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Soldano Ferrone *Editors*

Cancer Stem Cell Resistance to Targeted Therapy

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Cancer Stem Cell Resistance to Targeted Therapy

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Aims and Scopes

Cells with stemness/cancer-initiating properties (CSCs/CICs) have been isolated from tumors with different histological properties. These cells have been shown to be responsible for tumor formation and progression and represent the component of tumor resistance to standard therapies and immunotherapy. During the last decades, targeted cancer therapies have been developed, including drugs, antibodies, chemical inhibitors, etc., targeting specific tumor-associated genes, antigens, and blood vessels that regulate tumor growth and progression. More recently, FDA- and EMA-approved immunotherapy agents targeting the host immune responses have shown clinical activity by improving patient's survival. Targeted cancer therapies are being used alone or in combination either with other targeted therapies or with standard therapies.

Tumor cell resistance to targeted therapies remains a major problem, and several strategies are being considered to reverse the resistance to these therapies. The biological, molecular, and immunological characterization of CSCs/CICs contributed to identify key signaling pathways involved in controlling their properties.

Therapeutic strategies selectively targeting cancer cells with stemness properties can overcome tumor resistance.

The volume "Cancer Stem Cell Resistance to Target Therapy" part of the special series *Resistance to Targeted Anti-Cancer Therapeutics* will focus on the molecular and biological properties of cancer stem cells rendering these cells resistant to target therapy. Moreover, insights regarding novel CSC-/CIC-specific target molecules and their interaction with the host immune system will be provided, and possible solutions to the problem of tumor resistance to therapies will be discussed.

About the Editors

Cristina Maccalli MSc, PhD is a Principal Investigator at the Division of Translational Medicine, Sidra Medicine, Doha, Qatar.

Cristina obtained in 1990 a Master's Degree in Biological Sciences and then, in 1996, a PhD in Applied Genetics at the University of Milan, Italy. Her research interest lies in the area of immunology, tumor immunology, and immunotherapy, with main focus on the functional characterization of immune responses, cancer stem cells, and identification of biomarkers predictive of patients' clinical outcome and responsiveness to therapies.

She has carried out her postdoctoral research programs at the National Cancer Institute in Milan, Italy, and, then, at the Surgery Branch, National Cancer Institute, NIH, Bethesda, MD, USA. From 2003 to mid-2007, she worked as investigator at the Istituto Superiore di Sanita', Rome, Italy. In 2007, Cristina joined the Unit of Immuno-biotherapy of Melanoma and Solid Tumors at the San Raffaele Foundation Scientific Institute, Milan, Italy, and she has been dedicated to novel studies on the immunological characterization of cancer stem cells from glioblastoma and colorectal cancer patients and to perform monitoring of immune responses in cancer patients undergoing immunotherapy. In 2013, she contributed in the role of senior investigator to set up in the context of the Italian Network for Biotherapy of Tumors (NIBIT), a novel laboratory dedicated to the identification of biomarkers for cancer patients through the design of standardized and validated immune-monitoring assays. In

October 2014, Cristina joined the Translational Medicine Department at Sidra Medicine, Doha, Qatar, where she is involved in development of innovative studies in the context of biomarker discovery and immunotherapy. Her principal project areas are (1) the generation of chimeric receptor-engineered T cells to target hematological malignancies, (2) the molecular and functional characterization of cancer stem cells isolated from colorectal and breast cancer patients, and (3) the identification of biomarkers predictive of clinical outcome of patients with inflammatory bowel disease.

Cristina is author/coauthor of about 60 original peer-reviewed publications and 3 chapter books. She is associate editor of the *Journal of Translational Medicine* and reviewer of a variety of international scientific journals (e.g., *The Journal of Immunology*, *Cancer Research*, *Clinical Cancer Research*, *Cancer Immunology, Immunotherapy*, *Frontiers in Immunology*, etc.). She acted as reviewer for fellowship programs of the Society for Immunotherapy of Cancer (SITC). She has been involved as lecturer in educational courses in immuno-oncology in the context of NIBIT. She is a member of the SITC. In the context of SITC, she has served as member of working groups on immunotherapy biomarkers.

Matilde Todaro MD, has contributed to cancer research by developing new anticancer therapies. Recently, her interest has been focused on the most promising and innovative biomedical research, namely, cancer stem cells and their role in the onset and progression of breast cancer. The publication of many of her studies in prestigious journals has granted her inclusion in a national and international research network.

Dr. Todaro's group was one of the first to isolate and propagate cancer stem cells from tumors of epithelial origin. This advanced contribution to the scientific community led her to establish collaborations with prestigious international pharmaceutical companies such as Eli Lilly, Roche, Pfizer, Tristar, Merck, and Trevigen, who have financed part of her research aimed at the development of therapies against cancer stem cells.

She has supervised and mentored many graduate students, and she was the tutor in charge of several postgraduate and postdoctoral fellows.

Recently, Dr. Todaro's scientific activity is aimed at studying the biology of breast cancer in order to identify new molecular targets that facilitate early diagnosis and to improve the treatment options against breast cancer that nowadays are restricted to conventional therapies.

Dr. Todaro is currently an associate professor, SSD MED/04, General Pathology, University of Palermo. She is the medical director and head of Endocrinology and Metabolic Disease, Intensive Metabolic Care Unit, Institute of Clinical Medicine, University of Palermo. She is also responsible for the onco-hematological diagnostics laboratory, Central Laboratory of Advanced Diagnosis and Biomedical Research (CLADIBIOR), University of Palermo.

Soldano Ferrone MD, PhD, joined the Massachusetts General Hospital, Harvard Medical School, in 2012 as professor in the Department of Surgery. He has held faculty positions at many Academic Institutions in the United States and at the Medical School of the University of Milan, Milan, Italy.

His research program focused on the development of antibody-based immunotherapeutic strategies for the treatment of solid tumors and on the characterization of the role of defects of HLA class I antigen processing machinery as an escape mechanism utilized by tumor cells to avoid immune recognition and destruction. These studies are greatly facilitated by the large panel of HLA antigen- and human tumor antigen-specific monoclonal antibodies he has developed and shared with the scientific community over the years.

He has described the results of his studies in more than 600 papers published in peer-reviewed journals. Moreover, he has been the editor of 14 books and the guest editor of 5 special issues of oncology journals.

Dr. Ferrone has received many awards and honors. For the last 30 years, he has been the member of many review committees including NIH Study Sections and of the editorial boards of many scientific journals. Furthermore, he is the member of several external scientific boards.

Preface

Cancer represents one of the leading causes of death. Advances on the genetic and molecular characterization of cancer with different histological origins allowed to improve therapeutic treatments such as chemotherapy, radiotherapy-targeted agents, and their combinations. This led to increasing the overall survival for patients with some type of tumors. However, a significant proportion of cancer patients is unresponsive or develops resistance to treatments.

Factors contributing to failure of therapeutic interventions are genetic and epigenetic variability, interactions with tumor microenvironment and differences in patient's genomics, leading to both intratumor and intertumoral heterogeneity.

Cancer stem cells or cancer-initiating cells (CSCs/CICs) have been identified in both hematological and solid tumors. They are present with relatively low frequency within tumor lesions and are endowed with self-renewal, multipotency, and tumorigenic features. These rare cells have been shown as tumors' components responsible for tumor formation, resistance to therapies, and tumor progression and metastatization.

The tumorigenic properties of cancer stem cells have been demonstrated through the usage of xenotransplantation in immune-deficient mice. These systems have proven that upon transplantation of cells with "stemness" features, the neoformation of malignant lesions representing the phenocopy of the original tumor is observed. These models have allowed to demonstrate that tumors have a hierarchical organization and following serial transplantation, phenotypically different subpopulation can be identified including stem-like cells. Nevertheless, the biological characterization of CSCs/CICs provided evidence of their high grade of heterogeneity and plasticity. These properties are influenced by the interaction between CSCs/CICs and tumor microenvironment, in which the CSC-associated niche, which is needed for their survival and maintenance, is localized. Multiple "bona fide" CSC-/CIC-associated markers have been identified depending on the tissue of origins. These markers are mostly overexpressed by CSCs/CICs but also shared with either differentiated tumor cells or normal stem cells. Nevertheless, some of these markers have been used either to isolate stem-like cells from neoplastic tissues or to localize these cells within tumor tissues. Moreover, the identification of cells positive for

these molecules correlated with tumor prognosis, and, in some cases, their enrichment was observed following the failure in responsiveness to therapies. However, the lack of standardized assays and tools to isolate CSCs/CICs and their high grade of plasticity led to not yet definitive conclusions regarding the fate of these cells and their impact in tumor outcomes.

Immunotherapy represents a breakthrough therapeutic intervention which given the clinical development of novel strategies, such as immune checkpoint blockade or adoptive cell therapy, resulted in improvement of patients' overall survival for some type of tumors. However, a significant proportion of patients are unresponsive or develop resistance to this type of therapy. Evidences showed that CSCs/CICs display immunomodulating properties leading to the evasion from cell-mediated immune responses. Indicating that, from one hand, these cells can be responsible for immunological dormancy and remain quiescent until cross-talk with tumor microenvironment drives their entrance into cell cycling, with the final results of formation of either tumor recurrences or metastasis. On the other hand, CSCs/CICs can represent the tumor components responsible for the resistance to immunotherapy. The identification of therapeutic strategies targeting CSCs/CICs together with the differentiated counterparts of tumors is a requirement in order to achieve the complete eradication of tumors. To this aim, a comprehensive characterization of genomic, epigenetic, phenotypic, and immunological profile of CSCs/CICs could contribute to better understand the mechanisms orchestrating their biological properties and to design more effective therapeutic interventions for cancer patients.

This volume represents an overview of the state of the art in understanding the principle molecular pathway regulating "stemness" properties of tumor cells and in identifying the mechanisms of resistance to both standard and immune-based therapies.

Part I of this volume provides an introduction to the definition of CSCs/CICs, their ability to remain in quiescent state, and their metabolic adaptation to tumor microenvironment. The usage of xenograft tumors as "unique" tools to demonstrate the tumorigenicity of these cells will be discussed. Moreover, altered signaling pathways occurring in these cells and the available tools for their targeting will be described.

The epithelial-to-mesenchymal transition (EMT), which induces epithelial cells to acquire mesenchymal properties, represents a relevant process to drive epithelial cells toward stemness functions. Interestingly, these mechanisms are strictly influenced by pro-inflammatory signaling and immune responses. EMT is involved in mediating cell invasiveness and metastatization representing a link between CSCs and formation of metastases.

The identification of a "stemness" gene signature within tumor tissues has been shown to play a role in patients' prognosis and susceptibility to drug treatments. The identification of "stemness" cellular program could represent a relevant tool to characterize tumor heterogeneity and lead to provide a more accurate identification of CSCs/CICs that are responsible of therapeutic resistance. Moreover, CSCs/CICs display high grade of plasticity in relationship with tumor microenvironment; these properties are regulated by the dynamic accumulation of genetic and epigenetic

alterations that influence crucial "stemness" properties, such as "slef-renewal", proliferation, and differentiation. These features are also pivotal for the formation of the minimal residual disease upon drug treatment. The optimization of drug efficacy in the light of CSC/CIC concept will be discussed.

Along this line, studies leading to the isolation and characterization of CSCs/CICs in different type of solid tumors, such as colorectal, lung, and prostate cancer, melanoma, and glioma, are presented in association with their clinical role. The mechanisms driving the resistance of these cells to standard therapies include DNA damage repair, anti-apoptotic signaling, increased levels of drug transporters, self-renewal pathways, and quiescence mechanisms.

Interestingly, the role of the transcription factor Yin Yang 1 (YY1) is dissected as associated with the regulation of cell proliferation, viability, EMT, metastasis, and chemo-immune resistance. This transcription factor can determine the regulation of other transcription factors in CSCs/CICs and of drug resistance, opening the opportunity to target this molecule as a new therapeutic approach to reverse CSC-/CIC-associated resistance.

Part II of the volume provides an introductory overview and basic knowledge of different immune-based strategies that have been clinically developed. Among these strategies, the most novel approaches representing the breakthrough of immunotherapy and providing for the first-time improvement of survival for cancer patients are highlighted.

The interaction of leukemia-derived CSCs/CICs with tumor microenvironment, representing the niche for these stem-like cells, is discussed together with the immune evasion mechanisms develop by these cells. These represent relevant implications for immunotherapeutic intervention to target these cells.

The interaction of CSCs/CICs with tumor microenvironment is addressed also for the prostate model with particular focus on the immunological profile of these cells and the interactions with both innate and adaptive immune responses. In addition, a comprehensive analysis of the mechanisms of immune resistance of CSCs/CICs is provided with particular emphasis on the interaction with T-cell-mediated responses. The multiple immune evasion mechanisms are discussed in relation to the available immunotherapeutic strategies, highlighting the reasons of limited clinical efficacy and possible tools to achieve the successful targeting of CSCs/CICs.

In summary, this volume represents a detailed state of the art of the biological and immunological characterization of CSCs/CICs with particular focus on clinical implications and challenges of targeting these cells. Specific functional characteristics including genomic and immunological properties of CSCs/CICs should be fully identified with the aim to assess their role as prognostic and predictive biomarkers of responsiveness to therapies and to identify therapeutic interventions that might lead to complete eradication of tumors and durable patients' clinical responses.

Doha, Qatar — Cristina Maccalli
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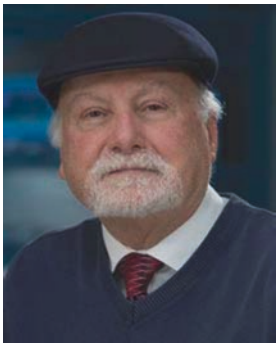
Series Editor Biography



Benjamin Bonavida PhD (series editor), is currently distinguished research professor at the University of California, Los Angeles (UCLA). His research career, thus far, has focused on basic immunochemistry and cancer immunobiology. His research investigations have ranged from the mechanisms of cell-mediated killing, sensitization of resistant tumor cells to chemo-immunotherapy, characterization of resistant factors in cancer cells, cell-signaling pathways mediated by therapeutic anticancer antibodies, to characterization of a dysregulated NF-κB/Snail/YY1/RKIP/PTEN loop in many cancers that regulate cell survival, proliferation, invasion, metastasis, and resistance. He has also investigated the role of nitric oxide in cancer and its potential antitumor activity. Many of the above studies are centered on the clinical challenging features of cancer patients' failure to respond to both conventional and targeted therapies. The development and activity of various targeting agents, their modes of action, and resistance are highlighted in many referenced publications.

Acknowledgments

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Cancer Stem Cells: From Birth to Death

Alice Turdo, Miriam Gaggianesi, Aurora Chinnici, Giorgio Stassi, and Matilde Todaro

Abstract Conspicuous investigations have proven the role of cancer stem cells (CSCs) in the onset and progression of a plethora of liquid and solid neoplasms. CSCs are endowed with the capability of initiating tumor growth and becoming dormant at distant organ sites just waiting for optimal conditions amenable for metastatic outgrowth. This cancer subpopulation is inherently resistant to anticancer therapeutics, and its targeting could avoid metastatic disease, which is largely incurable, and clinical relapses. CSCs are considered the Achilles heel of cancer. However, many efforts are necessary to identify univocal CSC markers as well as specific CSC biomarkers of therapeutic response.

Here, we summarize CSCs' peculiarities and highlight novel anticancer compounds coping with the hallmarks of CSCs, comprising the resistance to cell death, their quiescent state, the immune suppression, the epithelial to mesenchymal transition (EMT), and their metabolic adaptation to a hostile microenvironment.

Keywords Cancer · Metastasis · Cancer stem cells · Drug resistance · Anticancer drugs

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The authors declare no potential conflicts of interest

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Abbreviations

ABC	ATP-binding cassette
ALDH	Aldehyde dehydrogenase
AML	Acute myeloid leukemia
ATM	Ataxia telangiectasia mutated
BCRP	Breast cancer resistance protein
BET	Bromodomain and extra-terminal
BMI-1	B lymphoma Mo-MLV insertion region 1 homolog
BMP	Bone morphogenetic protein
CAF	Cancer-associated fibroblast
CCR2	Chemokine (C-C motif) receptor 2
c-FLIP	Cellular FLICE-like inhibitor protein
CHK	Checkpoint kinase
CSC	Cancer stem cell
CSF1R	Cell receptors colony-stimulating factor 1 receptor
DDL	Delta-like protein
DKK	Dickkopf
DNMT1	DNA methyltransferase 1
DVL	Dishevelled
EMT	Epithelial to mesenchymal transition
FoxO	Forkhead Box O
FZD	Frizzled
GSTO1	Glutathione S-transferase omega 1
HDAC	Histone deacetylase
HDM	Histone demethylase
HFSC	Hair follicle stem cell
HH	Hedgehog
HIF	Hypoxia-inducible factor
HMT	Histone methyltransferase
HR	Homologous recombination
HSC	Hematopoietic stem cell
JAG	Jagged
LRP	Lipoprotein receptor-related protein
MDR	Multidrug resistance
MRP	Multidrug resistance-associated protein
NHEJ	Non-homologous end joining
NICD	Notch intracellular domain
NSCLC	Non-small cell lung cancer
OXPHOS	Oxidative phosphorylation
PCP	Planar cell polarity
PD-L1	Programmed cell death ligand 1
P-gp	P-glycoprotein
PTCH	Patched
ROS	Reactive oxygen species
sFRPs	Secreted Frizzled-related proteins

SMO	Smoothened
TA	Transient amplifying
TAM	Tumor-associated macrophages
TAZ	Transcriptional coactivator with PDZ-binding motif
TCA	Tricarboxylic acid
TCF/LEF	T cell factor/lymphoid enhancer factor
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor α
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
WIF-1	WNT inhibitory factor 1
YAP	Yes-associated protein

1 Origin and Identity of Cancer Stem Cells

The existence of tumor heterogeneity was firstly observed in the nineteenth century by several pathologists. The histological heterogeneity was associated with the variation of marker expression, genetic landscapes, cancer progression, and response to therapy [1–8]. More recently, clonal studies demonstrated that a neoplasm is composed of an assembly of individual cells that together contribute to sustain tumor growth [9]. Thank to this diversity, cancer cells gain the ability to survive and adapt to changing environments [10].

1.1 Models of Tumor Growth

In order to explain cancer heterogeneity, two major models have been theorized: the clonal evolution model and the cancer stem cell (CSC) model (Fig. 1).

The clonal evolution model was postulated on the basis of Darwinian theories. In fact, it asserts that a tumor is composed of a multitude of individual cells, differing one from another for genetic and epigenetic alterations. The mutant clones, resulting from beneficial mutations, gain selective advantage and are able to prevail and sustain tumor growth, progression, and resistance to therapy [11, 12]. Interestingly, this theory does not assume the existence of a hierarchical organization for tumors [13]. Because of this, the model is suitable for the types of cancer without a precise hierarchy, but not for those that are markedly characterized by a subpopulation of tumor-initiating cells able to generate the other tumor compartments. It is noteworthy that both the clonal evolution and the CSC models can be applied to the same cancer, for example, mouse acute myeloid leukemia (AML) was described according to the clonal evolution model by some groups and the CSC model by others [14–18].

