The potential of nanomedicine to alter cancer stem cell dynamics: the impact of extracellular vesicles

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The presence of highly resistant cancer stem cells (CSCs) within tumors as drivers of metastatic spread has been commonly accepted. Nonetheless, the likelihood of its dynamic phenotype has been strongly discussed. Importantly, intratumoral cell-to-cell communication seems to act as the main regulatory mechanism of CSC reversion. Today, new strategies for cancer treatment focusing into modulating tumor cell intercommunication and the possibility to modulate the composition of the tumor microenvironment are being explored. In this review, we summarize the literature describing the phenomenon of CSC reversion and the factors known to influence this phenotypic switch. Furthermore, we will discuss the possible role of nanomedicine toward altering this reversion, and to influence the tumor microenvironment composition and the metastatic spread of the disease.

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Cancer stem cell models (the hierarchic model, stochastic model & dynamical tumor initiation model)

Remarkable similarities between embryonic development and tumor growth were acknowledged as early as the 19th century [1]. Later, in 1953, Dixon and Moore postulated for the first time that tumor cells possess characteristics similar to germinal cells and concluded that, in most cases, undifferentiated cells are the origin of tumor development [2]. However, the real breakthrough regarding the cancer stem cell (CSC) theory of tumor development came 40 years later from studies on the origin of acute myeloid leukemia [3]. Those studies demonstrated that only few rare cells (CSCs) were capable of initiating leukemia in mice. A strong resemblance of these cells with hematopoietic stem cells (HSCs) was also shown. They expressed typical HSC markers ($\text{lin}^- \text{CD34}^+ \text{CD38}^-$) and showed high capacity for self-renewal and differentiation. Notably, tumors that appeared after the *in vivo* transplantation of only few CSCs in mice were composed of a mixture of cells that was very similar to the one found in the initial tumor [3,4]. Since the first description of leukemic stem cells, CSCs have been identified in many types of cancers, including solid tumors [5–9]. Ever since, the CSC theory has been constantly modified based on growing knowledge, mostly disputing CSC characterization principles and the basis of tumor initiation [10–13].

The CSC model was originally referred to as a hierarchic model in which cancer cells originate from a precursor stem cell (CSC). This precursor cell divides either symmetrically, giving two identical CSCs or two differentiated cancer cells (DCCs), or asymmetrically, giving one CSC and one DCC [3]. Accordingly, the distinctive ability of these cell types regarding tumor initiation has been considered as an indispensable feature to distinguish CSCs from DCCs [3,14,15]. The presence of CSCs that fits into the hierarchic model has been demonstrated in some models of breast cancer where as few as a hundred CSCs were able to form tumors in mice, whereas tens of thousands of cells

with alternate phenotypes failed to form tumors [16]. Additional studies on brain and colon cancers also identified such subpopulations of rare cells that produce tumors *in vivo* [17,18].

In this context, even though clinicians often observe tumor shrinkage at the first stages of multimodal treatment, often remaining resistant clones of CSCs survive and eventually cause tumor relapse and very aggressive tumor types with limited treatment alternatives [19–21]. According to the hierarchic model, only the complete abolition of CSCs can eradicate a tumor [22,23]. Thus, considerable efforts have been invested to find what distinguishes CSCs from normal stem cells and/or DCCs, to allow specific therapeutic targeting of CSCs without affecting normal cells. However, it has not been possible so far to find a marker that would solely identify cancer cells with 'stem' characteristics in distinctive tumors and tumor cell lines. Most markers identified in some cell lines that segregated cells with tumor-initiating capacity were not confirmed in other cell lines, even from the same origin. Several CSC markers have been discarded since cells expressing them have not been able to show higher tumor-initiating capacities than other tumor cells. Often, identified markers distinguish cells of mesenchymal characteristics (i.e., $CD44^+/CD24^-$ in breast cancers) that confer tumor cells' more aggressive potential and the ability to induce metastatic growth. These markers, however, are not useful in tumor cell lines of mesenchymal origin. Some additional inconsistencies of the hierarchic model came from the observation that in some cases, even if CSCs were successfully eradicated, tumor growth and spread was evident [24,25]. The original CSC hypothesis has been further challenged by studies demonstrating that in some leukemias, more mature progenitor cell types or even cells expressing lineage markers can serve as leukemia-initiating cells as well [26–28].

These discrepancies raise the possibility that tumor-initiating cells might also come from differentiated cells that reacquire self-renewal capabilities [29]. Accordingly, an alternative stochastic model of tumorigenesis has been suggested. This model postulates that under a permissive environment, every cancer cell has the ability to self-renew and can recapitulate the phenotype of the disease because of the possibility of interconversion among cell phenotypes [30–33]. Indeed, under stress conditions or after eradication of the CSC repository, some DCCs can switch their phenotype into a stem-like phenotype to ensure tumor survival and progression [15,30,34–36].

