Title: Biomaterials to suppress cancer stem cells and disrupt their tumoral niche

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Funding:

Highlights:

- Cancer recurrence is attributed to the presence of a tumoral stem cell population located in a specific tumoral niche.
- Cancer treatment should address both cancer stem cells and the niche that supports and protects them.
- Advanced drug delivery systems provide clear benefits for the design of therapeutic systems targeted against cancer stem cells and their niche.

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Biomaterials to suppress cancer stem cells and disrupt their tumoral niche

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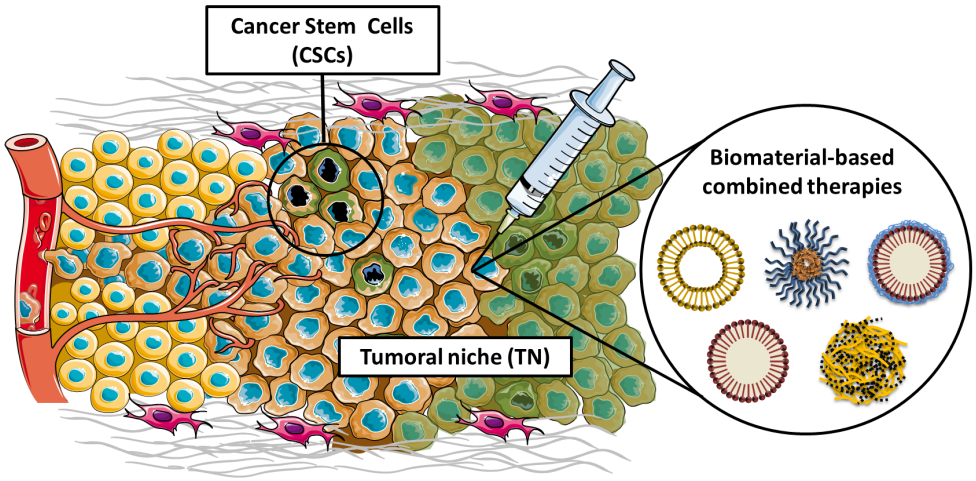
GRAPHICAL ABSTRACT



ABREVIATIONS

Aldehyde dehydrogenase (ALDH), acute promyelocytic leukemia (APL), all trans retinoic acid (ATRA), basic fibroblast growth factor (b-FGF), bone morphogenetic protein (BMP), cancer stem cells (CSC), extracellular matrix (ECM), epidermal growth factor receptor (EGFR), Enhanced Permeation and Retention (EPR), granulocyte macrophage colony stimulating factor (GM-CSF), interferon (IFN), interleukins (IL), hypoxia inducible factor (HIF), N-(2-hydroxypropyl)methacrylamide (HPMA), lysyl oxidase (LOX), lipopolysaccharide (LPS), mesenchymal stem cells (MSCs), poly[α-(4-aminobutyl)-L-glycolic acid] (PAGA), poly(ε-caprolactone) (PCL), pigment epithelium-derived factor (PEDF), poly(ethylene glycol) (PEG), polyethyleneimine (PEI), poly-glutamic acid (PGA), poly-lactic acid (PLA), poly(lactic-co-glycolic acid) (PLGA), poly(N-methyldietheneamine sebacate) (PMDS), polyvinyl pyrrolidone (PVP), reactive oxygen species (ROS), tumor associated-macrophages (TAM), transforming growth factor-*beta* (TGF-*beta*), toll-like receptor (TLR), tumor initiating cells (TICs), tumor niche (TN), tumor necrosis factor-alpha (TNF-α), tyrosine related protein 2 (TRP2), vascular endothelial growth factor (VEGF), Von Hippel−Lindau (VHL).

ABSTRACT

Lack of improvement in the treatment options of several types of cancer can largely be attributed to the presence of a subpopulation of cancer cells with stem cell signatures and to the tumoral niche that supports and protects these cells. This review analyses the main strategies that specifically modulate or suppress cancer stem cells (CSCs) and the tumoral niche (TN), focusing on the role of biomaterials (i.e. implants, nanomedicines, etc.) in these therapies. In the case of CSCs, we discuss differentiation therapies and the disruption of critical signaling networks. For the TN, we analyze diverse strategies to modulate tumor hypervascularization and hypoxia, tumor extracellular matrix, and the inflammatory and tumor immunosuppressive environment. Due to their capacity to control drug disposition and integrate diverse functionalities, biomaterial-based therapies can provide important benefits in these strategies. We illustrate this by providing case studies where biomaterial-based therapies either show CSC suppression and TN disruption or improved delivery of major modulators of these features. Finally, we discuss the future of these technologies in the framework of these emerging therapeutic concepts.

KEYWORDS: cancer stem cells; tumor initiating cells; tumor niche; biomaterials; tissue engineering; drug delivery; nanomedicine

Introduction

Conventional cancer treatment is based on two premises: first, that cancer cells are a homogeneous population that displays a distinct phenotype as compared to healthy cells, and that medicines can take advantage of these differences to eliminate the disease (Clevers, 2011). The second premise is that the tumoral niche is a clinically advantageous feature, at least for nanomedicine-based therapies, since its enhances permeability to macromolecules and nanocarriers and promotes their accumulation in the tumor (Schätzlein, 2006). Nowadays, there is growing evidence demonstrating that these two premises are incorrect or at least incomplete.

Tumor cell heterogeneity is now a widely accepted feature of cancer and can be discussed at the genetic and developmental levels, being both of these tightly connected. At the genetic level, tumor cells present intrinsic genetic variability, which results in several cancer subclones that evolve following Darwinian processes in an attempt to adapt towards the environment. This process leads to an enrichment of cells presenting advantageous mutations and more aggressive phenotype (Greaves and Maley, 2012). At the developmental level, it has been confirmed that tumor initiation and relapse is driven by a selected tumor cell subpopulation that has high resistance towards conventional therapies and that takes advantage of stem cell-specific features (Farrar, 2009)(Marotta and Polyak, 2009). Antitumorals are designed to target rapidly cycling cells such as those

from the tumor bulk, but will spare the quiescent (but deadly) cancer stem cells (CSCs) that will generate tumor relapse and metastasis (Clevers, 2011).

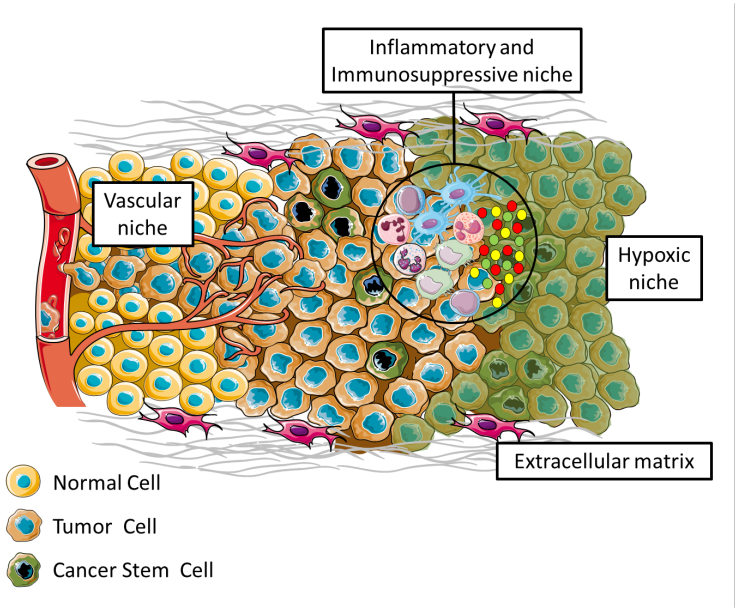


Figure 1. General overview of the organization of cancer stem cells (CSCs) and their tumor niche (TN). CSCs dwell in complex colonies together with differentiated cancer cells and other non-tumoral cell types. The tumoral niche presents several features that are critical for CSC physiology and relevant for the design of new therapies. These are: (i) a disorganized and hypertrophic vascular niche, (ii) a highly dynamic, remodeled extracellular matrix, (iii) the formation of hypoxic regions and (iv) the generation of an inflammatory microenvironment.

Tumor niche (TN) refers to the microenvironment that interacts with tumor cells and regulates their fate. TN has been revealed as a critical barrier for cancer treatment and it is analyzed in this manuscript through four different features: the vascular niche, the inflammatory and immunosuppresive niche, the hypoxic niche and the extracellular matrix, all of which are closely related among themselves, with the CSC phenotype (Figure 1). The tumor microenvironment was mostly seen as a potential advantage in the past since it enhances the permeability and retention of the nano-sized drugs in the tumor (i.e. the EPR effect). However, tumor vasculature is highly irregular and could be tight in some regions, while being leaky in others. This irregular growth of tumor vasculature

also generates non-functional branches, leading to poorly irrigated regions that cannot be easily accessed with chemotherapy (Jain, 2005). Besides, the accumulation of stroma in the tumor and the high intratumoral pressure also prevent drug transport to the inner regions. A recent survey of the literature has indicated that only a 0.7% of the nanocarrier dose is delivered to solid tumors (Wilhelm et al., 2016), a results that suggests the failure of the overall concept of passive targeting as it is understood nowadays.

Besides its barrier effect to drug delivery, the tumor niche also provides important signaling, often related to the cancer stem cell phenotype, that promotes tumor spreading and protection. CSCs and their niche have been recognized as critical features of cancer progression in the last years, and are currently in the focus of intense programs for drug development. Indeed, some prototypes have been developed to the stage of clinical implementation or are in advanced clinical trials (Figure 2). Most of the programs, however, are still focusing on separate aspects of CSCs and the TN, and as it will be illustrated in this review, those features are tightly interconnected (Figure 1) and might not be effectively addressed separately.