The father of modern cellular pathology, Rudolf Virchow, was the first to hypothesize that tumor growth derives from an alteration of an immature cell [19]. In the next decades, this idea will be implemented using the *seed and soil* hypothesis by

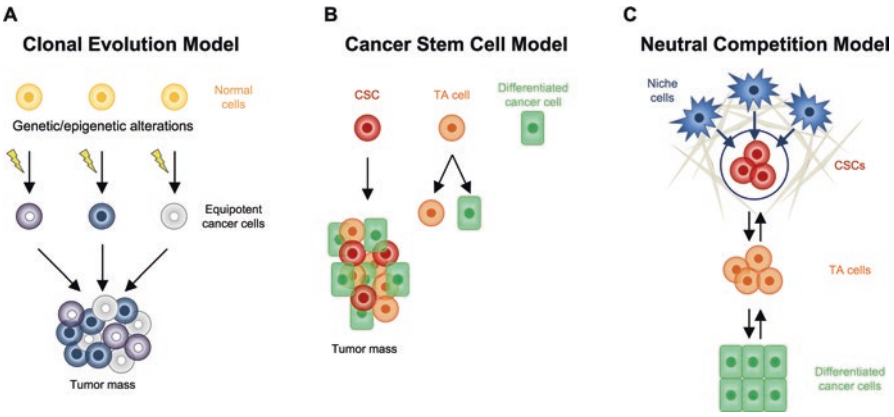

Fig. 1 Models of tumor growth. (**a**) According to the clonal evolution model, equipotent cancer cells are generated by the acquisition of genetic and/or epigenetic alterations. The tumor mass is therefore composed of distinct clones with different characteristics. (**b**) The cancer stem cell (CSC) model posits that only a subset of cancer cells, namely, the CSCs, is able to sustain tumor growth, thanks to marked self-renewal properties, whereas transient amplifying cells (TA cells) are able to give rise to nonproliferative cancer cells. (**c**) In the neutral competition model, CSCs reside in the limited space of the niche (blue circle), where they are subjected to the niche signals (blue arrows). These signals may also reprogram TA cells and differentiated cancer cells into CSCs

Stephen Paget, who asserted that certain cancer cells possess an intrinsic ability to root in distant organs characterized by an environment suitable for the growth of a secondary tumor [20]. In the past years, several studies focused on the existence of normal and malignant stem cells. In 1961, Till and McCulloch were the first to identify hematopoietic stem cells (HSCs). Thanks to a clonal in vivo repopulation assay, they showed that a single hematopoietic cell owns both a multilineage differentiation potential and self-renewal capacity [21]. Subsequent studies on malignant blood cells, squamous cell carcinomas, and teratocarcinomas and chronic myelogenous leukemia contributed to the identification of a subpopulation able to sustain tumor growth [19, 22, 23]. In 1994, John Dick's group for the first time isolated and characterized leukemia stem cells, describing the existence of a hierarchy even in the context of a malignancy [24].

The CSC concept assumes that tumors are characterized by a hierarchical organization, which is often similar to the normal tissue of origin, with a small subpopulation of CSCs at the apex of the hierarchy [13]. It hypothesizes that the CSCs are quiescent and are able to maintain the CSC pool, through symmetric division, or to generate a CSC and a transient amplifying (TA) cell through asymmetric division. The TA cells are characterized by rapid growth rate and constitute the majority of tumor bulk, although they are incapable of sustaining long-term growth since they eventually undergo differentiation. Conversely, CSCs are responsible for tumor initiation, since they represent the only compartment able to generate xenografts in immunocompromised mice and for resistance to chemo- and radiotherapy [25, 26].

Due to the difficulty in explaining tumor heterogeneity by using either one or the other model, it was recently necessary to unify the clonal evolution and the CSC model.

Neoplasms are generated by the acquisition of gene mutations in the founder cell, which forms a first subclone composed by CSCs and TA cells. Other subclones can be generated by genetic alterations occurring during tumor expansion, and, since CSCs could undergo genetic and epigenetic modifications, the tumor could contain different independent subclones with a different balance in its hierarchy, as well as different characteristics [10]. Lately, Batlle and Clevers applied the "neutral competition model" on CSCs. They affirm indeed that, as in adult stem cell niche, CSCs compete to occupy the space available in the limited niche size. In this way, the classical knowledge regarding the balance between symmetric and asymmetric divisions is unsettled, because CSCs' fate depends on the signals deriving from the niche. Moreover, niche stimuli can also reprogram TA and differentiated cancer cells into CSCs [25] (Fig. 1).

1.2 Cancer Stem Cell Isolation

The first studies on the isolation of CSC subpopulation were inspired by the methods developed by Weissman and colleagues, who exploited cell surface proteins for the isolation of normal blood stem cells [27, 28]. As stated before, Dick, Lapidot, and Bonnet demonstrated for the first time the existence of a hierarchy in human AML [24, 29]. They showed that the cells capable of propagating the malignancy in a recipient mice were characterized by the expression of the same surface markers of adult HSCs ($CD34^+/CD38^-$), whereas the others were mature blood cells at different levels of differentiation [26]. Recently, this characterization has been implemented by Blair et al., updating AML CSC population to $CD34^+/CD38^-/CD90^-/IL\text{-}3R^+/CD71^-/HLA\text{-}DR^-/CD117^-$ [30–32].

In the past years, several groups contributed to the identification of CSCs in solid tumors. The first marker was CD133 (prominin-1), a transmembrane glycoprotein expressed on neurospheres [33]. Interestingly, it was shown that $CD133^+$ population originated a tumor containing both $CD133^+$ and $CD133^-$ cells, suggesting that this population is able to generate itself and a differentiated progeny. The use of CD133 as CSC marker was intensively debated, since its role was not totally clarified and was linked to bioenergetic stress [34]. Moreover, CD133 is frequently expressed by endothelial progenitors of normal tissue [35]. Notwithstanding, this protein is now widely adopted for the identification of CSCs in several cancers, such as hepatocellular carcinoma [36], prostate cancer [37], melanoma [38], and colorectal cancer [39].

In general, most CSCs are defined by the expression of different markers with regard to tissue of origin. For example, a surface protein useful for CSC identification is CD44, the receptor for hyaluronic acid expressed on several types of tumorigenic cells, as well as its splicing variants (CD44v). Furthermore, another tool used to recognize CSCs is the activity of the aldehyde dehydrogenase (ALDH), reported to be elevated in cells able to sustain tumor growth in vivo [40].

The combination of these markers allowed to isolate CSCs from a plethora of tumors, such as the abovementioned prostate CSCs, usually identified by the $CD44^+/\alpha_2\beta_1^{hi}/CD133^+$ population [41]; colorectal CSCs, represented by $CD133^+/CD44v6^+/Lgr5^+/ESA^+/ALDH^{hi}$ population [42]; and breast CSCs, with a phenotype of $CD44^{hi}/CD24^{low/-}/ESA^+/ALDH^{hi}$ [40, 43].

Along with the abovementioned proteins, CSCs are detectable by the overexpression of drug efflux transport proteins [44], namely, the ATP-binding cassette (ABC) transporter P-glycoprotein (P-gp), the multidrug resistance-associated proteins (MRP) [45], ABCB5 glycoprotein [46], MRP1 (ABCC1) [47], and breast cancer resistance protein (BCRP/ABCG2) [48], the latter being correlated with the overexpression of CD133 and the regulation of PI3K-AKT signaling pathway [49].

In the past years, Vlashi's group described a reduced activity of the 26S proteasome in CSCs [50, 51]. This reduced activity was correlated with poor outcomes in head and neck skin cancer [51], glioblastoma [52], and breast cancer [53].

The expression of markers able to unambiguously identify CSCs is still missing. However, the reported tools are indeed useful for an enrichment in the CSC subpopulation.

Thus, nowadays, the most reliable assay to evaluate CSC frequencies is the in vivo limiting dilution assay described below.

1.3 Xenotransplantation Versus Genetic Tracing and Lineage Ablation Approaches

Starting from the CSC theory, in the past decades, the gold standard for in vivo assays was CSC transplantation. This assay allows to evaluate the presence of CSCs by generating xenografts in immunodeficient mice. CSCs are firstly isolated on the basis of specific surface markers and are then injected at serial dilutions. The obtained xenografts can be digested and re-injected in other immunocompromised mice several times. This technique was firstly used for hematological malignancies and then adapted to solid tumors, but, in the second case, it presents considerable limitations. Undeniably, the mechanical and enzymatic digestion of solid tumors fails to preserve contacts with the other cells, the extracellular matrix, and the tumor microenvironment. Moreover, before the in vivo injection, cancer cells are isolated from tumor bulk according to putative CSC surface markers and expanded in vitro [25, 54].

To overcome the problems deriving from the mechanical dissociation, lineage-tracing approaches were adopted widely. Genetic-lineage tracing was firstly used to identify the fate of normal stem cells, such as hair follicle stem cells (HFSCs). HFSCs generated all-epidermal lineages after transplantation [55, 56], but upon lineage tracing, they gave rise only to hair-follicle lineage [57].

Genetic-lineage tracing technique allows to label cells expressing a specific marker, which in turn induces the expression of a recombinase (e.g., Cre) that is able to activate the relative reporter. Nassar and colleagues studied chemically

induced skin tumors in mice labeled with keratin-14-Cre diver allele and *Rosa*-YFP reporter. They induced the recombinase with tamoxifen and observed that only a small subpopulation of labeled cells survived, demonstrating that CSCs can undergo asymmetric division, generating TA cells that are committed to terminal differentiation, or symmetric division to maintain CSC fraction. Moreover, they observed that, at a clonal level, these divisions occur in a stochastic manner [58].

In order to extend genetic-lineage tracing to human tumors, Cortina et al. edited human patient-derived colorectal cancer organoids. Using CRISPR/Cas9 technique, they integrated a GFP reporter after the LGR5 locus in order to identify colorectal CSCs and, subsequently, a CreERT2 knock-in to monitor labeled cells over time. As a result, they noticed that $Lgr5^+$ cell number directly correlated with tumor size and that $Lgr5^+$ population produced progeny able to survive for a long period and eventually prone to differentiate [59].

A third method to verify the role of a certain subpopulation is genetic- or laser-induced lineage ablation, which targets cells expressing a selected gene [60]. The ablation of CSCs allows the eradication of established tumors, for example, the targeting of Sox2-expressing cells was able to induce tumor regression in skin tumors [61]. Overall, molecular biology supported the evolution of increasingly cutting-edge approaches to understand CSC behavior in tumor initiation and progression.

1.4 The Hallmarks of Cancer Stem Cells

Cell quiescence is described as the persistence in a non-cycling state, represented by G0 phase, and is defined reversible because cells may reenter in cell cycle in response to external stimuli [62]. Quiescence, a characteristic of both disseminated and intratumoral CSCs, is responsible for the dormancy of primary and secondary malignancies as well as for the resistance to standard chemotherapy, which targets only actively proliferating cells [63]. CSCs' quiescence can be promoted by several factors. The main proteins involved are the cyclin-dependent protein kinase inhibitors p21 and p27, the tumor suppressors p53 and Rb, as well as a large number of micro-RNAs [21]. Other effectors contributing to cell quiescence are the components of Notch-related pathways and Forkhead Box O (FoxO) transcription factor. The latter is also correlated to the adaptation to environmental stress, thanks to its role in regulating PI3K-AKT pathway and reactive oxygen species (ROS) levels [21].

Self-renewal is one of the main properties of CSCs by defining their ability to sustain tumor growth. The principal evidence of self-renewal is the engraftment of serial transplantations of CSCs in immunocompromised mice [64]. One of the central pathways involved in self-renewal ability is the polycomb complex protein B lymphoma Mo-MLV insertion region 1 homolog (BMI-1), involved in the division of HSCs. BMI-1 was found overexpressed in several types of malignancies such as leukemia or mantle cell lymphomas [65], as well as in gliomas [66] and ovarian

cancer [67]. The CSC subpopulation must not be considered as a perceivable number of cells independent from tumor mass; it is indeed influenced by stimuli coming from the other cancer cells or the tumor microenvironment [68]. This plasticity can be induced by both genetic and epigenetic modifications that can be subsequent to chemotherapy or to microenvironmental signals [69]. Tumor microenvironment can indeed influence CSC potential to self-renew and migrate through several factors, such as hypoxia, acidity, extracellular matrix remodeling, nutrient supply, and immune cell recruitment [63]. Recent studies on CSC plasticity involved in vivo cell ablation experiments. The groups of Shimokawa and de Sousa independently showed that the ablation of $Lgr5^+$ population resulted in an initial reduction of tumor size. The termination of the ablation was followed by a repopulation of $Lgr5^+$, providing evidence of CSC plasticity [70, 71].

The most important reason for the arduous attempt to eradicate the CSCs is their resistance to therapy. CSCs are indeed resistant to genotoxic drugs. This is due to the elevated DNA damage response of CSCs as compared to the other components of tumor bulk [72]. Moreover, it was demonstrated that another way to survive to genotoxic therapy is the activation of checkpoint kinases [73]. Therefore, compared to normal stem cells that undergo differentiation or senescence in response to DNA damage, CSCs survive in spite of the acquired genetic alterations.

The first interest toward cancer metabolism is ascribed to the physiologist Otto Warburg, who noticed that, even in the presence of external oxygen, cancer cells increase their glucose demand for the preferential production of lactate [74]. This phenomenon, termed "Warburg effect," is definitely the metabolic feature of the majority of cells comprising tumor mass.

As stated before, the tumor mass is composed of a heterogeneous mixture of cancer cells, among which we can find the CSC subpopulation, discernible by a different metabolic phenotype [75]. The metabolic hallmarks of CSCs are still intensively debated. The first studies linked the metabolism of CSCs to the normal tissue hierarchy, where multipotent stem cells mainly show a glycolytic phenotype. This hypothesis is supported by the evidence that glycolysis is the preferred energetic source of breast, liver, and nasopharyngeal CSCs [76–78].

Other investigators described oxidative phosphorylation (OXPHOS) as the primary energy-production process in lung [79], glioblastoma [80], leukemia [81], and pancreatic ductal adenocarcinoma CSCs [82]. Compared to OXPHOS, glycolysis is less efficient and more dependent on extracellular nutrients. Because of this, CSCs preferring mitochondrial metabolism could gain a selective advantage in condition of limited nutrient availability [74]. Moreover, the high amount of lactate produced by differentiated cells may be used for OXPHOS machinery, forming a metabolic symbiotic system [83]. Recently, Maria Peiris-Pagès and colleagues [84] proposed a model of metabolic adaptation of CSCs to microenvironment. They based their assertion on the observation that CSCs are able to switch from an oxidative to a glycolytic phenotype after external stimuli such as hypoxia and glucose deprivation [85, 86]. The plasticity of CSCs is therefore crucial. When mitochondrial metabolism is blocked or inefficient, CSCs are able to acquire a combined phenotype, in

Fig. 2 The hallmarks of cancer stem cells. Schematic representation of the six major properties of cancer stem cells (CSCs). CSCs undergo the epithelial to mesenchymal transition (EMT) in order to be able to migrate, invade, and form metastasis. At metastatic sites, CSCs can modify their metabolism, according to the new hostile milieu, and they enter a state of quiescence, waiting to reenter the cell cycle and form macrometastasis. Moreover, CSCs can counteract anticancer immune responses. As their normal counterpart, CSCs self-renew to maintain the CSC pool or can differentiate to constitute the tumor bulk. CSCs are more prone to survive as compared to differentiated cancer cells by the upregulation of anti-apoptotic genes or by enhancing the DNA repair damage machinery in response to chemotherapy

which glycolysis and OXPHOS work together for their survival and resistance to a hostile milieu. On the basis of these assumptions, targeting tumor metabolism may lead to novel therapeutic strategies for CSC eradication (Fig. 2).

2 Transcriptional Networks and Pluripotent Genes

Developmental signaling pathways, such as WNT, Hedgehog (HH), Notch, Hippo, and transforming growth factor beta (TGF-β), govern cell proliferation, self-renewal, and cell differentiation in normal stem cells. For this reason, they need to be tightly regulated in order to sustain embryonic development and organ homeostasis. In human cancers, these pathways are commonly mutated or deregulated, increasing self-renewal and tumorigenic capacity of CSCs [87–89]. Compelling evidence indicates that these molecular signaling pathways interact with other tumorigenic pathways, among which are PI3K/AKT, MAPK, and NF-kB [90].

2.1 The WNT Signaling

The WNT pathway controls crucial processes associated with embryonic development and tissue homeostasis, since it is involved in cell survival, proliferation, and differentiation. Thus, many components of the WNT signaling pathway were found mutated in neurodegenerative and metabolic disorders and also in tumors [91]. WNT family is highly evolutionarily conserved, and it comprises 19 ligands (cysteine-rich glycoprotein) and many receptors or co-receptors. In particular, genes encoding WNT ligands are found in both vertebrate and invertebrate and display high homology between different species [92].

There are three major pathways that compose WNT signaling: the canonical or "β-catenin dependent" pathway, the non-canonical planar cell polarity (PCP) pathway, and the non-canonical WNT/calcium pathway. The last two are termed "β-catenin-independent pathways."

In the canonical pathway, WNT ligands bind to Frizzled (FZD), a seven-transmembrane receptor, and to a co-receptor, the low-density lipoprotein receptor-related protein 5/6 (LRP5/6), inducing Dishevelled (DVL) phosphorylation. Once phosphorylated, DVL recruits AXIN, eliciting β-catenin nuclear translocation. In absence of WNT signaling, β-catenin is sequestered into the cytoplasm by a degradation complex, composed of APC, the scaffold protein AXIN, and two kinases, CK1 and GSK-3β. These two serine/threonine kinases phosphorylate β-catenin in its N-terminus domain, leading to its polyubiquitination and subsequent proteasomal degradation. The presence of active WNT signaling promotes cytoplasmic β-catenin stabilization and its subsequent nuclear translocation, where it binds the T cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors to activate the transcription of WNT target genes, among which are c-Myc and Cyclin D1 [93, 94]. The canonical WNT pathway primarily regulates cell self-renewal and proliferation, whereas the two β-catenin-independent pathways are mainly associated with cell polarity and migration. In the PCP pathway, the WNT ligand-FZD complex activates small GTPase proteins, such as RhoA, RAC, and Cdc42, through DVL activation [95]. In the calcium-dependent pathway, WNT ligands bind FZD and RYK or ROR receptors boosting cell migration. Moreover, this pathway mediates WNT canonical pathway inhibition via increasing intracellular calcium flux and activation of CaMK2, JNK, and PKC [96, 97].

Usually, canonical WNT signaling is activated by WNT1, WNT3A, WNT8, and WNT8B, whereas the non-canonical ones by WNT4, WNT5A, and WNT11. In addition to WNT ligands, also other proteins, as Norrin and R-spondin, can bind FZD receptors or a FZD-LGR4/5 complex, leading to WNT pathway activation [98]. Moreover, WNT signaling is regulated by other modulators, including secreted Frizzled-related proteins (sFRPs), WNT inhibitory factor 1 (WIF-1), and the Dickkopf protein family (DKK). sFRP family is composed of five members that can interact with WNT ligand, hampering the binding with FZD receptors, or with FDZ receptors, generating a nonfunctional complex [99]. WNF-1, like sFRPs, binds

WNT ligands, reducing their activity [100]. Otherwise, DKK family impairs the canonical WNT signaling pathway by binding to the LRP5/6 co-receptors, blocking the formation of WNT-FZD-LRP5/6 complex [101].

Compelling evidence demonstrates that aberrant activation of WNT signaling pathway is crucial for regulating CSCs in many tumors, including colorectal, breast, hematologic, skin, and lung cancers [89, 102].

The majority of colorectal cancers harbor genetic alterations in the WNT pathway. In particular, the abnormal WNT pathway alteration interferes with the balance between stem and differentiated cells in the colon crypts, giving rise to the development of CSC phenotype [103]. In squamous cell carcinoma, WNT signaling is crucial for the tumorigenic potential of $CD34^+$ CSCs [104]. Hence, targeting of WNT signaling pathway could be a therapeutic strategy to impair CSC survival [105].

2.2 The Hedgehog Pathway

The HH signaling pathway exerts its mitogenic and morphogenetic functions during embryonic development, while in the adult it is implicated in controlling the proliferation and plasticity of stem cells and progenitor cells of some organs, including the brain, skin, prostate, and bladder [106].

In absence of ligands (Indian, Sonic, and Desert), the receptor Patched (PTCH) inhibits the transmembrane protein Smoothened (SMO), and target gene transcription is impaired by Gli3 and Gli2-R (Gli2 in its repressor form). Upon ligand-PTCH binding, SMO is released, leading to the activation of Gli transcription factors (Gli1 and Gli2-A) and the transcription of target genes, such as BMI-1 and n-Myc [107].

Mutations of PTCH1 and SMO genes lead to ligand-independent HH pathway activation, and, based on the role of the HH signaling pathway in adult stem cell proliferation, deregulations of this pathway have a pro-tumorigenic role in colon, lung, and skin cancers [108, 109]. Furthermore, the aberrant activation of the HH pathway has been observed in CSCs from different cancer types, such as glioblastoma, lung squamous cell carcinoma, myeloma, and chronic myeloid leukemia (CML) [88, 110–112].

