2005).

Another ECM protein playing, among other functions in tumor development, an active role in the control of apoptosis is the Secreted Protein Acidic and Rich in Cystein (SPARC).

SPARC is an important regulator of cell growth and malignancy and enhances apoptosis in various tumors including ovarian cancer, where its absence significantly diminished cell death. The mechanism is still elusive but involves the activation of caspase-8, the initiator caspase of the extrinsic apoptotic pathway independently of death receptor activation and leads to downstream involvement of Bid. Interestingly, this effect results from the interaction between SPARC and the N-terminus of the procaspase-8 DED-containing domain (Tang & Tai, 2007). The secreted small leucine-rich proteoglycan decorin has also been shown to inhibit cancer growth by impairing EGFR function and triggering apoptosis through caspase-3 activation (Seidler et al., 2006).

EMILIN2, another ECM glycoprotein, adopts a peculiar mechanism in affecting tumor development and apoptosis. Elastin Microfibril Interface-Located proteINs (EMILINs) constitute a family of ECM glycoproteins characterized by the presence of an EMI domain at the N-terminus and a gC1q domain at the C-terminus (Doliana et al., 2000; Mongiat et al., 2000). Recent studies show that this molecule affects tumor cell survival leaving normal cells unharmed; the mechanism involves the direct activation of the death receptors and in particular DR4. The activation of the extrinsic apoptotic pathway leads to a dramatic decrease of tumor cell viability associated with DNA fragmentation and caspase-8 and caspase-3 activation. More recently a discrete sequence of this molecule was found to be involved in these anti-tumorigenic effects (Mongiat et al., 2007; Mongiat et al., 2010).

2.3 Cells of the microenvironment

Cancer associated fibroblasts (CAFs) Several studies have demonstrated that normal fibroblasts contribute to maintaining epithelial homeostasis by suppressing proliferation and oncogenic potential of adjacent epithelia. Once they are recruited to the tumor, most of them switch to a permanently activated phenotype, and become CAFs. The phenotype of CAFs resembles that of normal fibroblasts during wound healing where they are activated and generate ECM components and pro-inflammatory cytokines. After the wound is healed, the normal phenotype is restored; on the contrary, in the tumor microenvironment their status remains permanently activated and for this reason tumors are frequently termed as "wounds that do not heal". By creating an altered microenvironment through the overexpression of cytokines such as TGFβ, PDGF, FGF and connective tissue growth factors, a growing tumor can co-opt fibroblasts and turn them into CAFs (Erez et al., 2010).

CAFs are not only target cells whose activity is modulated by environmental factors, but also active cells that significantly contribute to tumorigenesis and metastasis. Abundant growth-promoting factors have been found in the conditioned media of CAFs isolated from metastatic colon cancer patients, compared to the conditioned media taken from normal skin fibroblasts (Xing et al., 2010). In addition, CAFs are able to induce epithelial cells transformation following injection of normal human breast epithelial cells mixed with irradiated or not-irradiated fibroblasts in immunocompromised mice (Kuperwasser et al., 2004). The tumorigenic potential of CAFs depends also on the production of the mutagenic reactive oxygen species (ROS) under conditions of pH and hypoxia that characterize the tumor microenvironment. CAFs are known to produce Insulin-like Growth Factor-1 and -2 (IGF-1 and -2), thus promoting tumor growth providing survival signals allowing tumor cells to evade programmed cell death. CAFs are also supporters of tumor angiogenesis being an important source of VEGF (Li et al., 2007). Finally, CAFs produce high levels of the Stomal cell-Derived Factor 1α (SDF-1α), the ligand of C-X-C chemokine Receptor type 4

(CXCR4), which is highly expressed by cancer cells. Once activated, CXCR4 can promote mammary epithelial proliferation, migration and invasion and angiogenesis (Orimo et al., 2005).

Another important role of CAFs is their active participation in ECM remodeling during tumor development. The increase production of fibronectin, collagens, proteoglicans and glicosaminoglicans promote survival stimuli from integrin engagement and impair apoptosis induced by chemotherapeutic drugs. Another event affecting the drugs uptake is the increased interstitial fluid pressure. The deposition of a dense ECM network leads to fibroblasts' proliferation which may acquire a contractile phenotype to become myofibrobrast. This results in a constant increase in tension within the tumor connective network and increase of interstitial fluid pressure leading to an impaired uptake of therapeutic agents (Bouzin & Feron, 2007).

It has been demonstrated that in some cases increased fibronectin expression correlates with tumorigenicity amplifing integrin survival signaling (Han et al., 2004; Marastoni et al., 2008). Collagen I also contributes to the decrease of the uptake and regulates the sensitivity of tumor cells to these drugs.

The ECM remodeling by MMPs is one of the most crucial steps for cancer progression where the equilibrium between MMPs and their inhibitors is completely disrupted. It has been shown that CAFs secrete various matrix-degrading proteases as well as their activators such as uPA, thus promoting tumor invasion. The breakage of the basement membrane allows the epithelial cancer cells to intravasate into the circulation system and also in this context the cross-talk between the cancer cells and CAFs seems to be crucial.

Tumor associated macrophages (TAMs) and inflammatory cells. It is known that tumors are generally infiltrated by immune cells with which the host tries to contrast tumor progression, a process known as immune surveillance. Several studies report a correlation between the inflammatory infiltrates and an improved prognosis or a better lifespan for patients. However, some evidences contradict this paradigm identifying the presence of immune cells as a negative prognostic factor particularly for breast, prostate, ovarian and cervical cancers (Whiteside, 2007). The association between a chronic inflammation and cancer was first formulated by Dr. Virchow in 1863. This concept has been recently reconsidered when specific inflammatory cell types and in particular TAMs, the major inflammatory component of the stroma of many tumors, have been associated to neoplastic transformation (Houghton, 2010). In less than 10% of the cases, the presence of TAMs is associated with good prognosis. A possible explanation to this apparent paradox is that tumor cells are able to recruit immune cells and, similarly to what happens with CAFs, to activate their status. The cytokine profile in the tumor microenvironment is considered extremely important in determining the phenotype of the local inflammatory cells (Pollard, 2004).

Compared to reactive macrophages, TAMs are alternatively activated, acquiring an M2 phenotype, thus promoting tumor growth, angiogenesis, metastasis and suppressing potential anti-tumor immune activities, a phenomenon known as immunoediting.

A central event determining TAMs activity is NF-kB activation. In response to different pro-inflammatory signals TAMs display defective NF-kB activation that causes impaired expression of cytotoxic mediators, such as Nitric Oxide (NO), Tumor Necrosis Factor α (TNFα), and Interleukin 1 and 12 (IL1 and IL12), (Mantovani et al., 2006). In a normal tissue, pathogenic infection or a wound healing triggers the local expression of an array of inflammatory cytokines and growth factors, such as Colony Stimulating Factor 1 (CSF-1),

Granulocyte–Macrophage CSF (GM-CSF), Macrophage-Stimulating Protein (MSP) and Transforming Growth Factor β1 (TGF-β1). These factors, together with the products of tissue breakdown, recruit circulating monocytes which are stimulated to differentiate into macrophages. Macrophages, in turn, mediate immune responses, kill pathogens, stimulate angiogenesis and participate in tissue repair (Pollard, 2004). This physiological phenomenon is uncontrolled in tumors given the persistent stimuli from both tumor cells and CAFs. CSF-1, the main growth factor responsible for survival, proliferation, differentiation and chemotaxis of macrophages, is over-expressed in different types of cancers. In addition tumor-derived anti-inflammatory molecules such as IL4, IL10, TGFβ-1, and prostaglandin E2 are essential for TAMs' function and this finding suggested that exposure to IL4 and IL10 may induce monocytes to develop into polarized M2 macrophages (Mantovani et al., 2002).

TAM infiltration positively correlates with tumor cell proliferation in breast, endometrial and renal cell carcinoma. In fact, various studies have shown that these cells express a number of factors that stimulate tumor cell proliferation and survival, including EGF, PDGF, TGF-β1, HGF, and bFGF. TAMs are also important supporters of tumor angiogenesis being a source of VEGF; TAMs cluster in avascular areas, recruited by chemokines that are secreted in response to hypoxia. This condition up-regulates hypoxia-inducible factor-2α (HIF-2α) which, in turn, induces VEGF expression. VEGF expression by TAMs is induced by CSF-1 and the cytokine in a positive feedback loop recruits other macrophages thus increasing the rate of vascularization (Lewis & Pollard, 2006). TAMs also produce other pro-angiogenetic factors and proteolitic enzymes including IL8, FGF, PDGF, TNF-α, MMP-2, MMP-7, MMP-9, MMP-12, and cyclooxygenase 2 (COX-2) (Lewis & Pollard, 2006). TAMs enhance the invasive capacity of cancer cells (Lewis & Pollard, 2006). This effect may depend on the expression of MMPs and uPA and its receptor uPAR which enhances the motility and invasion of cancer cells (Guruvayoorappan, 2008).