In cell cultures in attachment conditions, CSCs divide asymmetrically, reaching a final steady state with a stable CSC population rate characteristic for each cancer cell line. In addition, when DCCs are plated alone, CSCs appear after a few days in culture to regenerate the CSC population [24]. In low attachment conditions, where DCCs are not able to survive, these cells can also undergo de-differentiation to ensure survival. However, when cell culture conditions are again favorable (i.e., in attachment), the equilibrium of CSCs versus DCCs is re-established. This is accompanied by an adjustment of the stemness gene expression and invasive potential of these cells. Accordingly, de-differentiated cells in low attachment conditions rapidly express high levels of CSC genes, whereas in attachment conditions, the stemness expression signature of these DCCs is reverted. Similarly, cells cultured in low attachment are more invasive [24]. DCC to CSC reversion can be also observed *in vivo* [24,37]. There is evidence that intestinal epithelial cells and hepatocytes have CSC potential in colon and liver cancer models, respectively [24,38,39].

Of note, this plasticity process seems much more common than previously believed. In fact, a similar feature also occurs with healthy differentiated cells, which are known to reach a stemness state under traumatic conditions [40].

Importantly, since tumors are composed of various cell types and not only of cancer cells, the interconversion does not affect only DSCs and CSCs. In fact, a complex tumoral microecosystem that includes immune cells and stromal cells exists within a tumor, forming the so-called tumor microenvironment (TME) [41]. Cancer cells directly interact with other cells within the TME through paracrine signaling and/or direct cell-to-cell contact signaling to create a favorable physical and molecular signaling environment, to ensure tumor progression [42].

Cancer cells interact with other cells through a combination of mechanical interactions, enzymatic modification of the structure of the extracellular matrix (ECM) and signaling interactions, all of which have an effect on tumor progression and the dissemination of cancer cells (Figure 1) [43,44]. To acknowledge all of these important players, recently a new dynamical tumor initiation model for a mixed population has been proposed. It involves CSCs, DCCs and other cell types, such as quiescent cells, immune cells and fibroblasts, among others (Figure 1) [45]. In this dynamic model, each cell population becomes a nonlinear dynamical system based on its own proliferation rates controlled by chemical and/or biological pathways, and physical interactions. The dynamics are governed by the possibility of full tumor extinction, but also of overshooting dynamics after a period of remission or postsurgical relapse [45]. Importantly, implementation of the realistic complexity of tumor growth regulation changed the view of its effective eradication. Based on the dynamical tumor initiation model, the most promising strategies to fight cancer spread should be the ones that target the activator of the CSC phenotype rather than CSCs because of the feedback loops between CSCs and DDCs. New therapeutics (including nanomedicines) should thus emerge in

Figure 1. Dynamic cancer stem cell model. A tumor is a highly complex heterogenic dynamic entity that involves cellular components (CSCs, DCCs, fibroblasts, immune cells, mesenchymal cells, etc), cell-secreted functional molecules (growth factors, hormones, metabolites, cytokines, chemokines, etc), EVs (exosomes, vesicles, etc) and the extracellular matrix. Cancer cell subpopulations (CSCs, DCCs, PCCs) within tumors are characterized by their respective densities and rates, and the alteration of these densities and rates alters the equilibrium state of the tumor as a whole biological system. Self-renewal and differentiation rates of cancer cells are finely tuned by all tumor microenvironment players (fibroblasts, immune cells, mesenchymal cells, the extracellular matrix) to ensure tumor progression. CAF: Cancer-associated fibroblast; CSC: Cancer stem cell; DCC: Differentiated cancer cell; EV: Extracellular vesicle; PCC: Pluripotent cancer cell.

this direction [46,47]. With numerical simulations, the model highlights how modifications of the environment, in which the tumor expands, can abruptly change its long-time behavior [45].

Regulation of phenotypic dynamism via intratumoral communication

A role for extracellular vesicles

A tumor should be regarded as a highly complex heterogenic dynamic entity that evolves over time, best adapting to its local microenvironment and the immune system to ensure its survival. Cell-to-cell interactions, affecting CSCs' dynamic phenotype and tumor immunological response, should thus be carefully considered when proposing new anticancer nanomedicine-based therapies. Indeed, tumors from patients with recurrent resistant tumors show higher numbers of CSCs [48]. This clearly suggests the need to develop new effective treatments targeting CSCs, since CSCs account for therapy resistance and represent a cellular depot responsible of tumor regrowth and its metastatic spread [11]. Moreover, modifiable immune cells at the niches also have an important role in tumor spread, cell adaptability and survival [49].