In multiple myeloma, SMO and Gli1 are highly expressed in CSCs, and the inhibition of HH signaling pathway impairs CSC self-renewal capacity [113]. In other studies, a higher activation of HH signaling has been also reported in lung squamous cell carcinoma and glioma CSCs [110, 114]. Moreover, in a murine CML model, the deletion of SMO reduces CSC compartment, and, conversely, SMO overexpression enhances CSCs and boosts CML progression [112].

A similar role of HH pathway was observed in colorectal CSCs. Indeed, the knockdown of SMO reduces colorectal CSC survival, whereas PTCH1 silencing increases their number [115].

2.3 The Notch Pathway

Notch signaling pathway is highly conserved, and it is crucial for the embryonic development of different tissues by regulating stem cell differentiation. In adults, Notch signaling regulates SCs of the skin, hematopoietic system, and intestine [116].

The Notch signaling cascade is mediated by the interaction between surface-bound ligands and transmembrane receptors. Five ligands, three delta-like proteins (DLL1, DLL3, and DLL4), two Jagged proteins (JAG1 and JAG2), and four Notch receptors (Notch1, Notch2, Notch3, and Notch4) have been identified in mammals. Both ligands and receptors are transmembrane protein, and thus Notch pathway activation requires cell-cell contact. The interaction between the ligand and the extracellular portion of the receptor prompts two consecutive proteolytic cleavages at the receptor intracellular portion by ADAM protease and γ-secretase, releasing an active Notch intracellular domain (NICD). Then, NICD translocates to the nucleus and activates transcription of target genes via the CBF1, suppressor of hairless, LAG-1/recombination signal-binding protein for immunoglobulin k J region (CSL/RBPJ) transcription factor [90, 117].

The Notch pathway is altered in many tumors, among which are leukemia, glioblastoma, and colorectal and breast tumors [118]. The role of Notch signaling in CSCs has not yet been completely elucidated, as it could act as tumor promoter or tumor suppressor in different tissue types. In colorectal cancer Notch signaling acts as a tumor promoter, because its inhibition induces the differentiation of adenoma APC-mutated cells into goblet cells [119]. In another study, pancreatic CSCs express high levels of Notch and JAG1 and the activation of Notch pathway is necessary to maintain CSC features [120]. Moreover, in a xenograft model of esophageal adenocarcinoma, the pharmacological inhibition of the Notch inhibitor reduces tumor growth and decreases CSC population [121]. The activation of Notch pathway is also required for CSC survival, as the use of a γ-secretase inhibitor on ductal carcinoma cells reduces mammosphere formation [122]. Conversely, in a murine model, the knockdown of Notch1 enhances the development of BCC skin tumors [123].

2.4 The Hippo Pathway

Hippo pathway regulates many cellular processes in embryonic development, and it is involved in tissue homeostasis and regulation of organ size.

In mammals, the activation of the Hippo pathway induces the phosphorylation cascades of serine/threonine MST1/2 and LATS1/2 kinases and then LATS1/2 phosphorylates Yes-associated protein (YAP) on Ser127. Phosphorylated YAP is sequestered in the cytoplasm and subsequently degraded in a ubiquitin proteasome-dependent manner. When dephosphorylated, YAP interacts with TEAD family transcription factors and promotes transcription of target genes, such as CTGF, AXL, *and* SURVIVIN. The transcriptional coactivator with PDZ binding motif

(TAZ) is a paralog of YAP in mammals and is regulated by the LATS1/2 in a similar manner. YAP/TAZ could also interact with other transcription factors, including Smad, Runx1/2, and Pax3 [124]. The Hippo pathway has a key role in embryonic development. In fact, YAP induces pluripotent gene expression in ESCs and hence needs to be inactivated during differentiation. Moreover, the knockdown of YAP/TAZ impairs embryonic stem cell phenotype [125, 126]. For these reasons, the effectors of the Hippo pathway are considered as tumor suppressors and their mutation can lead to an uncontrolled cell proliferation. Furthermore, YAP and TAZ are aberrantly upregulated in tumors, and their activation sustains CSC phenotype, metastatic potential, and resistance to chemotherapy [127, 128].

In breast cancer the overexpression of TAZ increases CSC compartment and their metastatic capacity in low-grade breast cancer cells [129, 130]. Moreover, glioma cancer cells display a high expression of YAP, and this correlates with reduced survival [131]. In another study, YAP is upregulated in gastric adenocarcinomas, and its nuclear localization is correlated with worse outcomes [132].

In addition to their role in tumorigenesis and metastatic progression, the activation of YAP/TAZ confers chemoresistance. Indeed, an elevated YAP/TAZ activity confers resistance to 5-fluorouracil in colorectal cancer cells and to doxorubicin and taxol in breast cancer cells [129, 133, 134].

2.5 The TGF-β Pathway

The TGF-β superfamily is mainly involved in the control of cell growth and differentiation. In particular, it regulates embryonic stem cell differentiation and commitment during embryonic development, while in adult stem cells, it is involved in the regulation of growth, apoptosis, and tissue repair [135]. TGF-β family is composed of 33 ligands and includes TGF-βs (1–3); activins; nodal, growth, and differentiation factors; and bone morphogenetic proteins (BMPs) [136]. TGF-β ligands bind to two types of transmembrane serine/threonine kinases, type I and type II receptors. Type II receptors hold a constitutive kinase activity and phosphorylates type I receptors, which propagate the signal by phosphorylating SMAD transcription factors. Eight different SMAD proteins (SMAD1–8) have been identified in mammals. In particular SMAD2–3 are activated by TGF-β, activin, and nodal receptors, while BMP receptors phosphorylate SMAD1/5/8, forming receptor-activated SMAD (R-SMAD) complexes. These complexes interact with SMAD4 (co-SMAD), common for all the pathways, and translocate to the nucleus. All the SMAD proteins, except SMAD2, own a DNA/binding activity but, in order to activate or repress the transcription of target genes, need to cooperate with coactivators (CPB and p300) or repressor (histone deacetylases) [137]. This pathway is known as canonical or SMAD-dependent pathway. TGF-β signaling could also be transduced by a non-canonical or SMAD-independent pathway. In fact, type I and II receptors could interact with different tyrosine kinase pathways, among which are p38/JNK, MAPK, and PI3K/AKT signaling [138].

Among the SMAD family, SMAD7 displays inhibitory activity through a feedback loop mechanism. In particular SMAD7, after TGF-β stimulus, translocates from the nucleus to the membrane, blocking R-SMADs' binding with type I activated receptors. SMAD7 can also interact with Smurf1/2 ubiquitin ligases and promote receptor degradation. Moreover, it could bind to DNA, preventing the formation of SMAD-DNA complex [139].

TGF-β acts as a tumor suppressor pathway in normal cells or in the early stages of tumor growth, whereas it has a pro-metastatic role in advanced cancers. TGF-β pathway components are frequently mutated in many tumor types, in order to escape from tumor growth inhibition mediated by TGF-β signaling. In fact, inactivating mutations in *TGFβRII*, *TGFβRI*, *SMAD2*, and *SMAD4* have been reported in colorectal, gastric, pancreatic, and prostate tumors. *TGFβRII* mutations are often found in tumors showing microsatellite instability, due to mutations in mismatch repair genes [140].

Conversely, *SMAD4* is mutated in sporadic colorectal cancers without microsatellite instability [141]. Germline mutation in *SMAD4* gene is found in a subset of juvenile polyposis syndrome patients. Moreover, promoter hypermethylation of *TGF-β* receptor genes leads to decreased expression and hence reduced pathway activity [142].

Specifically, TGF-β exerts its tumor suppressor role by (i) blocking cell cycle progression through the induction of CDK inhibitors and suppression of c-Myc expression and (ii) inducing apoptosis by increasing pro-apoptotic gene transcription. Conversely, TGF-β promotes tumor progression acting on tumor microenvironment and on cancer cells. Specifically, TGF-β signaling induces (i) immunosuppression by impairing cytotoxic T cell functions and inhibiting NK cell activity, thus favoring tumor immune escape, (ii) angiogenesis by stimulating the production of VEGF and enhancing vessel permeability, and (iii) the formation of cancer-associated fibroblasts (CAFs) that secrete growth factors and cytokines, inducing cancer cell mobilization and their metastatic spread. The role of TGF-β in cancer cells is primarily to increase epithelial to mesenchymal transition (EMT) and to promote the formation of distant metastasis [137, 143].

3 Developing Novel Tools to Target the Therapy-Resistant CSC Population

Researchers' battle of all time against cancer aims to discover targetable hallmarks that permit to distinguish cancer versus normal cells. This has been a tough challenge considering also the novel evidence about intratumoral and intertumoral heterogeneity, which makes the choice of a univocal marker even harder. Moreover, the prospective isolation of CSCs and the awareness of their role in conventional anticancer therapy failure complicated the development of novel compounds that selectively affect this cell subpopulation (Fig. 2).

3.1 What Makes CSCs Resistant to Conventional Treatments?

Several reports showed that CSCs are spared by the majority of conventional anticancer drugs, including target therapy, and thus are capable to expand and drive the re-growth of a new tumor with a more aggressive behavior. Indeed, CSCs are enriched in patients who underwent radiation therapy, chemotherapy, and target therapy. Lu et al. demonstrated that the chemotherapeutic compound carboplatin induces the expression of the glutathione S-transferase omega 1 (GSTO1) via the hypoxia-inducible factor 1 (HIF), triggering the activation of STAT3 signaling, which in turn leads to the expression of pluripotent genes and the enrichment of breast CSCs [144]. Treatment with the tyrosine kinase inhibitor gefitinib caused the expression of ALDH1A1 and the appearance of stem-like properties in lung carcinoma cells [145]. In glioblastoma multiforme, both temozolomide and the monoclonal antibody targeting VEGF, bevacizumab, provoked an increase of the CSC pool by a cellular dedifferentiation process and the expression of neuropilin-1 that strengthened VEGFR2/VEGF signaling, respectively [146, 147].

Recent discoveries pointed the finger against the capability of CSCs to repair DNA damage. For instance, $CD90^+$ breast CSCs were able to survive and expand following radiation therapy due to their enhanced free radical scavenger machinery, resulting in low ROS levels, which are the crucial mediators of ionizing radiation-mediated cell death [148]. Glioblastoma-initiating cells, when exposed to radiation therapy, showed a highly efficient DNA homologous recombination (HR) repair [149] as well as an activation of the non-homologous end-joining (NHEJ) pathway [150]. Breast CSCs surviving to DNA damage caused by ionizing radiations display high levels of RAD51. Knockdown of RAD51 sensitized CSCs to the PARP inhibitors even in the absence of *BRCA1/2* mutations [151]. Also CSCs isolated from glioblastoma patients showed elevated RAD51 levels and a highly efficient PARP1 activity that confers radioresistance [152]. A powerful strategy to make CSCs susceptible to DNA-damaging agents is to interfere with the activity of checkpoint kinases (CHKs). In this context, the pioneer work from Bao et al. demonstrated that $CD133^+$ glioma cells have a proficient activity of the ataxia telangiectasia mutated (ATM) protein and the CHK2 to counteract the formation of DNA lesions induced by radiotherapy [153]. Inhibition of CHK1 in non-small cell lung cancer (NSCLC) stem cells mediates the response to chemotherapy regardless of their TP53 mutational status [73]. On the other hand, the screening of an FDA-approved panel of drugs pointed out CHK1 as a biomarker of therapy response in colorectal CSCs. The authors also showed that the optimal effect was seen in hyperdiploid and TP53-deficient colorectal CSCs independently of RAS mutations [75]. For a comprehensive overview of all major mechanisms protecting CSCs from DNA-damaging agents, we refer you to an exhaustive review by Vitale et al. [72].

CSCs are in general resistant to all cellular mechanisms responsible for triggering cell death. Our group demonstrated that colorectal CSCs are protected from apoptosis through the upregulation of anti-apoptotic molecules such as survivin, under the control of IL-4/STAT6 signaling pathway. Neutralization of IL-4

or STAT6 pathway inhibition caused nuclear localization of survivin, which is correlated with good prognosis in colorectal cancer patients [154]. High levels of survivin and low levels of caspase 8 have been observed in other CSCs from brain tumors [155, 156]. Moreover, the combinatorial administration of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and suppression of cellular FLICE-like inhibitory protein (c-FLIP) affected breast CSCs' viability, self-renewal, and capability to form metastasis in vivo [157].

As discussed earlier in this chapter, CSCs share with their normal counterpart the ability to either impede the uptake of drugs or efflux them due to their presence on the plasma membrane of transporters, causing the so-called multidrug resistance (MDR). Overexpression of ABC transporters has been described in CSCs isolated from almost all cancer types [158].

Four generations of ABC transporter inhibitors have been developed and they are currently at different stages of clinical trials. While the first three generations of ABC transporter inhibitors showed enhanced toxicity in cancer patients because of high doses needed and the accumulation of chemotherapeutic drugs in the brain and kidney, the fourth generation of compounds possesses more specificity and promises less side effects [159].

Interestingly, an increasing number of studies showed that the administration of chemotherapy, which is the gold standard treatment for those cancers without any option of target therapy, enriches resistant dormant/quiescent cancer cell clones that repopulate the tumor. The fact that quiescent cells are spared by chemotherapy, because they are not actively proliferating, is not self-sustaining. Indeed, one reliable explanation is that surrounding dying cancer cells release cytokines and other molecules that act as mitogens for quiescent cancer cells, allowing them to reawake [160]. Thus, it is urgently needed to develop novel compounds targeting quiescent CSCs to impede tumor repopulation.

3.2 Stemness Modulator Drugs

Several molecules targeting CSC self-renewal, such as BMI-1, HH, EGF, PI3K, MAPK, NF-kB, WNT/β-catenin, and Notch pathway inhibitors, have been extensively studied and used in clinical trials [63]. Multiple combinations of these compounds are under testing in order to overcome mechanisms of resistance and rescue generated by single treatments.

Quiescence is a cellular state used by CSCs to stay in the G0 phase of cell cycle, waiting for appropriate stimuli to re-enter the cell cycle. CSCs can remain in a quiescent state for a long period of time because (i) as non-cycling cells they are spared by chemotherapy or (ii) they engraft at distant metastatic sites and are preparing for macrometastatic outgrowth. The latter phenomenon is defined as tumor dormancy. One possible approach to target quiescent CSCs is to force them to reenter the cell cycle, even if it is a risky procedure, in case anticancer drugs would not efficiently kill awakened cells, causing disease progression. Interestingly, JARID1B, which is a H3K4 demethylase that binds to the Notch ligand JAG1 promoter, inhibiting its

transcription, demarcates a slow-cycling melanoma population responsible for the maintenance of the tumor. Knockdown of JARID1B impaired the metastatization of melanoma cells in pre-clinical studies [161].

The BMPs are growth factor proteins belonging to the TGF-β family. The BMPs regulate the proliferation and differentiation of normal and cancer cells from several tissues. For this reason, the BMPs have been used in an attempt to induce the differentiation of CSCs, making them more susceptible to chemotherapy and target therapy. Indeed, the administration of BMP4 caused the differentiation of colorectal CSCs and their efficient killing by 5-fluorouracil and oxaliplatin [162]. Moreover, a BMP7 variant (BMP7v) inhibits tumor angiogenesis in glioblastoma CSC xenografts [163]. Another ideal example is represented by the all-trans retinoic acid utilized to cause terminal differentiation of acute promyelocytic leukemia stem cells [164].

The deregulation of epigenetic pathways can be at the base of CSC aggressive behavior. Inhibitors of the DNA methyltransferase 1 (DNMT1) dampened the tumorigenic potential of lung and breast CSCs [165, 166]. DNMT1 inhibitors function also as differentiation therapy, rendering cancer cells more vulnerable to conventional chemotherapy [167]. The second class of epigenetic modulators is constituted by the histone deacetylase (HDAC) inhibitors. HDAC inhibitors induce differentiation and apoptosis of CSCs [168, 169]. Moreover, the histone methyltransferase (HMT) and the histone demethylase (HDM) inhibitors showed efficacy in depleting the CSC compartment [167]. Nowadays, increasing attention has been paid to bromodomain and extra-terminal (BET) proteins that recognize acetylated histones and regulate gene expression of oncogenes, one among all is MYC. JQ1 is one of the most studied BET inhibitor for its capability to block MYC activity and induce apoptosis and differentiation of cancer cells [167].

3.3 Targeting the "Metabostemness"

According to the canonical CSC model, stemness is governed by genetic and/or epigenetic modifications. However, recent evidences showed that cellular metabolism plays a crucial role in the acquisition or loss of stem-like peculiarities. Metabolic shifts can occur as early events in normal or differentiated cancer cells, making cells more prone to the expression of pluripotent genes and epigenetic reprogramming. Additional insults can terminally define the stem-like features of a given cancer cell and its prominence in the cancer cell hierarchy and more in general in cancer evolution. The term "metabostemness" has been coined to indicate the metabolic processes controlling the epigenetic and genomic hits driving normal and differentiated cancer cells toward a stem-like state [170]. Indeed, metabolites from the OXPHOS, glycolysis, tricarboxylic acid (TCA), and mitochondrial fatty acid oxidation are used as cofactors for epigenetic DNA changes such as methylation and acetylation. Moreover, the production of oncometabolites can induce histones and DNA modifications leading to tumorigenesis [170].

As previously discussed in this chapter, whether CSCs are glycolytic or depend on OXPHOS is context dependent [74]. However, the most promising strategy to eliminate CSCs seems to be the use of OXPHOS inhibitors. Several investigations showed that CSCs do not satisfy their energetic demand from glycolysis, may be due to low glucose levels in tumors, and rely on OXPHOS. For instance, the antibiotic salinomycin that inhibits OXPHOS was effective in eliminating breast CSCs [171]. Other compounds inhibiting mitochondrial respiration and translation have been developed and selectively target the CSC compartment [74]. Although being an antidiabetic drug, metformin has been repositioned as an anticancer drug. Indeed, it is effective against CSCs by inhibiting the mitochondrial complex I impairing OXPHOS [172]. Many efforts are being made to selectively deliver these compounds uniquely to the mitochondria of cancer cells.

3.4 Targeting Tumor Microenvironment

Tumor microenvironment is composed of stromal cells, which synthesize the extracellular matrix and secrete pro-tumorigenic factors, and also by immune cells that play opposite roles either as tumor promoters or as tumor suppressors. Altogether these components constitute the tumor niche, which is a spatial entity where CSCs reside to thrive, preserve their stemness traits, and be protected from anticancer therapeutic compounds. Thus, it is now clear that a really powerful approach to direct CSCs' fate consists of targeting their microenvironment. On the other hand, chemotherapy can induce modifications in the tumor microenvironment. For instance, in a study by Lotti et al., CAFs, isolated from colorectal cancer patients, were treated with fluorouracil, oxaliplatin, and leucovorin, causing CAFs' secretion of IL-17A that, in turn, promoted CSCs' self-renewal and tumorigenic properties [173].

Tumor microenvironment is a reservoir of resources for CSCs that can allow CSCs to undergo EMT. The latter is a process that fosters the invasive and metastatic behavior of CSCs. The EMT and therapy resistance can be promoted by Zeb1 in CSCs from a variety of tumor types. Fibroblast-secreted HGF, OPN, and SDF-1 increased the expression of CSCs' biomarker CD44v6 and an EMT signature on colorectal CSCs through the activation of WNT/β-catenin pathway [42]. Besides directly targeting the WNT pathway, it was shown that the BMPs induce sensitization to standard anticancer therapy and counteract β-catenin activation [42, 162]. Another important effector of the EMT process is the TGF-β. The TGF-β has a peculiar behavior throughout the multiple steps of tumor development, being usually a tumor suppressor at the very beginning of tumor formation and an inducer of EMT in advanced cancers. Inhibitors of HGF, TGF-β, and WNT pathways are in phase II and III of clinical trials as well as other inhibitors of extracellular molecules such as IL-6, Hedgehog, and Notch. CSCs are able to switch from one pathway to another accordingly to the tumor milieu and the exogenous administration of inhibi-

tors of one specific pathway. In the attempt to identify an effective therapeutic target able to hamper the EMT process, several compounds have been developed also to target tumor hypoxia (HIF1α inhibitors), intracellular pathways (SRC, FAK, PI3K/AKT, and RAS/RAF/MAPK inhibitors), and transcription factors (YAP/TAZ, NF-kB, and STAT3 inhibitors). Marcucci et al. have recently reported an exhaustive overview about the state-of-the-art EMT inhibitors in clinical development [174].