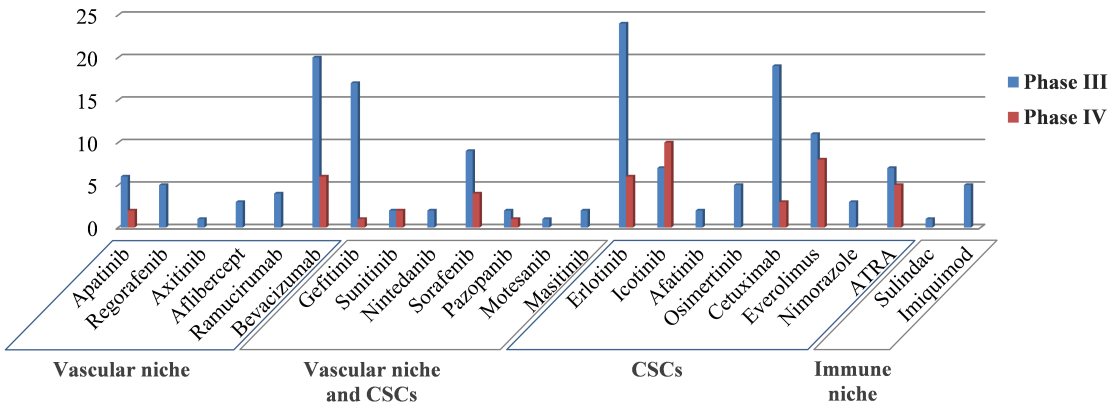


Figure 2. Advanced clinical trials (phase III or higher) of therapies against CSCs and/or their niche (only years 2011-2016). Further information on Supplementary Information (Table S1). Source: ClinicalTrials.gov (www.clinicaltrials.gov).

The field of biomaterials and drug delivery, mostly in tissue engineering, has focused on pulsing important cell signaling routes, particularly those related to stem cell development, and understanding and mimicking the biological substrate (the "niche"). Concretely, scaffolds and other tissue engineering devices are frequently used to: (i) induce stem differentiation (Prabhakaran et al., 2009), (ii) deliver cell-cycle modulators (Nayab et al., 2007) , (iii) modulate the inflammatory niche (Lisignoli et al., 2006), (iv) modulate tissue vasculature (Stegemann and Nerem, 2003) and (v) induce extracellular matrix remodeling (Schneider et al., 2010). This spectrum of biological activity fits perfectly the requirements of a new generation of antitumorals capable of modulating CSCs and their niche.

The objective of this review is to analyze the properties and implications of the CSC phenotype and the TN, and to cover the main therapies designed to address

these characteristics, focusing on the potential role of biomaterial-based technologies (i.e. implants, nanomedicines, etc.) in such therapies.

Cancer stem cells

Cancer stem cells (CSCs) have been defined as a cell subpopulation in the tumor bulk that possesses stem cell capacities. CSCs may be derived from adult stem cells or progenitor cells, but also from terminally differentiated cells that undergo epigenetic changes (Marotta and Polyak, 2009)(Hermann et al., 2010). In any case, malignant cells take advantage of stem cell-specific signaling to drive tumor development.

CSCs were isolated for the first time in the 1990´s in acute myeloid leukemia, and were named "tumor initiating cells" (TICs) because they were able to start by themselves a tumor. Later, CSCs were isolated in several types of solid tumors (colon, glioma, pancreatic, lung, breast etc.). The fundamental traits of CSCs can be listed as: (i) ability for self-renewal and tumor reactivation, even in the absence of growth signals; (ii) evasion of apoptosis by secreted factors; (iii) increased activity of drug efflux transporters that enhances their resistance to chemotherapy and radiation; (iv) quiescence; (v) capacity to differentiate into any cell of the tumor population; (vi) ability to migrate and metastasize to other tissues, and (vii) increased capacity for DNA repair (Wicha et al., 2006) (Kaiser, 2015). From a molecular biology perspective, CSC traits are driven by the activation of specific signaling pathways (Table 1), many of them present also on non-pathological stem cells.

Pathway	Mechanism	Reference
Hedgedog (Hh)	Cell proliferation, differentiation, survival, self-renewal, CSCs maintenance, epithelial mesenchymal transition (EMT).	(Dean et al., 2005) (Lu et al., 2012)
Wnt	Cell proliferation, differentiation, self-renewal and migration.	(Reya and Clevers, 2005) (Takebe et al., 2015)
Notch	Cell proliferation, differentiation, self-renewal, communication cell-to-cell and apoptosis.	(Liu et al., 2005) (Pannuti et al., 2010)
NF-κB	Cell proliferation, migration and apoptosis	(Dolcet et al., 2005) (Hoesel and Schmid, 2013)

Table 1. Important cell signaling pathways implicated in the CSCs phenotype.

The key implication of CSCs is that a reduced number of these cells have the capacity to regenerate the tumor. Therefore, any therapy that aims at successfully increasing survival needs to be effective in fully eliminating these cells, which are more resistant to conventional cytotoxic drugs. A corollary to this is that tumor reduction is only informative on the capacity of the drug to eliminate the bulk tumor cells, and might not correlate with medium or long-term survival. There are, however, some drugs that treat specifically CSCs, as described in seminal works in oncology (Clement et al., 2007)(Visvader and Lindeman, 2008)(Hambardzumyan et al., 2008)(Zhao et al., 2009)(Wang et al., 2010)(Singh and Settleman, 2010) (Merchant and Matsui, 2010)(Takebe et al., 2011)(Yu et al., 2012)(Pattabiraman and Weinberg, 2014)(Skvortsov et al., 2015)(Takebe et al., 2015)(Takebe et al., 2015). Although some overlapping is admitted, for clarity, we classify these CSCs-specific therapies by two action mechanisms: (i) CSC differentiation and (ii) targeting CSC signaling pathways. The most studied drugs that act by these two mechanisms are presented in the following sections,

together with biomaterial-based systems that have shown the capacity to enhance their activity in cancer models or at least improve their delivery profile.

2.1 Differentiation therapy

2.1.1. Retinoid derivatives

Since cancer stem cells take advantage of specific cell programs to boost their malignancy, the CSC pool can be depleted by inducing differentiation towards a mature phenotype (Figure 3). The use of differentiation therapies is intrinsically linked with the discovery of CSCs in hematopoietic cancers, where the most studied drugs have been retinoid derivatives. The mechanism of action of retinoid derivatives is related to ALDH, an enzyme that oxidizes intracellular aldehydes and retinol to retinoic acid, which induces cell differentiation. .

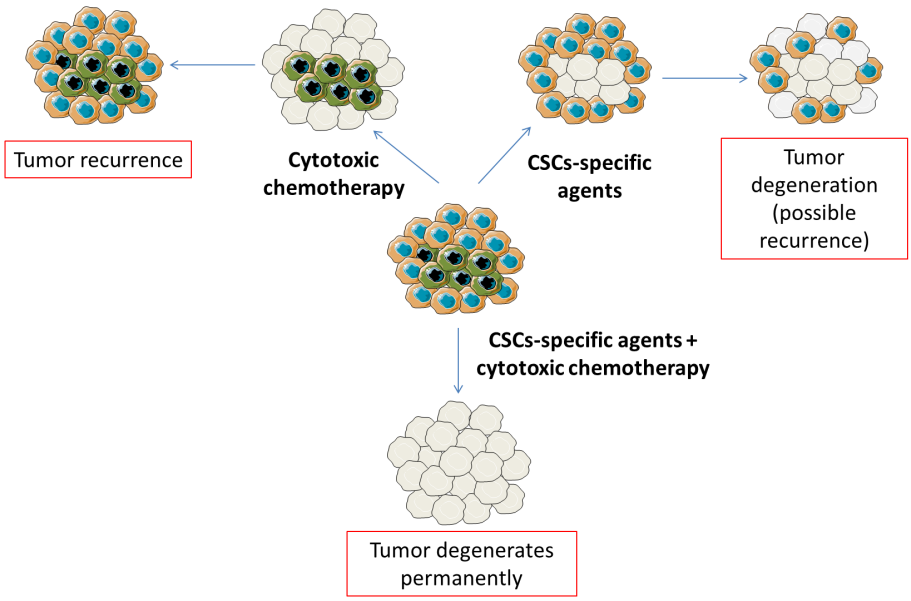


Figure 3. The significance of treating cancer stem cells (CSCs). The figure illustrates the outcome of conventional cytotoxic therapies that eliminate the bulk tumour (orange cells) and spare CSCs (green cells) vs. the outcome of therapies specifically directed against CSCs. Tumors depleted from CSCs might degenerate temporarily, but if some cells from the tumor bulk dedifferentiate cancer might recur. The combination of both strategies could lead to a more efficient elimination of the tumor.

Acute promyelocytic leukemia (APL) has benefited the most from treatments based on inducing cell differentiation. All-trans retinoic acid (ATRA) has dramatically turned APL therapy, and has further benefited from the introduction of another differentiation and pro-apoptotic agent, arsenic trioxide, that is given as a combined therapy with ATRA (Spira and Carducci, 2003). Traditionally considered a lethal disease, APL is nowadays one of the most treatable cancers, with the ATRA/arsenic trioxide combination achieving around 90% remissions. Other cancers where retinoid-based differentiation could be useful are melanoma, teratocarcinoma, squamous cell carcinoma, neuroblastomas and colon carcinoma, although the clinical efficacy of these treatments is still under investigation (Leszczyniecka et al., 2001)(Kawamata et al., 2006).

ATRA has been encapsulated in several advanced formulations to address its low aqueous solubility and improve its stability, to enable its controlled release, to reduce hematological toxicity, and to improve its pharmacokinetic profile *in vivo*. These formulations include microemulsions, nanoparticles and liposomes. Liposomal formulations have reached clinical trials (phase I and II) for the treatment of solid tumors (clinical trial references NCT00195156, NCT00005969 and NCT00003656). A microemulsion encapsulating ATRA was developed using PEGylated-phospholipids as surfactants, providing improved stability and solubility for the drug (Hwang et al., 2004). Similarly, different polymeric nanoparticle compositions have been tested for ATRA delivery, most based on PEGylated polymers that prolong circulation time and optimize tumor targeting. These nanoparticles offer more opportunities for controlling drug

release; for example poly(ε-caprolactone)-poly(ethylene glycol) (PCL-PEG) nanoparticles showed controlled release of ATRA, and the pharmacokinetic profile of the formulation could be modified through changes in the polymer molecular weight, drug loading and polymer concentration (Jeong et al., 2004). Encapsulation of ATRA in nanocarriers is also beneficial to prevent the hemolysis produced by the free drug (Lim et al., 2004), and to improve the antitumoral activity *in vivo*, an effect probably associated to the improved pharmacokinetic and biodistribution profile (Mehta et al., 1994)(Hwang et al., 2004)(Jeong et al., 2004)(Li et al., 2011).

Because ATRA only induces the differentiation of the CSCs, it is necessary to combine it with other drugs as it is done in the clinical setting to get total relapse of the tumor. Nanocarriers are ideal platforms to host multiple drugs, and poly-lactic acid -poly(ethylene glycol) (PLA-PEG) nanoparticles have been loaded with ATRA and doxorubicin to get a synergistic effect that targets both CSCs and the bulk tumor cells. This effect was found to be stronger than that achieved by the co-administration PLA-PEG nanoparticles encapsulating both drugs separately, and markedly better than the monotherapies formulated in nanoparticles (Sun et al., 2015).