Nonetheless, the exact molecular mechanisms that control the dynamic phenotype of CSCs still remain widely unknown. In melanoma, maintenance of the slow-cycling (JARD1B-positive) stem cell subpopulation has been proved to be dynamic. Sorted JARD1B positive cells (CSCs) gave a rise to JARD1B-negative cells, and, conversely, a single JARD1B-negative cell (DCC) originated in a heterogeneous progeny including JARD1B-positive cells [53]. Similarly, in breast cancer, it was demonstrated that basal-like mammary epithelial cells are able to spontaneously de-differentiate into stem-like cells [54]. This further reinforces the hypothesis of the existence of a controlled balance

between CSC and DCC populations, and suggests that cancer cells opt for de-differentiation as a survival mechanism under stress conditions (i.e., chemotherapeutic treatments) [30,34]. In fact, it has also been shown that several external factors, cell interactions and paracrine cell-to-cell communication are involved in the regulation of this process. As an example, in bladder cancer, prostaglandin E2 that is secreted by apoptotic cancer cells after chemotherapy promotes proliferation of adjacent CSCs [55]. Similarly, in breast cancer, IL-6 is secreted by DCCs to induce CSCs, to maintain this dynamic equilibrium via IL-6-JAK1-STAT3 signaling [56]. Further, various aspects like cell pluripotency, invasion, proliferation, differentiation, tumor recurrence and resistance to apoptosis/treatment, among others, are regulated by constant paracrine cell-to-cell communications in both tumors and metastatic sites [13,57]. Late evidence suggests that this communication is at least partially mediated by EVs (Figure 2).

EVs encompass a very heterogeneous population of lipoproteic vesicles that are released into the extracellular space [58]. The main subtypes of EVs are microvesicles (MVs), exosomes and apoptotic bodies. EV subtypes differ substantially in their origin and biogenesis and thus also in their composition and size. MVs are formed by direct outward budding of plasma membrane, whereas exosomes are formed by an inward budding of the early endosomes. The maturation into multivesicular bodies is necessary during this process. However, apoptotic bodies are formed by a separation of the plasma membrane from the cytoskeleton as a result of an apoptotic process, during which dying cell contract due to increased hydrostatic pressure [59].

Although specific features have been proposed for each EV subpopulation, the identification of specific markers of each subgroup is still under debate [60]. Recently, the minimal feature requirements regarding EV characterization and a guideline to standardize studies performed with EVs have been summarized and consented [61]. Exosomes, MVs and apoptotic bodies are currently categorized based on size (small, medium or large), density (low, medium or high) and some specific markers (Alix, TSG101, HSC70 and HSP90 β) [61].

Due to their biogenesis, EVs may contain a high variety of molecular cargo of their cell of origin, such as DNA, proteins, mRNA, microRNA and lipids, which can affect multiple signaling pathways inside target cells [62]. Subsequently, EVs may trigger phenotypic changes after the interaction with their target cells, whereas single molecules that bind to a single receptor at the cell surface would often activate a single molecular pathway. Indeed, tumor cells secrete EVs bearing immunosuppressive molecules and facilitate the immunogenic escape of a growing tumor [63]. For these reasons, EVs are now considered as leading actors of the intercellular communication signaling, mediating both physiological and pathological responses.

Within EVs, big interest is centered toward exosomes, as it was shown that exosomes participate in cell-to-cell communication, cell maintenance and tumor progression. In addition, exosomes have been found to stimulate immune responses by acting as antigen-presenting vesicles [58,64]. Importantly, the contents of the exosomes are not a random array of signaling molecules from undergoing intracellular events but a specific set of proteins, genetic information and lipids that essentially come from the plasma membrane and the cytosolic compartment, with very limited amounts of compounds from other intracellular compartments, such as the nucleus, the endoplasmic reticulum and the Golgi apparatus. This makes exosomes clearly different from other membrane vesicles (i.e., vesicles released by apoptotic cells). Thus, the so-called hEXO motif (an RNA-binding protein) that is responsible for miRNA sorting toward exosomes via direct binding has been recently identified [65]. Further, Wani and Kaul reported that miR-2909 is packaged in tumor exosomes by a specific 30-end post-transcriptional modification of the miRNA [66]. However, knowledge regarding exosome constitution is still missing, and strong efforts are currently devoted to unraveling how to target specific content to exosomes and EVs.