Immune cells are a fundamental component of tumor microenvironment. CSCs can "hide" themselves from T cell-mediated cell death through the expression on the cell membrane of programmed cell death ligand 1 (PD-L1) [175], whose binding to its receptor PD-1, on T cells, inhibits immunological cytotoxicity and facilitates T cell apoptosis. IL-4 is secreted by the tumor microenvironment and blocking of IL-4 signaling reduces the expression of PD-1 on T cells as well as promotes stem-like traits, proliferation, invasion, and tumorigenic capabilities of breast CSCs [176]. Both the tumor necrosis factor α (TNF-α) and IL-6 secreted by immune cells upregulate the expression of EMT-associated genes, and the IL-22 activates STAT3 signaling and the transcription of stemness genes [63]. A combination of IFN-γ and oxaliplatin was effective against colorectal CSCs [63]. In pancreatic cancer, inhibition of chemokine (C-C motif) receptor 2 (CCR2) to myeloid cell receptor colony-stimulating factor 1 receptor (CSF1R) on tumor-associated macrophages (TAMs) or inflammatory monocytes increased T cell responses against tumors, augmented the efficacy of chemotherapy, and fostered stem-like traits and metastatic capabilities of pancreatic cancer cells [177].

4 Concluding Remarks

Cancer patient deaths are mostly caused by the outgrowth of metastasis rather than by the clinical consequences of the primary tumor. The dissemination of cancer cells from the primary tumor site to distant organs represents a crucial mechanism to be targeted by innovative anticancer therapies. Indeed, metastatic disease still presents limited therapeutic options.

CSCs are responsible for the aforementioned process of metastatic spreading and they are resistant to conventional anticancer treatments. Moreover, CSCs are able to suppress immune-mediated cell death and can reprogram their metabolism accordingly to their needs. Although CSCs are considered the root of many cancers, there is a lack of markers that exclusively identify the CSC subpopulation.

Several novel anticancer compounds selectively targeting CSCs are obtaining promising clinical responses. However, the main unsolved issue in clinical cancer research is the absence of specific CSC biomarkers able to dictate cancer patients' outcomes and, additionally, methods to follow up the disease with regard to the quality and quantity of CSCs, as the post-treatment reduction of a tumor is not indicative of targeting CSCs.

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A Cancer Stem Cell Perspective on Minimal Residual Disease in Solid Malignancies

Maartje van der Heijden and Louis Vermeulen

Abstract Minimal residual disease (MRD) is a major difficulty in clinical oncology. It refers to the situation in which seemingly successful therapy is followed by a period of clinical complete remission after which the tumor eventually relapses. This course of events suggests that a small number of tumor cells evaded the therapeutic schedule and give rise to the recurrence, sometimes even after many years. Multiple processes have been advocated to underlie MRD including genetic heterogeneity, cellular dormancy, and also simple stochasticity. In the past decennium, another potential contributing explanation emerged, which is related to the notion that many tumors are hierarchically organized tissues such as advocated by the cancer stem cell model. In particular since it became increasingly clear that cancer stem cells are highly resistant toward chemotherapeutic interventions, this model provides a very elegant framework for MRD. However, the cancer stem cell theory is still an intense field of study, and many challenges remain that each has its own impact on how MRD can be explained as a consequence of therapy-resistant cancer stem cells. This chapter will provide an overview of the developing cancer stem cell model and will outline which important questions remain with respect to the cancer stem cell nature of MRD. In addition we will explore the consequences of drug development of the most recent insights in the cancer stem cell field, such as the central role of the microenvironment and the plasticity of the cancer stem cell population. To conclude we will provide several suggestions as how to optimize clinical evaluation of novel drugs keeping in mind the lessons from the cancer stem cell concept.

Keywords Cancer · Colorectal cancer · Cancer stem cells · Cancer stem cell markers · Stem cells · Minimal residual disease · Cancer stem cell niche · Therapy resistance · Side population

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1 Introduction

Cancer is a disease in which the body's own cells proliferate in a non-regulated fashion, have the ability to invade through tissue barriers, and have obtained ways to evade cell death. These properties of cancer cells are acquired during a stepwise process that spans usually years to decades. In the majority of cases, the underlying causes of these changes in cell behavior are mutations in genes that encode for crucial proteins that regulate proliferation, differentiation, migration, and apoptosis. These mutations result in modified proteins, which either results in continuous activity (oncogenes) or impairs the function of the protein (tumor suppressor genes) [32]. A typical cancer cell is believed to contain approximately seven to ten driver mutations, i.e., mutations that are of crucial importance in the development, expansion, and spread of the tumor [32]. The driver mutations result in activation of cellular programs that resemble features that are active during particular stages of development (e.g., proliferation) or in other cell types (e.g., migration of mesenchymal stem cells) but which are inappropriate for the specific cell in the given context.

Although the above account is a highly simplified view of malignancies, it already comprehends the essence as to why cancers are so difficult to cure in situations where surgery alone is insufficient in removing all malignant cells: while, for example, antibacterial agents are directed against foreign components of the bacteria that entered the human body, anticancer drugs are aimed at targeting cells that are in essence highly similar to the normal, non-transformed cells. As a consequence, the therapeutic window of most anticancer drugs is relatively narrow.

Despite this inherent difficulty in targeting cancer cells, during the latest decades, very effective therapies have been developed that have resulted in a major increase in both survival rate and quality of life of patients suffering from many types of cancer. The anticancer drugs currently in use as the standard of care are mostly aimed at characteristics that are distinctive between normal, untransformed, cells and cancer cells such as an increased proliferation rate (e.g., oxaliplatin) or the dependence of cancer cells on particular cellular signals (e.g., c-Kit inhibitors, monoclonal antibodies against epidermal growth factor receptor (EGFR). Application of these types of drugs commonly results in tumor reduction, better disease control, and prolonged survival [16, 34]. Often the primary malignancy or the metastases appear to have disappeared fully. This is referred to as complete response. Unfortunately, even in the majority of cases in which a complete response is recorded, tumor eventually relapses and has acquired resistance against the initially applied drug [35, 38, 57]. The state of total clinical remission preceding a tumor relapse is referred to as minimal residual disease (MRD), as it is assumed that a small fraction of cells have evaded therapy. Many different biological processes have been suggested to underlie MRD which we will briefly discuss in this chapter. In recent years much interest has gone to the cancer stem cells as the potential culprits responsible for MRD [40]. This chapter mainly focused on reviewing how the cancer stem cell theory relates to MRD and how recent advances

in cancer stem cell research impact on the notion these cells are responsible for MRD. In particular, new insights in the way cancer stem cells are dependent on the interaction with the microenvironment (the cancer stem cell niche), even to the point that signals emanating from the environment can install a cancer stem cell phenotype in more differentiated cancer cells, will be investigated. In the final part, we will explore the consequences of advances in the cancer stem cell field for the development and clinical evaluation of novel drugs. We will advocate that the current strategy to assess therapy efficacy in early clinical trials is potentially inept to determine the therapeutic potential of compounds in prolonging disease-free survival and improving quality of life of cancer patients. To conclude, we will suggest several avenues to explore further to improve selection of the most promising drugs for further clinical testing in randomized controlled trials.

2 The Developing Cancer Stem Cell Model

The notion that cancers are heterogeneous tissues composed of cells which vary in morphology, marker expression, and proliferation rate is widely established and around for over a century [91]. These phenotypical differences are related to the genetic background of the cells, comprising the mutations they acquired during the development of the malignancy, as well as due to distinct signals cells received from the (micro-)environment. Moreover, more recent observations have revealed that differentiation grade of individual cells importantly contributes to cellular heterogeneity in tumors. Following these insights, it was proposed that malignancies, like normal tissues, contain a small population of stem cell-like cells with an undifferentiated, immature phenotype that displays properties associated with normal stem cells including the abilities to self-renew and to generate more differentiated tumor cells. A consequence of this hypothesis is that tumor growth is dependent on these cancer stem cells and that more differentiated tumor cells have lost the ability to contribute to tumor growth and progression. Only in the last decades it became feasible to experimentally test this hypothesis successfully, a development greatly facilitated by technological progress in the areas of monoclonal antibody generation and fluorescence-activated cell-sorting equipment [52, 91].

Initially the cancer stem cell concept was developed in hematological malignancies for the apparent reasons that cell populations are easier to separate and the cell surface markers associated with immature cell types much better characterized [96]. More recently the discoveries in that field have been generalized to solid malignancies as well. In this respect, breast cancer, colorectal cancer, and brain tumors were the first solid tumors in which rare tumor-initiating cells were identified [2, 59, 73]. In a typical assay to assess cancer stem cell properties of a subset of cells, the tumor is dissociated, and after staining with antibodies directed against cell surface molecules that are associated with immature cell types (e.g., CD133, CD44), the cells are separated by flow sorting or by magnetic bead separation. The purified cell populations are subsequently injected in immunodeficient mice in a limiting dilution assay

and the frequency of tumor initiation is determined. Using this approach colorectal cancer cells expressing CD133 were identified to comprise the tumorigenic fraction in this malignancy, and therefore it is suggested that the $CD133^+$ population of cells contain cancer stem cells [59, 63, 83, 85]. This notion is corroborated by the finding that single cell-cloned cancer cells expressing cancer stem cell markers display both multi-lineage differentiation potential and self-renewal, two main characteristics of stem cells [8, 18, 92, 98]. Much of the debate on cancer stem cells centers around the question what fraction of tumor cells function as cancer stem cells, as in case the cancer stem cell population is a large proportion of tumor cells, viewing a malignancy as hierarchically organized is obviously less significant [1, 52]. In this respect the transplantation assay to determine cancer stem cell properties is often criticized. For example, it is suggested that the ability of cells to induce new tumors does not reflect the potential of these cells to fuel expansion and progression of an established malignancy. In addition this assay depends on complete tissue disruption and any level of tissue organization that might be vital during tumor growth is lost. To conclude, also the fact that human cells are injected in immunodeficient mice (xenotransplantation) raises concerns, as clearly in human cancers the immune system plays a crucial—albeit a yet incompletely understood—role. This criticism is reflected in the finding that the particular immunodeficient mouse strain that is used to assess the tumor-initiating cell frequency greatly influences the frequency of these cells [62].

In recent years new techniques have become available for studying cellular hierarchies within malignant tissues. For example, recent research has identified hierarchical cell lineages in oligodendroglioma with the use of data derived from RNA sequencing of primary tumors. Here, the majority of cells displayed a differentiated glial cell program, whereas a rare group of cells showed stem cell characteristics [82]. Also, genetic lineage tracing is an increasingly popular method to study stem cell behavior of normal stem cells and cancer stem cells [90]. This method has proved very beneficial to examine the stem cell dynamics in normal intestinal tissues in homeostasis and during tumor initiation in different organ systems [49, 53, 74, 75, 89]. Additionally, genetic lineage tracing in normal tissue has enabled the detection of multiple new stem cell markers. [5, 58]. Subsequently, these stem cell markers have been adopted to identify cancer stem cell populations of the corresponding tissue of origin [72]. Furthermore, several research groups have identified a population of cancer stem cell-like cells in unperturbed premalignant lesions or tumors by using lineage tracing in mouse models of various malignancies, including intestinal adenomas, squamous skin tumors, and glioblastoma multiforme (GBM) [9, 13, 23, 56, 58, 68]. Although these mouse models only reflect the corresponding human malignancies to a certain degree, these studies highlight, that at least in early neoplastic expansions cells with properties of stem cells are present. Moreover, studies with a marker-free lineage tracing approach in adenoma mouse models have also revealed a multi-lineage cellular hierarchy in these lesions by showing only a limited number of stem cells [44, 68]. In contrast to the cancer stem cell marker method, a marker-free tool enables studying cancer stem cell dynamics in an unbiased manner, and therefore further studies in cancer models that employ this

approach are awaited. However, unfortunately, lineage tracing as performed in mouse models and xenografts will not be feasible in human tumors in situ.

Another point of criticism is the potential plasticity of the cancer stem cell population during time. Our group has provided experimental data that suggest that more differentiated tumor cells can adopt a cancer stem cell phenotype upon exposure to factors produced by the tumor microenvironment [88]. These data suggest that the cancer stem cell population is not stable over time but instead is continuously shaped by the microenvironment. We refer to this as the dynamical cancer stem cell model, in contrast to the strictly hierarchical model that was often proposed initially (Fig. 1) [87]. It is clear that this modified model directly impacts the notion that

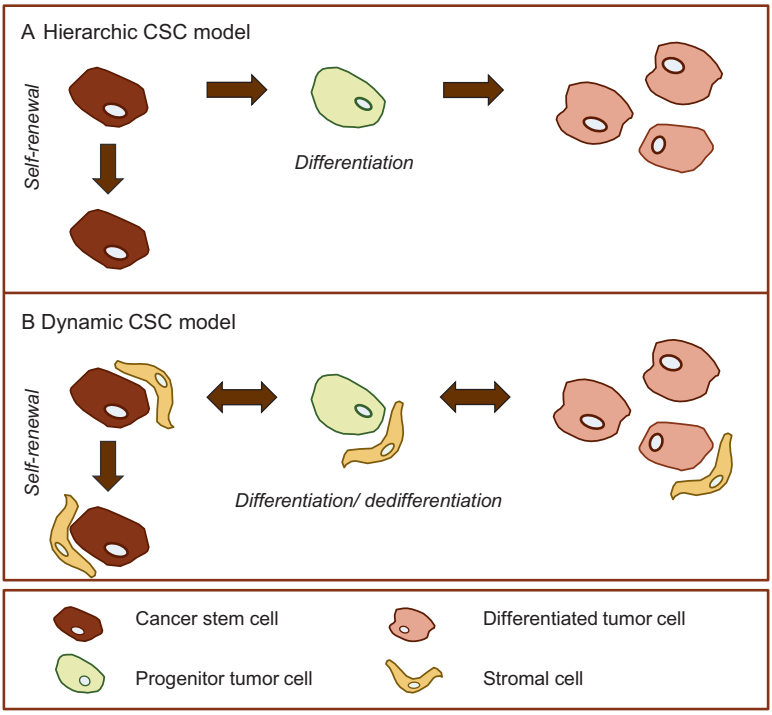

Fig. 1 Hierarchical versus dynamic cancer stem cell model. (**a**) Initial cancer stem cell model that has been proposed as strictly hierarchical. This encompasses the idea that solely cancer stem cells promote tumor growth by both self-renewal divisions, expanding the cancer stem cell pool and generating progenitor-like cells. These progenitor-like cells subsequently give rise to terminally differentiated cells, which are considered to make up the bulk of the tumor. (**b**) In past years, the hierarchic model has substantially evolved to a more fluid interpretation of the concept. The cancer stem cell fraction is a dynamic population, and more differentiated cancer cells can reacquire stem cell features (dedifferentiation) if the right cues are present. A major role in this process is reserved for the microenvironment that modifies cells by providing signals that both promote and sustain cancer stem cells but also generate novel cancer stem cell by inducing dedifferentiation (Stromal cells in the figure represent myofibroblasts in case of colon cancer or endothelial cells in case of GBM)

cancer stem cells are the cells responsible for MRD and on the suggestion that effective and specific targeting of these cells will cure the patient. We will discuss these consequences in more detail later.

3 Cancer Stem Cells and Minimal Residual Disease

Cancer stem cells are thought to share many features with normal tissue stem cells [18, 94]. Besides self-renewal and multi-lineage differentiation potential, these include inherent resistance to chemotoxic compounds and radioresistance. This is concluded from experiments in which human tumors transplanted in mice are treated resulting in an increased fraction of cells expressing markers associated with cancer stem cells [24, 47, 83]. Using this approach it has been established that colorectal cancer cells displaying the cancer stem cell marker CD133 are enriched during following oxaliplatin treatment [83]. Alternatively, studies have been performed that directly evaluate the clonogenic potential of malignancies that underwent treatment estimating the cancer stem cell fraction of tumors independent of cell surface marker expression. Human colorectal cancer xenografts after treatment with irinotecan display an increased tumorigenic potential when cells were injected in immunodeficient mice in a limiting dilution assay [24]. Comparable results have been obtained in several different malignancies, including leukemia, GBM, breast, lung, bladder, urothelial, and prostate cancer, establishing that tumorigenic cancer cells with stem cell properties are indeed relatively resistant to therapeutic interventions in most malignancies [14, 21, 40, 45]. Moreover, recent reports in mouse models of squamous cell and pancreatic carcinomas have also shown enrichment for the cancer stem cell populations, respectively the $BMI1^+$ and MSI^+ cells, upon chemotherapy [12, 28]. Notably, in a GBM mouse model, it is found that the quiescent cancer stem cell population, as is evidenced by lineage tracing, reenters the cell cycle and shows more clonal growth upon temozolomide treatment [97]. These recent studies provide additional evidence that targeting self-renewal of cancer stem cells in combination with the standard therapy regimens could be an important aspect of anticancer treatment in order to prevent relapse.

To date, there are only few studies in which the effect of therapeutic intervention on the cancer stem cell population is directly assessed in patients. In this respect, colorectal cancer patients treated with 5-fluorouracil in a neoadjuvant setting display enrichment for cancer stem cells, as evidenced by an increase in ABCB5-expressing cells [95], and similar findings were reported in a series of breast cancer patients [80]. Caution is warranted though as not in each malignancy cancer stem cells seem to be equally resistant to therapy. For example, in the case of testicular cancer, the undifferentiated tumor cell population that is responsible for tumor growth seems to be more sensitive to cisplatin compared to the more differentiated cells [54]. It is likely that this particular feature is related to the generally very good therapeutic outcome in this type of malignancy.

The widespread relative resistance of cancer stem cells toward therapeutic interventions is not exclusively related to chemotoxic compounds as also novel targeted agents fail to successfully eradicate this population in many cases. In a study in patients with gastrointestinal stromal tumors (GIST) that receive imatinib (c-Kit and BCR-ABL inhibitor) treatment, it was revealed that discontinuation of the drug results in rapid relapse of the disease [33, 46]. This finding supports the notion that imatinib fails to target GIST stem cells, as was established using a murine GIST model [4]. Similarly, it has been found that Her-2+ breast cancer stem cell are resistant to trastuzumab and that application of this drug might even increase the size of this population by an inflammatory loop [43].

Additionally, recent findings show that in lung, breast, and pancreatic carcinomas, the same signaling route, namely, the KRAS-RalB-NF-κB pathway, drives cancer stemness and resistance of cancer stem cells to erlotinib, an EGFR inhibitor [71]. Altogether these findings indicate that cancer stem cells could also be responsible for residual disease after treatment with targeted therapies. However, it remains to be established to what extent the plethora of other targeted therapies that are available are targeting the stem cell populations effectively.

The biological features that underlie the superior resistance of cancer stem cells to therapeutic interventions are only partially resolved and a field of intense study. One of the often-featured mechanisms is the proposed quiescence of cancer stem cells. However, conflicting data exists on the cycling rate of cancer stem cells. In a study using a genetic mouse model of GBM, it was reported that tumor stem cells are relatively quiescent and that this feature contributes to their resistance to temozolomide [13]. However, in other malignancies, for example, in breast cancer, it is demonstrated that cancer stem cells cycle at a significantly higher pace compared to normal stem cells, instead [17]. It was established that in the case of colon cancer, both rapidly cycling and more quiescent cancer stem cells could be identified that display differential sensitivity to treatment [29]. This finding suggests that the proliferative properties of cells remain important with respect to the chemosensitivity, also within the cancer stem cell population.