2.1.2. TGF-β superfamily modulators

Other strategy to induce differentiation is based on the premise that CSCs are sensitive to developmental signaling such as bone morphogenetic proteins (BMP) and transforming growth factor-*beta* (TGF- *beta*) inhibitors. The actions of BMP-signaling are complex and cell-specific, often resulting in contradictory

outcomes in different cancers (Kallioniemi, 2012). However, for some tumors BMP-signaling shows marked capacity to stop tumor proliferation and to drive CSCs towards more benign phenotypes, potentially treatable by conventional chemotherapy (Gonzalez-Gomez et al., 2014). Another member of the same signals superfamily, TGF-β, has been unveiled as an important inductor of CSCs stemness, as a regulator of the epithelial-mesenchymal transition (EMT) and as a promoter of CSC self-renewal. Treatments with TGF-β inhibitors result in reduced CSC markers in the tumor and in cell migration (Anido et al., 2010)(Singh and Settleman, 2010).

Advanced formulations for BMPs have been designed for managing glioblastoma stem cells. The therapeutic concept here was the generation of a local reservoir for the controlled release of the CSC suppressor BMP-7, which could be implanted at the time of primary tumor resection. The controlled release properties of this formulation are essential due to the physiological half-life of BMP-7 that is limited to a few minutes. A poly(lactic-co-glycolic acid) (PLGA) microsphere formulation was optimized towards this aim, where BMP-7 was encapsulated in the form of a nanocomplex with heparin and poloxamine. The formulation achieved over 90% encapsulation, minimal burst and sustained BMP-7 release for over two months in bioactive form (Reguera-Nuñez et al., 2014). Further *in vivo* studies using human primary glioblastoma stem cell lines confirmed that the implantation of this formulation was able to activate the BMP-canonical pathway in the tumor for over two months, and that this activation

results in reduced tumor development and downregulation of malignancy markers related to the CSC phenotype (Figure 4) (González-Gómez et al., 2015).

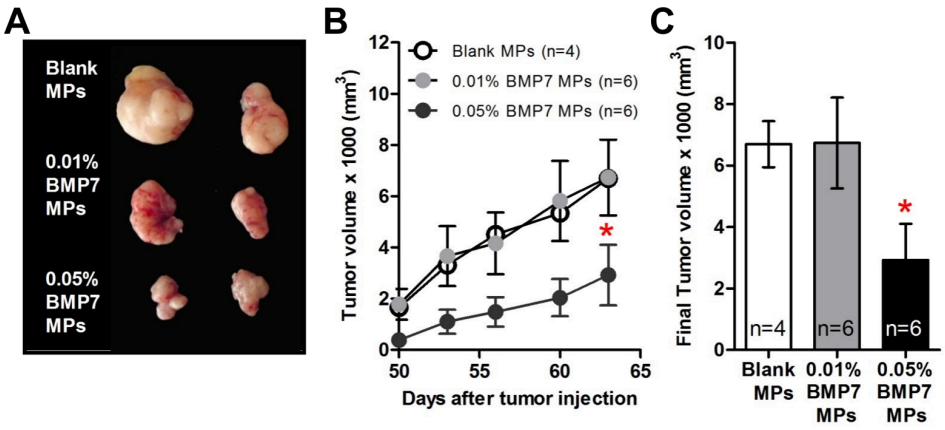


Figure 4. BMP-7 microspheres decreased tumor growth in a primary human glioblastoma stem cell xenograft model. Three groups were compared, blank microspheres (Blank MPs), and microspheres loaded either with 0.01% or 0.05% (w/w) BMP-7 (0.01% BMP7 MPs and 0.05% BMP7 MPs, respectively). (A) Representative picture of the tumors on the last day of the experiment. (B) Tumor volume measured at different times. (C) Final tumor volume measurements. *p < 0.05. Reproduced from (González-Gómez et al., 2015) under a Creative Commons Attribution License.

2.2 Targeting CSC signaling pathways

In general, CSCs are considered as hyper-resistant to chemotherapy. However, due to their intrinsic signaling pathways, some molecules might have specific effects on CSCs. These molecules generally have broad effects in cell function, but most converge towards an apoptotic effect in the CSCs. We will cover here the most relevant examples of these drugs, which include Hedgehog, Wnt, NF-kB and PI3K/Akt/mTOR inhibitors.

2.2.1 Hedgehog pathway inhibitors

The Hedgehog pathway inhibitor HPI-1 has shown promising activity for suppressing CSCs (Coni et al., 2013), but its clinical use is limited by low aqueous solubility and bioavailability. To overcome these problems, HPI-1 has been encapsulated in PLGA-PEG nanoparticles (NanoHHI) (Chenna et al., 2012). NanoHHI showed 3 to 4-fold higher oral and intraperitoneal bioavailability than the same drug in a conventional formulation (i.e. parent compound). NanoHHI showed marked suppression of tumor growth *in vivo* and the attenuation of metastasis. This advanced formulation showed a remarkable reduction of the CD133+ cell subpopulation, which is identified as the CSC pool (Y. Xu et al., 2012). Besides, NanoHHI has also been combined with gemcitabine, and this association was able to inhibit tumor growth without systemic toxicity in a pancreatic xenograft model (Chenna et al., 2012).

2.2.2 Wnt inhibitors

Wnt signaling regulates the proliferation, differentiation and survival of cancer cells, and can be divided into two pathways: canonical and non-canonical. The canonical pathway, also known as WNT-β-catenin pathway, is correlated with poor prognosis in cancer, and its inhibition reduces tumor progression through the downregulation of cell cycle proteins cyclin D1, c-Myc and c-jun (Persano et al., 2013) (Kahn, 2014). Inhibitors of the Wnt/β-catenin signaling pathway include antioxidants (quercetin, resveratrol, curcumin and EGCg), anti-inflammatories (tetrandine, Sulindac) and other synthetic compounds (PRI-724,

OMP-18R5), some of them currently in clinical trials (Kahn, 2014)(Amado et al., 2014).

Quercetin, resveratrol and tetrandrine are three validated Wnt inhibitors that share some biopharmaceutical and therapeutic limitations: low aqueous solubility, important toxicity and low oral bioavailability. Because of that, nanoformulations have been proposed with two aims: (i) to improve their biodistribution following i.v. administration and (ii) to improve their oral bioavailability.

PEGylated nanocarriers have been the most used systems to improve the biodistribution of these molecules, although other carriers such as magnetic nanoparticles have also been investigated (Barreto et al., 2011). Among PEGylated nanocarriers, the most investigated formulations have been liposomes and nanoparticles based on biodegradable polyesters. PEGylated liposomes have been used to encapsulate quercetin and resveratrol, and these formulations have shown better pharmacokinetics, higher tumor accumulation, improved antitumoral effect and reduced toxicity in healthy tissues (Yuan et al., 2006) (X.-Y. Lu et al., 2012). In order to get a synergistic cytotoxic effect, a second drug can be loaded in the liposomes. The combination of quercetin and vincristine produces higher effect than the monotherapy with either drug alone, and requires lower drug doses and has lower systemic toxicity (Wong and Chiu, 2011)(Sun et al., 2014). Resveratrol and curcumin were included in a liposomal formulation targeted with an antibody against HER-2, and the system showed

remarkable efficacy while decreasing the toxicity of the drugs (Catania et al., 2013).

Other important nanoformulations used to improve the biodistribution of Wnt inhibitors are core-shell type nanoparticles that have been used to deliver resveratrol and tetrandrine. Studies of resveratrol encapsulated in mPEG-PCL or PCL-PLGA-PEG nanoparticles and studies of tetrandrine encapsulated in polyvinylpyrrolidone-block-poly(ε-caprolactone) (PVP-b-PCL) nanoparticles indicate that these nanoformulations improve the intracellular transport of these drugs and increase their therapeutic efficacy (Sanna et al., 2013)(Karthikeyan et al., 2013)(Xu et al., 2013) (Shao et al., 2009). *In vivo* studies, performed in a ectopic human ovarian tumor xenograft, with resveratrol–bovine serum albumin nanoparticles have shown higher accumulation of the drug in tumor, liver and kidney, and an increased pro-apoptotic effect as compared with the reference formulation (Guo et al., 2010). Nanoparticles can also be used to deliver drug combinations such as tetradrine with paclitaxel loaded in mPEG-PCL nanoparticles. This formulation enhanced reactive oxygen species (ROS) accumulation in the tumor and produced a higher pro-apoptotic effect *in vitro* (X. Li et al., 2012).

Another objective of nanoformulations is improving oral bioavailability. A nanomicellar formulation of quercetin based on 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol (DSPE-PEG) has been developed, and it showed improved drug solubilization, protection of the drug from intestinal enzymes and enhanced drug permeability through the intestinal

barrier. An *in vivo* assay in a lung tumor xenograft mouse model showed significantly increased anti-tumor efficacy for quercetin nanomicelles compared to quercetin suspensions when administered orally (Chang et al., 2012).

2.2.3 NF-kB inhibitors

NF-kB signaling regulates the apoptosis, adhesion and migration of cancer cells, through a balance in the expression of pro-apoptotic proteins (FLICE-like inhibitory protein) and inhibitors of apoptosis (anti-apoptotic Bcl-2 family). Moreover, it also participates in the immune response against the tumor (Helbig et al., 2003)(Dolcet et al., 2005). Some inhibitors of this pathway are curcumin and disulfiram (Dolcet et al., 2005)(Zha et al., 2014) and both have been formulated in nanocarriers, but with different objectives.

Curcumin is an inhibitor of NF-kB, but also transduces its effect through other networks such as Wnt, covered before. Curcumin has a plethora of interesting properties for cancer since it is pro-apoptotic, antiangiogenic, anti-inflammatory, immunomodulatory and antimitogenic. Curcumin is also poorly soluble in water and this conditions its oral bioavailability, a limitation that has been addressed by its incorporation into mucoadhesive systems, concretely polyacrylic acid-based nanoparticles (Lim et al., 2011) and stearic acid-g-chitosan micelles (Wang et al., 2012). These systems have shown the capacity to improve curcumin solubilization and bioavailability. Importantly, they are also able to improve the pro-apoptotic, antitumoral effect of the drug following oral

administration while reducing systemic side effects. *In vivo* studies also confirmed a specific effect on the CSC subpopulation (Wang et al., 2012).