In 2007, a group led by Jan Ltvall revealed that mRNA and/or microRNA from EVs (exosomes) could be translated into proteins in target cells, providing the first demonstration of a genetic information cell-to-cell transfer mediated by exosomes [67]. Equally, EVs released by embryonic stem cells can reprogram hematopoietic progenitors by horizontal transfer of specific mRNA and proteins. Comparable ‘information exchange’ represents one of the critical components of conditioning premetastatic niches and is involved in the control of CSC self-renewal and tumor expansion. Released EVs can directly stimulate surrounding cells by interacting with specific surface receptors or transferring from one cell to another various bioactive molecules, including membrane receptors, proteins, mRNA, microRNA and even whole organelles (Figure 1).

The colonization by metastatic cells requires the existence of a hospitable microenvironment in distal sites that allows an adequate hosting of the metastatic arriving cells, fostering their survival. Accordingly, EVs derived from tumor cells can remain at the extracellular space, can remain at the site of origin or can enter into biological fluids, reaching distal sites and participating in the creation of premetastatic niches. Moreover, exosomes from other cells involved in the TME (i.e., fibroblasts, stromal cells, immune cells) influenced CSC–DCC balance in a bidirectional

paracrine setting (Figure 2). Fibroblasts have been reported to secrete diverse bioactive factors such as cytokines (IL-6, IL-8) that can mediate the conversion of DCCs into CSCs [68]. Further, the communication between tumor cells and stromal cells in the metastatic niche, mediated by EVs released by the primary tumor, has a critical role in facilitating cancer metastasis. It has been shown based on specific expression patterns of integrins and glycans that tumor-derived EVs are uptaken by tissue-specific cells to prepare premetastatic niches [69,70].

Furthermore, the resistance of tumor cells to chemotherapeutic treatments also involves cellular intercommunication mediated by EVs, as intratumoral CSC/DCC content is relevant to define the treatment response and recurrence of tumor. Released EVs evacuate pro-apoptotic proteins or directly withdrawn therapeutic drugs from cells. Indeed, resistant CSCs release significantly more EVs than cells responsive to antitumor therapy. Moreover, EVs also appear to favor CSC survival by reducing the responsiveness of T cells or directly inducing apoptosis in activated antitumor T cells [57]. In fact, fibroblasts and immune cells at the tumor niches are substantially involved in tumor spread, adaptability and survival [49]. Understanding the tight relationship among CSCs, the TME and the immune response is essential for developing effective anticancer nanomedicine-based treatments (Figure 2).

EVs as natural nanocarriers engaged in cell-to-cell intercommunication

Despite that nanomedicine is regarded as one of the most promising fields of research to bring new treatment strategies in cancer care, only very few (2%) nanocarrier-based treatments have successfully entered into clinical trials. Moreover, only a limited handful (15) of these formulations have reached clinical practice [4]. There are a variety of reasons for this still suboptimal translation from bench to bedside, like inadequate physicochemical features of nanomaterials, improper preclinical efficacy and safety, and regulatory and manufactory issues. Further, changes of the properties and complexity of the nanomaterials when in biological fluids (i.e., protein corona, cargo release, opsonization) are difficult to compute and significantly alter the functionality of nanomedicines after administration [71]. There are multiple factors that modulate the toxicity of nanomaterials, such as size, shape, porosity and surface charge. Another major hurdle is the feasibility of manufacturing nanomedicines under good manufacturing practices. Although most clinical and preclinical studies have been made from small amounts of materials, large-scale production requires great effort to maintain their physicochemical properties and release capabilities. Currently, a lot of effort is put into amelioration of large-scale synthesis (i.e., CELLLine Bioreactor Flask) that is required for the EV's clinical translation. The other option is the production of synthetic EV-based drug delivery systems (DDS) that could be produced as a large-scale homogeneous population with standardized protocols [72]. In this regard, EVs demonstrate clear prospects as nanocarriers, as they have a biological origin, little toxicity and lack of immunogenicity [73]. EVs are also very promising as delivery systems due to their capacity to directly release their cargo into the cellular cytoplasm of targeted cells. After internalization, EVs can also fuse with the endosomal membrane and release their content [58,64]. Their structure resembles that of liposomes, but their lipid layer has a more complex composition. They are characterized by the presence of multiple families of proteins such as tetraspanins (CD63, CD81, CD9), heat shock proteins (HSC70), lysosomal proteins (Lamp2p) and fusion proteins such as CD9, flotillin and Annexin (Figure 3). The complexity of their membrane composition may help EVs escape endosomes, thus allowing delivery of material into the nucleus, whereas artificial nanocarriers will require complex protein and peptide modifications [74]. However, EVs are known to be nonimmunogenic, which is certainly an advantage to reduce potential treatment resistance if using protein-based therapies, particularly as enzyme replacement therapies. Moreover, EVs might be the best option when dealing with nondruggable target candidates. Further, one of the challenges of drug delivery systems is to optimize their targeting properties to release the therapeutic compounds only in specific organs/tissues of the body, avoiding or reducing systemic toxicity. In this regard, there is strong data in the literature reporting preferential delivery of EVs (exosomes) to specific target tissues according to their integrin and glycan receptor profile of their membrane surface [69,70]. This is one of the reasons that might explain why EVs from different tumor types have a tendency to modify and condition specific distal tissues to host tumor cells as metastatic niches for each particular tumor type.