Neutrophils. Once recruited to the tumor microenvironment, neutrophils have also been demonstrated to change their phenotype and promote tumor progression. Various transgenic mice models and clinical data show that the presence of tumor-associated neutrophils often correlates with poor prognosis, given their ability to stimulate tumor growth, angiogenesis and metastasis. IL8, which is frequently over-expressed by tumor and stromal cells, is a strong chemokine for neutrophils, which express the IL8 receptors CXCR-1 and -2. Once inside the tumors, neutrophils can secrete factors such as HGF and oncostatin M which induces tumors cells to enhance the VEGF expression and increases their invasiveness. IL8 also stimulates neutrophil degranulation with the release of MMP9 (Gregory & Houghton, 2011; Murdoch et al., 2008).

Neutrophil elastase (NE) is an important protease released by neutrophils. Recent data show that it promotes tumor growth via an alteration of tumor cell signaling that ultimately leads to PI3K hyperactivity (Gregory & Houghton, 2011; Houghton, 2010). Reactive Oxygen Species (ROS) produced by neutrophils as a host defense against microorganisms could act as pro-tumorigenic agents. As CAFs and TAMs, neutrophils can also facilitate tumor cells metastasis. It is likely that the neutrophils phenotypic switch, which is defined N1-N2 polarization similarly to the M1-M2 polarization of macrophages, is mediated by the key cytokine TGFβ (Fridlender et al., 2009).

Tumor ECs and pericytes. Vessels are a requisite for tumor growth and also are necessary for tumors to metastasize. Thus, the ability to recruit vessels is an important and growth limiting step for tumors. The release of pro-angiogenetic factors in the tumor microenvironment results in the recruitment and enhanced proliferation of ECs. Members of

VEGF family and their receptors are considered the major key mediators of angiogenesis. The binding of VEGF to VEGFR triggers an intracellular signaling that is mainly mediated by MAPK and PI3K/Akt/mTOR pathways. This results in the expression of HIF-1α and induction of PDGF, FGF, G-CSF, TGFβ and angiopoietins, thus enhancing angiogenesis (Boehm et al., 2010).

Another important cellular component of the blood vessels are pericytes whose recruitment is highly dependent on PDGFβ-R expression as well as on the production of PDGFβ ligand by ECs (Pietras & Ostman, 2010). Tumor pericytes are rarely dispersed and loosely along the vessels compared to their normal counterparts and their role in tumor development is not yet well defined. Some studies have demonstrated that increased coverage of pericytes enhances tumor growth, by promoting neo-vascularization. In contrast, other cancer models show a pericyte-mediated angiostatic effect, thus the outcome of pericyte-derived signals appears to be highly context dependent (Pietras & Ostman, 2010).

2.4 ECM proteins and tumor angiogenesis

The network of fibrous proteins and glycosaminoglycans (GAGs) of the ECM affects tumor vessel formation both in direct and indirect way. The GAGs carbohydrate polymers of the proteoglycans are involved in both keeping the ECM and surrounding cells hydrated and trapping and storing growth factors. Therefore, GAG molecules may exert a variety of regulatory effects on the accessibility of angiogenic factors (Rouet et al., 2005). Heparan Sulfate (HS) GAGs include the syndecans, glypicans, perlecans and agrins. HSGAGs that are present on the EC surface have the ability to either inhibit or promote neovascularization by mediating signalling through VEGF receptors (Iozzo & San Antonio, 2001) or bFGF (Walker et al., 1994).

Most of the ECM proteins, such as fibronectin, collagen and laminin, mediate angiogenesis through integrin-binding RGD motifs. In a resting quiescent state ECs exhibit the lowest cell mitotic index of all the cells of the body (Folkman, 2006). Induction of angiogenesis and ECM remodeling is characterized by an increased proliferation and permeability as well as cytoskeletal and cell-to-cell contact changes which results in newly formed focal contacts primarily mediated by integrins. Fibronectin is produced by both activated ECs and smooth muscle cells (SMC), and its levels are augmented during angiogenesis as fibronectin leaks from the blood due to the increased vascular permeability. The RGD domain of fibronectin binds to the integrin $\alpha_5\beta_1$, which is markedly up-regulated during angiogenesis and is over-expressed in ECs within the tumors. The collagen integrin receptors $\alpha_1\beta_1$ and $\alpha_2\beta_1$ also play a positive role during angiogenesis. Moreover, α_1 chain laminin peptides mediate angiogenesis *in vitro* (Malinda et al., 1999). As previously mentioned, the proteolytic activity triggered during angiogenesis facilitates degradation of the basement membrane, matrix remodeling and cell migration and invasion which are essential for the development of novel blood vessels, hence this process is tightly regulated. There are two major classes of enzymes: the PA/plasmin system and MMPs. There are at least 20 members of the MMP family most of them being implicated in tumorigenesis and a large number of these proteases degrade the vascular basement membrane and matrix in order to allow vascular sprouting (Pepper, 2001). The activity of these proteases is regulated by endogenous tissue inhibitors of metalloproteinases (TIMPs) which generally display anti-angiogenic properties. By degrading the matrix, MMPs not only provide physical space within the matrix for cell migration, but also provide ECs with proliferation and differentiation signals due to the release of cryptic sites present on ECM molecules and the release of soluble growth factors.

Various MMPs cleave heparin bound growth factors such as VEGF and bFGF, thus stimulating the formation of new blood vessels (Lee et al., 2005; Hawinkels et al., 2008). Stromal recruitment of fibroblasts and immune cells such as macrophages can also affect angiogenesis as analyzed above. Recently it was found that loss of PTEN signaling in stromal fibroblasts results in the induction of ECM remodeling through the increase of the transcriptional factor Ets2 which is an upstream target of MMP-9 (Trimboli et al., 2009). Protease-mediated cleavage of the ECM also results in the release of cryptic domains that regulate angiogenesis. Cleavage of collagen XVIII and the α1, α2 and α3 chains of collagen IV release the angiogenic inhibitors endostatin, arrestin, canstatin and tumstatin, respectively (Kalluri, 2003). For this reason MMP inhibitors have an impact on tumor angiogenesis. For instance, TIMP-1 over-expression suppresses tumorigenesis, in part due to its effects on the tumor vasculature. Treatment of ECs with TIMP-1 alters the formation of tubules on Matrigel while proliferation is not affected. TIMP-2 decreases EC' proliferation and inhibits angiogenesis *in vivo* (Seo et al., 2003). TIMP-3 has also been shown to decrease angiogenesis, particularly through EC disaggregation and impairment of Matrigel tubulogenesis (Chetty et al., 2008).

Another group of ECM proteins which have been recently linked to tumor angiogenesis are the membrane-associated disintegrin and metalloproteinases (ADAMs), including those carrying Thrombospondin Motifs (ADAMTs) which are instead secreted in the microenvironment (Dunn et al., 2006). These proteins regulate angiogenesis directly or through expression of MMPs. For instance, ADAM-17 alters EC' morphology of decreases cell proliferation. ADAMTS1 and ADAMTS8 contain the thrombospondin antiangiogenic domain (TSR1) and inhibit EC proliferation and suppress growth factor induced vascularization (Vazquez et al., 1999).

The Bone Morphogenic Proteins (BMPs) have also gained recognition for their potential role in tumor angiogenesis. BMPs belong to the TGFβ superfamily of proteins and were found to influence angiogenesis by stimulating the secretion of proangiogenic growth factors such as VEGF (Deckers et al., 2002). BMP expression correlates with increased EC migration and tubulogenesis on Matrigel (Rothhammer et al., 2007). The role of BMPs in angiogenesis is controversial and some reports demonstrate an anti-angiogenic effect. Further understanding of the role played by BMPs in the ECM and tumor angiogenesis will benefit therapeutic studies which target angiogenesis, tumour growth, and metastatic spread of disease.

2.5 ECM proteins involved in invasion and metastasis

The main cause of cancer related deaths is the metastatic disease, which is due to the widespread dissemination is often impossible to successfully eradicate. Metastasis results from a complex molecular cascade comprising many steps, all of which are interconnected through a series of adhesive interactions and invasive processes as well as responses to chemotactic stimuli, through which cancer cells leave the site of the primary tumor mass and disseminate to distant anatomic sites where they proliferate and form secondary tumor foci. To complete this process tumor cells must invade the tissue surrounding the primary tumor, enter either the lymphatics or the bloodstream, survive and eventually arrest in the circulation, extravasate into a tissue and grow in the new site. A crucial element in the metastatic process is the ECM whose composition, structure and biophysical forces exerted may influence cell invasion and migration.