In the case of disulfiram, its effects are dependent on the presence of copper in the medium (P. Liu et al., 2012), and for this reason, their co-encapsulation is advantageous. A liposomal formulation of disulfiram and copper in liposomes has been developed and has shown extended plasma half-life as compared to the free drug. The liposomal disulfiram/copper formulation also had antitumoral effect in mice breast cancer xenografts and minor systemic toxicity *in vivo* (Liu et al., 2014).

2.2.4 PI3K/Akt/mTOR inhibitors

The phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) network is an essential pathway for cell proliferation, differentiation and survival, and is considered critical for CSC function (Xia and Xu, 2015). Rapamycin is the classical inhibitor of this pathway, although currently there are several new and more specific compounds in clinical trials (Benjamin et al., 2011). Rapamycin has low water solubility, low specificity for tumor cells and important side effects. For these reasons, several nanoformulations for this drug have been tested, mostly based on PEGylated polyester nanoparticles and liposomes.

PEGylated polyester micelles based on PLA or PCL have been developed for rapamycin delivery. In general, rapamycin in PEGylated micelles has better solubility, intracellular uptake and anti-proliferative effect (Chen et al., 2013).

Rapamycin delivered in PEGylated micelles also enhances the efficacy of concomitant radiotherapy (Woo et al., 2012) and paclitaxel administration (Tian et al., 2015)(Mishra et al., 2013). *In vivo*, PEG-PCL micelles have demonstrated higher accumulation ratio of both drugs in the tumor compared to the accumulation in liver and spleen. Rapamycin and paclitaxel encapsulated in PEG-PCL micelles can suppress tumor growth completely in a breast cancer murine model by acting specifically through the mTOR pathway (Blanco et al., 2014).

Rapamycin has been encapsulated in PEGylated liposomes in combination with the cytotoxic drug paclitaxel. This formulation was more effective *in vitro* than any or the combination of the free drugs. *In vivo* results confirmed that the nanoformulation was effective in controlling tumor growth in a breast cancer murine model (Eloy et al., 2016).

Another interesting drug association is that of rapamycin and perifosine, integrated in albumin-bound nanoparticles. An *in vivo* study in a multiple myeloma murine model indicated mutual suppression of the PI3K/Akt/mTOR pathway by rapamycin and perifosine, inducing synergistic tumor cell elimination in multiple myeloma xenograft mouse model (Cirstea et al., 2010).

Rapamycin biodistribution can also be improved through the active targeting of the nanocarriers. PLGA nanoparticles targeted with a EGFR-antibody on their surface showed better cellular uptake and a superior anti-proliferative activity compared to the free drug or to non-conjugated nanoparticles (Acharya et al., 2009).

Tumoral niche

3.1 Aberrant Vasculature

The formation of abnormal blood and lymphatic vessels is one of the critical hallmarks of solid tumors, which is driven by the secretion of high concentrations of angiogenic factors by the tumor cells. On one hand, the role of this abnormal vasculature is the nutrition and oxygenation of the tumor, but it also has a regulatory role in the secretion of growth factors and cytokines that spur tumor growth. Tumor-induced angiogenesis results in different vessels from those in normal vasculature as they are more tortuous, irregular and highly permeable, even to large proteins (Anderson et al., 2000). The irregularity of the vessels generates sometimes non-functional branches (Baluk et al., 2005), while in other cases, the interstitial pressure strangles functional vascular branches. This leads to areas with poor irrigation within the tumors that explains the resistance to radio- and chemotherapy (Jain, 2005). Besides, these low perfusion areas result in hypoxic microenvironments that will be the subject of a further section in this manuscript (section 3.3). There are several molecules implicated in the regulation of angiogenesis. The most prominent are: the vascular endothelial growth factor family (VEGF), pigment epithelium-derived factor (PEDF) and basic fibroblast growth factor (b-FGF) (Carmeliet and Jain, 2000).

Targeting the vascular niche is advantageous because: (i) tumor endothelial cells are homogenous across different tumors, and they are less prone to genetic instability than cancer cells, and (ii) the tumor vascular endothelium is accessible

to systemically administered drugs and thus, does not require drug transport through the tumor tissue. Because of these advantages, it is in principle more straightforward to design biological-based therapies (e.g. monoclonal antibodies) towards these targets, and reduce the cytotoxic side effects associated to conventional chemotherapy that is directed against the tumor (Kobayashi and Lin, 2006).

Traditionally, antiangiogenic therapies were aimed at completely destroying the tumoral vascular niche to deprive tumors from the oxygen and nutrients required for its growth. In the last decade, this concept has coexisted with that of "vascular normalization", where the active agents ''normalize'' the abnormal vasculature to make it more efficient for oxygen and drug delivery, avoiding drug resistance and the formation of the hypoxic niche (Jain, 2005) (Figure 5). Even though these concepts have different implications in overall therapy, they largely use the same active compounds and both mediate their effect by sabotaging the vascular niche. Therefore, within the particular scope of this review, we do not consider further this distinction in antiangiogenic therapies.

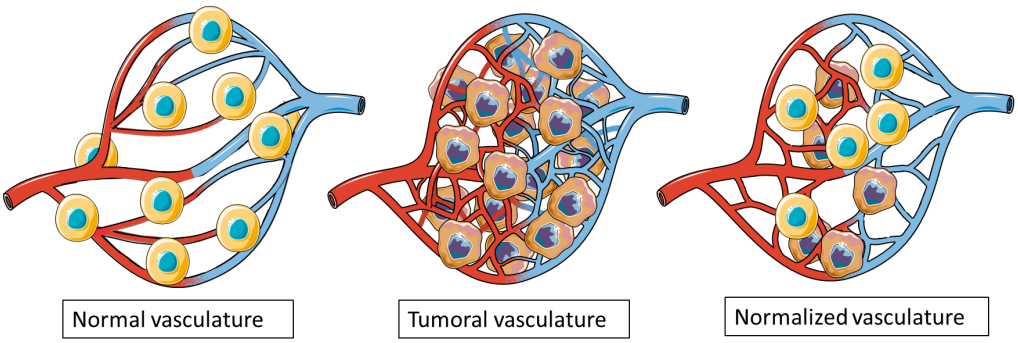


Figure 5. The tumor vascular niche. Compared to physiologically organized vascular branches (left), tumor vasculature is chaotic and hyperthrophic (middle). This lack of organization leads to non-homogeneous oxygen supply, and the formation of hypoxic regions. Anti-angiogenic therapies can eliminate tumor vasculature, but in excess, they can promote hypoxia and the acquisition of a CSC phenotype. At controlled doses, these compounds can "normalize" tumor vasculature (right), resetting physiological organization, improving drug delivery and destroying the nurturing features of the perivascular niche for CSCs.

Targeting the vascular niche can be achieved through two possible strategies: (i) interfering with pro-angiogenic molecules, their receptors or downstream signaling, or (ii) upregulating or releasing endogenous inhibitors. The conventional therapy used in the clinic for this purpose is based on anti-VEGF monoclonal antibodies (bevacizumab), tyrosine kinase inhibitors such as imatinib, sorafenib, sunitinib, or combinations of them (Al-husein et al., 2012). Some of these molecules have already reached extended clinical use that is expected to expand even further as the results from many clinical trials validate their potential in many other indications (Figure 2).

Biomaterial-based therapies against the vascular niche have also been investigated based on the same two strategies outlined above. As compared to other therapies, these systems try to provide added value fundamentally through their improved targeting. As mentioned before, molecular targets have a

particularly high exposure in the tumor endothelium, which makes actively targeted nanocarriers especially useful. Most drugs used for modulating tumor vasculature are non-selective by nature, and therefore, could benefit from encapsulation in an actively-targeted nanocarrier that increases the local concentration of the antiangiogenic agent, prolongs its release and decreases drug concentration at off-target sites.

Even integrating active compounds that could be considered targeted such as antibodies, nanocarriers can provide some distinct advantages derived from the fact that many ligands increase cooperatively or synergistically their affinity when binding at several sites or by several receptors ("clustering"). This effect has spurred the interest on investigating multivalent carriers where ligand spacing is optimized for ligand-receptor interaction (Yu et al., 2010). In summary, actively-targeted nanocarriers could have the main benefit of concentrating both specific ligands and a high drug payload in distinct carriers.

Another region of interest in the solid tumor vascular system are lymphatic vessels, whose defective function contribute to the enhanced tumor interstitial pressure (Padera et al., 2004), but also constitutes a possible route of metastatic cell spreading (Swartz and Skobe, 2001)(Hirakawa, 2009). To maximize lymphatic drainage of drug nanocarriers after subcutaneous administration, it is important to optimize their physicochemical properties, which should comprise a highly passivated surface and small particle size (Abellán-Pose et al., 2015). Moreover, Abellán-Pose et al. have studied the biodistribution of poly-glutamic acid-poly(ethylene glycol) (PGA-PEG) nanocapsules loaded with docetaxel

upon i.v. administration in a mice model, and observed that maximum extravasation to the lymphatic vessel was obtained with particles below 150 nm (Abellan-Pose et al., 2016). This prototype has been administered in a metastatic lung cancer murine model and has shown higher effect than the commercial formulation Taxotere® both in the primary tumor and in the lymphatic metastasis (Borrajo et al., 2016). Nanocarriers can also be actively targeted to the lymphatics by conjugation with VEGFR-3, podoplanin, and the hyaluronan receptor LYVE-1 (Hirakawa, 2009)(Swartz and Skobe, 2001).

3.1.1 Nanocarriers that interfere with pro-angiogenic signaling

VEGF is the main factor that promotes angiogenesis, thus blocking this signaling pathway is an attractive strategy to modulate tumor vasculature. Gold and silver nanoparticles have intrinsic antiangiogenic properties because of their interaction with endothelial heparin-binding glycoproteins associated to the VEGF receptor that inhibits their activity (Gurunathan et al., 2009)(Mukherjee et al., 2005). Moreover, gold nanoparticles bind vascular permeability factor and b-FGF, which are two other angiogenic mediators. These mechanisms result in inhibited endothelial and fibroblast proliferation (Mukherjee et al., 2005). Other metallic nanoparticles with antiangiogenic properties are silver and cerium oxide nanoparticles. These nanoparticles have the capacity to modify ROS intracellular levels, inhibiting the PI3K/Akt/mTOR pathway. The inhibition of this pathway reduces the production of pro-angiogenic growth factors, including VEGF and

FGF, ultimately reducing cell invasion and migration (Gurunathan et al., 2009)(Giri et al., 2013)(Kosmidou et al., 2001).