Other groups have aimed to elucidate resistance of cancer stem cells by the presence of drug-efflux pumps [37, 47, 48]. Indeed, populations with cancer stem cell properties can be identified in several tumors by a dye exclusion assay (side population), which is a functional analysis to test drug transporter activity [20]. Although it has been proven difficult to link the expression of individual drug transporter to resistance, it was reported that colon cancer stem cells express ABCB5 and that these cells are enriched following therapy with fluorouracil, as we have already touched upon earlier [95]. An additional mechanism, and one that might also explain radioresistance of cancer stem cell populations, is related to the way cancer stem cells deal with the inflicted damage. In this respect, evidence is accumulating that cancer stem cells express high levels of anti-apoptotic molecules that result in a higher apoptotic threshold which can be evaded by combination treatment with small molecules that are blocking these anti-apoptotic proteins [19, 83]. Similarly, genes involved in DNA damage response pathways are highly expressed in cancer stem cells facilitating the DNA repair after an insult. GBM stem cells, characterized

by high CD133 surface levels, have an enhanced activation of checkpoint proteins (e.g., ATM, Chk1, and Chk2) following DNA damage compared to the CD133-low cell population [66].

To conclude, the location of cancer stem cells within the tissue might influence the effect of treatment on that population. In this respect, the study of the tumor microenvironment, and in particular the cancer stem cell niche, is of major importance, as we will discuss in the section below.

4 Cancer Stem Cells: The Microenvironment and Dedifferentiation

Normal tissue specific stem cells reside in a niche that consists of specialized cells providing defined cues to support self-renewal divisions and maintain an immature state [51, 55]. For example, intestinal stem cells lose their stem cell identity when they migrate upwards away from the crypt niche factors provided by Paneth cells [65]. In addition, extracellular matrix components and physical properties such as the local oxygen concentration can contribute to preserving a functional stem cell population at defined positions within the organ [41]. Niche signals are also considered to fulfill a critical role in directing a proper response of stem cells in situations of tissue damage or other external influences that warrant an appropriate response of the stem cell population [67]. For example, intestinal differentiated cells reexpress Wnt signaling in an inflammatory environment and thereby acquire stem cell potential. As a result, these dedifferentiated cells also gain the stem cell ability to initiate tumor formation [70]. Analogously, cancer stem cells are believed to be influenced by signals form the environment that mimic the relation of normal stem cells with their niche. For example, it has been established that GBM stem cells reside close to vascular endothelial cells [10, 15]. These cells produce nitric oxide, which has the ability to activate the Notch signaling pathway in GBM cells, and this signal maintains the stem cells in an undifferentiated state [11].

The relationship with cancer cells and their niche seems to be a bidirectional one as cancer stem cells can actively attract endothelial cells by producing VEGF [3]. This suggests that part of the beneficial effects of VEGF inhibitory therapy might be related to disturbance of the cancer stem cell niche. Moreover, the intimate relationship of cancer stem cells with their niche is highlighted by the finding that GBM stem cells can differentiate in cells mimicking endothelial cells, thereby directly creating their own niche [64, 93].

The niche has also profound clinical importance as evidence is accumulating that the cancer stem cell microenvironment plays a critical role in a tumor's response to therapy. For example, GBM stem cells that reside in close contact to vascular endothelial cells demonstrate increased radioresistance [36]. Interestingly, this feature of endothelial cells could be reversed by application of a Notch inhibitor, further supporting the importance of this pathway in GBM stem cells [93]. In a squamous cell

carcinoma mouse model, it is found that cancer cells on the tumor border are installed with active TGFβ signaling by the microenvironment. Subsequently, these cells are driven into a more resistant state to cisplatin [60]. In the case of colon cancer, it was established that IL-4, produced by infiltrating immune cells and differentiated tumor cells, results in upregulation of anti-apoptotic molecules in cancer stem cells [83, 85]. In addition, another report shows that signals emanating from differentiated tumor cells are able to enhance the clonogenic capacity, which is a cancer stem cell feature, of undifferentiated stem cell-like tumor cells. Here, also a similar effect has been found for hypoxic conditions. This increased clonogenicity of cells can be suppressed by imatinib treatment, indicating that in both cases, KIT signaling is regulating this effect [27].

Previously, our laboratory established that besides maintaining stem cell properties in cancer stem cells, the microenvironment can also install these features in more differentiated tumor cells [88]. Myofibroblasts that reside in the stroma of colorectal cancers produce growth factors, i.e., hepatocyte growth factor (HGF), osteopontin, and stromal-derived factor 1α, which activate the Wnt pathway and reverts differentiated tumor cells back to a cancer stem cell state. These dedifferentiated cancer cells display all features associated with cancer stem cells, including the ability to induce tumors upon transplantation. Also, enhanced Wnt signaling, as induced by stromal cells, results in an increased CD44v6 expression in tumor cells which makes them more amendable for migration and eventually metastasis [84]. Interestingly, another study has shown that in colorectal cancer, under the influence of chemotherapy, the array of growth factors that is secreted by fibroblasts changes, which leads to a cancer stem cell phenotype in more differentiated tumor cells [50].

The next illustration shows the importance of the interplay between cancer stem cells and differentiated cancer cells in their niche upon chemotherapy. In colorectal cancer, it is found that a small percentage of differentiated cells display a high amount of drug-efflux pumps, whereas the stem cell-like cells lack this feature. This characteristic makes this subgroup of differentiated cells more resistant to irinotecan treatment. Subsequently, these cells generate a protective effect on the stem cell-like cells that are in close proximity by keeping drug concentrations low upon treatment [26].

These findings have important implications for the way MRD is relating to the cancer stem cell concept, as it indicates that MRD does not necessarily need to consist of clonogenic cancer stem cells at any time. Alternatively, after application of the therapy, more differentiated cells that have evaded the treatment adopt a cancer stem cell state and fuel the relapse, as a consequence of microenvironmental signals (Fig. 2a). Since cancer stem cells are not completely insensitive to commonly used drugs but only *relatively* more resistant, it is likely the scenario above underlies many of the observed relapses, especially as the differentiated tumor cells outnumber the cancer stem cells manifold. A further consequence of the notion that differentiated tumor cells can adopt a cancer stem cell phenotype is that therapeutic interventions specifically aimed at targeting the cancer stem cell population are likely to fail. In that sense it remains crucial for successful therapies to also effectively target the more differentiated tumor cells. Alternatively, signals emanating

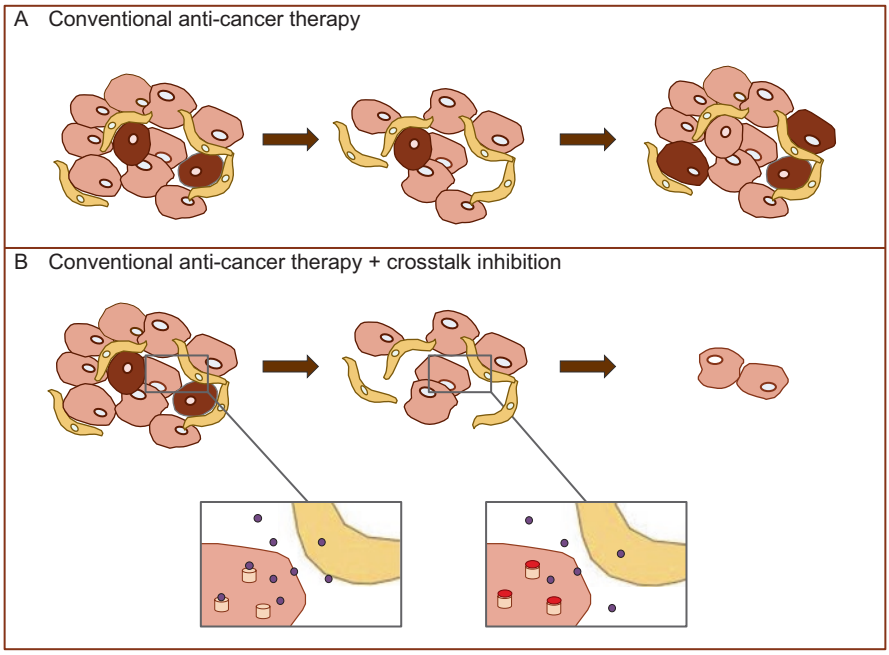

Fig. 2 Optimizing therapy by interfering with signals emanating from the cancer stem cell niche. (**a**) Current therapies are effectively targeting differentiated cells but unfortunately are much less capable of killing cancer stem cells. In light of the dynamic cancer stem cell model, tumor recurrence and therapy resistance are due to relative resistance of cancer stem cells in combination with more differentiated cells that survived therapy adopting a cancer stem cell phenotype. The latter is due to signals that are produced in the microenvironment, e.g., HGF and osteopontin. (**b**) By blocking signals emanating from the microenvironment that sustain and induce stem cell features in cancer cells, the efficacy of therapy could potentially be increased

from the microenvironment that install a stem cell phenotype in differentiated cancer cells could be the target of promising novel therapeutic strategies (Fig. 2b). Indeed, inhibition of HGF in a xenograft model of human colorectal cancer has proven to be a promising new therapeutic approach [86]. It remains to be explored, however, if using neutralizing antibodies against this growth factor does indeed prevent dedifferentiation of differentiated tumor cells following therapy. Whereas, in our view, selective targeting of cancer stem cells is unlikely to result in lasting therapeutic effect, it is evident that in any case, cancer stem cells need to be targeted successfully to achieve curative therapy. Crucially, tumor growth is driven by a small clonogenic core of stem cell-like tumor cells which are sustained by microenvironmental signals. These novel insights in tumor biology, that is tumor growth, warrant reconsideration of the way that novel therapeutic schedules are currently evaluated.

5 Cancer Stem Cells and Clinical Trials

Recent clinical studies have aimed to study the effectiveness of therapies that directly target pathways that are associated with a cancer stem cell phenotype, namely, signaling pathways that are involved in embryonic and adult tissue development. Analogously, these pathways, which include the Hedgehog, Notch, and Wnt signaling cascades, are thought to be preserved in cancer stem cells. Therefore, the idea is that inhibition of these pathways would impact on the survival, proliferation, and (de-)differentiation of cancer stem cells [78]. For example, there is some evidence that breast cancer patients treated with a more specific anticancer stem cell treatment, i.e., Notch inhibitor, show a reduction in the number of cancer stem cell, as is evidenced by a decrease in $CD44^{+}/CD24^{-}$ and $ALDH^{+}$ populations in post-treatment biopsies [69]. However, until now, many of these compounds showed lack of any clinical activity in different kinds of cancers [79]. Two issues might be underlying to this observation:

1. Cancer stem cells might not be dependent on only one of these pathways and therefore a combinatorial target regime would be more beneficial.
2. Each cancer type can be subdivided into different molecular subtypes [31].

However, the origin of these distinct molecular diseases within similar tumor types is still unclear. Consequently, there might also be inter-tumor heterogeneity in the cancer stem cell characteristics, which then most likely require different therapy strategies for successful targeting. Therefore, also more insights are necessary about the molecular basis of these subtypes.

Another challenge is the detection of the post-therapeutic tumor load. Currently, novel drugs for the treatment of solid malignancies in the initial stages of clinical testing are evaluated using the Response Evaluation Criteria in Solid Tumors (RECIST) system [25]. This set of rules evaluates the effects of a novel drug on the number and size of the so-called target lesions. In short, therapy is regarded to be effective when the target lesions shrink and if no novel tumor localizations develop on radiological imaging. Only after passing this test successfully in phase II further clinical testing in phase III will establish if the novel drugs have any significance for prolonging (disease-free) survival by comparison against the current golden standard. Regularly, the development of a particular drug is discontinued when it fails to result in tumor shrinkage in phase II as companies in the pharmaceutical industry tend to be highly selective for which drugs they take to phase III as this is arguably the most costly part of drug development. The question is, however, if the criteria used in phase II to predict the efficacy of drugs are optimal, especially when keeping the cancer stem cell model in mind. As we highlighted earlier, any therapeutic intervention to be effective needs to (1) target the cancer stem cell population and (2) prevent dedifferentiation of more differentiated cells toward the cancer stem cell state by either killing all cancer cells or inhibiting the dedifferentiation process itself. These features are not evaluated directly by simply focusing on target lesions shrinkage. To illustrate this point, we present two extreme situations: Compound A

is highly active in reducing tumor volumes on radiological evaluation, which is achieved by this drug's efficacy against the bulk of the more differentiated tumor cells. Unfortunately this drug is not very effective in targeting the cancer stem cell population that remains as MRD and is not detected using conventional imaging techniques. Compound B, in contrast, successfully targets the cancer stem cell population and moreover prevents the acquisition of cancer stem cell features in more differentiated cells; regrettably the more differentiated cells themselves making up the majority of the tumor are relatively insensitive, thereby resulting in no or only limited effect on target lesion size. Based on these outcomes, drug A is very likely to be selected for further development in phase III and drug B likely to be discarded as being ineffective. Ironically, in further clinical testing, compound A is likely to fail as it does not result in increased disease-free survival rates, while drug B might be superior as it demonstrates effectiveness against the cell population which is responsible for disease dissemination and relapse. That this is not just a theoretical consideration is highlighted by the fact that direct tumor response only marginally correlates with survival, something that is referred to as the treatment paradox [38]. Moreover, studies using computational models of MRD and tumor relapse have established that in case cancer stem cells are left untargeted, this not only results in failing to cure the patient but even results in relapses that in some ways are more aggressive compared to the primary tumor and demonstrate accelerated expansion and enhanced invasive growth [76, 77], thereby stressing the need to successfully target this subset of cancer cells.

These examples make it clear that innovative methods need to be developed that successfully assess the efficacy of novel compounds against the cells that comprise the clonogenic core of the malignancy as it directly correlates with the most crucial clinical features including tumor expansion and progression and metastasis formation [87]. In addition, also therapeutic failure and tumor recurrence directly relate to the self-renewal ability of cancer cells. Subsequently, direct assessment of clonogenicity of tumor cell populations after therapy provides a promising readout of drug efficacy. Indeed, in multiple myeloma, it has been demonstrated that evaluation of post-treatment clonogenicity could inform about therapeutic efficacy [7]. The feasibility of this approach in solid malignancies is demonstrated by a study concerning GBM, as the level of in vitro clonogenicity was determined to successfully predict prognosis [61]. Obviously, determining clonogenicity after treatment in solid malignancies is only feasible in a neoadjuvant setting. In other cases, surrogate markers need to be identified. In this respect cancer stem cell markers serve great potential, as, per definition, expression of these molecules associated with self-renewal and clonogenic capacity of cells. Biopsies of the primary tumor or metastasis can be taken and the fraction of cancer stem cell marker expressing cells determined. A less invasive approach would be to use fine needle aspirations of the same lesion, before and after therapy, to determine the expression levels of cancer stem cell marker genes or determine the activity of pathways associated with the cancer stem cell phenotype, e.g., Wnt in colorectal cancer or Notch in GBM. For example, a recent pilot clinical study with an Hedgehog pathway inhibitor tested for pancreatic carcinoma has conducted biopsies before and after treatment to study the effectiveness of

this treatment on the cancer stem cell population [42]. Moreover, evidence is accumulating to suggest that circulating tumor cells (CTCs) are enriched in cells with a cancer stem cell phenotype, potentially reflecting the importance of these cells in metastasis formation [22]. For example, in breast cancer patients 18–35% of the CTCs express cancer stem cell markers ($CD44^+/CD24^-$) [81]. In addition, a report on colorectal cancer patients high levels of CD133 mRNA in blood samples correlated with poor disease outcome [39]. We propose that related techniques could be applied in the future to determine therapy responses and allow for the tracking of CTC numbers with a cancer stem cell phenotype before, during, and after therapy. This would have major benefits as the analysis can be performed on peripheral blood and is therefore noninvasive. Evidently, before the implementation of these methods in clinical drug testing, much more fundamental research needs to be performed to justify its use and ascertain the reliability.

6 Outlook

To date, the cancer stem cell field relies heavily on marker detection to identify cancer stem cells despite the lack of reliable markers and significant heterogeneity. As we and others have found, normal tumor cells and cancer stem cells have appeared to be plastic entities that can de- and differentiate depending on their microenvironment and other factors like the use of cytostatic drugs. Therefore, determining absolute numbers of cancer stem cells in space and time with a single marker has proven to not be as straightforward as it seemed in earlier years [90]. Consequently, addressing all of the questions about chemoresistance in cancer stem cells remains to be a major challenge.

Nevertheless, this chapter aimed to give an overview of the therapeutic consequences and opportunities that are related to the discovery that many tumors are driven by a small population of stem cell-like cells. Clearly many challenges are ahead to translate the wealth of biological insight we gathered with respect to the pathways and the microenvironmental interactions that sustain these cells into improved therapies to avoid MRD. In addition, although the cancer stem cell concept appears to be a powerful model to explain MRD, it fails to satisfyingly explain a related clinical phenomenon at this stage; acquired drug resistance often accompanies tumor relapse after MRD and is generally assumed to be related to a clonal trait present in a subpopulation of cancer cells [6]. For example, it is well established that in chronic myeloid leukemia, resistance to imatinib develops from secondary mutations in a region of the BCR-ABL gene that encodes for the drug interaction domain [30]. This finding provides compelling evidence that MRD and drug resistance are not simply consequences of drug-resistant cancer stem cells but instead result from a complex interplay between resistant, genetically distinct clonal lineages within the tumor and relatively resistant cancer stem cells that sustain these clones. Which of these two mechanisms is most important and contributes most to therapy failure is probably different for each type of drug and malignancy and

perhaps even each individual tumor. Disentangling these two contributing mechanisms of therapy failure will be of critical importance to optimize therapy efficacy, as both need a fundamentally different approach to avoid. Evidently, more basic research is needed, and more advanced drug evaluation protocols are required to improve treatment for the next generation of cancer patients.

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Cancer Stem Cells in Lung Cancer: Roots of Drug Resistance and Targets for Novel Therapeutic Strategies

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Abstract Lung cancer represents the leading cause of cancer-related deaths worldwide due to its high incidence and the lack of effective therapies. Current pharmacological strategies for the treatment of advanced stage disease are in fact largely ineffective mostly due to the emergence of drug resistance. The cancer stem cell (CSC) hypothesis suggests that treatment failure and tumor relapse may be explained by the existence of a subset of self-renewing cancer cells endowed with tumor-initiating potential which are able to escape conventional and targeted therapies and to regenerate tumors.

In this chapter we will first focus on the description of studies which led to identification and characterization of CSCs in lung cancer according to their expression of specific markers and/or functional properties and will discuss the potential clinical value of CSC-related markers to predict patients' outcome and response to therapies. We will next review evidences supporting the proposed mechanisms of resistance of CSCs to chemotherapy and targeted therapies and in particular intrinsic CSCs' properties such as enhanced activation of the DNA damage repair machinery and anti-apoptotic signaling, increased expression of drug transporters, activation of self-renewal pathways, and quiescence status. The ability of tumor microenvironment (TME)-derived signals to modulate CSC phenotype, especially through the induction of epithelial mesenchymal transition, has also been demonstrated to contribute to drug resistance. Here we will discuss the interconnection among TME signals, modulation/generation of CSC, and development of resistance to both conventional and targeted therapy in lung cancer. Finally we will present novel strategies based on targeting of specific pathways activated in CSCs or able to impair the cross talk between TME and CSCs and aimed at eradication of the CSC subsets, which have been already tested or are currently under investigation in clinical trials in advanced lung cancer.

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Despite some still ongoing controversies regarding the best strategy/markers to define the stem cell population in lung cancer, several evidences support the resistance of lung CSC to conventional and targeted therapies providing a new perspective for the understanding of drug resistance mechanisms and indicating the path for development of innovative targeted therapies that may ultimately improve clinical outcome of lung cancer patients.