ECM and invasion. Most solid tumors exhibit a distinctive ECM-protein profile, compared to the normal counterparts and many of these proteins interact directly with tumor cells influencing their migration and invasion ability. A number of proteins, such as tenascin-C (TNC), fibronectin, and SPARC are consistently up-regulated in tumors (Allen & Louise, 2011). TNC is a multifunctional protein expressed at low levels in normal adult tissues but over-expressed in conjunction with cell migration occurring for instance during embryogenesis, wound healing or cancer development. TNC is highly expressed in a variety of invasive and metastasizing cancers, including breast, colon, lung and brain (Guttery et al., 2010). Multiple alternatively-spliced isoforms of TNC are known and seem to be tumor-specific. For example, the full length protein is expressed in pancreatic and prostate cancers, while in breast and ovarian carcinomas the domain A and D containing isoforms are the most abundant. Besides its composition, also the tensile strength of ECM can regulate epithelial cell growth, transformation and migration; the tumor-associated stroma is usually more stiff than the normal tissue, and this peculiar characteristic of the tumor microenvironment may influence tumor progression (Tilghman et al., 2010). For example, the reduction of ECM stiffness can inhibit the malignancy of transformed mammary epithelial cells (Allen & Louise, 2011). One way in which tensile strength can be modulated is the cross-linking of collagens and elastin due to lysil oxidase activity (LOX). Studies conducted using mouse models of breast cancer allowed to correlate the high LOX expression, increased collagen cross-linking and elevated ECM stiffness with the invasive progression of the disease. The role of LOX has been well established in colorectal (Baker et al., 2011) and breast (Erler & Giaccia, 2006) cancer progression, in this last case it was demonstrated that hypoxic primary tumor cells increase LOX expression and secretion, enabling cell movement to more oxygenated and thus nutrient-rich areas. LOX increases cell invasion and migration through regulation of cell-matrix adhesion and focal adhesion kinase (FAK) activity. LOX may be involved in the cell-matrix adhesion interactions required for intravasation and extravasation and, finally, is required for the formation of a mature ECM at the secondary site, allowing survival signaling and possibly bone marrow derived cells recruitment.

The fibrillar collagens surrounding tumors are often remodeled and this is associated with the metastatic progression. The linearization and reorientation of collagen fibers at the invasive front of cancer cells is a classic example of malignant transformation and metastatic dissemination (Cox & Erler, 2011).

In most human tumors there is abundant accumulation of HA. A large number of studies performed during the last three decades have demonstrated a close correlation between malignancy and hyaluronan-rich ECM, as well as with the expression of some specific variants of its CD44 receptor (Misra et al., 2011). The CD44 present at the surface of cancer cells interacts with HA-rich microenvironments, thus affecting cell signaling pathways and enabling malignant cells to migrate, invade basal membranes and ECM and to lodge at distant sites from the primary tumor. The HA content in the tumor stroma impacts on the overall outcome of the disease and correlates with tumor grade and poor prognosis. HA, HA synthetases, hyaluronidases and HA receptors are involved in a wild range of carcinomas, as breast, lung, skin, and gastrointestinal (Sironen et al., 2011).

Cell-cell and cell-ECM interactions. Invasion, which characterizes the metastatic process, consists of changes in tumor cell adherence to the other cells and to the ECM and is accompanied by the proteolytic degradation of the surrounding tissue and by the motility that propels tumor cells through the tissue.

Loss of cell-cell adhesion within the primary tumor mass permits disaggregation of tumor cells and hence aids the initial dissemination. Compared with normal epithelial cells, cancer cells show diminished cell-cell adhesiveness, due to the alteration of cadherin/catenin expression. The process leading from a polarized epithelial non-motile phenotype to a more motile and non-polarized phenotype is called epithelial to mesenchymal transition (EMT). This event is associated with the cadherin switch consisting in the loss of E-cadherin expression, normally present on the epithelial cell surface, and the gain of N-cadherin expression which associates with a metastatic phenotype. The loss of E-cadherin expression has been shown in several types of epithelial cancers including breast, gastrointestinal, lung, stomach, prostate, and kidney (Hazan et al., 2004).

The interaction between tumor cells and the stroma is mostly mediated by integrins, which not only mediate cell-ECM adhesion, but also play a role in intracellular signaling stimulating events as proteolysis and angiogenesis (Brooks et al., 2010). Integrins are emerging as important factors in metastatic progression and their expression levels correlate with pathological outcomes, for example colon, breast and prostate cancers often display altered integrin expression which can vary considerably between normal and tumor tissues (Desgrosellier & Cheresh, 2010). Most notably, integrins $\alpha_V\beta_3$, $\alpha_5\beta_1$ and $\alpha_V\beta_6$, are expressed at low levels in most adult epithelia but can be highly up-regulated in tumors. On the other end, $\alpha_2\beta_1$, is down-regulated in cancer leading to an increase of tumor cell dissemination; when this integrin is re-expressed in breast cancer cells, the phenotype is reverted, suggesting that it behaves as a tumor-suppressor integrin.

Proteases. The engagement of integrins and other adhesion molecules is accompanied by the recruitment of proteases which degrade the ECM and help providing a pathway for invasion. During the metastatic dissemination of tumor cells, the degradation of components of the ECM is achieved through the action of several hydrolytic enzymes which are released either by the tumor cells themselves or by cells surrounding the tumor. The enzymes play a role during metastasis include MMPs, cathepsines, uPA and heparanases. All these molecules once exogenously over-expressed increase cell invasion (Brooks et al., 2010). The proteolityc activity of these enzymes facilitates the migration of tumor cells in different ways; physical barriers of dense matrix are removed, latent proteases, growth factors and chemotactic agents are often activated, integrin-binding sites within the ECM molecules are exposed and transmit migratory and survival signals, finally bioactive ECM fragments are generated as, for example, from collagen IV and laminin-5 (Geho et al., 2005).

The expression and role of the different MMPs is quite diverse during cancer progression. They can promote or inhibit cancer development depending on the tumor stage, tumor site, enzyme localization and substrate profile. For example, MMP-9 functions as a tumor promoter during carcinogenesis but at later stages of the disease and under specific conditions it can also function as an anticancer enzyme (Gialeli et al., 2011).

Another important regulator of tumor invasion is the uPA system consisting of the specific protease uPA and its cellular receptor uPAR. The enzyme is released by tumor or tumor-associated cells, it binds to the receptor leading to plasmin activation. This mechanism results in the amplification of ECM proteolysis by both the degradative ability of plasmin and the initiation of an activation cascade involving MMPs. Elevated levels of uPA have been reported in many types of cancer, and anti-uPAR targeting therapy is currently being attempted (Brooks et al., 2010).

The pre-metastatic niche. Once tumor cells reach the secondary site, they undergo apoptosis, lie dormant or proliferate to form the secondary tumor. Recent evidences suggest that distant sites, called pre-metastatic niches, may be pre-conditioned to become a fertile ground for the establishment of metastasis (Coghlin & Murray, 2010). Hematopoietic cells migrate through the tissues and cause the remodeling of the ECM and growth factors milieu. Such altered microenvironment can attract tumor cells and support their proliferation (Brooks et al., 2010). Elevated fibronectin expression by resident fibroblasts at the pre-metastatic site represents a critical factor in the development of the pre-metastatic niche. The key tumor-secreted factors that determine the formation of this niche have yet to be identified, although TNF, TGF-β and VEGF are likely to be involved (Erler et al., 2009). ECM remodeling enzymes like MMPs and LOX seem to play a role also in the pre-metastatic niche formation. For example, the primary tumor-driven VEGFR-1 activation is required for the secretion of MMP-9 by the ECs and macrophages, thus deeply affecting the metastatic environment of the lungs. This is thought to make the lung microenvironment more hospitable for the subsequent invasion and survival of metastatic cells. The activity of tumor-secreted LOX is essential for the recruitment of bone marrow derived cells, because of ECM remodeling and MMPs' activation induced by collagen cross-linking. Exactly what determines the site of the pre-metastatic niche remains elusive, although some recent studies have proposed that they might occur in sites exposed to frequent mechanical strain (Cox & Erler, 2011).

3. Therapeutic strategies

3.1 Receptor tyrosine kinase inhibition

Given the critical role that RTKs play in signal transduction profoundly affecting tumor progression, several molecules to block the aberrant activation of these pathways have been developed (Zwick et al., 2002). Due to the high level of complexity of these pathways, investigators have attempted to interfere at different levels of the signaling cascade, from the extracellular ligands, to the membrane receptors, to the intracellular non-receptor tyrosine kinases (Fig.2). Several modalities are currently employed to inhibit kinase activity such as small inhibitors, molecular antibodies, but also peptide mimetics and antisense oligonucleotides (Dancey & Sausville, 2003; Imai & Takaoka, 2006).