Gene therapy is another option for the design of antiangiogenic therapies, and several groups have designed nanocarriers for silencing the gene expression of VEGF. For instance, He et al. designed an efficient calcium carbonate nanocarrier for abrogating VEGF expression in colon cancer (He et al., 2008)(He et al., 2009). In another study, a siRNA against a VEGF receptor was included in alginate-modified polyethyleneimine (PEI) nanoparticles. These nanoparticles were designed to profit from the complexing and endosomal escape properties of PEI, but improving the efficacy/toxicity ratio of carriers through mixing with alginate (Li et al., 2014). Sakurai et al. have also designed liposomes to silence VEGFR2, composed by a pH sensitive cationic lipid, known as YSK05, and conjugated with an integrin ligand αvβ3 (cRGD). *In vivo* assays showed a selective action in tumor endothelial cells, but no effect in normal endothelial cells. This formulation also produced a reduction in the tumor volume in a renal cell carcinoma mouse model (Sakurai et al., 2013).

In recent decades, the antiangiogenic effect of glucocorticoids (GCs) has also been described. GCs are implicated in transcriptional responses to the majority of inflammatory, angiogenic, immunomodulatory and apoptotic genes, either by binding directly to DNA or to transcription factors involved in gene regulation. The antiangiogenic effect of GCs is mainly achieved by downregulating the secretion of pro-angiogenic factors. To reduce the toxicity of GCs, these molecules have been encapsulated in PEGylated-liposomes.

Concretely, four different GCs were encapsulated in this system: budesonide, dexamethasone, methylprednisolone, and prednisolone. Liposomes efficiently delivered the GCs to the tumor, reduced the system side effects, and prolonged tumor growth inhibition. Budesonide encapsulated in liposomes resulted the most potent regarding their antiangiogenic effects. Liposome PEGylation was critical to realize this prolonged drug circulation and to achieve enhanced drug extravasation to the tumor in a melanoma murine model (Banciu et al., 2006)(Banciu et al., 2008).

3.1.2 Nanocarriers that enhance antiangiogenic signaling

Pigment epithelium-derived factor (PEDF) is a protein that has antiangiogenic, anti-inflammatory, antitumoral and antioxidant effects (Ek et al., 2006)(Ek et al., 2007)(J. T. Liu et al., 2012). Therefore, several works in the literature have attempted to deliver this protein, frequently in the form of gene therapy. Dass et al. described the first delivery system for a cancer therapy based on a PEDF, which was based on chitosan microparticles loaded with a PEDF-encoding plasmid. Animals treated with this therapy showed a reduction in bone lysis and in tumor progression, as observed in an osteosarcoma mouse model. Moreover, it also showed a reduction in the number of lung metastases, probably due to reduced invasiveness of the cells (Dass et al., 2007). After this first report, other nanocarriers were also developed from PLGA, PEI and their PEGylated analogues (Cui et al., 2010)(L. Li et al., 2012)(Xu et al., 2016). Targeting moieties can also be added on these nanoparticles to improve selective

accumulation at the tumor site. For example, PEG–PEI nanoparticles conjugated to the integrin ligand αvβ3 (cRGD) showed enhanced transfection efficiency in comparison with non-targeted PEG–PEI nanoparticles, despite of a worse capacity to complex DNA (L. Li et al., 2012).

A combined therapy based on paclitaxel and PEDF-encoding plasmid, both loaded in PEG−PLGA nanoparticles was described by Xu et al. *In vivo* experiments indicated a superior anticancer effect for nanoparticles containing the combined therapy as compared to nanoparticles loaded with the drugs separately. Specifically, the combined therapy reduced tumor weight and improved survival in a colon adenocarcinoma mouse model. This positive effect was accompanied by a clear reduction on the tumor microvessel density (L. Li et al., 2012)(Xu et al., 2016).

Another molecule with antiangiogenic activity is TNP-470, a fumagillin analogue that is considered to be one of the most potent and broad-spectrum angiogenesis inhibitors (Satchi-Fainaro et al., 2005)(Benny et al., 2008). TNP-470 has been conjugated to a N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer to prolong its plasma half-life after intravenous administration and to improve its biodistribution by passive targeting mechanisms. Indeed, conjugation to HPMA prevents drug transport across the blood brain barrier (BBB), reducing the neurotoxicity typical of the free drug in glioblastoma, melanoma, pancreatic adenocarcinoma and breast cancer mouse models (Satchi-Fainaro et al., 2004)(Satchi-Fainaro et al., 2005). TNP-470 has also been encapsulated in mPEG-PLA micelles to improve its solubility and to enhance its

intestinal absorption. An *in vivo* study in a murine melanoma model showed that the micellar formulation achieved good oral bioavailability and sustained plasma levels of the drug (Benny et al., 2008).

3.2 Aberrant Extracellular Matrix

The extracellular matrix (ECM) is responsible for maintaining the architecture and homeostasis in normal tissue and it serves as support for tissue specific cells, immune cells, capillaries and fibroblasts (Kalluri and Zeisberg, 2006). It is composed by proteins (i.e. collagen, elastin and fibronectin) and polysaccharides such as hyaluronic acid and proteoglycans (i.e. perlecan, agrin, syndecans and glypicans) (Kim et al., 2011)(P. Lu et al., 2012). Cells bind to the ECM through functional structures, integrins, and the mechanical forces transduced can have important signaling roles (Geiger et al., 2001). The ECM also has polysulfated regions to bind growth factors and other signaling proteins, and acts as reservoir of these molecules whose release is triggered by ECM degradation (Schultz and Wysocki, 2009). Globally, both growth factor binding, release and cell-ECM interactions have critical roles in cancer development. In cancer, the ECM is deregulated, disorganized and enriched in pro-tumoral molecules (Figure 6) (Cox and Erler, 2011)(Oskarsson, 2013). From a therapeutic standpoint, the ECM in the CSC niche raises two issues. First, ECM components in conjunction with enhanced intratumoral pressure hinder drug movement and protect CSCs from chemotherapy. The second issue is that the highly dynamic ECM environment promotes tumor development through increased signaling and could favor cancer cell migration (P. Lu et al., 2012)(Oskarsson, 2013).

Biomaterial-based technologies to modulate both of these properties are currently under investigation.

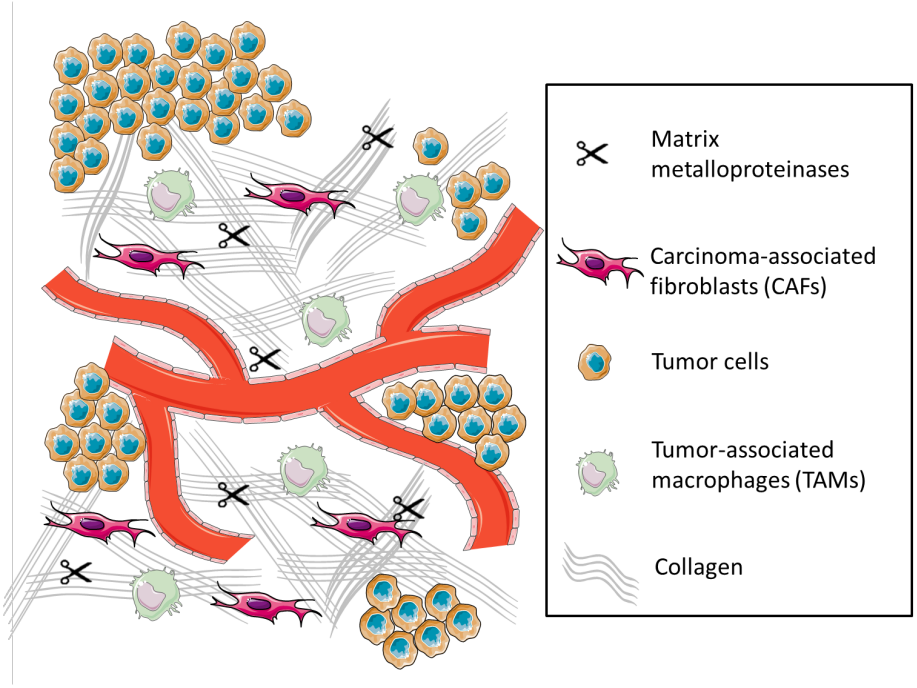


Figure 6. Tumoral extracellular matrix (ECM) has distinct features that contribute to tumor development. These include disorganized collagen fibers and high concentrations of ECM-degrading enzymes (metalloproteinases) that trigger the release of growth factors from cryptic ECM-sites. The ECM hosts other non-tumoral cells such as tumor-associated macrophages (TAMs) and carcinoma-associated fibroblasts (CAFs) that contribute to the properties of the TN.

3.2.1 Biomaterials that modulate ECM permeability

Collagen IV, one of the principal constitutes of ECM, interacts anomalously with proteoglycans in cancer. Proteoglycan concentration is also higher in tumor than in normal tissue, and this feature is directly correlated with tumor aggressiveness (Iozzo and Sanderson, 2011). These characteristics result in a compact ECM that constitutes a physical barrier for drug transport, and particularly for macromolecules (Netti et al., 2000). For regional delivery routes, the hindrance to drug transport can be reduced by co-administration of ECM degrading

enzymes (e.g. collagenase or hyaluronidase). Unfortunately, this strategy cannot be easily implemented for intravenous administration, since the enzymes would be distributed throughout the body. Alternatively, these enzymes could be integrated onto the surface of nanoparticles. As a proof-of-concept, Goodman et al. have reported the bioconjugation of collagenase on polystyrene nanoparticles. The 100 nm polystyrene nanoparticles with collagenase showed four times higher transport through the ECM than 100 nm albumin-coated nanoparticles in vitro, in a multicellular spheroid culture of human cervical carcinoma (Goodman et al., 2007). The concept is pending of *in vivo* validation and of implementation in more pharmaceutically acceptable materials.

Lysyl oxidase (LOX) is an enzyme that increases collagen cross-linking, thereby enhancing ECM rigidity. It is overexpressed in cancer (Kanapathipillai et al., 2014), and therefore, its inhibition is a potential strategy to enhance the ECM-penetration. LOX inhibiting antibodies have been conjugated onto PLA-PEG nanoparticles. *In vivo* studies, in an orthotopic breast cancer mouse model, revealed that antibody-nanoparticle conjugates have a higher enzyme inhibitory effect and lower toxicity than the free antibody. In addition, the tumor treated with the antibody-nanoparticle conjugates exhibited lower stiffness than those treated with the free antibody. The enhancement in efficacy observed for the nanoparticle conjugate allowed a 15-fold reduction of the administered dose, and is likely a consequence of ligand clustering effect (Kanapathipillai et al., 2012).