In this context, modifying EV-producing cells may lead to the design of EVs with adequate profiles that are required to target specific tissues. Previously, EVs donor cells were transfected with pDisplay™ encoding the transmembrane domain of platelet-derived growth factor receptor fused to a peptide that specifically binds to the EGFR. Subsequently, released EVs efficiently delivered miRNA to EGFR+ cancer cells [75]. Alternatively, to further enhance the display of targeting peptides on the EV's surface, a donor cell may be transected with the engineered vectors that contain a gene of a protein of the exosomal membrane (e.g., Lamp2b) fused with targeting peptides. EVs released from such modified cell lines will display the targeting peptides on their surface [76–78].

Figure 3. Structure of extracellular vesicles. Extracellular vesicles are lipid vesicles with a complex membrane composition. They express multiple families of proteins, such as tetraspanins (CD63, CD81, CD9), heat shock proteins (HSC70), lysosomal proteins (Lamp2p) and fusion proteins, such as CD9, flotillin and annexin, at the membrane. Within the inner core, they may contain mRNA, miRNA, proteins and organelles, among others.

This approach was first used by Alvarez-Erviti *et al.* to produce EVs displaying the neuron-specific RVG peptide on their surface [79]. Importantly, the authors demonstrated brain-targeting capability of the engineered EVs that delivered stable siRNA after systemic administration in mice. A similar approach has been used to deliver imatinib and siRNA to leukemic cells, using EVs with overexpressed IL-3 on their surface [80].

Another way to tailor-make EV surface targeting peptides is through membrane functionalization by covalent modification. Accordingly, this technique modifies directly isolated EVs rather than modifying their cells of origin. In this regard, EVs with both imaging and therapeutic properties by loading paramagnetic iron oxide nanoparticles and curcumin have been proposed for a glioblastoma theranostic approach. Targeting of glioma has been increased by adding the neuropilin-1-targeted peptide to the exosomal membrane by click chemistry [81]. Alternatively, the membrane of EVs was functionalized by a cationic lipid and pH-sensitive peptide (GALA) conjugation to improve EV cellular uptake and cytosolic release [82]. It was also demonstrated that it is possible to increase the specificity of EVs to target cancer cells through an RNA nanotechnology approach. To this aim, cholesterol-conjugated RNA aptamers harboring a specific targeting domain for cancer cells were developed. Resultant EVs conjugated with RNA nanoparticles harboring EGFR aptamers and loaded with survivin siRNA were able to inhibit breast cancer growth in mice [83]. Some examples of successful preclinical EV development are summarized in Table 1. Nonetheless, even though EVs have demonstrated clear prospects as natural delivery systems, many

Table 1. List of modified extracellular vesicles designed to alter the tumor microenvironment.

Extracellular vesicle source	Extrinsic cargo	Extrinsic targeting	Cancer type	Ref.
Bone marrow-derived MSCs	TRAIL	N/A	Various cancers	[84]
Various carcinoma cells	Doxorubicin, cisplatin, methotrexate	N/A	Various cancers	[85]
B16-F10 melanoma cells	SPIONs	N/A	Melanoma	[86]
LNCaP, PC-3 cancer cells	Paclitaxel	N/A	Prostate cancer	[87]
Immature dendritic cells	Doxorubicin	iRGD-Lamp2b	Breast cancer	[88]
Bovine milk	Paclitaxel	N/A	Lung cancer	[89]
B16BL6 melanoma cells	CpG DNA	N/A	Melanoma	[90]
HEK293T	Imatinib, BCR-ABL siRNA	IL3-Lamp2b	Leukemia	[76]
H22 hepatocarcinoma cells	Doxorubicin, 5-FU	N/A	Hepatocarcinoma	[91]
Raw 264.7 macrophages	Doxorubicin	DEAP	Colon cancer	[92]
HeLa cervical cancer cells	Dextran	GALA	Cervical cancer	[82]
Primary dendritic cells	VEGF siRNA	AS1411 aptamer (nucleolin targeting)	Breast cancer	[93]
Divers origins	SPIONs and curcumin	RGERPPR (neuropilin1 targeting)	Glioma	[81]
Macrophages	Paclitaxel	N/A	Lewis lung carcinoma	[94]
HEK293T	TPD52 siRNA	LAMP2b-DARPin G3 (HER2/Neu targeting)	Breast cancer	[78]
Mesenchymal stromal cells	Paclitaxel	N/A	Pancreatic cancer	[95]
Red blood cells	miR-125b-2, Cas mRNA	N/A	Leukemia, breast cancer	[96]
Plants (grapefruit)	miR-17, miR18s	N/A	Brain and colon cancer	[97,98]