Small molecule tyrosine kinase inhibitors - Tyrosin kinases are present in an active or inactive form. Active kinases are characterized by high structural homology whereas the structures of the inactive forms are dissimilar. This characteristic suggests that molecules able to recognize and bind to the inactive form of the enzyme, could present higher efficacy because of lower cross-reactivity and increased specificity. Despite the fact that more specific inhibitors allow to precisely control cellular activity, several studies have demonstrated the importance of inhibiting multiple critical nodes in order to increase treatment response. Most cancers in fact are characterized by multiple aberrant signaling pathways and thus may require inhibition of a number of signaling endpoints for an optimal response. In addition, the ability of multi-targeted drugs to inhibit different kinases can represent an advantage in case of the development of drug resistance. In some occasions cells treated with a selective kinase inhibitor activate alternative pathways to maintain their activity. Hence, a chemical compound able to simultaneously inhibit several kinases bares the potential to be less prone to drug resistance. On the other hand, the use of multitargeted inhibitors is usually accompanied by worse side effects and limits on the maximum dose

that can be used for treatment, thus limiting the real benefits. Most of the small molecule kinase inhibitors currently in preclinical development and in clinical use are ATP-competitive inhibitors. These molecules are characterized by a high affinity for the kinase ATP-binding sites and their interaction with these sites prevents ATP binding and therefore kinase activation. One of the limitations of this kind of inhibitors is represented by the susceptibility to mutations that enhance target kinase activity, which is common in several types of cancer. In addition to ATP-competitive inhibitors, several non-competitive inhibitors have also been developed. These molecules bind to an allosteric site distant from the ATP-binding site and induce a conformational change of the receptor thus preventing the ATP binding. These kinds of inhibitors are active independently on the ATP binding site sequence, thus maintaining their efficacy also in the presence of mutant proteins.

Monoclonal antibodies - An alternative approach to impair the RTKs' activation is represented by the use of blocking monoclonal antibodies (mAbs) (Hinoda et al., 2004). These antibodies act directly preventing the ligand-receptor interaction and therefore inhibiting ligand-mediated intracellular signaling and increasing RTK down-regulation and internalization. In addition, mAbs act also indirectly by stimulating the immune response. Following their binding to the cell surface receptors the subsequent activation of the immune system causes antibody-dependent cellular cytotoxicity through the activation of macrophages or natural killer cells. Finally, recent studies demonstrated that some monoclonal antibodies are able to influence tumor growth by modulating the receptor signaling activity and therefore altering the intracellular signaling inducing apoptosis and/or growth inhibition.

Compared to the small molecule inhibitors, mAbs are more expensive to produce and, due their large molecular weight, the tissue penetration, tumor retention and blood clearance is less efficient especially in some tissues such as the brain. Moreover, because of their inability to penetrate through the cellular membrane, they can only be used to target cell surface molecules and not intracellular proteins. Despite these limitations, in clinical trials the mAbs have encountered higher approval success rates compared to new drugs, including small-molecule agents (Reichert et al., 2005). It must be pointed out that the activation of the immune system is likely crucial to efficiently eliminate tumor cells and for this reason, the use of small molecule inhibitors in combination with mAbs should maximize the therapeutic effects.

RTKs as Antiangiogenic targets - The prevention of angiogenesis is a potentially successful mean to impair tumor growth and progression. In contrast to standard chemotherapy, inhibition of angiogenesis generally does not lead to tumor regression but induces long-term stable disease creating a favorable window of opportunity for radiotherapy or chemotherapy to exert stronger tumor-killing effects. Growth factors such as VEGF-A, FGF2, IL8 and PDGF play an important role in promoting blood vessels formation and several antiangiogenic molecules have been developed to target either growth factors or their receptors.

The use of Bevacizumab (Avastin®), a humanized mAb able to specifically bind to VEGF-A and blocking its interaction with VEGFR2, in combination with 5-fluorouracil-based chemotherapy was approved for the first-line treatment of metastatic colorectal cancer (CRC) by FDA in February 2004 (Ferrara et al., 2005). Sunitinib (Sutent®) is an orally active small molecule inhibitor of VEGFR2, PDGFRβ, KIT and FLT3 (Mendel et al., 2003; Abrams et al., 2003) and is used in clinical trials for patients with advanced solid malignancies (Motzer et al., 2006; Motzer & Bukowski, 2006).

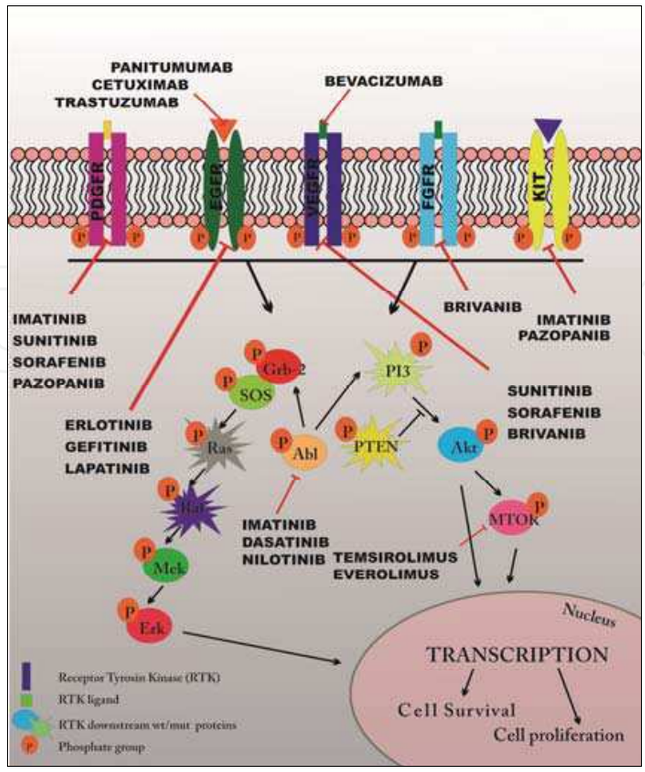

Fig. 2. Schematic representation of the therapeutic strategies targeting receptor tyrosine kinases; PDGFR: Platelet-derived growth factor Receptor; EGFR: Epidermal Growth Factor Receptor; VEGFR: Vascular Endothelial Growth Factor Receptor; FGFR: Fibroblast Growth Factor Receptor; KIT: c-Kit Receptor aka CD117.

Sorafenib (Nexavar®) is a multi-kinase small molecule inhibitor able to block CRAF, BRAF, VEGFR2, PDGFRβ, KIT and FLT3; is used in clinical trials as monotherapy or in combination with standard therapy (Wilhelm et al., 2006; Liu et al., 2006). Brivanib, a dual tyrosine kinase inhibitor of VEGFR and FGFR signaling, was active that in phase I clinical trials against metastatic solid tumors refractory to standard therapy (Park et al., 2011).
Imatinib mesilate (Gleevec®) binds to the inactive form of the BCR-ABL fusion protein inhibiting its activity and is used for the treatment of chronic myeloid leukemia. It also functions as a multi-target inhibitor of KIT and PDGFRβ. Due to this specificity it is also used in clinical trials for the treatment of metastatic GISTs and glioblastoma.
Other anti-VEGF therapeutic approaches are under pre-clinical and clinical trials (Roskoski, Jr., 2007; Kowanetz & Ferrara, 2006).
Anti-EGFR therapy - EGFR receptor was one of the first tyrosine kinase to be linked to tumor development and the dysregulation of one or more components of its pathway are a common trait of several tumor types. For this reason many molecules have been developed to try to inhibit the EGFR aberrant activity at different levels. Many of the mAbs and small molecules directly target the EGF receptor and some are currently used in clinical practice.
Gefitinib (Iressa®) was the first EGFR small molecule inhibitor to enter clinical trials (Schiller, 2003). In combination with standard therapy its activity has been evaluated in several tumor types such as non-small cell lung cancer and squamous cell carcinoma of the head and neck although no improvement in overall survival and response rate has been reported yet.

Cetuximab (Erbitux®), a human-mouse chimeric antibody able to bind and inhibit EGF receptor is used for the treatment of metastatic colorectal cancer (Goldberg, 2005). A higher response rate to the therapy was observed in combination with irinotecan, oxaliplatin or radiotherapy in different tumor types, highlighting the importance of acting on different cellular activities to inhibit tumor growth.
Panitumumab (Vectibix®) is another anti-EGFR antibody (Giusti et al., 2007) that has shown efficacy in chemotherapy refractory colorectal cancers and is being used in clinical trials for the treatment of esophageal, head and neck and lung tumors. Trastuzumab (Herceptin®) is a mAb approved in 1998 for the treatment of HER2-positive breast cancer patients in combination with paclitaxel and doxorubicin. This molecule is able to bind to the extracellular domain of ERBB2 preventing receptor dimerization and the subsequent signaling cascade.
Most of the failures with the use of some of these drugs may be due to a wrong selection of the patients. Indeed the utility of a personalized therapy derived from a patient selection based on tumor molecular profile has been demonstrated for several of these targeted therapeutics, including cetuximab and trastuzumab. The presence of a specific genomic and/or proteomic profile in fact correlates with the response to the therapy. Finally, other molecules that target multiple members of the EGF receptor family are currently under investigation (Reid et al., 2007) and some of them such as XL647 and AEE788 have shown to be active *in vitro* and are in different phases of clinical trials.
Considering the complexity of the mechanisms regulating tumor progression, the most effective approach in impairing tumor development is to simultaneously target different pathways. The increasing knowledge about the crosstalk between cancer cells and tumor microenvironment will allow the development of new drugs in order to block different players of the signaling cascades. The goal is that with the use of therapies involving complementary and synergistic combinations will increase drug efficacy and reduce toxicity as well as the risk of development of resistance to the treatment.