3.2.2 Biomaterial-based devices to suppress ECM-mediated protumoral signals

Matrix metalloproteinases are enzymes that degrade the ECM, and are involved in cell proliferation, migration, and tumor development. These enzymes need the presence of Cu or Zn ions to preserve their catalytic properties. Treatments to prevent tumoral ECM remodeling are based on matrix metalloprotease inhibitors such as chelating agents capable of sequestering the metal ions from the enzymes active-sites. Some investigated metalloprotease inhibitors include β-aminopropionitrile (Cox and Erler, 2011), marimastat (Rosenbaum et al., 2005)(Goffin et al., 2005) and batimastat (Rintoul and Sethi, 2002). Another example is doxycycline, an antibiotic that non-selectively inhibits metalloproteinases by an unknown mechanism. Administration of these agents is limited by significant side effects, because metalloproteases are also mediators of platelet and endothelial function, and participate in other processes such as scarring, inflammation and atherosclerosis. Considering the severe side effects of metalloproteinase inhibitors, the benefits of integrating these agents in selective delivery systems is clear. For instance, liposomes and Eudragit-based nanoparticles encapsulating doxycycline showed no toxicity, but were able to induce clear metalloprotease inhibition *in vitro* (Yücel et al., 2013).

3.3 Hypoxic foci

While tumors are characterized by extensive vascularization, the presence of aberrant, non-functional vascular branches and poor blood flow derived from the high intratumoral pressure often generate hypoxic foci inside the tumor (Figure

7). This feature is important because presence of tumor hypoxia is linked to poor clinical prognosis (Vaupel and Mayer, 2007). Indeed, several studies have shown that activation of hypoxia-related intracellular signaling results in higher expression of efflux pumps, reduced pro-apoptotic signaling, and higher tendency towards quiescence (Cosse and Michiels, 2008). Tumor cells in hypoxic regions are more resistant to radiotherapy since the generation of ROS that mediates its effect is hindered due to the reductive environment (Keith and Simon, 2007)(Bartholomeusz et al., 2009). Tumor cells under hypoxia are also more aggressive and have higher tendency for metastasis. All these unfavorable features are related to the acquisition of an undifferentiated, stem cell-like phenotype with a gene expression pattern that is a direct target of the intracellular transduction signals of hypoxia (Heddleston et al., 2009).

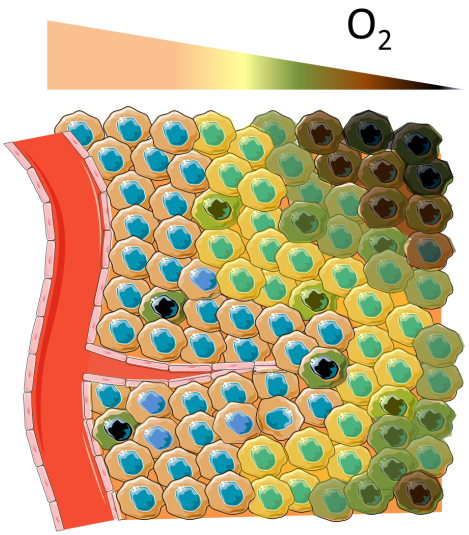


Figure 7. The hypoxic niche. Irregular or non-functional branches generate regions characterized by low oxygen tension (hypoxia) that promote a CSCs phenotype. CSCs are also hosted in the perivascular space, but are mainly enriched in these hypoxic foci with restrictive therapeutic access.

The cellular response to hypoxia is mediated by hypoxia inducible factors (HIFs). There are two types of HIFs, HIF-1 and HIF-2, and they are composed by 2 subunits: α (catalytic subunit) and β (constitutively expressed subunit). The function of HIF-1α is to regulate the cellular adaptation to low oxygen levels, including the survival of tumor cells under hypoxic conditions. HIF-1α activates the transcription of genes involved in angiogenesis, glycolytic metabolism, reduced oxygen consumption, cell migration, and tumor cell invasion (Rapisarda et al., 2009). Under normoxic conditions HIF-1α is hydroxylated and interacts with the tumor suppressor Von Hippel−Lindau (VHL) protein, which produces the degradation of the factor inside the proteasome. When cells are in hypoxic conditions, non-hydroxylated HIF-1α translocates to the nucleus where it activates the transcription of numerous target genes (Lu et al., 2009)(X. Q. Liu et al., 2012). HIF-2α participates in the regulation of stem cell self-renewal and multipotency, but it is expressed only in some specific cell types (Keith and Simon, 2007). HIF-2α acts through Oct4, the key transcription factor regulating cell "stemness" (Mohyeldin et al., 2010)(Seidel et al., 2010).

Two major therapeutic approaches are being investigated to counteract hypoxic effects. From a clinical standpoint, the more advanced option is the use of vascular normalization agents (see Section 3.1), which are supposed to improve perfusion homogeneity under controlled conditions. Vascular normalization has demonstrated to improve vessel lining and its maturation, resulting in enhanced tumor perfusion and oxygenation, together with inhibited tumor cell invasion, intravasation, and metastasis. Furthermore, the good oxygenation of the tumor

improves its sensitivity to the chemotherapy and radiation (Mazzone et al., 2009).

The second strategy relies on the selective inhibition of HIFs. Since these are intracellular proteins, interfering RNA strategies based on siRNAs or miRNAs have been the most frequently investigated (Piao et al., 2012)(Bartholomeusz et al., 2009). The major problem of RNA interference is the delivery of the therapeutic sequence to the cell cytosol, and due to this reason, nanocarriers have a major role in these therapeutic strategies (Thomas et al., 2003). Liu et al. designed cationic micellar nanocarriers composed by a combination of amino-phosphate poly(ε-caprolactone)-block-poly(2-aminoethylethylene phosphate) (PCL-b-PPEEA) and poly(ε-caprolactone)-block-poly(ethylene glycol) (PCL-b-PEG), and used these materials to complex siRNA against HIF-1α. *In vitro* studies showed that these micellar systems inhibit HIF-1α, and by this mechanism, they reduce the secretion of proangiogenic factors, tumor growth and cell migration. A synergic antitumoral effect was observed *in vivo*, when these micelles were administered in combination with doxorubicin in a prostate tumor xenograft model (Allen et al., 1998)(X. Q. Liu et al., 2012). SiRNA targeting HIF-1α has also been formulated in a new biodegradable copolymer, D-α-tocopheryl polyethylene glycol 1000 succinate-b-poly (ε-caprolactone-ran-glycolide). *In vivo* experiments in a xenograft nasopharyngeal carcinoma murine model confirmed that the nanoparticles did not produce a cytotoxic effect per se, but were able to decrease 2-fold the expression of HIF-1α in 24 hours.

This reduction in HIF-1α resulted in a proportional reduction in tumor volume (Chen et al., 2015).

3.4 Inflammatory and immunosuppresive environment

The tumor microenvironment contains heterogeneous non-tumoral cell populations that include both innate and adaptive immune cells (Grivennikov et al., 2010). These immune cells have an important role in tumor development since they produce growth factors, cytokines, chemokines, prostaglandins and ROS (Barcellos-Hoff et al., 2013). Tumor-promoting agents, including CXCR4/SDF1α signaling and Gremlin-1-expressing mesenchymal stem cells (MSCs) that stimulate the recruitment of cells implicated in inflammation. Among these, immature myeloid cells and carcinoma-associated fibroblasts positive for α-smooth-muscle-actin are of special interest (Quante et al., 2011). Tumor associated-macrophages (TAMs) are also present in the tumoral niche during all the stages of tumor progression. Through the secretion of growth factors, chemokines, interleukins, enzymes and other mediators, these cells enhance the proliferation and invasion of cancer cells, promote angiogenesis, and trigger immunosuppressive effects that prevent the attack of natural killer and T-cells (Noy and Pollard, 2014).

The tumor microenvironment is particularly rich in pro-inflammatory molecules, mainly cytokines. Cytokine receptors are present both bound to the cell membrane and in soluble form. They are transduced through G-proteins and regulate cell behavior including chemotaxis, growth, differentiation, and immune stimulation or immune suppression. Depending on the type of cytokine,

they can promote or inhibit cancer progression and metastasis (Dranoff, 2004). Chemokines are group of over 50 chemotactic cytokines with only 18 chemokine receptors, implying overlapping in ligand-receptor specificity. For cancer, the most important is CXCR4, a receptor with low expression in most healthy tissues, but overexpressed in many highly metastatizing tumors (Balkwill, 2004)(Kang et al., 2005). The interaction of the CXCR4 ligand (CXCL-12) produces the activation of migratory, proliferative and survival signaling pathways (Wicha et al., 2006). Preclinical studies in several murine cancer models have shown that blocking these receptors reduces metastasis and tumor invasion (Liang et al., 2004)(Kakinuma and Hwang, 2006).

Other important cytokines present in the tumor niche are interleukins (IL) (Dranoff, 2004), tumor necrosis factor-α (TNF-α) and interferons (IFNs) (Grivennikov et al., 2010)(West et al., 2015). These proteins have very diverse functionalities that result in pro- or antitumoral effects, depending on the specific cytokine and the concomitant signaling activated.

Disconnecting the tumor immunosuppressive environment is a critical step to rescue immune responses against tumors, and is now considered a major strategy to fight cancer including metastasis. The main drug delivery strategies to reactivate immunity in the tumor are described below and are classified in: (i) biomaterials as “danger signals”, (ii) cytokine delivery systems, (iii) biomaterials that inhibit chemotaxis and (iv) integrative systems.

3.4.1 Biomaterials as "danger signals"

The integration of "danger signals" in biomaterials, i.e. toll-like receptor (TLR) ligands, is a method to stimulate the immune system. The presentation of such ligands can revert TAM polarization to an immunostimulatory phenotype and provide effective antitumoral cytotoxic responses through CD8+ T-cell activation. The most used "danger signal" is lipopolysaccharide (LPS) but others such as PolyI:C or CpG are also widely reported (Ali et al., 2014)(Goldberg, 2015).