Keywords Lung cancer · Cancer stem cells · Chemoresistance · CD133 · ALDH Targeted therapy · Tumor microenvironment · EMT · ABC transporters · Notch Wnt

Abbreviations

ABC	ATP-binding cassette
ADC	Adenocarcinoma
ALCAM	Activated leukocyte cell adhesion molecule
ALDH	Aldehyde dehydrogenase
AML	Acute myeloid leukemia
ATRA	All-trans retinoic acid
CAF	Cancer-associated fibroblast
CSC	Cancer stem cells
CXCR4	Chemokine receptor type 4
DDR	DNA damage response
DFS	Disease-free survival
Dhh	Desert hedgehog
DSBs	Double-strand breaks
Dvl	Disheveled proteins
EGFR	Epidermal growth factor receptor
EGFR-TKI	Epidermal growth factor receptor tyrosine kinase inhibitor
EMT	Epithelial to mesenchymal transition
EPCAM	Epithelial cell adhesion molecule
Hh	Hedgehog
Ihh	Indian hedgehog
IL-6	Interleukin-6
LC	Large cell carcinoma
MIC	Metastasis initiating cell
MMP	Metalloproteinase
NSCLC	Non-small cell lung cancer
PDX	Patient-derived xenograft
PTCH	Patched

SCC	Squamous-cell carcinoma
SDF1	Stromal cell-derived factor 1
Shh	Sonic hedgehog
SP	Side population
TGF-β	Transforming growth factor beta
TKIs	Tyrosine kinase inhibitors
TME	Tumor microenvironment
VEGFR	Vascular endothelial growth factor receptor
ZEB	Zinc finger E-box-binding

1 Lung Cancer Stem Cells (CSCs): Introduction

Lung cancer represents the leading cause of cancer-related mortality worldwide and is estimated to be responsible for more than 1.5 million deaths/year [1]. Despite recent advances in early detection strategies and development of novel pharmacological treatments, prognosis remains poor especially for advanced stage disease in which current strategies result in 5-year survival rates of less than 15% mainly due to inefficient control of relapsing disease and metastatic dissemination [2]. Inherent and acquired drug resistance represents therefore a significant clinical challenge in the treatment of lung cancer and, in particular for its most frequent type, non-small cell lung cancer (NSCLC) which accounts for 80–85% of all lung cancers. Drug resistance is a multifactorial phenomenon dependent on several characteristics of both cancer cells and surrounding microenvironment [3]: in this chapter we will review the role of cancer stem cells in this mechanism.

The cancer stem cell (CSC) model suggests that tumors are arranged in a hierarchical structure, at the apex of which a small subset of stem-like cells are responsible for tumor initiation and maintenance [4]. Mounting evidence suggests that CSCs play a critical role not only in tumor formation but also in metastasis and drug resistance [5]. Most current anticancer therapies in fact may fail to eradicate CSC clones due to their inherent drug resistance, resulting in their selection. CSCs spared by therapy may regenerate the original tumor (local relapse) or disseminate to distant organs driving tumor recurrence and metastasis. CSCs are characterized by a strong resistance to currently adopted therapies, such as chemotherapy and radiotherapy, due to their slowly proliferating nature, the intrinsic high levels of anti-apoptotic molecules, their relative resistance to DNA damage and the high activity of the detoxification machinery involved in drug extrusion [4, 6, 7]. Moreover CSCs can also resist to molecular targeted therapy due to the activation of specific pathways able to bypass drug activity [8].

The identification of CSC-specific markers and/or subsets, as the first step to devise novel therapeutic targets to specifically hit CSCs, is therefore becoming a compelling issue to overcome drug resistance and tumor recurrence to ultimately improve clinical outcomes of lung cancer patients.

1.1 Stem Cells in Lung Cancer

The cancer stem cell model proposes that tumors are organized into an aberrant "organ-like" hierarchy driven by a subset of cells endowed with the ability to self-renew and generate the heterogeneous cell population representing the tumor bulk [4]. The first experimental evidence supporting the existence of CSCs came from human acute myeloid leukemia (AML) with the demonstration that only rare malignant cells had the ability to reform the original disease over several transplantations, implying self-renewal and differentiation ability [9] [10]. Hierarchical organization in solid tumors was later experimentally demonstrated in breast cancer [11], and subsequently CSCs have been identified in other solid tumors including cancers of the brain [12, 13], colon [14–16], head and neck [17, 18], pancreas [19–21], melanoma [22–24], liver [25, 26], stomach [27], prostate [28, 29], ovary [30], and lung [31, 32].

Indeed the first experimental evidences for the existence of a stem-like clonogenic subpopulation in lung cancer were provided in the 1980s [33, 34]. These pioneering studies demonstrated that a very small proportion (<1.5%) of SCLC and lung adenocarcinoma cells from patient samples possessed the ability to generate colonies in soft agar that demonstrated tumor initiation potential when transplanted into nude mice. More recently the identification and isolation of lung CSCs have relied on the expression of specific surface markers [31, 32] or on their functional properties. Several markers have been proposed to identify lung CSCs, and up to now there is not a common consensus about the definition of the unique or combination of markers for identification of CSCs. Several studies reported similar CSC features for lung tumor cell subpopulation expressing different markers, and other works demonstrated that these cell subsets are not overlapping, presumably suggesting the existence of different lung CSC subpopulations [35].

The origin of CSCs remains a controversial issue: they may come from neoplastic transformation of normal stem cells in which the self-renewal machinery is already endogenously activated or from progenitor/differentiated cells that have reacquired properties of stem cells during the oncogenic transformation process [36]. A prerequisite for experimental investigation of CSCs is that these cells should be prospectively identified and isolated to test their functional properties: it follows that adequate cell-specific markers are needed and these can be sometimes inferred by properties of normal stem cells of the tissue of origin. Compared to other cancer types, however, less is known about the biology of lung cancer stem cells. This is in part due to the complexity of this disease in terms of phenotypic diversity and anatomically distinct sites of cancer origin in the pulmonary airways. The existence of distinct subsets of lung stem cells responsible for homeostasis of different anatomically defined regions of the respiratory tract which may represent the cells of origin of the different lung cancer histological subtype has been proposed to explain the diversity encountered in lung cancers. In support of this hypothesis, it has been demonstrated in murine models that sites of origin of the different histological subtypes of NSCLC (i.e., squamous-cell carcinoma (SCC),

adeno-/bronchoalveolar carcinomas (ADC), and large cell carcinomas (LC)) coincide with distinct airway stem cell niches [37]. Indeed exploiting transgenic mouse models in which lung cancer was induced by oncogene activation or tumor suppressor knockout under the control of lung epithelial cell-specific promoters, several studies have demonstrated that genetic modifications in the stem cell subsets specific for proximal airway (basal cells), mid-level airway (Clara cells), and distal airway (bronchoalveolar stem cells) give rise to histologically different lung tumors [38–40], thus supporting the concept that normal airway stem cells can act as cell of origin for lung cancers [41].

Until now, resident lung stem/progenitor cells of different anatomically defined regions of the airway epithelium had only been identified in the lungs of mice. However the evidence derived from murine model suffers from the constraint of genetic manipulation and cannot be easily translated to humans also because of species specificity of some of the markers adopted to identify murine stem cells (e.g., Sca-1) for which no human counterpart is known. Recently Kajstura et al. [42] presented the first evidence for the existence in adult human lungs of multipotent resident lung stem cells that could induce lung repair following injury. These cells, identified by the *c-kit* maker, are able in vivo to originate novel airway structures and vasculature successfully rebuilding the complete lung architecture. The existence of a multipotent lung stem cells in humans remains however controversial, and alternative evidence has been provided showing that c-kit(+) cells did not contribute to lung epithelium regeneration and homeostasis, but rather represented the progenitor endothelial cells able to reconstruct damaged lung vasculature [43].

Therefore until now no consensus has been reached on the definition of the human lung stem or progenitor cells specific for different regions of the respiratory tract. This lack of knowledge regarding normal lung stem cells also hampers the possibility to uniquely define lung cancer stem cells; indeed many controversies are still ongoing regarding the best strategy/markers to identify stem cell population in lung cancer.

An additional layer of complexity comes from recent evidence showing that differentiated tumor cells may also revert to CSCs' status under specific stimuli from tumor microenvironment [44–46]. The CSCs' compartment might even in itself be heterogeneous and comprise different subsets responsible for primary tumor initiation/maintenance, drug resistance, and metastatic dissemination [5, 20]. The intrinsic plasticity of tumor cells, which are capable of acquiring CSCs' properties under specific conditions, together with CSCs' heterogeneity makes therefore challenging the effort to design specific therapies able to efficiently target this evolving and dynamic population.

1.2 Lung CSC Markers

Identification of CSCs is mostly based on the expression of surface marker able to discriminate the stem-like subset from differentiated cells and allowing physical separation of different subpopulations using FACS sorting. Other strategies rely on

functional activities of CSCs exploiting their intrinsic elevated levels of drug transporters and enzymes deputed to detoxification. The ability to form clonal spheroids in vitro and the tumorigenic potential in mice represent the assays general use to test properties of isolated cells with the latter representing the gold standard to ascertain tumor-forming potential.

Side Population

The first isolation of lung CSCs was performed exploiting side population (SP) assay [47]. This assay was firstly described by Goodell et al. to identify hematopoietic stem cells [48] and relies on the ability of ABC transporters, highly expressed in stem cell populations, to drive efflux of the fluorescent dye Hoechst 33342. Cells able to exclude Hoechst 33342 dye are termed "side population" since they are identified in flow cytometry plots as a (generally) small fluorescence-negative subpopulation. Ho and colleagues demonstrated that the side population identified in lung cancer cell lines showed cancer stem-like characteristics such as tumor-initiating abilities, high invasiveness, chemoresistance, increased telomerase (hTERT) activity, and quiescence, compared to non-SP population. They also reported the existence of a small fraction of SP in primary lung cancer. Further evidence also confirmed the existence of the SP in NSCLC cell lines showing stem-like features including self-renewal ability and expression of embryonic stem cell transcription factors such as Oct4, Sox2, and Nanog [49].

Despite the fact that the side population assay is widely exploited to identify cells with CSCs' properties, there is some criticism regarding the use in this assay of a fluorescent DNA-intercalating dye: under certain conditions non-SP cells, unable to extrude the dye, may in fact suffer from cytotoxic effects due to the presence of this agent misleading the interpretation of functional assays comparing the behavior of SP vs. non-SP populations. Evidence in lung cancer and other tumor types supports the notion that the side population assay may identify cancer stem cells, but experimental variables such as incubation time, dye (and cell) concentration, and gating strategy may result in different frequencies of SP detection among experiments [50]. Therefore, a standardized and more stringent experimental procedure is needed to produce comparable and solid results and to determine the ability of this assay to accurately quantify and isolate CSCs.

ALDH

Another "function-based" method to isolate lung CSCs exploits their elevated activity of aldehyde dehydrogenase (ALDH) enzyme. ALDH family members are deputed to detoxification and are involved in chemoresistance process [51]. ALDH activity, that defines normal stem cells and CSCs, is generally measured by the Aldefluor assay (Stem Cell Technologies).

In NSCLC two aldehyde dehydrogenase isozymes, ALDH1A1 and ALDH3A1, were identified overexpressed in atypical pneumocytes possibly following malignant transformation after chronic carcinogen exposure [52]. Next, Sullivan et al. demonstrated for the first time the increased tumorigenic ability of $ALDH^+$ cells isolated from NSCLC cell lines compared to the negative counterpart. $ALDH^+$ cells showed an enhanced activation of the NOTCH pathway, and its targeting using

ɣ-secretase inhibitor resulted in a drastic decrease of ALDH$^+$ cells [53]. Following this seminal paper, other reports have substantiated the CSCs' properties of ALDH$^+$ cells. Akunuru et al. showed that ALDH^{high} cells isolated from NSCLC cell lines have an increased potential to generate spheroids in vitro and tumorigenic and metastatic activity in vivo [35]. Similarly Jiang et al. proved the self-renewal potential and high tumorigenic ability of NSCLC cells with high ALDH1 activity, as well as their resistance to chemotherapy [54].

CD133

The main method for identification and isolation of lung CSCs is based on FACS sorting of tumor cells expressing specific stem cells-related markers.

CD133 (Prom1) is a cell surface glycoprotein with five transmembrane domains and two large glycosylated extracellular loops [55] . The glycosylated epitope of CD133, AC133, has been shown to select normal human hematopoietic and neural stem cells and next to identify CSCs in several solid tumors such as glioblastoma and colon and pancreas carcinomas [55–57].

The first evidence for identification of lung CD133$^+$ CSCs in primary NSCLC tumors was provided by Eramo et al. who identified self-renewing and highly tumorigenic CD133$^+$ cells that could be cultured and expanded in vitro as floating spheroids. CD133$^+$ lung tumor spheroids were characterized by the expression of embryonic stem cells transcription factors (Oct4 and Nanog) and by their ability to generate tumor xenografts in immunocompromised mice with features resembling original patients' tumors. Spheroids induced to differentiate lost CD133 expression, stem-like features and tumorigenic ability. CD133$^+$ spheroids were additionally shown to resist in vitro to chemotherapy treatment [31]. We provided further evidence for the existence of CD133$^+$ lung CSCs using prospective isolation from freshly dissociated primary NSCLC samples or patients' derived xenografts (PDXs). CD133$^+$ cells were shown to possess high tumorigenic ability when injected at low dose in immunocompromised mice and to be able to give rise to tumors that recapitulate the complexity of primary tumors. Notably, we showed that both acute and chronic exposure of lung cancer cells to cisplatin resulted in the selection of chemoresistant CD133$^+$ cells and identified in this subpopulation frequent coexpression of the ABCG2 transporter and the CXCR4 chemokine receptor [32]. Recently we also reported that the subset of CD133$^+$CXCR4$^+$ lung CSCs possesses increased ability to disseminate and initiate metastasis, thus representing metastasis-initiating cells (MICs). Furthermore we demonstrated that this specific cellular subset shows mesenchymal features and can be directly modulated by tumor microenvironment signaling, providing support to the hypothesis of a tight interplay between microenvironment, stemness, and chemoresistance [46, 58].

Following another possible strategy, Levina and coworkers exploited chemotherapy to enrich for resistant CSCs in lung cancer cell lines. Tumor cells able to survive cisplatin, doxorubicin, and etoposide treatments were enriched for CD133$^+$ cells, lost expression of differentiation markers, and showed high tumorigenic and metastatic potential in vivo [59]. Several other papers also reported the existence of a CSC subset defined by CD133 expression. CD133$^+$ lung CSCs

identified in primary NSCLC tissue were shown to express high level of Oct-4 transcription factor. Oct-4 knockdown was able to prevent tumor sphere formation in vitro and inhibit CD133$^+$ cells' ability for tumor formation and also to chemosensitize CSCs thus increasing the efficacy of chemotherapy [60]. Similarly, Chiou et al. showed that Oct4 and Nanog transcription factors are key regulators of CD133$^+$ cell maintenance. Their ectopic expressions in lung ADC increased the percentage of CD133-expressing subpopulation and sphere formation, enhanced drug resistance, and promoted EMT. Double knockdown of Oct4 and Nanog suppressed the expression of Slug, reversed the EMT process and blocked the tumorigenic and metastatic ability [61].

Despite the wide use of CD133 marker, many controversies are still ongoing regarding its value as optimal marker for CSCs' isolation in different types of cancer, since several discordant evidences have been provided. One of the major issues to be considered is related to the glycosylation of CD133 antigen, since indeed only AC133 glycosylated epitope has been proven to select for CSCs and thus antibodies recognizing different CD133 isoforms and epitopes may be not properly distinguish between CSCs and differentiated tumor cells. In lung cancer it has been reported that also CD133$^-$ cells sorted from NSCLC cell lines maintained tumor-initiating potential and ability for self-renewal [62]. We however provided robust evidence indicating that even if CD133$^-$ cells could initially generate tumors in vivo, they failed to sustain tumor initiation in serial transplantation assays because of their limited tumorigenic potential, whereas CD133$^+$ cells endowed with sustained self-renewal ability can indefinitely propagate tumors [32].

CD44

CD44 is a cell surface glycoprotein that functions as a receptor for hyaluronic acid, an extracellular matrix-related glycosaminoglycan. It is expressed both in normal and in cancer stem cells, and it is involved in multiple cellular processes such as proliferation, differentiation, migration, and angiogenesis [63]. CD44 represents an important marker for definition of CSCs in breast, prostate, pancreatic, squamous head and neck, and more recently also lung cancer [64]. Leung et al. demonstrated that CD44$^+$ cells isolated from NSCLC cell lines possessed an enhanced self-renewal ability, were able to generate spheroids in vitro, expressed pluripotency genes (Oct-4, Nanog, and SOX2) and EMT makers (SNAI1, CDH2, and VIM), and showed an increased in vivo tumor-initiating ability compared to the subpopulation of CD44$^-$ cells. Tumors derived from CD44$^+$ cells recapitulated the same heterogeneity of the parental tumor indicating the ability of CD44$^+$ cells to give rise to all differentiated cells composing the tumor bulk. Moreover CD44$^+$ cells could resist cisplatin treatment [65]. Combination of the CD44 marker with ALDH activity also discriminated a subset of lung cancer cells with enhanced tumorigenic potential and drug resistance. The ALDH(hi)CD44(hi) subset sorted from NCSLC cell lines showed the highest invasion rate, pluripotency genes expression, tumor initiation ability with shortest latency and highest growth rates compared to ALDH(hi) CD44(lo), ALDH(lo)CD44(hi), ALDH(lo)CD44(lo) cells and unsorted controls. ALDH(hi)CD44(hi) were moreover able to efficiently survive chemotherapy and

targeted therapy, and in accordance, clinical lung cancer samples with high frequency of ALDH- and CD44-coexpressing cells were correlated with shorter recurrence-free survival [66].

CD166

Another surface marker described to select for the lung CSC population is represented by CD166, also known as activated leukocyte cell adhesion molecule (ALCAM). CD166 is a member of the immunoglobulin superfamily of cell adhesion molecules, and it is involved in angiogenesis, differentiation, homing, and maintenance of hematopoietic stem cells. It is known to be a marker for normal hematopoietic stem cells as well as for CSCs of colorectal and prostate cancer [67, 68].

More recently Zhang et al. identified CD166 as a novel marker for lung CSCs isolated from primary NSCLC tumors. $CD166^+$ $EpCAM^+$ cells were shown to be endowed with the ability to self-renew and to initiate primary and secondary xenograft tumors representing the phenocopies of parental patients' tumors when injected at low doses in immunocompromised mice. In vitro $CD166^+$ cells were able to form spheres, and as few as 1–5 single cells from dissociated lung spheres were capable to initiate tumors in vivo. Finally $CD166^+$ expression was also found to be a poor prognostic indicator for shorter overall survival in NSCLC patients [69].

2 Lung CSCs and Drug Resistance

2.1 Clinical Relevance of CSCs for Lung Cancer Treatment

The CSCs' paradigm has profound implications for cancer therapy but also represents a formidable challenge for clinical validation since our current understanding of tumor response during treatments mainly relies on imaging techniques that may not capture the complexity of the dynamics of small subpopulations. The clinical application of CSC-related concepts requires therefore evaluation of available evidences under a new perspective. In this chapter we will discuss potential implications of CSCs in light of the efficacy of current pharmacological treatments and the clinical value of CSC markers.

2.1.1 Lung Cancer Treatments, Drug Resistance, and CSCs

Surgery still represents the best option for long-term survival of NSCLC patients when the disease is detected at an early stage and results in 5-year survival rates of more than 70% in pathological stage Ia. The potential use of adjuvant platinum-based chemotherapy after surgery has also been widely investigated [70, 71], but its efficacy in stage I–II disease, the criteria for selection of patients that could benefit from this type of treatment, and the potential for novel drugs in this setting still

remain unclear [72]. Unfortunately, however, approximately 70% of patients are diagnosed with unresectable disease (locally advanced or metastatic). Combination chemotherapy, usually based on platinum doublets, is currently the first-line therapy of choice for advanced NSCLC with selective use of radiotherapy. The prognosis for chemo-/radio-treated patients remains disappointingly low with a 5-year survival rate less than 5%, largely due to the emergence of drug resistance (intrinsic or acquired) during treatments [73].

In recent years, new therapies directed against specific molecular targets (targeted therapy) have entered clinical trials for the treatment of lung cancer. Tyrosine kinase inhibitors (TKIs) against epidermal growth factor receptor (EGFR) or oncogenic fusion events (EML4-ALK) are currently used in clinical practice for specific patient subgroups as well as anti-angiogenic agents against vascular endothelial growth factor receptor (VEGFR) [74]. However, targeted therapies often result in short-term improvements of survival in responsive subsets and have a marginal impact on overall mortality since eventually most patients experience tumor recurrence [75]. More encouraging results have recently emerged from immunotherapeutic strategies based on the use of drugs targeting immune checkpoint inhibitors (anti-PD1/PDL-1) [76] which have been shown to induce relevant and long-lasting clinical responses especially; however more conclusive data on the real efficacy of immunotherapy in lung cancer are needed [77].