3.2 Apoptotic molecules

The ability of tumor cells to evade apoptosis is one of the hallmarks of cancer progression and can play a significant role in their resistance to conventional therapeutic regimens. To overcome resistance to apoptosis through the development of therapeutic drugs tackling the single components of the pathway is one of the efforts that is being attempted to potentiate the anti-tumoral therapy (Reed & Pellecchia, 2005). Researchers have also focused the attention on different agents able to indirectly modulate apoptosis (Fig. 3). Histone deacetylase (HDAC) inhibitors, for example, can reduce transcription of anti-apoptotic molecules of the Bcl2 family and are currently under investigation in clinical trials. Small-molecule drugs and mAbs directed against various RTKs analyzed in the previous section also affect this pathway and induce tumor cells to become more sensitive to apoptosis. Personalized treatments can be achieved given that the tumor-specific genetic lesions that affect sensitivity or resistance to apoptosis are well known (Zivny et al., 2010). In addition, different therapeutic strategies have been hypothesized or are currently under evaluation taking into account all the microenvironment molecules found to affect apoptosis. For instance, the *in vitro* anti-proliferative effect of exogenous SPARC could be exploited therapeutically by administering recombinant SPARC or one of its derivate peptides to patients. Pre-clinical studies are encouraging and demonstrate that subcutaneously

administered SPARC can inhibit the growth of neuroblastoma xenografts in mice (Chlenski et al., 2002). Moreover, exogenous SPARC can reverse the acquired resistance to chemotherapeutic agents (Tai et al., 2005). As previously explained, integrins are involved in anchorage independent growth, anoikis resistance and metastasis formation, which are all characteristics of malignant tumor cells. For this reason small molecule antagonists able to block the integrin binding to the ECM molecules are currently under evaluation. As an example RGD-mimetic peptides have been extensively used in pre-clinical studies (Eble & Haier, 2006). Cilengitide, a cyclic peptidic antagonist of $\alpha_v\beta_3$ and $\alpha_v\beta_5$, to block also RGD-independent effects and to achieve a more efficacious anti-tumoral effect, for example (Paolillo et al., 2009). Furthermore, thanks to the capacity of the HS side chains of HSPGs to bind a multitude of growth factors, chemochines, cytochines and enzymes, neutralizing antibodies or antisense oligonucleotides have been demonstrated to enhance apoptosis in a number of tumor cells and may represent promising therapeutic approaches (Fukumoto et al., 2000). The abnormal PG expression in cancer and stromal cells may serve as a biomarker for tumor progression and patient survival. In this context, the better understanding of role of PGs in cancer progression may represent a novel approach in directly targeting the tumor microenvironment (Theocharis et al., 2010).

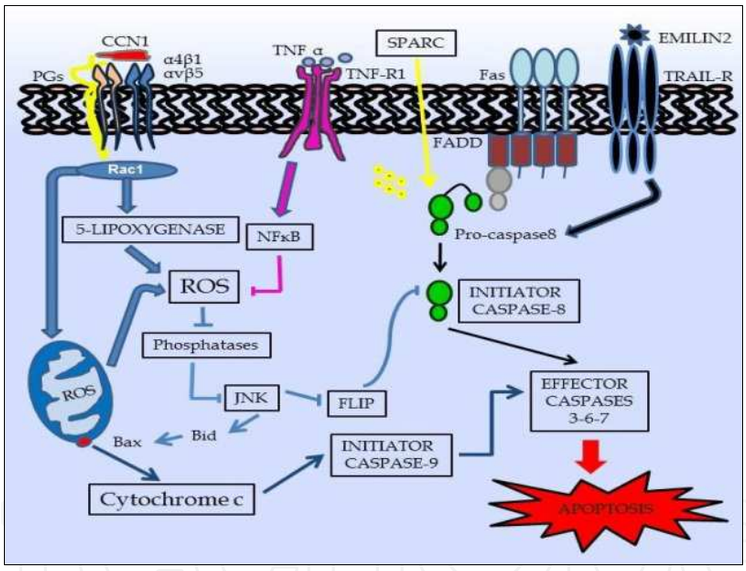

Fig. 3. Schematic representation of the therapeutic strategies targeting apoptosis. PGs: Proteoglycans; $\alpha_4\beta_1$ and $\alpha_v\beta_5$: integrin receptors; CCN1, SPARC and EMILIN2: extracellular matrix molecules; TNF α: Tumor Necrosis Factor α; TNF-R1: Tumor Necrosis Factor Receptor 1; Fas and TRAIL-R (TNF-Related Apoptosis Inducing Ligand Receptor) death receptors; FADD (Fas-Associated protein with Death Domain): adapter molecule of the extrinsic apoptotic pathway; ROS: Reactive Oxygen Species; FLIP: FLICE-like inhibitory protein.

Finally, also the ECM molecule EMILIN2 may represent a good target in this context and increasing its levels at the tumor site may suggest a therapeutic use since EMILIN2 exerts pro-apoptotic effects for tumor cells enhancing the extrinsic apoptotic pathway but leaves normal cells unharmed. This hypothesis is well supported by pre-clinical studies (Mongiat et al., 2007; Mongiat et al., 2010).

3.3 Cells of the microenvironment

Targeting CAFs - CAFs are the primary cells within the tumor stroma that determine the tumor dynamics. Despite their frequent genomic instability, they are more sensitive to chemotherapeutic drugs than tumor cells and thus are considered potential innovative therapeutic targets (Fig. 4). Many epithelial tumor cells secrete PDGF but lack PDGFR that is expressed by CAFs and its activation is frequently associated with metastases in colon carcinomas (Pietras et al., 2001; Bouzin & Feron, 2007; Haubeiss et al., 2010). In a recent study, the activity of four different FDA approved PDGF inhibitors such as Dasatinib, Imatinib, Nilotinib and Sorafenib has been compared and all were found to reduce CAFs' viability. Dasatinib resulted the most efficacious and inhibited not only CAFs' viability, but also induced a quiescent state and partially reverting their phenotype to normal. In addition, the incubation of cancer cells with conditioned media from CAFs pre-incubated with Dasatinib resulted in a significant reduction of tumor cell proliferation (Haubeiss et al., 2010). PDGF inhibitors also reduced the contractility of myofibroblasts in the tumor, thereby reducing interstitial fluid pressure and increasing chemotherapeutic drugs uptake. Encouraging results in inhibiting PDGFβ-R were also obtained using CDP860, a Fab' fragment-polyethylene glycol conjugate (Bouzin & Feron, 2007).

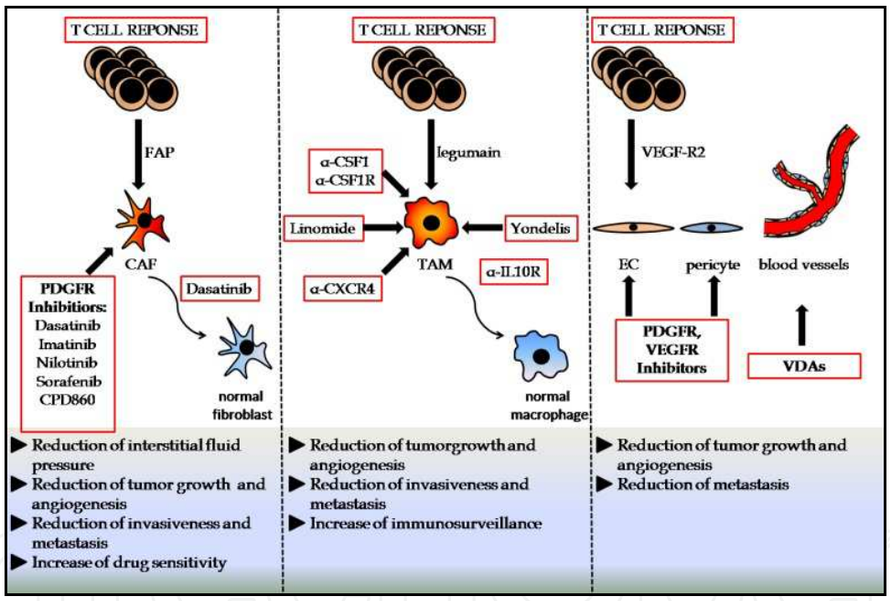

Fig. 4. Schematic representation of the therapeutic strategies targeting tumor associated cells FAP: Fibroblast Activating Protein; CAF: Cancer Associated Fibroblasts; PDGFR: Platelet-Derived Growth Factor Receptor; α-CSF1 and α-CSF1R: antibodies against the Colony Stimulating Factor 1 and its receptor; α-CXCR4: antibodies against the C-X-C chemockine receptor type 4; α-IL10R: antibodies against the Interleukin 10 Receptor ; VEGFR2: Vascular Endothelial Growth Factor 2; VDAs: Vascular Disrupting Agents.