MSC: Mesenchymal stem cell; N/A: Not applicable.

aspects of their stability, integrity and reproducibility need to be considered during scale-up production. In fact, the great interest and expectations toward the use of EVs as nanocarriers is hampered by a current low-production rate to high-production cost ratio. In addition, some regulatory aspects regarding the clinical use of modified EVs have not been entirely defined. Specific guidelines and regulation regarding EV isolation and characterization (content, surface, isolation method) is thus required to speed up the translation from bench to bedside of EV-based nanomedicines.

Nanomedicines to modulate the tumor microenvironment & reduce tumor spread

As mentioned earlier, the interaction between stromal and tumor cells within a specific TME plays a critical role in tumor growth and dissemination. Cancer cells require mechanical support, blood supply and growth factors secreted by stromal cells to guarantee continuous growth [99]. In fact, the initiation of tumor growth and metastatic growth is linked with the alteration of the healthy stroma and the appearance of a pro-tumoral environment (i.e., inflammatory signatures). This process includes neoangiogenesis, infiltration of fibroblasts and immune cells, and extensive remodeling of the ECM, and exhibits strong similarities to normal wound healing [100]. Cancer stroma thus not only supports but rather directs cancer progression and dissemination.

The most abundant cells of the tumor stroma are cancer-associated fibroblasts (CAFs), which significantly influence cancer growth and progression through TME control [101]. As mentioned previously, a bidirectional paracrine communication between CAFs and CSCs/DCCs is indispensable to maintain the required balance of cell populations within the TME. Of note, normal fibroblasts are activated into overproliferative CAFs by sonic hedgehog proteins that CSCs secrete into the niche. These in turn activate CAFs via upregulation of the WNT/hedgehog signaling pathway [102]. However, CAFs are the main source of multifunctional cytokines (IL-6, IL-8) that regulate CSC–DCC balance and neoangiogenesis within the TME [103–105]. Importantly, paracrine CAF/tumor cell signaling could also be mediated by EV signal transfer as described previously. In addition, CAFs secrete a variety of growth factors (i.e., transforming growth factor β (TGF β), vascular endothelial growth factor and platelet-derived growth factor, interleukins and ECM components (i.e., collagens, fibronectins and metalloproteinases) that support tumor growth, generate a physical barrier against drugs and immune infiltration, and facilitate cancer invasion [101,103–105]. Indeed, CAFs may directly influence tumor infiltrating immune cells and modulate the immune microenvironment.

Table 2. List of alternative nanomedicines that modulate the tumor microenvironment/immune system.

Nanoparticles	Therapeutic cargo	Targeting	Cancer type	Ref.
Artificial extracellular matrix	N/A	RGD-YIGSR motif (cancer cell targeting)	Breast cancer	[113]
Binary prodrug nanoparticles	Oxaliplatin, NLG919	N/A	Breast cancer	[114]
Carboxymethylcellulose nanoparticles	Docetaxel	N/A	Pancreatic cancer	[115]
Coreshell structured peptide nanoparticles	Doxorubicin	FAP- α antibody (CAF targeting)	Prostate cancer	[116]
Ferritin nanoparticle cage	ZnF 16Pc	Anti-FAP scFv (CAF targeting)	Breast cancer	[117,118]
Hydrogel	Losartan	N/A	Breast cancer	[119]
Immunoliposomes	N/A	Anti-FAP scFv (CAF targeting)	Fibrosarcoma	[120]
IND-liposomes	Indoximod (IND), doxorubicin	N/A	Breast cancer	[121]
Lipid bilayer nanoparticles	Gemcitabine, cisplatin	N/A	Bladder cancer	[122]
Lipid calcium phosphate nanoparticles	Quercetin	Anisamide motif (CAF targeting)	Bladder cancer	[123]
Lipid calcium phosphate nanoparticles	CXCL 12 trap plasmid	N/A	Colon, breast cancer (liver metastasis)	[124]
Lipid nanoparticles	Paclitaxel	Sulfatide	Breast cancer	[125]
Lipid nanoparticles	CXCL 12, PD-L1 trap plasmids	N/A	Pancreatic cancer	[126]
Lipid-protamine-DNA nanoparticles	IL-10, CXCL 12 trap plasmid	N/A	Breast, pancreatic cancer	[127]
Lipid-protamine-DNA nanoparticles	sTRIAL plasmid DNA	Anisamide motif (CAF targeting)	Bladder, pancreatic cancer	[128]
Liposome-protamine-hyaluronic acid nanoparticles	Wnt16 siRNA	Anisamide motif (CAF targeting)	Bladder cancer	[129]
Liposomes	Paclitaxel, marimastat	Hyaluronic acid	Breast cancer	[130]
Nanoemulsion	Fraxinellone	N/A	Melanoma	[131]
Peptidic spherical nanoparticles	Paclitaxel	Anti-FAP peptide (CAF targeting)	Breast cancer	[132]
pH-responsive nanocores	PHIS/R848, doxorubicin	Hyaluronic acid	Breast cancer	[133]
PLGA nanoparticles	Tumor antigens	N/A	Breast cancer	[134]
PLGA-PEG nanoparticles	Doxorubicin	Hyaluronidase	Breast cancer	[135]
SPIONs	Relaxin 2	N/A	Pancreatic cancer	[136]