Resistance to therapy is one of the major hurdles in clinical management of lung cancer patients and contributes largely to disease progression, recurrence and mortality. Several mechanisms concur in mediating drug resistance including reduced drug uptake (or increased efflux) due to enhanced activity of drug transporters, the increased activity of detoxifying enzymes, the increased activity of the DNA damage repair machinery, and the enhanced resistance to apoptosis or altered cell-cycling properties [78]. The presence of specific subpopulations of cancer cells endowed with both increased tumor-forming potential and chemoresistance (all features of cancer stem cells) has also been suggested to be responsible for the observed tumor recurrence after therapy [79]. In particular in NSCLC patients, it was clinically demonstrated that induction chemotherapy induces a faster tumor regrowth in the waiting period between chemotherapy treatment and subsequent radiotherapy due to an accelerated regrowth of surviving tumor cells with deleterious implications for curative intervention [80]. This observation may support the concept that conventional therapies eliminate the bulk of tumor cells but may spare the subpopulation of CSCs able to survive treatment and to proliferate to reconstitute the tumor, thus explaining tumor recurrence and treatment failure following an apparently successful first line of therapy [81].

Several lines of evidence in experimental models have demonstrated that both conventional and targeted therapies may enrich for CSC subset through a positive selection of pre-existing and intrinsically resistant CSCs or through the induction of epithelial to mesenchymal transition (EMT) program linked to generation of cells with CSC-like features [82]. Different mechanisms have been proposed to confer CSCs' resistance to treatments that will be extensively discussed in Sects. 3.2.2 and 3.2.3, including the intrinsic high expression levels of ATP-binding cassette (ABC)

drug pumps or anti-apoptotic molecules, their relative resistance to DNA damage, and their quiescent/slowly proliferative nature [7].

In this context we postulate that only a deeper understanding of the mechanisms underlying CSCs' drug resistance and the development of novel combination treatments able to target both the tumor bulk and CSC subsets may lead to the eagerly awaited improvements in NSCLC patient outcome.

2.1.2 Prognostic Significance of Lung CSC Markers

Several studies have tried to correlate the expression of CSC-related makers with NSCLC patients' outcome and response to therapy. However, due to discordant results, the potential clinical impact of CSC markers is still controversial, and they have not yet entered the clinical practice. This is not surprising considering that these efforts are confronted by two great challenges: (i) the selection of validated CSC markers (discussed in Chap. 1) and (ii) the limitations of the techniques generally used to evaluate marker expression in clinical samples. Currently the most practical applications for prognostic markers in solid tumors rely on immunohistochemical (IHC) staining performed on archival tissues: this technique however may not adequately capture the CSCs' subpopulation (and its subsets), and we may have to wait for a paradigm shift and implementation of flow cytometry also in this setting (as in hematological malignancies) before CSC markers can usefully be applied in the clinic.

The prognostic/predictive value of CD133 expression has been extensively investigated in NSCLC. Woo et al. analyzed the expression of CD133 by IHC in 177 surgically resected stage I lung adenocarcinoma and found that CD133 is independent prognostic marker for shorter disease-free survival (DFS); moreover the combination of CD133 with proliferating marker Ki-67 could predict postoperative recurrence [83]. Similarly Li H et al. demonstrated in a case series of 145 stage I NSCLC patients that the coexpression of CD133 and ABCG2 is predictive of high risk of postoperative early relapse [84]. Mizugaki et al. reported, in a case series of 161 surgically resected NSCLCs, the correlation of CD133 expression with pathological advance stages and identified CD133 as an independent factor for poor prognosis [85]. Conversely, Salnikov et al. demonstrated in a retrospective series of 88 untreated NSCLC that CD133 does not represent a prognostic parameter for patient survival but is strongly correlated with the expression of chemoresistance-related proteins and therefore can potentially be useful to predict efficacy of anticancer therapies [86]. In NSCLC patients treated with platinum-containing regimens, we also observed a tendency toward shorter progression-free survival when $CD133^+$ cells were detected by IHC in pretreatment samples [32]. Interestingly using flow cytometry, we have been recently able to show that identification of the $CD133^+CXCR4^+EpACM^-$ lung CSC metastatic subset in primary tumors correlates with tumor recurrence and poor outcome [46].

Many other studies investigate the clinical and prognostic significance of CD133 in NSCLC reporting different results. This discrepancy may be due to

differences in clinical pathological features and size of patients cohort analyzed as well as to methodological differences such as the use of different antibodies to detect CD133 or different IHC scores used to evaluate CD133 positivity. A meta-analysis of 13 studies, with a total of 1004 NSCLC patients, proved that CD133 expression was associated with overall survival (OS) but not with disease-free survival (DFS) or any other clinicopathological parameters except tumor differentiation [87]. Another meta-analysis including 23 studies confirmed that CD133 level was significantly correlated with the overall survival of NSCLC patients but not with the disease-free survival; considering clinicopathological features, CD133 level was positively correlated with lymph node metastasis, but not with histological classification. Overall these meta-analyses support the possible use of CD133 as a biomarker for worse prognosis in NSCLCs [88] .

The ABCG2 drug transporter pump, one of the determinants of the "side population" phenotype, was demonstrated to be associated with a shorter survival in advanced NSCLC treated with platinum-based chemotherapy, although it did not predict response to chemotherapy [89]. A similar observation was reported in an independent study demonstrating that in NSCLC patients receiving cisplatin-based adjuvant chemotherapy, high ABCG2 expression as assessed by qPCR was correlated with short progression-free survival but not with response to treatment [90].

Different studies also investigated the prognostic potential of ALDH1 protein expression in NSCLC. Jinang and coworkers showed that high expression of ALDH1 was associated with poor prognosis in NSCLC patients and with a more aggressive and advanced pathological grade and stage [54]. Similarly, Sullivan et al. confirmed that tumors with higher numbers of $ALDH^+$ cells had a significantly poorer overall survival and this association was present also in patients with stage I and N0 disease [53]. Interestingly combined analysis of ALDH1A1 and CD133 revealed strong association with poor survival in resected early-stage NSCLC [91]. Furthermore, CD133 or ALDH1 positivity in NSCLC undergoing induction chemoradiotherapy was significantly correlated with worse overall survival and resulted as an independent prognostic factors for disease relapse [92].

Some evidence also demonstrated prognostic value of CSC-associated transcription factors. The increased expression of embryonic stem cells transcription factors Oct4 and Nanog together with Slug, an EMT-related marker, was found to be associated with worse prognosis in lung adenocarcinoma patients [61]. A retrospective analysis of 226 patients with lung adenocarcinoma showed that high Nanog expression was independently associated with a poor prognosis [93]. On the same lines, Vrzalikova et al. demonstrated that in NSCLC patients who had received adjuvant therapy, the expression of BMI-1, an oncogene belonging to the Polycomb group of ring finger transcription factors, was correlated with shorter DFS in stage I and II tumors [94].

Taken together these evidences sustain the prognostic and predictive significance of different lung CSC markers, even if some discordant results have been published, likely due to methodological variability and to selection criteria used in different studies. Moreover since no consensus has been reached regarding the use of optimal

markers to identify lung CSC, a combination of different markers possibly identifying distinct CSC subsets might improve the predictive/prognostic value of a potential CSC-based biomarker for clinical application.

2.2 *Molecular Pathways Sustaining Intrinsic Drug Resistance of Lung CSC*

The intrinsic drug resistance of CSC can be viewed as the consequence of several biological mechanisms that are constitutively activated in CSC including (i) enhanced activity of the DNA damage repair machinery and the ability to escape apoptosis; (ii) expression of specific transmembrane transporters with drug-extruding capability; (iii) activation of stemness pathways regulating and sustaining self-renewal; and (iv) quiescence status.

2.2.1 DNA Damage Response and Anti-apoptotic Pathways

Many chemotherapeutic drugs such as platinum-based agents as well as radiotherapy exert their anticancer activities by inducing lethal levels of DNA damage. Conversely, cancer cells can survive treatments by activating DNA damage response (DDR) pathways that allow DNA repairing. DDR mechanisms determine cell cycle arrest at specific checkpoints and recruitment of the DNA repair machinery leading to damage control: in-depth investigation of DDR pathways activity in cancer cells could therefore give information on basic principles of cancer development and also result in novel therapeutic strategies [95].

Enhanced DNA repair capacity has been demonstrated to contribute to increased resistance to therapy in the CSC population. The first evidence came from a pioneering study by Bao et al. showing that $CD133^+$ glioblastoma CSCs preferentially activate DNA damage checkpoint response and DNA repair mechanisms contributing to radioresistance and tumor regeneration. Accordingly, specific inhibitors of checkpoint-related kinases Chk1 and Chk2 could overcome CSCs' radioresistance [96]. In a seminal study, the CSC population in NSCLC was also found to strongly activate Chk1 kinase in response to chemotherapy compared to the counterpart of differentiated cells representing the tumor bulk. A combination of Chk1 inhibitors (AZD7762) with chemotherapy dramatically determined a reduction in CSCs' survival by inducing premature cell cycle progression and mitotic catastrophe. Furthermore in vivo combination treatment with Chk1 inhibitors and chemotherapy was able to abrogate the ability of CSCs to form tumor in immunocompromised mice [97]. Enhanced DNA repair ability was also reported in $CD133^+$ cells sorted from A549 NSCLC cell line due to the upregulation of DNA double-strand break (DSB) repair genes that caused an increase resistance to radiotherapy [98].

Overexpression of anti-apoptotic molecules represents another mechanism by which tumor cells can escape damage induced by therapy. Tumor cells can express high levels of anti-apoptotic Bcl-2 family proteins, including Bcl-2, Bcl-XL, and Mcl-1 that contribute to chemotherapy resistance [99]. In NSCLC primary tumors, the CSC subset was shown to express the anti-apoptotic Bcl-XL at particularly high levels. Treatment with a selective inhibitor of Bcl-XL, ABT-737, showed a preferential cytotoxic activity toward slowly proliferating CSCs in vitro and was able to impair tumor growth of CSC-derived xenografts and reduce CSCs' content in vivo, indicating its specificity in CSCs' targeting [100].

2.2.2 Proteins Involved in Drug Efflux and Detoxification

One of the most investigated mechanisms for anticancer treatment failure is the activity of specific transmembrane transporters mediating drug efflux. ATP-binding cassette transporter proteins (ABC transporters) are recognized as one of the main families of such transporters with the ability to drive the extrusion of a wide range of chemotherapeutic drugs such as doxorubicin, etoposide, paclitaxel and cisplatin using ATP hydrolysis as a source of energy to overcome chemical gradient [101].

The cancer resistance protein ABCG2, one of the members of ABC transporters family, is responsible for the efflux of Hoechst dye defining the "side population" (SP) enriched for CSCs and is one of the main transporters mediating CSCs' resistance to therapy in different cancers [102]. ABCG2 actively effluxes a wide variety of xenobiotic compounds from cells, and its overexpression in tumor cells confers multidrug resistance to several chemotherapeutic agents and targeted therapies [103]. Moreover in lung cancer patients, high expression of ABCG2 is also associated with lower response to carboplatin and cisplatin and poor overall survival [89, 104].

The first evidence proving that ABC transporters could confer chemoresistance properties to lung CSCs came from the study by Ho et al.; in this work SP cells, sorted for six lung cancer cell lines, showed stem-like features, an enhanced tumorigenic potential in vivo, and an increased resistance to various chemotherapeutic drugs such as cisplatin, gemcitabine, and vinorelbine, all of which are commonly used as first-line therapies for lung cancer, due to the high expression of ABC transporters [47]. In line with these observations, we also reported that $CD133^+$ lung CSCs expressed high level of ABC transporters compared to the $CD133^-$ counterpart. Coherently with this finding, we showed both in cell lines and in patient-derived xenografts (PDX) that cisplatin treatment resulted in a selection of $CD133^+$ CSCs that coexpressed the ABCG2 pump proving the contribution of this drug transporter in CSC-mediated chemoresistance [32].

ALDH are a group of $NAD(P)^+$-dependent enzymes that catalyze the oxidization of aldehydes into carboxylic acids, and their intrinsic detoxifying action can contribute significantly to the development of drug resistance [105]. ALDH11A and ALDH3A1 enzymes were demonstrated to identify CSC subpopulation in different

tumors, including lung cancer, and they can act as drug-detoxifying enzymes mediating CSCs' therapeutic resistance [105]. In particular in lung cancer, tumor cells with high ALDH1 activity isolated from cells line displayed CSC features and greater resistance to chemotherapeutic drugs commonly used as first-line therapy in clinical setting compared to ALDH1$^-$ cells [54]. Knockdown of ALDH1A1 and ALDH3A1 isozymes in NSCLC cell lines confirmed an increased sensitivity to cyclophosphamide and a decreased tumorigenic potential [106]. Treatment of H460 and H1299 lung cancer cell lines with paclitaxel resulted in the selection of resistant ALDH1$^+$ CSCs' population. Notably, in vivo treatment of xenografts with paclitaxel resulted in reduction of primary tumor growth but promoted the selection and priming of ALDH1-positive CSCs with a consequent increase in the number of metastatic nodules [107]. Resistance to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKI) is a major issue in the treatment of EGFR-mutated lung cancer, and ALDH1 CSCs were proven to resist targeted therapy [108]. To mimic in vitro the acquisition of resistance to EGFR-TKI gefitinib, Shien and coworkers generated resistant sublines from four EGFR-mutated NSCLC cell lines, through stepwise escalation and high-concentration exposure to gefitinib. Resistant sublines exhibited an overexpression of ALDH1, increased EMT-associated markers, self-renewal potential, and higher tumor-initiating capability in vivo suggesting that acquired resistance to TKI may also rely on the expansion of drug-refractory CSC population. Moreover gefitinib-resistant sublines also displayed an enhanced resistance to the chemotherapeutic agents docetaxel and paclitaxel, an effect that may be mediated by the expansion of the ALDH1 CSC population [108]. Similar results were reported in another study showing that ALDH1-positive lung cancer cells isolated from EGFR-mutant PC-9 cell line displayed resistance to gefitinib and to conventional chemotherapeutic drugs such as cisplatin, etoposide, and fluorouracil, compared to ALDH1-negative cells. Remarkably, analysis of clinical sample confirmed a correlation between high expression of ALDH1 and resistance to both EGFR-TKI and chemotherapy drugs [109].

2.2.3 Self-Renewal Pathways

In normal stem cells' self-renewal, proliferation and differentiation processes are tightly controlled by several pathways including the embryonic Notch, Hedgehog, and Wnt/β-catenin signaling pathways. The same pathways are found aberrantly activated in cancer and may contribute to CSCs' generation and maintenance [110].

Notch Signaling Pathway

The Notch signaling pathway is crucial for cell fate determination [111]. Notch signaling is initiated by the binding of ligands of the Delta-like (DLL1/2/3) or Jagged-like (JLL1/2) families to the transmembrane receptors Notch1, Notch2, Notch3, and Notch4, which induce proteolytic cleavage of the receptor intracellular domains by enzymes of the γ-secretase complex. The intracellular domain is then

translocated into the nucleus where it induces the transcription of Notch target genes involved in cell fate determination [111].

Notch signaling plays an important role in embryogenesis, organogenesis and maintenance of adult lung homeostasis through fine regulation of the differentiation process of stem cells [112]. Suppression of Notch signaling during lung development determines premature tissue differentiation [113], whereas its overexpression results in accumulation of stem cells and arrest of differentiation [114]. Overexpression of Notch signaling has been frequently observed in lung cancer; however some controversies are still ongoing regarding the oncogenic or tumor-suppressive function of this pathway. Several evidences proved that blockade of Notch signaling pathway using γ-secretase inhibitor resulted in cancer cell growth arrest and increased apoptosis, supporting the role of Notch signaling as an oncogenic driver promoting tumor cell survival [115–117]. Conversely, other studies have demonstrated that overexpression of Notch in NSCLC cell lines determined cell death and reduction of tumor growth in vivo, suggesting that Notch may also act as a tumor suppressor [118].

Numerous evidences indicate the role of Notch pathway in maintenance of CSCs in different tumor type, including in lung cancer [119, 120]. Concerning the role of Notch in mediating CSC drug resistance, Liu et al. demonstrated that treatment of NSCLC cell lines with low-dose cisplatin was sufficient to enrich for chemoresistant $CD133^+$ CSC and that this selection was mediated by activation of the Notch pathway. Indeed pretreatment with a γ-secretase inhibitor or a Notch-targeted shRNA was able to reduce cisplatin-induced enrichment of $CD133^+$ cells and to enhance sensitivity of CSCs to chemotherapy. In vivo combination treatment with γ-secretase and cisplatin significantly reduced $CD133^+$ CSCs confirming that activation of Notch signaling is pivotal in mediating cisplatin-induced enrichment of resistant CSCs [121]. The pivotal role of Notch in maintenance of lung CSCs' properties was substantiated in a study by Hassan et al. showing that NSCLC cells with high Notch activity, identified using a Notch GFP reporter construct, displayed stem-like features, have enhanced in vivo tumorigenicity, and can survive cisplatin and docetaxel chemotherapy. Tumor xenografts treated in vivo with γ-secretase inhibitor and docetaxel failed to regenerate tumors in serial transplantation assays indicating exhaustion of the CSC subset [122]. Interestingly, Notch was also shown to mediate the resistance of CSCs to targeted therapy. Arasada et al. reported that treatment of EGFR-mutated lung cancer cell lines with erlotinib mediated selection and expansion of resistant ALDH-positive CSCs and that this enrichment was dependent on direct activation of Notch signaling [123].

Hedgehog Signaling Pathway

The Hedgehog (Hh) signaling pathway is involved in the regulation of cell differentiation and proliferation in embryonic development and in the maintenance of adult stem cells [124]. The Hh ligands (i.e., Sonic hedgehog, Shh; Indian hedgehog, Ihh; and Desert hedgehog, Dhh) bind to the Patched (PTCH) receptor triggering derepression of Smoothened (SMO) protein within the cell membrane and activation of GLI transcriptional regulators of Hh target genes [125].

The Hh pathway coordinates lung development during embryogenesis; indeed, knockout of Shh in transgenic mice determines aberrant lung development [126, 127]. According to some evidence, the Hh pathway remains active in adult lung stem cells as a mechanism for regeneration of tissue in response to airway epithelial injury [128]. Hedgehog pathway can be aberrantly activated in cancer, resulting in tumor growth, proliferation, and metastasis [129]. Activation of the Hh pathway has been shown in lung cancer, where GLI1 expression was found in a large percentage of primary NSCLC samples and in 85% of SCLC tumor samples, indicating constitutive activation [130, 131].

In particular in SCLC, Hh signaling pathway was demonstrated to play an important role in tumor initiation, and it may possibly represent a therapeutic target to prevent cisplatin resistance [132]. Constitutive activation of the Hedgehog signaling promoted the clonogenic ability of SCLC cells in vitro and the initiation and progression of SCLC in vivo. Conversely pharmacological blockade of Hh determined growth arrest of SCLC cells, also after chemotherapy treatments that are usually followed by quick recurrence and disease progression. These findings suggest a crucial role of Hedgehog signaling in the development and maintenance of SCLC and propose Hh inhibition as a therapeutic strategy to keep in check tumor progression and delay cancer recurrence [132]. In lung adenocarcinoma Hh inhibition was demonstrated to cause growth arrest and to significantly decrease the frequency of the side population endowed with tumor-initialing potential and chemoresistance. As a result, combination treatment with inhibitor of the Hh pathway and cisplatin resulted in an increased cytotoxic effect linked to depletion of the CSC population [133]. Additionally it has been shown that induction of EMT in NSCLC confers resistance to both EGFR-tyrosine kinase inhibitors and chemotherapy: interestingly, inhibition of the Hh pathway in NSCLC cell lines resistant to EGFR-TKI erlotinib resulted in attenuation of the EMT phenotype, decrease of CSC marker expression, and sensitization of cancer cells to erlotinib and cisplatin, thus further substantiating a connection between Hh signaling, CSC, and drug resistance [134].