Another potential therapeutic target is the Fibroblast Activating Protein (FAP), a serine protease actively implicated in ECM remodeling and over-expressed in different tumors. Several studies underline its involvement in immunosuppression, promotion of tumor growth and increased metastatic potential (Bouzin & Feron, 2007; Loeffler et al., 2006; Kraman et al., 2010). Apart from a FAP-antibody an oral DNA vaccine against FAP is also

under evaluation. Through a specific CD8+ T cell-mediated killing of CAFs, this vaccine successfully affected both primary tumor growth and metastases of multidrug-resistant murine colon and breast carcinomas. Accordingly, FAP vaccinated mice display a reduction of intra-tumoral collagen I and thus an increased doxorubicin uptake leading in many cases to tumor rejection and increased lifespan (Loeffler et al., 2006).

Targeting TAMs - Possible therapeutic strategies targeting TAMs are aimed at reducing the number and the function of these cells and/or increasing their antitumor activity. Different groups reported that TAM are committed to produce high levels of the inhibitory cytokine IL10, which mediates defective IL12 production and NF-kB activation in TAMs, an event which is very important in determining TAMs activity and that is frequently correlated with poor prognosis. It has been demonstrated that the employment of anti-IL10 receptor antibodies and Toll-like receptor 9 ligand CpG can restore NF-kB activity and revert the macrophages phenotype from M2 to M1 status, thus activating an innate response against tumor (Guiducci et al., 2005).

Another way to reduce the number of TAMs is to target molecules that are involved in the recruitment of macrophages. In this perspective, the employment of antibodies against chemokines or chemokines receptors such as CFS1/CSF-1R could have important therapeutic effects.

Given the important role of TAMs in supporting angiogenesis, antiangiogenic drugs also represent a therapeutic tool against TAMs. Tumor infiltrating macrophages are a significant source of VEGF that act as a loop by recruiting other macrophages. Thus targeting this growth factor could reduce the number of TAMs in the tumor stroma. Linomide is an interesting antiangiogenic drug that is effective in the reduction of tumor growth in a murine prostate cancer model. Its activity includes the inhibition of macrophages recruitment and the increase of antiangiogenic molecules in TAMs such as IL12, IL18, CXCL10 and CXCL9. Hypoxia induces HIF, and hence VEGF expression in TAMs and HIF could represent another interesting target. Hypoxia signaling also leads to an increase in CXCR4 expression both on TAMs and ECs and CXCR4 antibodies such as AMD3100 inhibit tumor angiogenesis *in vivo* (Mantovani et al., 2004; Mantovani et al., 2006).

Anti-tumor agents with selective cytotoxic activity on monocyte-macrophages would be ideal therapeutic tools for their combined action on tumor cells and TAMs. Yondelis (Trabectedin), a natural product derived from the marine organism *Ecteinascidia turbinate* displays a potent anti-tumor activity and is specifically cytotoxic to macrophages and TAMs, while sparing the lymphocyte sub-set (Sessa et al., 2005). In addition, Yondelis inhibits the production of CCL2 and IL6 by TAMs which in turn contribute to growth suppression of inflammation-associated human tumors (Allavena et al., 2005).

A legumain-based vaccine represents another strategy aimed at inducing the host to contrast the infiltration of TAMs in the tumor. In fact, legumain, a member of the endopeptidase family, is highly over-expressed by TAMs in murine and human breast tumor tissues, and hence provides an ideal strategy for breast tumor targeting (Lewen et al., 2008; Xiang et al., 2008). The vaccination of mice against legumain provides a robust CD8+ T cell response against TAMs, significantly reducing tumor growth. Moreover, a dramatic reduction in TGFβ, TNFα, MMP9 and VEGF expression is induced, with a consequent reduction of both angiogenesis and metastatic rate (Luo et al., 2006).

Targeting ECs and pericytes - Besides targeting pro-angiogenetic growth factors and cytokines as mentioned above it is also possible to specifically target ECs and pericytes. The selective disruption of tumor vessels through the employment of the so called vascular disrupting

agents (VDAs) is a potentially useful alternative strategy. These compounds that predominantly affect the tumor periphery and small tumor masses have a different mechanism of action and tolerability profile compared with conventional antiangiogenic drugs and thus may provide an additional clinical benefit for a wide patient population. VDAs act to disrupt the established tumor vasculature in order to create an extensive necrosis in the tumor core. On the basis of their mechanism of action, VDAs are divided into 2 groups. The first includes the tubulin-binding agents that selectively target tumor ECs by disrupting their cytoskeleton. The second VDAs group includes compounds related to flavone acetic acid and they act in two ways; they induce ECs apoptosis and indirectly increase the intra-tumoral concentrations of TNFα and other cytokines, as well as nitric oxide, leading to an inhibition of tumor flow (Boehm et al., 2010; McKeage & Baguley, 2010).

3.4 ECM and angiogenesis

As previously mentioned, anti-angiogenic therapy represent a promising strategy for cancer therapy and offers the chance to avoid resistance to chemotherapeutic treatments. J. Folkman's long-standing vision of angiogenesis as a therapeutic target has been increasingly validated in both traditional transplant tumor models and genetically engineered mouse models of cancer (Szentirmai et al., 2008). Important targets in this context are ECM molecules (Fig. 5) which can exert both pro-angiogenic or anti-angiogenic effects (Tabruyn & Griffioen, 2007).

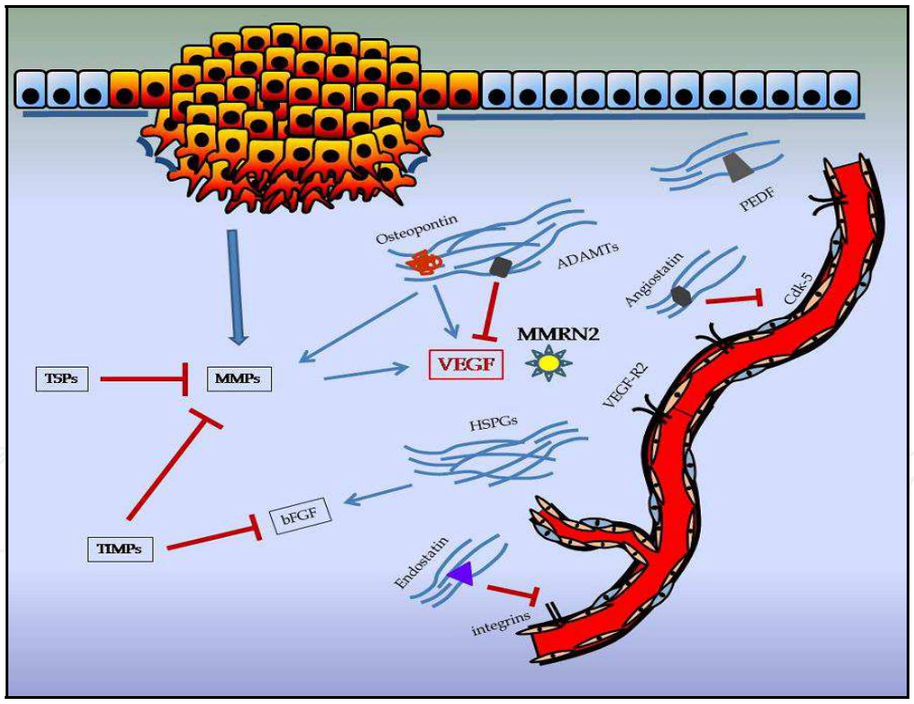

Fig. 5. Schematic representation of the therapeutic strategies targeting angiogenesis hinging on ECM molecules; TSPs: thrombospondins; MMPs: metalloproteinases; bFGF: basic Fibroblast Growth Factor; HSPGs: Heparan Sulfate Proteoglycans; VEGF and VEGFR2: Vascular Endothelial Growth Factor and one of its receptors; MMRN2: MULTIMERIN2; ADAMTS: A Disintegrin And Metalloproteinase with Thrombospondin Motifs; PEDF: Pigment Epithelial-Derived Factor; cdk-5: cyclin dependent kinase 5.

Pro-angiogenic ECM proteins - Many ECM proteins surrounding the vasculature including collagens, laminins, and fibronectins are pro-angiogenic promoting EC survival, proliferation, migration and tube formation. Pro-angiogenic factors such as VEGF, bFGF and TGF-β are bound and sequestered by the ECM via the heparan-like glycosaminoglycans. Fibronectin, a fairly ubiquitous and abundant ECM protein that becomes assembled into fibrils at the cell surface, is necessary for vasculogenesis, in fact if this gene is knocked out mice die before birth due among others, to vascular bed defects. An alternatively-spliced form of fibronectin that contains an extra B domain has been found in fetal and neoplastic tissues but not in normal adult tissues; this isoform was found to be synthesized by the vascular cells in malignant astrocytoma (Castellani et al., 2002) and its expression appears to be a precise diagnostic marker of the highest grade of glioma or glioblastoma. Also other studies demonstrate that this isoform may be a good marker of angiogenesis (Santimaria et al., 2003) and could also be a therapeutic target.