CAF: Cancer-associated fibroblast; FAP: Fibroblast activation protein; N/A: Not applicable.

Activated CAFs express TGF- β , α -SMA, FAP, PDGFR- α/β and S100A4, among others. After activation, CAFs fail to reacquire an inactive status, and therefore they constantly exert a pathophysiological response producing a desmoplastic TME. Further, CAFs may induce epithelial to mesenchymal transition by secreting carbonic anhydrase IX, which causes acidification of the ECM and further enhances the activity of MMP-2 and MMP-9 metalloproteases, which in turn facilitates the local invasion of cancer cells into their surrounding tissues [106]. This has been observed in pancreatic cancer, prostate cancer, squamous cell cancer and melanoma, although with a variable degree of desmoplasia [107–110]. Because of this, new strategies that either reduce stromal desmoplasia or inactivate CAFs have been explored over the past years (Table 2). In this regard, a recent study showed that the anticancer compound minnelide is able to disrupt TGF- β signaling and revert activated CAFs into a quiescent form by decreasing α -SMA expression. Moreover, the application of all-transretinoic acid promotes a quiescent form of pancreatic stellate cells by modulating Wnt- β catenin signaling, reducing tumor progression [111]. Furthermore, hydrophobic drugs with activity against CAFs have been also encapsulated into nanocarriers to improve their solubility. Accordingly, various types of nanocarriers with distinct functions have been proposed against CAFs, including liposomes, polymeric nanoparticles, nanoemulsions and inorganic nanoparticles. In addition, surface moieties have been conjugated to nanocarriers that have been modified to increase their stability in circulation and to enhance targeting efficiency (Table 2). Alternative strategies have been also tested to modify the ECM to enhance the tumor penetration of drugs while reducing metastatic spread of the disease. An example is the use of a PEGylated recombinant formulation of human hyaluronidase (PEGPH20) aimed to improve the tumor penetration efficiency of chemotherapeutics. In fact, hyaluronic acid is a major component of the ECM and increases the interstitial pressure inside tumors. Hyaluronic acid degradation by PEGPH20 normalizes the interstitial pressure within the tumor and enhances the delivery of cytotoxic agents. In this context, PEGPH20 has been successfully tested in combination with cytotoxic drugs in clinics (i.e., nab-paclitaxel/gemcitabine and doxorubicin). Although phase

I–II studies showed promising results in patients with high hyaluronic acid-expressing tumors, the phase III study of PEHPH20 was not as successful and thus its further development is currently on pause. Additional intrinsic factors, such as lack of significant neoantigens, low tumor mutational burden, and epithelial to mesenchymal transition, should be considered [112]. Currently, PEHPH20 is still tested in combination with pembrolizumab (MK-3475) for patients with previously treated hyaluronan-high metastatic pancreatic ductal adenocarcinoma (NCT03634332) and in combination with FOLFIRINOX in patients with newly diagnosed metastatic pancreatic cancer (NCT01959139).

Recently, in addition to chemotherapy, nanocarriers have also been designed to improve the efficiency of cancer immunotherapy. Lipid-calcium-phosphate (LCP) nanoparticles have been successfully used as a peptide vaccine delivery system to stimulate anticancer T-cell response [137]. Another strategy has elegantly combined an LCP-based vaccine with targeted silencing of TGF- β expression at the TME using liposome-protamine-hyaluronic acid nanoparticles. Since TGF- β acts as immune suppressor within the TME, its silencing enhances the systemic immune response against the tumor. A combination of these two nanoparticles offers a flexible and powerful promising platform for immunotherapy [138]. In this regard, nanoparticles have been also conjugated to the surface of transferred T cells, acting as persistent autocrine-like signals to stimulate the immune response against tumors [139]. Further, inflammation regulators such as cytokines have been loaded into CAFs by using targeted nanoparticles to modify the TME [131,140,141]. Another example is the use of a PEGylated liposome (LPD) with a plasmid-encoding CXCL12 fusion protein (CXCL12 trap), combined with PD-L1. CXCL12 trap facilitates the penetration of T cells into the TME, whereas PD-L1 trap allows infiltrated T cells to kill the cancer cells. A similar setting of lipid-coated calcium phosphate nanoparticles (LCP) also resulted in enhanced infiltration of T lymphocytes while causing a drop in the expression of CXCL12 and immunosuppressive cells in the liver, clearly suggesting a potential impact of nanomedicine in modulating anticancer immune response [126].