PLGA and polyurethan-urea nanoparticles have been used to integrate TLR ligands tyrosine related protein 2 (TRP2), 7-acyl lipid A or LPS. The administration of these formulations *in vivo*, in a melanoma mouse model, resulted in antitumoral immune responses with recruitment of dendritic cells and increased secretion of pro-inflammatory cytokines (Hamdy et al., 2008)(Zhang et al., 2011). To produce a more localized immunostimulation with reduced systemic side effects, these particles can be targeted with antibodies against adhesion molecules present in inflamed endothelial cells (VCAM-1 and ICAM-1) (Morral-Ruiz et al., 2013). If a potent, direct antitumoral effect is desired, a drug such as paclitaxel can be co-encapsulated with a LPS to obtain a synergistic effect. *In vivo* studies in a melanoma mouse model showed that treatment with nanoparticles with this combined therapy achieved a 40% reduction in tumor size compared to mice treated only with Taxol®. A clear improvement in animal survival was also observed (Roy et al., 2013).

Cationic polymers such as cationic dextran and PEI are also capable of interacting with TLRs and shifting TAMs polarization, resulting in higher IL-12 expression. This effect reduces angiogenesis, produces immunoactivation, inhibits tumor progression, and ultimately, increases survival in murine cancer models (Huang et al., 2013).

3.4.2 Cytokine delivery systems

Cytokines have very short half-lifes *in vivo*, and therefore, it is critical to provide sustained levels to achieve a therapeutic effect. The two major ways to achieve these sustained levels are to use controlled release devices and/or to use gene therapies encoding these cytokines.

IL-12 is a cytokine that can produce tumor regression by enhancing natural killer and cytotoxic T-lymphocyte activity. Due to this potent activity, IL-12 has been encapsulated in controlled release systems. Liu et al. designed implantable biodegradable gelatin hydrogels for subcutaneous delivery. This formulation showed controlled release of IL-12 over 12 days and efficient suppression of colon carcinoma growth in mice (Liu et al., 2003). Chitosan and cholesterol-bearing pullulan nanoparticles have also been used for the controlled release of IL-12. These formulations showed low toxicity, capacity to induce antitumoral immunity, and the capacity to target metastasis in colon carcinoma murine model (Shimizu et al., 2008)(Q. Xu et al., 2012).

IL-12 delivery is also interesting for combination therapy. For example, PLA microspheres loaded with IL-12, TNF-α and granulocyte-macrophage colony-

stimulating factor (GM-CSF) were used as antitumoral therapy in a murine breast cancer model. The microspheres were administered by intratumoral injection, where they initiated a major infiltration of polymorphonuclear cells and CD8+ cytotoxic T-cells that resulted in increased the number of tumor free mice at the end of the study. This therapy also resulted in specific memory T-cells that could prevent tumor relapse in a murine model of breast cancer (Sabel et al., 2004).

In another combined therapy case, Park el al. designed a liposome-type carrier loaded with IL-2 and the TGF-β antagonist SB505124. The concept was to combine IL-2 immunostimulation and SB505124 blockage of TGF-β mediated immunosuppression. These carriers were based on an innovative concept where both drugs were encapsulated in a photopolymerized nanogel, and this was subsequently coated by a PEGylated lipid bilayer. The liposome-type formulation was able to increase the cytokines´ half-life in circulation, to induce innate immunity and to inhibit tumor growth in melanoma-bearing mice (Park et al., 2012).

Nanoparticles are also frequently used for gene therapies aimed at inducing local cytokine expression due to their capacity to stabilize plasmids and improve their intracellular delivery. Examples of polymeric biomaterials used to improve the transfection of IL-12 encoding plasmids are the amphiphilic block copolymers poly[α-(4-aminobutyl)-L-glycolic acid] (PAGA) (Maheshwari et al., 2000) and poly(N-methyldietheneamine sebacate) (PMDS) (Wang et al., 2006). The general idea was validated by Maheshwari et al. who encapsulated IL-12 coding plasmids in PAGA nanoparticles. The formulation showed activation of host

immunity and an antitumoral effect in a colon adenocarcinoma mouse model, an effect which could not be observed with the naked plasmid (Maheshwari et al., 2000). In another study, PDMS-nanoparticles were used for combined therapy comprising an IL-12 encoding plasmid and paclitaxel. The results indicated a synergistic effect of the active agents, where enhanced tumor sensitivity to paclitaxel was achieved after a reduced number of administrations (Wang et al., 2006).

Another cytokine of interest for T-cell activation and cancer treatment is IL-18, and a nanoemulsion-based gene delivery strategy for sustained expression of this cytokine has already been reported. This nanoemulsion formulation was composed of the standard cationic lipids DOPE and DOTAP, the PEGylated surfactant Tween 80 and different oils. In a lung tumor mouse model, this formulation generated a stable system with higher transfection activity and higher capacity for T-cell activation than the commercial agent Lipofectamine (Kang et al., 2009).

3.4.3 Biomaterials that inhibit chemotaxis

Another important strategy to treat the inflammatory niche is to inhibit chemokine activity. Due to its relevance in many tumors, silencing CXCR4 expression is a particularly interesting idea where interfering RNA strategies can play an important role. Abedini et al. associated CXCR4-siRNA to dextran-spermine nanoparticle. This formulation was selected for its beneficial efficacy/toxicity ratio as compared to other reference materials (i.e. PEI and

DOTAP/cholesterol) tested in the same study (Eliyahu et al., 2005). A *in vivo* test performed in a colon carcinoma mouse model showed that these siRNA nanoparticles were able to produce improved CXCR4 inhibition as compared to the naked siRNA (Abedini et al., 2011)(Abedini et al., 2012).

Another approach to inhibit CXCR4 is to use the synthetic drug antagonist perixaflor. Misra et al. designed PLGA-acrylate nanoparticles for the controlled release of this drug. The formulation has shown effective receptor inhibition *in vitro*, with a better dose/response curve than the free molecule (Misra et al., 2015).

3.4.4 Integrative systems

Particularly powerful devices can be designed when biomaterials are used to integrate some of the elements described in the previous sections in a rational manner. An early example of this integration was provided by Hori et al. who included activated dendritic cells and IL-15 in an alginate gel for peritumoral injection in a mouse melanoma model. The matrix was able to reduce tumor size and improve survival (Hori et al., 2009).

Some of the best devices for integrative immunomodulation, often involve the combined presence of "danger signals", cytokine release and antigen presentation, all integrated in scaffolds that provide a cellular context. Ali et al. prepared PLGA-scaffolds loaded with GM-CSF, tumor cell lysates and CpG-ODN. This scaffold produced an inmunostimulatory response that increased recruitment of CD8+ T-cells and production of proinflammatory cytokines in the

tumor and dendritic cells at the vaccination site. The device achieved a complete regression of the tumors and enhanced survival of mice bearing established melanomas. Finally, this study confirmed the synergic effect of the three elements of the immunomodulatory material (Ali et al., 2009). Afterwards, similar results were obtained with another prototype where CpG-ODN was changed for poly(I:C). In this study, the effect of the integrated system was compared with the intratumoral injection and the injection of the free components in a rat glioma model. Only the immunomodulatory material achieved complete tumor remission and improved mice survival. This result clearly indicates that the scaffold is not a passive substrate, but rather a part of immunomodulatory microenvironment that can surveyed by immune cells (Ali et al., 2011).

Outlook and conclusions

While there have been considerable advances in oncology over the last decades, several types of cancer still present very low survival rates. Many of these are the cancers where a tumor initiating cell subpopulation with stem cell-like has been reported. Despite the existence of this population, therapies that where under clinical development in the last decade failed to recognize the importance of cancer stem cells (CSCs), while they also neglected the importance of the main traits of the CSC niche including abnormal vasculature, hypoxia, ECM-dysregulation and inflammation.

In the last years, the interest on CSCs has translated from the molecular biology laboratories to pharmaceutical industry and drug delivery science. Together with this change, there has been a wider recognition of the importance of the tumor niche that supports these CSC features. This has resulted in a very high number of therapies in clinical trials that are directed to CSCs for solid tumors, and even some therapies in clinical practice for leukemia. Many of these therapies, however, might face important challenges before they can advance towards medical use. Their main limitations are due to their sub-optimal delivery characteristics and the unmet necessity to be integrated in combined therapies, together with regulatory and industrial challenges.

Delivery issues for these of therapies are important because many dysregulate signaling pathways or environmental features that, although critical for CSCs and their niche, can also affect other important populations such as non-cancer stem cells. Thus, side-effects for these therapies are almost inevitable, and delivery systems capable of improving selective drug biodistribution towards tumoral cells are a must. Additionally, many of these therapies combine this fine biodistribution requirements with fast degradation times. Such characteristics call for controlled release systems with active targeting, or if cell spreading can be neglected, to regional delivery using controlled release devices. This last concept has been the basis in CSC-specific strategies such as the use of controlled release microspheres for the regional delivery of BMP-7 in the treatment of glioblastoma (Reguera-Nuñez et al., 2014). On the other hand, gene therapy could be important to manipulate CSCs signaling pathways (Hedgehog,

Wnt, etc.) or effectors of environmental cues such as HIF. Gene therapy in the context of CSC treatment shares most of its challenges with other *in vivo* applications, with the provision that the nanotherapeutics need to reach the CSCs in their niche. Considering that CSCs are a minor part of the tumor, and that they are often in its most inaccessible regions, this becomes and additional difficulty. In general terms, any therapy that is not capable of being transported through the tumor ECM or that binds tumor cells non-selectively, will either be retained at the periphery of the tumor or will be absorbed by terminally differentiated cancer cells acting as sacrificial barriers.

Another future challenge of future therapies against CSCs and their niche is their necessity to be integrated in combined therapies to have substantial effects. Since cellular processes are typically redundant, any effect on a targeted pathway might be compensated by additional activation of other pathways. For drug combinations to be effective, however, it is critical that there is mechanistic cooperation or synergism between the two agents. Acute promyelocytic leukemia provides a clinically successful example of how a very potent differentiation agent (ATRA) would be ineffective without a pro-apoptotic inducing agent, since the cells would become resistant to this medication in a short time span. When combined with a proapoptotic agent, the cells differentiated by ATRA are easily removed before any resistance is generated. Such roles of combination with ATRA were traditionally provided by anthracycline-based agents, and since 2013, by arsenic trioxide that combines a pro-apoptotic with a further pro-differentiation effect.