Osteopontin, a secreted cell attachment protein, seems to be over-expressed in association with increased tumor angiogenesis (Hirama et al., 2003). The proangiogenic function of osteopontin can be attributed to its ability to promote VEGF-directed dermal microvascular EC migration (Senger et al., 1996) and to increase MMP-2 levels in an RGD-dependent manner (Teti et al., 1998). Additionally, osteopontin-bound integrin $\alpha_v\beta_3$ inhibits NF-kB-dependent EC apoptosis (Cooper et al., 2002).

Tenascin-C is a glycoprotein composed of six subunits covalently associated by disulfide bonds. Different human Tenascin-C isoforms are generated by alternative splicing with aberrantly regulation in neoplastic tissues (Jones & Jones, 2000). It is expressed around angiogenic vessels in many tumors and there is evidence that it promotes and regulates angiogenesis *in vitro* and *in vivo*. Indeed the antibodies directed against Tenascin-C in glioma patients induced a significant inhibition of tumor angiogenesis.

HSPGs are necessary for stable binding of the proangiogenic growth factor bFGF to its receptor (Ornitz et al., 1992) and alterations in HS glycosamminoglycan in breast carcinoma have been shown to result in an increase in bFGF binding and receptor complex assembly.

Perlecan deposited along blood vessels basement membrane is thought to mediate structural and functional interactions with different molecules (Iozzo, 2005; Mongiat et al., 2003a). Intact perlecan is thought to be a pro-angiogenic HSPG as: *i)* its expression is altered during embryonic vasculogenesis and in neoplasia (Tapanadechopone et al., 2001; Zhou et al., 2004) perlecan-null mice show severe and sometimes fatal vascular and chondrogenic defects (Costell et al., 1999); *iii)* antisense targeting of perlecan blocks tumor growth and angiogenesis *in vivo* (Sharma et al., 1998); and *iv)* increased perlecan expression stimulates angiogenesis (Jiang & Couchman, 2003). Tumor cell secreted perlecan is thought to promote EC sprouting and proliferation, thereby promoting angiogenesis (Jiang et al., 2004). Interestingly a degradation product of perlecan (endorepellin) exerts an opposite effect (Mongiat et al., 2003b).

Transmembrane chondroitin sulphate proteoglycan NG2 is another protein involved in tumor angiogenesis and has been shown to promote EC spreading perycite function (Ozerdem & Stallcup, 2003). An additional proangiogenic mechanism by which NG2 promotes angiogenesis includes its ability to bind and sequester angiostatin, thus blocking its antiangiogenic function (Chekenya et al., 2002). All these molecules may represent important tools for the development of drugs able to counteract blood vessel formation.

Anti-angiogenic ECM proteins - Among the molecules that counteract blood vessel formation thrombospondin-1 (TSP-1) and thrombospondin-2 (TSP-2) are the best studied and their

expression in tumor tissues is often inversely correlated with angiogenesis (Adams, 2001; Adams & Lawler, 2004; Armstrong & Bornstein, 2003). The mechanisms by which they can inhibit angiogenesis include: *i)* the induction of EC apoptosis by the binding of TSP-1, and potentially TSP-2, to CD36, thereby inducing Fas ligand expression (Jimenez et al., 2000; Nor et al., 2000); *ii)* the clearance of MMP-2 following lysosomal degradation of the TSP-2/MMP-2 complex (Armstrong et al., 2002; Rodriguez-Manzaneque et al., 2001) and *iii)* inhibition of EC proliferation (Armstrong et al., 2002).

Another extensively studied molecule is angiostatin, the cleavage product of plasminogen, the only not ECM antiangiogenic protein among the molecules described above. Angiostatin was shown to inhibit EC proliferation and motility and to down-regulate the protein level of cyclin-dependent kinase 5 (cdk5), a cdk absent in quiescent EC and induced by bFGF (Sharma et al., 2004). Angiostatin also acts by upregulating the mRNA levels of FasL and reducing the level of c-Flip which activates the extrinsic apoptotic pathway.

Other endogenous angiogenesis inhibitors include cryptic fragments from ECM molecules such as endostatin, a specific and potent anti-angiogenic factor generated from the proteolytic cleavage of collagen XVIII by matrix metalloproteinases, elastases or cathepsins (Kim et al., 2009). Endostatin inhibits EC proliferation and migration and causes EC G1 arrest and apoptotic cell death (Dhanabal et al., 1999). These properties have provided clues for development of an anti-tumor strategy with proven therapeutic effectiveness in numerous models of neoplasia.

Pigment epithelial-derived factor (PEDF) was initially identified as an antiangiogenic protein. Its high expression has been linked to decreased microvessel density and suppression of tumor growth in a number of tumors including glioma as well as prostate carcinoma and melanoma (Abe et al., 2004). In support of a role for PEDF in inhibiting angiogenesis, increased microvessel density is found in PEDF deficient mouse tissues (Doll et al., 2003), in addition PEDF inhibits cornea neovascularisation. The mechanism whereby PEDF acts to inhibit angiogenesis likely resides on its ability to bind collagen and to potentially interfere with the adhesion of cells to the collagen (Meyer et al., 2002) as well as triggering Fas up-regulation and down-regulation of the VEGF mRNA levels (Takenaka et al., 2005).

MULTIMERIN2 (MMRN2), also known as EndoGlyx-1, is an ECM glycoprotein exclusively expressed in the blood vessel in close proximity with the endothelium. In neoplastic tissues, MMRN2 is consistently found to be deposited along tumor capillaries and, in certain tumors, in the "hot spots" of neoangiogenesis (Belien et al., 1999). This protein is characterized by a short cluster of charged amino acids (10 out of 27 residues) located between the coiled-coil region and the C1q-like domain. The basic amino acids are arranged in a sequence similar to that of the consensus motifs responsible for the ionic interactions with glucosaminoglycans, such as heparin and heparan sulfate (Hileman et al., 1998) and are also found in heparin binding proteins such as the von Willebrand factor (Sobel et al., 1992). Given its deposition in tight contact with the EC surface, we have hypothesized the involvement of this protein in the regulation of angiogenesis and demonstrated that MMRN2 functions as a homeostatic molecule for ECs preventing their sprouting to form new vessels (unpublished results). This strong anti-angiogenic effect is reflected in a potent *in vivo* anti-tumor activity effect following treatment with MMRN2. For these reasons we are confident that this molecule represents a promising tool for the development of novel approaches to impair angiogenesis and tumor growth.

3.5 ECM molecules involved in invasion and metastasis.

The targeting of ECM components and ECM-remodeling enzymes that would prevent changes of the stroma homeostasis promoting cancer progression has received much attention and is becoming an increasingly attractive therapeutic approach for preventing cancer progression and metastasis (Fig. 6).

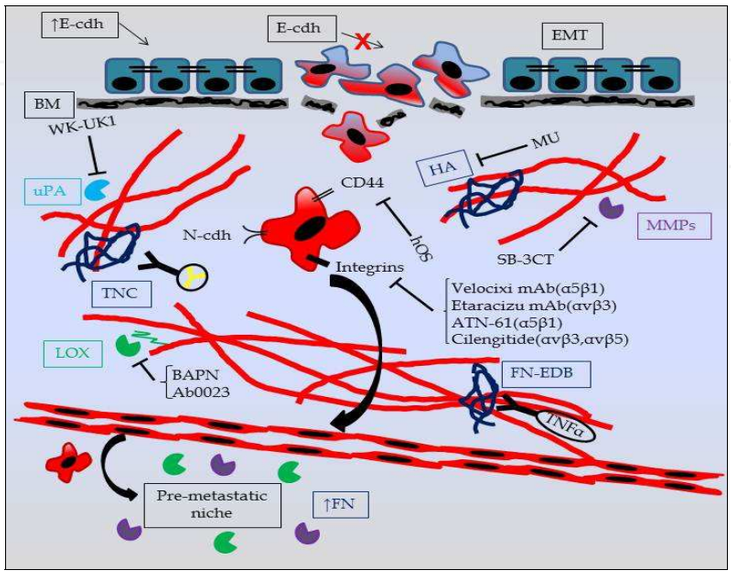

Fig. 6. Schematic representation of the therapeutic strategies targeting the metastatic processes. E-cdh: E-cadherin; EMT: Epithelial-Mesenchymal Transition; BM: Basement Membrane; WK-UT1: uPA (urokinase-type Plasminogen Activator) inhibitor; TNC: Tenascin-c; LOX: lysil oxidase activity; BAPN: β-aminoproprionitrile LOX inhibitor; Ab0023 inhibitory monoclonal antibody specific for the LOX family member LOXL2; N-cdh: N-cadherin; hOS: hyaluronan oligosaccharides; HA: Hyaluronan; MU: HA syntetase inhibitor 4-methylumbelliferone; MMPs: metalloproteinases; SB-3CT: MMP-2 and -9 inhibitor; FN: fibronectin; FN-EDB: oncofetal form of fibronectin; TNFα: Tumor Necrosis Factor α.