Overall, the list of alternative nanomedicines that have been successfully explored in preclinical settings as TME/immune system modulators to reduce tumor progression and metastasis is increasing (Table 2). There is no doubt that in the future, new nanomedicine-based strategies against cancer should focus on the use of nanomedicine to therapeutically intervene in the different aspects that define a tumor and its microenvironment, as well as in the immunological response of the immune system to combat metastasis and the preconditioning of metastatic sites.

Conclusion

A tumor should be regarded as a heterogeneous ensemble of different cell types that are in constant intercommunication to orchestrate the growth and dissemination of the tumor. In addition, cells with metastatic potential communicate with distant tissues to prepare niches for hosting and nesting cancer cells to allow spread of the disease. According to the current dynamic CSC model of tumor growth, new nanomedicine-based therapies under development should focus not only on eradicating cancer cells but should also consider all components of the TME. In fact, after the eradication of the CSC population, specific complex mechanisms (feedback loops, phenotypic reversion and paracrine messaging) become active to re-establish the cellular balance within the TME and guarantee the progression and dissemination of the tumor. In this scenario, a clear understanding of the information exchanged between CSCs and differentiated cells (DCCs) to surrounding cells and, further, the understanding of close interactions among players within the metastatic niche are of utmost importance to develop efficient treatments.

Future perspective

According to the state of the art exposed in this review, a deep characterization of the content from secreted EVs as the main responsible actors of the cell-to-cell intercommunication within the tumors and metastatic sites is needed. Deciphering of EV content would lead to a better understanding of the fundamental nature of CSC/DCC crosstalk, the reversion phenotype of DCCs into CSCs, and their impact on immune response, tumor recurrence, metastasis and future clinical treatment outcomes. Similarly, to decoding ENIGMA during World War II that helped allied troops decipher encoded messages from enemy forces and prompted the end of the conflict, EV cargo will provide new candidate targets for future clinical interventions. Deciphering messages emitted by cancer cells and using nanomedicine-based strategies to modulate these messages should counteract metastatic dissemination and treatment resistance, which are the principal causes of mortality of oncologic patients worldwide. Likewise, a better understanding of the immunological processes occurring at the TME should also open the possibility to design new strategies to combat cancer relapse and metastatic spread.

Moreover, standardized protocols for isolation, modification and loading of EVs are required to prompt EV use in clinical practice. Current lack of quality controls under good manufacturing practices for the evaluation of safety and functionalization of large-scale EV production is preserving their use as DDS.

For all of these reasons, it is necessary to gain insight into fundamental processes of EV biology, to understand the basic mechanisms through which these vehicles can target specific cell types and their precise roles in the TME and develop new methodologies to translate their potential as DDS into clinics. This new knowledge will definitely help in further improving EVs for drug delivery in cancer.

Executive summary

Cancer stem cell models (the hierarchic model, stochastic model & dynamical tumor initiation model)

- A tumor should be regarded as a highly complex heterogenic dynamic entity that evolves over time, best adapting to its local microenvironment and the immune system to ensure its survival.
- The dynamic tumor initiation model for a mixed population describes the cancer stem cell (CSC) dynamic phenotype and cancer initiation.

Regulation of phenotypic dynamism via intratumoral communication

- Cancer cells directly interact with other cells within the tumor microenvironment (TME) through paracrine signaling and/or direct cell-to-cell contact signaling to create a favorable physical and molecular signaling environment, to ensure tumor progression.
- Extracellular vesicles (EVs) play an important regulatory role in TME modulation and CSC dynamics.

EVs as natural nanocarriers engaged into cell-to-cell intercommunication

- EVs and nanomedicines designed to modulate the TME should be regarded as effective anticancer therapy rather than CSC elimination.

Nanomedicines to modulate the TME & reduce tumor spread

- To inhibit tumor growth and dissemination, nanomedicines should directly target cancer cells and should also modulate the TME.

Author contributions

P Gener drafted the work and figures. P Gonzalez Callejo, J Seras-Franzoso, F Andrade and D Rafael contributed to the writing, and I Abasolo and S Schwartz Jr revised it critically for important intellectual content and approved the final version.

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