Because of their intrinsic flexibility, biomaterial-based devices are ideal platforms for combined therapies. For instance, Sun et al. have taken advantage of a rationally selected drug combination (ATRA and doxorubicin) and a suitable delivery platform to deliver this combined therapy (Sun et al., 2015). A more sophisticated example of the use of biomaterials to maximize the therapeutic value of drug combinations is provided by the work of Mooney´s group, which combines anticancer vaccines, TLR agonists and controlled release of cytokines to design potent immunomodulatory materials. However, a critical aspect of these technologies is that the diverse elements are integrated within the context of a polymeric scaffold that helps to generate a regional environment. The effects of these elements are thereby focalized while providing a mechanical and spatial context for the recruited immune cells (Ali et al., 2014). Because of their focus on stem cell differentiation, modulation of stem cell signaling and mimicking the cell microenvironment, tissue engineering devices can find surprising new applications in cancer suppression as it has been illustrated through some examples in this review.

A final challenge for therapies directed to CSCs and their niche could come from regulatory authorities and industry. For these actors, an important issue would be identifying experimental outcomes during screening and preclinical experimentation that correlate with positive clinical endpoints. Cytotoxicity in cell panels and reduction of tumor volume are routinely used for anticancer drug screening, but are not necessarily indicative of CSC elimination and survival. Also, tumor niche modulation might not be able to eliminate the tumor by itself,

but could have instrumental effects in well-chosen combination therapies. Identifying and measuring the effects of these combinations at early stages and selecting combined therapies with great potential from less interesting ones, could prove to be difficult.

Addressing cancer by suppressing the CSCs and their supporting niche is still far from being a validated general strategy. In the next years, there will be more data available from clinical trials of drug candidates aimed at addressing some of these relatively new aspects of tumor biology. We expect, however, that higher benefit will be achieved with combined therapies that address simultaneously the CSC phenotype and the niche, and probably, in the presence of another drug capable of eliminating differentiated tumor cells. Despite the open questions, it is already clear that the CSC phenotype and the tumor niche are essential features of cancer, and thus, it is reasonable to expect that addressing them will be essential for future therapies. In this sense, the design of devices capable of integrating these strategies in a coordinated and synergistic fashion could be critical to achieve maximum benefits.

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Conflict of interest

The authors declare no conflicts of interest.

Author Contribution

CGM and NC performed the bibliographic search. MGF designed the paper outline. All authors contributed to data analysis, writing and revision of the manuscript.

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Supporting Information

Table S1: Overview of the drugs in clinical trials against CSCs and/or their niche. Only trials from years 2011-2016 and in phase III or higher are presented. Source: ClinicalTrials.gov (www.clinicaltrials.gov).

Intervention	Clinical phase	Condition	Mechanism of action	Number
Apatinib	Phase III	Non-squamous non-small-cell lung cancer	VEGFR inhibitor	NCT 02824458 NCT 02332512
		Small cell lung cancer		NCT 02875457
		Hepatocellular carcinoma		NCT 02329860
		Gastric cancer		NCT 01512745 NCT 02537171
	Phase IV	Gastric cancer		NCT 02426034 NCT02776527
Regorafenib	Phase III	Colorectal cancer	VEGFR inhibitor	NCT 02664077 NCT 01584830 NCT 01853319 NCT 01939223
		Hepatocellular carcinoma		NCT 01774344
Axitinib	Phase III	Clear cell renal carcinoma	VEGFR inhibitor	NCT 01599754
Aflibercept	Phase III	Colorectal carcinoma	VEGFR inhibitor	NCT 01661270 NCT 01571284 NCT 01670721
Ramucirumab	Phase III	Hepatocellular carcinoma	VEGFR inhibitor	NCT 02435433
		Gastric and esophageal adenocarcinoma		NCT 02314117
		Non-small cell lung cancer		NCT 02411448
		Urothelial carcinoma		NCT 02426125
Bevacizumab	Phase III	Glioblastoma	Monoclonal antibody againstVEGF	NCT 01290939 NCT 02017717
		Breast cancer		NCT 01663727
		Non-small-cell lung cancer		NCT 02272413 NCT 01661790 NCT 01966003
		Non-squamous non-small-cell lung cancer		NCT 01364012 NCT 02633189 NCT 01763671 NCT 02366143
		Colorectal carcinoma		NCT 01351415 NCT 01878422 NCT 02563002 NCT 02394834 NCT 02314182 NCT 02162563 NCT 02934529
		Ovarian cancer		NCT 01462890 NCT 01802749
		Renal cancer		NCT 02420821
	Phase IV	Non-small-cell lung cancer		NCT 02316327
		Colorectal carcinoma		NCT 02582970 NCT 01972490 NCT 01695772
		Ovarian cancer		NCT 01706120
		Solid tumors		NCT 01588184
Gefitinib	Phase III	Advanced pulmonary adenocarcinoma	VEGFR and EGFR inhibitors	NCT 02929693 NCT 02889692
		Non-small cell lung cancer		NCT 02893332 NCT 02882984 NCT 02859077

				NCT 01405079 NCT 01774721 NCT 00322452 NCT 02588261 NCT 01404260 NCT 01544179 NCT 02714010 NCT 00322452 NCT 02296125 NCT 02824458 NCT 02518802 NCT 01024413 NCT 02031601
		Non-squamous non-small cell lung cancer Lung neoplasms Thoracic neoplasm		
	Phase IV	Non-small cell lung cancer		
Sunitinib	Phase III	Renal cell carcinoma	VEGFR and EGFR inhibitors	NCT 02535351 NCT 02231749
	Phase IV	Renal cell carcinoma Pancreatic neuroendocrine tumor		NCT 02555748 NCT 01525550
Nintedanib	Phase III	Non-small-cell lung carcinoma Colorectal carcinoma	VEGFR, PDGFR and FGFR inhibitor	NCT 02231164 NCT 02149108
Sorafenib	Phase III	Hepatocellular carcinoma	VEGFR and PDGFR inhibitor	NCT 01482442 NCT 02576509 NCT 01761266 NCT 02187081 NCT 01887717 NCT 02436902
		Renal cell carcinoma		NCT 01613846 NCT 02627963
		Leukemia cutis and myeloid sarcoma		NCT 01371981
	Phase IV	Hepatocellular carcinoma Acute myeloid leukemia Renal carcinoma		NCT 02504983 NCT 02474290 NCT 01339962 NCT 01728948
Pazopanib	Phase III	Renal Cell Carcinoma Sarcoma	VEGFR and PDGFR inhibitor	NCT 01575548 NCT 02049905
	Phase IV	Renal Cell Carcinoma		NCT 01521715
Motesanib	Phase III	Non-small cell lung	VEGFR and PDGFR inhibitor	NCT 02629848
Masitinib	Phase III	Gastro-intestinal stromal tumour Colorectal cancer	VEGFR and PDGFR inhibitor	NCT 02009423 NCT 02605044
Erlotinib	Phase III	Non small-cell lung cancer	EGFR inhibitor	NCT 01652469 NCT 00874419 NCT 01360554 NCT 01342965 NCT 02411448 NCT 01523587 NCT 02178397 NCT 02031744 NCT 01487174 NCT 02134015 NCT 02140333 NCT 01887795 NCT 02352948 NCT 02152631 NCT 02193282

		Non squamous non-small-cell lung cancer		NCT 02588261 NCT 02296125 NCT 02588261 NCT 01328951 NCT 01351415 NCT 00883779 NCT 02633189 NCT 01456325 NCT 01887886
	Phase IV	Non small-cell lung cancer		NCT 02031601 NCT 01402089 NCT 01287754 NCT 02000531
		Non squamous non-small-cell lung cancer Lung adenocarcinoma		NCT 01609543 NCT 02399566
Icotinib	Phase III	Non-small-cell lung-cancer	EGFR inhibitor	NCT 02714010 NCT 01719536 NCT 02448797 NCT 02486354 NCT 01724801 NCT 02125240
		Lung adenocarcinoma		NCT 01996098
	Phase IV	Non-small-cell lung-cancer		NCT 01926171 NCT 01465243 NCT 02778893 NCT 01665417 NCT 02404675 NCT 01646450 NCT 02031601 NCT 02103257 NCT 02194556
		Lung adenocarcinoma		NCT 02283424
Afatinib	Phase III	Non-small-cell lung carcinoma	EGFR inhibitor	NCT 01121393 NCT 00949650
Osimertinib	Phase III	Non-small cell lung carcinoma	EGFR inhibitor	NCT 02511106 NCT 02474355 NCT 02474355 NCT 02151981 NCT 02454933
Cetuximab	Phase III	Colorectal cancer	Monoclonal antibody against EGFR	NCT 01309126 NCT 02934529 NCT 02484833 NCT 01878422 NCT 02563002 NCT 01910610
		Squamous Cell Head and Neck Cancer		NCT 01810913 NCT 02383966 NCT 02551159 NCT 02236936 NCT 02105636 NCT 01884623 NCT 01969877 NCT 02252042 NCT 02358031

	Phase IV	Oropharyngeal Squamous Cell Carcinoma Nasopharyngeal Carcinoma Colorectal cancer Squamous Cell Head and Neck Cancer		NCT 01855451 NCT 01302834 NCT 01874171 NCT 02633176 NCT 00327093 NCT 01315990 NCT 02015650
Everolimus	Phase III Phase IV	Advanced neuroendocrine tumors Renal cancer Breast cancer Pancreatic tumor Renal cancer Breast cancer Neoplasms	mTOR and HIF inhibitor	NCT 01524783 NCT 01668784 NCT 01865747 NCT 01773460 NCT 01805271 NCT 02511639 NCT 02137837 NCT 01626222 NCT 01674140 NCT 02404051 NCT 02246127 NCT 01514448 NCT 02056587 NCT 01206764 NCT 02338570 NCT 01948960 NCT 01743560 NCT 02248571 NCT 01789281
Nimorazole	Phase III	Head and neck carcinoma	Inhibit glycolysis and the repair of radiation-induced cellular potentially lethal damage in hypoxic niche	NCT 01507467 NCT 01880359 NCT 01950689
ATRA	Phase III Phase IV	Acute myeloid leukemia Non-small cell lung carcinoma Acute promyelocytic leukemia	Expression of genes implicated in the differentiation	NCT 01237808 NCT 01067274 NCT 00151255 NCT 00599937 NCT 00146120 NCT 01226303 NCT 00482833 NCT 00504764 NCT 02200978 NCT 00504764 NCT 00465933 NCT 00408278
Sulindac	Phase III	Colorectal adenocarcinoma	Inmunomodulador	NCT 01349881
Imiquimod	Phase III	Nodular basal cell carcinoma Intraepithelial neoplasia	Hedgehog pathway inhibitor and immunostimulator	NCT 02242929 NCT 02329171 NCT 01283763 NCT 01861535 NCT 02059499