ECM components - The peculiar expression of tumor-associated ECM components may offer the opportunity to develop new strategies for cancer treatment, either targeting their function associated to tumor progression or by exploiting their specific localization to delivery bioactive molecules to the tumors. The ECM components are highly abundant in tumors and are often more stable than cell surface antigens. One example of this type of approach is offered by tenascin-C (TNC), which exerts different effects both on tumor cells and also on the many cell types within the tumor, thus affecting cancer progression. The effects on tumor and stromal cells are exerted very early in tumorigenesis and persist during tumor progression. Currently, the most promising approach is to target TNC with a specific mAb (81C6) coupled to radioactive molecules. This strategy has been successful in patients with recurrent primary and metastatic brain tumors, and is currently in clinical trials (Xing et al., 2010). A similar approach targeting the oncofetal form of fibronectin (FN-EDB) containing an extra-domain, and often up-regulated in tumors was particularly successful. Anti-FN-EDB antibodies show specific localization to a range of tumors, including brain, lung and colorectal cancers. This antibody has been used as a vehicle for TNFα and has been shown to induce necrosis in tumors (Allen & Louise, 2011).

Cell-cell and cell-ECM interactions - Integrin-targeted therapeutics have recently been proved beneficial in delivering chemotherapeutics, oncolytic viruses, proapoptotic peptides and redionucleotides to both tumor cells and the supporting vasculature. Recent studies showed that delivery of targeted nanoparticles loaded with doxorubicin to integrin $\alpha_V\beta_3$-positive tumor vasculature inhibited metastases while eliminating the toxicity and patients' weight loss associated with the systemic administration of this drug (Desgrosellier & Cheresh, 2010).

HA contributes to cancer progression in many types of carcinomas and it is up-regulated in tumor stroma; its activated CD44 receptor is over-expressed in solid tumors unlike the non-tumorigenic counterparts and this characteristic may also be exploited to develop new therapies. The membrane receptor CD44 can internalize HA, thus, HA-carrying drugs have the potential to be used as targeted drugs. HA–drug conjugates are internalized via CD44, and the drug is released and activated mainly by intracellular enzymatic hydrolysis. Several preclinical studies have shown that HA chemically conjugated to cytotoxic agents improves the anticancer properties of the agent in vitro (Sironen et al., 2011). Manipulation of HA synthesis alters the tumorigenicity of malignant tumors, including breast cancer. Recently, the HA syntetase inhibitor 4-methylumbelliferone (MU), which has been reported to inhibit HA synthesis dose-dependently in several cell types, was shown to exert anti-tumor effects inhibiting *in vitro* cell proliferation, migration and invasion (Urakawa et al., 2011). MU suppresses intra-osseous tumor growth, suggesting its potential as a therapeutic candidate for established breast cancer-derived bone metastasis. Additive effects of MU in combination with trastuzumab for the treatment of trastuzumab-resistant breast cancers have also been reported both in *in vitro* and *in vivo* experiments.

The expression of integrins by various cell types involved in tumor progression and their ability to crosstalk with growth factor receptors has made them appealing therapeutic targets. Integrin inhibitors have been extensively studied as anti-cancer agents because integrins play a dual function: they are involved in invasion of tumor cells out of the primary tumor site to the metastatic sites, but they also regulate ECs migration into the tumor mass during angiogenesis (Veiseh et al., 2011). Velociximab, a chimeric mAb that inhibits integrin $\alpha_5\beta_1$, has been used in clinical phase II trials for renal cell carcinoma, metastatic melanoma and pancreatic cancer. The rationale of using velociximab in cancer therapy is based on the fact that this specific integrin is expressed by endothelial cells and up-regulated in tumor vasculature (Jarvelainen et al., 2009). The use of etaracizumab, a blocking antibody for $\alpha_V\beta_3$ integrin, directly affects not only cancer cell growth and angiogenesis, but also osteoclast attachment, suggesting a possible efficacy in reducing bone metastasis (Desgrosellier & Cheresh, 2010).

Another strategy is based on the development of small-molecules compounds or peptide mimetics in order to block the integrin signaling pathways activated by ECM remodeling. A successful example of this strategy is cilengitide, a cyclic RGD peptide that inhibits integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$, that has been proven promising in lung and prostate cancer patients and for the treatment of glioblastoma (Allen & Louise, 2011). ATN-61 is a non-RGD-based peptide inhibitor of integrin $\alpha_5\beta_1$ blocking breast cancer growth and metastasis and, in combination with fluorouracil, significantly reduces liver metastasis in mouse models of colon cancer (Desgrosellier & Cheresh, 2010). Targeting other types of ECM receptors has also been attempted. For example the administration of hyaluronan oligosaccharides (hOS), which interfere with the CD44-hyaluronan interaction are effective inhibitors of the progression of several tumor types, including mammary and lung carcinoma.

Likewise, increasing the cadherins expression could inhibit cell invasion by strengthening cell-cell adhesions, thus preventing the cell from escaping the primary tumor. Indeed, forcing the overexpression of cadherins has been the focus of some anti-invasion therapy studies (Junxia et al., 2010).

Modification enzymes – The inhibition of LOX activity reduces primary tumor growth and mechanotransduction in the mammary epithelium. LOX inhibition prevents the invasion of tumor cells *in vivo* and abrogates bone marrow derived cells recruitment and the establishment of metastasis. It also destabilizes already-formed metastases reducing their growth and improves patients' survival (Cox & Erler, 2011). LOX inhibitors are currently in development for clinical use. For example, a small-molecule lysyl oxidase inhibitor called β-aminoproprionitrile (BAPN) is available for clinical studies and it prevents the invasion of metastatic breast cancer and melanoma cell lines *in vitro* (Baker et al., 2011). More recently the efficacy of BAPN was outperformed by the use of an inhibitory mAb (AB0023) specific for LOXL2, a member of the LOX family, that was efficacious in both primary and metastatic xenograft models of cancer, as well as in liver and lung fibrosis models (Barry-Hamilton et al., 2010).

Proteases - On the basis of the pivotal roles that MMPs play in several steps of cancer progression, many efforts have been spent with the aim of developing safe and effective agents targeting MMPs. Several generations of MMPs inhibitors have been tested in phase II clinical trials, including peptidomimetics, non-peptidomimetics and tetracycline derivates (Gialeli et al., 2011). Unfortunately so far the results have been disappointing; all the clinical trials involving the use of MMPs inhibitors have failed to show significant efficacy and to increase the rates of patients' survival (Cox & Erler, 2011). The poor success of this approach largely depended on the toxicity of the compounds for normal tissues, to the conflicting roles of MMPs in both promoting and reducing metastatic dissemination, and to the functional redundancy between the family members. Nevertheless, the ongoing challenge is the development of a new generation of highly specific MMPs inhibitors, as for example SB-3CT that binds to the active site of MMP-2 and -9 and re-establishes the pro-enzyme structure. These new compounds aim at targeting the enzymes involved in ECM remodeling avoiding unwanted side effects. To increase the specificity of MMPs inhibitors, the future of drug development comprises the use of molecules targeting specific exo-sites, specific binding sites outside the active domain of the metalloprotease involved in substrate selection (Gialeli et al., 2011).

Finally, also the uPA system (uPAS), due its involvement in many steps of cancer progression, including angiogenesis, cell proliferation, invasion and growth at the metastatic site, represents a suitable source for the development of new anti-cancer drugs. Several therapeutical approaches aimed at inhibiting the uPA/uPAR functions lead to anti-tumor effects in xenograft models, including selective inhibitors of uPA activity such as WK-UK1, antagonist peptides, mAbs able to prevent uPA/uPAR binding and gene therapy techniques aimed at silencing uPA/uPAR expression. However, although promising, all these strategies need definitive confirmation in humans as, up to now, only few uPA inhibitors have been used in clinical trial (Ulisse et al., 2009).

4. Conclusions

The cells and molecules of the tumor microenvironment hold the potential for the development of innovative therapeutic approaches. Many of the molecules and approaches

that we have taken into account in this chapter are currently in clinical trials, while some have only been suggested based on preliminary results and require further investigations. To avoid problems of toxicity and resistance to the treatments the future of cancer therapy is likely to involve the use of combinatorial treatments that will include conventional chemotherapeutic agents at lower doses and novel molecules able to halt tumor development with little or no toxicity for the patient.

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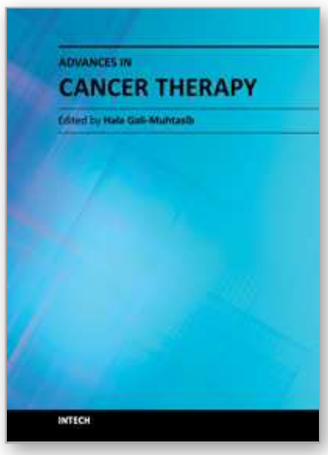

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The book "Advances in Cancer Therapy" is a new addition to the Intech collection of books and aims at providing scientists and clinicians with a comprehensive overview of the state of current knowledge and latest research findings in the area of cancer therapy. For this purpose research articles, clinical investigations and review papers that are thought to improve the readers' understanding of cancer therapy developments and/or to keep them up to date with the most recent advances in this field have been included in this book. With cancer being one of the most serious diseases of our times, I am confident that this book will meet the patients', physicians' and researchers' needs.

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