Wnt/β-Catenin Signaling Pathway

Wnt signaling is essential both for the control of cell proliferation and cell fate determination during embryonic development and in the maintenance of adult stem cell [135]. Briefly, the binding of Wnt ligands to the Frizzled receptor results in recruitment of Disheveled proteins (Dvl) that in turn block Axin/APC/GSK-3β complexes thereby derepressing β-catenin. The accumulation and translocation of β-catenin into the nucleus promote transcription of Wnt target genes [136].

The Wnt pathway is well known to be deregulated in several tumor types, including lung cancer [137]. Some studies have demonstrated the overexpression of Wnt-1 and Wnt-2 in NSCLC cell lines and primary cancer tissues; moreover inhibition of Wnt signaling caused cell growth arrest and induced apoptosis in NSCLC cell lines [138, 139]. Giangreco et al. reported that membranous staining for β-catenin was observed in normal and metaplastic lung specimens, whereas carcinoma in situ and severely dysplastic lung tissues showed nuclear localization

of β-catenin, indicating activation of Wnt/β-catenin signaling during cancer progression [140].

Regarding activation of the Wnt pathway in lung CSC subsets, Levina et al. showed that lung cancer cells able to survive chemotherapy were enriched for CD133 CSC marker and expressed high nuclear level of β-catenin compared to their corresponding parental counterparts [59]. Teng et al. reported high activation of Wnt/β-catenin signaling in cisplatin-selected A549 lung cancer cells concomitantly with an increased expression of OCT-4 embryonic transcription factor. Knockdown of β-catenin expression using RNA interference in lung cancer cells resulted in downregulation of the Wnt target genes and in a reduction of OCT-4-expressing cells concomitantly with decreased proliferation and reduced clonogenic potential, migration, and drug resistance [141]. Taken together, these studies provide evidence for the involvement of Wnt signaling in maintenance of lung CSC and chemoresistance.

2.2.4 Intrinsic Quiescence

Quiescence is another mechanism contributing to the chemoresistance of tumor cells. Quiescent cells are arrested in the G0 phase of the cell cycle; this dormant state is reversible and can be modulated in response to the activation of signaling pathways induced by microenvironmental stimuli [142]. Quiescence is regulated by different signaling molecules including the well-characterized tumor suppressors p53 and RB and several cyclin-dependent kinase inhibitors, in particular p21, p27, and p57, all able to induce cell-cycle arrest [142].

Quiescence is a distinctive feature of normal stem cells, and it was proved to characterize specific subsets of CSCs [143]. Tumor cells endowed with stem-like features can disseminate to distant sites and survive in nonproliferative quiescent state for long time. This process occurs at early time of tumor progression or following therapeutic intervention and awakening of dormant cells may lead to tumor progression and relapse after very long periods from primary tumor removal or treatment [144]. The mechanisms leading to quiescent cell awakening are not well understood, but this process appears to be tightly regulated by microenvironment signals [145] as clearly demonstrated in breast cancer where two microenvironment-secreted factors, thrombospondin-1 and periostin, have been shown to play a crucial role in dictating cancer cells' quiescence and metastasis outgrowth [146, 147].

Quiescent state also allows CSCs to escape conventional chemotherapy that targets actively proliferating tumor cells [143]. Subsets of nonproliferative and drug-resistant CSCs could therefore "respond" to tumor shrinkage caused by treatments through reactivation and reconstitution of the tumor bulk. Three different strategies could be exploited to eradicate the quiescent CSCs' subpopulation. The first one paradoxically consists in promotion of cancer cells' proliferation to sensitize CSCs to conventional therapies; however this approach may also promote cancer progression due to CSCs' awakening and possible CSCs' dissemina-

tion. The second and more conservative approach proposes to maintain CSCs in a quiescent state avoiding their awakening with the final aim to treat tumor as a chronic disease. The last strategy consists in CSCs' eradication while these are still in a quiescent state: this is a fascinating approach, but at present a deeper understanding of molecular pathway governing CSCs' dormancy is still needed before such strategy could be implemented [144].

Long-term label retention is a widely used strategy for the identification of stem cells by exploiting their slow-cycling nature, whereas rapidly dividing progenitor cells dilute their labels [148]. The use of membrane-labeling dyes such as PKH67/PKH26 has been reported to track slow-cycling cells including both normal and cancer stem cells [149]. In lung cancer we demonstrated the existence of slow-cycling PKH^+ cells enriched for $CD133^+$ CSC; within this subset it was possible to distinguish a long-term quiescent PKH_{Bright} population, strongly enriched for $CD133^+$CXCR4-CSCs deputed to primary tumor maintenance, and a short-term quiescent PKH_{Dim} population enriched for $CD133^+CXCR4^+$ lung metastatic CSCs [150]. Both PKH^+ cell fractions were resistant to cisplatin treatment, suggesting that quiescent $PKH^+/CD133^+$ subpopulation overlaps with the already reported cisplatin-resistant $CD133^+$ CSCs [32]. Pretreatment with the differentiating agent all-trans retinoic acid (ATRA) counteracted cisplatin resistance, preferentially sensitizing PKH_{Dim} cells to chemotherapy suggesting an effect on metastatic CSC subset as proven by in vivo decrease of tumor dissemination. By exploiting the quiescent properties of CSCs, this study revealed therefore the heterogeneity of lung CSCs and suggested the potential use of retinoic acid in combination with standard chemotherapy to counteract lung cancer metastatic spread [150].

2.3 Tumor Microenvironment Signaling Promoting CSC Drug Resistance

It is becoming increasingly clear that cancer development and progression cannot be fully understood without considering the major role played by the surrounding tissue microenvironment which actively participates to tumor growth [151]. The tumor microenvironment (TME) is a complex environment composed by extracellular matrix and several different cell types, including immune cells, vascular endothelial cells and cancer-associated fibroblasts, all of which participate in different aspects of tumor formation [152]. In this context it is easily appreciated that drug resistance can both be driven by the intrinsic ability of tumor cells to survive pharmacological treatment (intrinsic resistance) and by indirect mechanisms involving TME signals able to protect cancer cells from the damage caused by different drugs (extrinsic resistance) [153].

Induction of epithelial-mesenchymal transition (EMT) in tumor cells by TME-related signals is currently seen as one of the most crucial processes responsible for extrinsic resistance [154]. EMT is a reversible process active during develop-

ment by which epithelial cells acquire mesenchymal traits losing their apical-basal polarity and cell-cell adhesion: the same process is crucial for cancer cells in acquiring invasiveness and metastatic features [155]. The concepts of EMT and stemness are closely interconnected as many of the signals inducing EMT have also been shown to regulate stemness properties of cancer cells [44, 156, 157]. In this chapter we will therefore review experimental and clinical evidences related to EMT and drug resistance together with studies highlighting the link between EMT and acquisition of CSC phenotype.

Activation of EMT is associated with increased expression of mesenchymal markers including vimentin, fibronectin, N-cadherin, enhanced activity of matrix metalloproteinases such as MMP-2, MMP-3 and MMP-9, and decrease of epithelial markers such as E-cadherin [158]. The modulation of mesenchymal and epithelial gene expression during EMT is regulated by specific transcription factors (TF) acting as master regulators and in particular by Snail, Twist, and zinc finger E-box-binding (ZEB) [159]. The Snail family of zinc finger transcription factors, consisting of Snail1 (Snail), Snail2 (Slug), and Snail3 (Smuc), was demonstrated to play a crucial role in promoting EMT in cancer cells through the transcriptional repression of E-cadherin [160]. A role for Slug in lung cancer progression has also been proposed [161]. Twist is an highly conserved basic helix-loop-helix transcriptional factor that drives lineage determination in healthy tissue and has been shown to actively regulate EMT and metastasis in breast cancer [162]. In lung cancer Twist appears to play a pivotal role in promoting EMT by repressing E-cadherin and promoting N-cadherin expression thus inducing acquisition of metastatic traits through upregulation of MMP and FAK activity [163]. The Zeb family which includes ZEB1 and ZEB2 transcription factors can promote EMT through the repression of epithelial genes such as E-, P-, and R-cadherins and components of tight and gap junctions and desmosomes [164–166]. Moreover Zeb family TF can activate mesenchymal genes such as vimentin and N-cadherin and induce metalloproteinases such as MMP1, MMP2, and MMP14 [167, 168]. A correlation between high expression of ZEB1 and aggressiveness of the disease, defined by metastasis and chemoresistance occurrence, has been reported in lung cancer [169].

Different signals from the tumor microenvironment are able to trigger EMT process in lung cancer cells. The most well-known and studied inductor of EMT is the transforming growth factor beta (TGF-β) that explicates its effects through the activation of SMAD transcription factor complexes and regulation of target genes [170, 171]. The SMAD complex transduces extracellular signals from TGF-β to the nucleus where it interacts with Snail, Twist, and Zeb transcription factor families to repress epithelial genes and induce mesenchymal traits [172–174]. Another potent inducer of EMT is represented by the pro-inflammatory interleukin-6 (IL-6). In particular IL-6 plays a crucial role in regulating EMT in lung cancer through aberrant activation of STAT3 phosphorylation particularly in the context of KRAS activation [175, 176]; the inhibition of this axis can prevent distant metastasis formation in lung cancer xenograft models and reverse IL-6-induced EMT [177, 178]. Notably, IL-6 has also been shown to correlate with a poor clinical outcome and shorter overall survival in NSCLC patients [179], and elevated serum levels of IL-6

have been detected in lung cancer patients and correlated to lung cancer risk [180]. In different experimental settings, however, the inhibition of IL-6 has also been shown to enhance tumor progression highlighting the complex interplay and timing of the interactions within the TME [181].

2.3.1 Epithelial to Mesenchymal Transition and Drug Resistance

It has been increasingly recognized that cancer drug resistance is frequently accompanied by EMT in different types of cancer [182]. Strong experimental evidence supporting this link comes from recent studies exploiting genetically engineered mice models of pancreatic and breast carcinomas proving the crucial role for EMT in inducing chemoresistance [183, 184]. Challenging commonly held beliefs, EMT impairment did not affect metastasis development; however, EMT cells were shown to survive chemotherapy due to reduced proliferation, apoptotic tolerance, and increased expression of resistance genes and significantly contributed to drug resistance and even to metastasis formation after chemotherapy [184]. In pancreatic cancer the suppression of EMT did not decrease tumor dissemination and metastasis formation but led to an increase in drug transporter expression that contributed to enhanced sensitivity to gemcitabine treatment [183]. Overall these studies indicate the potential use of an EMT inhibitor to enhance efficacy of conventional chemotherapies.

Other studies have reported that induction of EMT was associated with overexpression of ABC transporters and of DNA repair proteins increasing resistance to chemotherapy [185, 186]. In lung cancer, analysis of cisplatin-resistant cells displayed the acquisition of an EMT phenotype and an increased invasion and migration ability [187]. The mechanism through which chemotherapy enriched for EMT cells may rely on the eradication of epithelial cells with a consequent relative increase of mesenchymal cells or on the direct promotion of EMT in cancer cells. Notably, chemotherapy treatments can induce the release of both stroma and tumor cytokines able to trigger pro-survival pathways in surviving tumor cells as well as induction of EMT, paradoxically sustaining chemoresistant cells and conferring increased metastatic ability [188]. In this respect cisplatin treatment of NSCLC was proved to increase the pro-inflammatory cytokine IL-6 that contributes to both EMT induction and chemoresistance of cancer cells due to the upregulation of anti-apoptotic proteins and DNA repair machinery [189]. Moreover different studies have reported the role of tumor microenvironment, particularly of cancer-associated fibroblast, to contribute to EMT induction and chemoresistance of NSCLC cells through a paracrine loop based on IL-6 [190, 191]. In particular treatment of lung cancer cells with cisplatin increased the expression of TGF-β that determined fibroblast activation and increased their IL-6 production. IL-6 in turn activated EMT in cancer cells and caused resistance to chemotherapy [191].

Accumulating evidence indicates that EMT activation is also linked to the acquisition of targeted therapy resistance [192]. In particular in NSCLC, the resistance to epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) has been

associated with different mechanisms including the mesenchymal phenotype of tumor cells [193]. In detail, gene expression profiling of a panel of 42 NSCLC cell lines screened for erlotinib sensitivity demonstrated the correlation between a gene signature associated with epithelial to mesenchymal transition (EMT) and insensitivity to erlotinib. Notably, NSCLC patients that showed strong E-cadherin expression and thus an "epithelial" phenotype experienced a longer DSF and OS with erlotinib plus chemotherapy treatment versus chemotherapy alone [193]. Similar results were reported by Thomson and coworkers showing in vitro and in vivo that sensitivity of human NSCLC cell lines to EGFR-TKI treatment was dependent on the degree to which cells have undergone an epithelial to mesenchymal transition (EMT). NSCLC lines expressing high levels of E-cadherin showed greater sensitivity to EGFR inhibition compared to cell lines expressing vimentin and/or fibronectin that were insensitive to the growth-inhibitory effects of EGFR-TKI [194]. The same group also reported that induction of EMT in NSCLC line H358 by TGF-β treatment caused loss of EGF family ligand expression, increased EGFR-independent Mek-Erk pathway activation, and reduced sensitivity to EGFR inhibition [195]. Finally, it was demonstrated that restoration of E-cadherin expression was able to increase cancer cell sensitivity to EGFR-TKIs [196]. All these evidences support the role of EMT as potential determinant for insensitivity to EGFR inhibition in NSCLC patients highlighting a common mechanism of resistance to both conventional and targeted therapies. In an attempt to investigate the cause of EGFR-TKI resistance, Yao and colleagues uncovered the existence of a subpopulation of lung cancer cells intrinsically resistant to erlotinib that displayed EMT phenotypes [197]. This subpopulation presented autocrine activation of TGF-β signaling that determined its mesenchymal features and secretion of IL-6, enabling cells harboring mutant EGFR to overcome their EGFR dependency, resulting in decreased sensitivity to erlotinib treatment. These findings imply that resistance to molecular targeted therapy can be driven both by tumor cell-autonomous mechanisms and/or activation of the tumor microenvironment [197].

2.3.2 Epithelial to Mesenchymal Transition and Acquisition of CSC Properties

It has been reported that EMT endows tumor cells with stem-like features, and thus the frequency of CSCs may also be partially regulated as a result of EMT activation induced by microenvironment cues in differentiated tumor cells. This observation implies that selection and expansion of CSCs conferring drug resistance may be due to both selective pressure and survival advantage of pre-existing CSCs and/or their de novo generation through the EMT process induced by TME signals.

The first seminal paper proving the connection between EMT and CSCs was published in 2008 by Weinberg's group [44]. Overexpression of EMT-related transcriptional factors, Snail and Twist, or TGF-β treatment induced in breast can-

cer cells an increase of CD44 ^{high}/CD24^{low} cancer stem cells and enhanced the capability to form mammospheres in vitro and to initiate tumor in vivo, two hallmarks of functional cancer stem cells [44]. In fact there is remarkable overlap in signaling pathways able to maintain CSC properties and to activate EMT such as Wnt, Hedgehog, and Notch pathways. Therefore drug resistance related to the activation of EMT (discussed in Sect. 3.2.3.1) can be also mediated by CSCs' generation through self-renewal signaling activation. For this reason EMT, CSCs, and drug resistance have been described as "an emerging axis of evil" for cancer treatment [154].

In lung cancer activation of Hedgehog pathway was demonstrated to induce EMT providing tumor cells with metastatic potential and resistance to chemotherapy [198]. Hh pathway can also confer resistance to EGFR-TKIs by inducing EMT in lung cancer cells [199] and, importantly, inhibition of the Hh pathway can reverse the EMT phenotype with a concomitant reduction of CSC markers and sensitize cells to EGFR-TKIs [134]. Notch signaling activation was also demonstrated to promote EMT in lung cancer cells, linked to the acquisition of resistance to EGFR-TKI [200].

Several studies reported that treatment of NSCLC cells with TGF-β induces EMT associated with the acquisition of CSC phenotype, demonstrated by the expansion (or de novo generation) of CD133$^+$ cells, enhanced migratory potential and tumorigenicity [45, 46, 201]. Interestingly, we also observed that the ability of lung tumor cells to "sense" TGF-β stimuli and to generate CD133$^+$ cells through the EMT process was linked to their plasticity that could be measured as a ratio between epithelial (E-cadherin) and mesenchymal (SNAI2) gene expression. Cells showing an intermediate EMT state, thus expressing both markers, were the most prone to generate CSCs under microenvironment stimuli both in vitro and in vivo [45].

Besides TGF-β, other cues from the tumor microenvironment can induce EMT and generation of CSC subsets. Cancer-associated fibroblasts (CAFs) were demonstrated to facilitate the conversion of differentiated lung primary tumor cells into CSCs, through the paracrine activation of EMT program and WNT, Notch, and Hedgehog signaling [202]. CAFs are crucial for CSC maintenance and regulation through the overexpression of growth factors such as IGF-II, HGF, and SDF1 and concomitant induction of the expression of their corresponding receptors in CSCs [45, 46, 203, 204]. Interestingly, tumor cells co-cultured with CAF also showed an enhanced resistance to chemotherapeutic drugs that was linked to microenvironment-generated CSC subsets [204]. In particular we recently reported that microenvironment stimuli eliciting EMT, including signals from CAFs, are able to generate the subset of CD133$^+$CXCR4$^+$EpCAM cells that represent the metastatic and chemoresistant fraction of CSCs [46]. Stromal-derived SDF-1/CXCL12 cytokine, the ligand of the CXCR4 receptor, is able to trigger EMT in lung cancer cells, and inhibition of CXCR4 signaling can partially block the EMT program induced by CAF-conditioned medium and prevent metastatic dissemination induced by chemoresistant CSCs. This observation points at the SDF-1/

CXCR4 axis as one of the crucial mediators of tumor-stroma cross talk responsible for EMT induction and generation of chemoresistant CSCs [46].

3 Novel Therapeutic Strategies Targeting Lung CSCs

The therapeutic implications of the cancer stem cell concept encompass different areas ranging from the potential use of CSC markers as prognostic and/or predictive factors (discussed in Sect. 3.2.1.2.) to the rationale design of novel therapies targeting these "seeds" of drug resistance and tumor recurrence [205]. Building on information gathered in preclinical studies dissecting CSCs' biology, the main approaches that can be considered are (i) direct targeting of pathways implicated in CSCs' maintenance or specific CSCs' functional properties (i.e., high expression of drug transporters, detoxifying enzymes, and anti-apoptotic molecules) and (ii) interference with tumor microenvironment communication [58, 206].

3.1 Targeting Intrinsic CSC Drug Resistance

With the aim to eliminate CSCs and possibly overcome drug resistance, different compounds specifically targeting self-renewal pathways involved in CSCs' maintenance have been tested in preclinical models and clinical trials. In particular several inhibitors of the Notch signaling pathway have been developed and tested, including γ-secretase inhibitors (GSIs), monoclonal antibodies (mAb) against Notch receptors or ligands, blocking peptides, and natural compounds [207, 208]. To date, GSIs are the most extensively developed and investigated class of Notch inhibitors. In lung cancer, the γ-secretase inhibitor R04929097, previously evaluated in other solid tumors [209, 210], has been tested in a phase II clinical trial for treatment of patients with advanced NSCLC who had completed treatments with front-line chemotherapy (clinicaltrials.gov, NCT01193868). The same compound has been under evaluation in combination with the EGFR-TKI erlotinib in advanced NSCLC (NCT01193881). Although both trials were terminated as a result of discontinued production of the study drug, administration of Notch-targeting compounds in combination with other drugs was evaluated as safe and feasible indicating potential for development of novel molecules [211]. In addition to γ-secretase inhibitors, the monoclonal antibody demcizumab (OMP-21 M18, OncoMed) has been developed to target Notch ligand DLL4. This antibody has been evaluated in NSCLC cancer in combination treatment with carboplatin and pemetrexed (NCT01189968), and encouraging early clinical activity has been observed and reported at the 2016 Annual Meeting of the American Society of Clinical Oncology [212].

The Hedgehog (Hh) pathway has long been implicated in CSC maintenance, and many of its components have received considerable interest as targets for Hh signaling inhibition [213]. In particular pharmacological targeting of SMO has been

widely explored, and GDC-0449 (vismodegib, Genentech) has been the first SMO inhibitor to enter clinical trials and to show its antitumor efficacy in solid tumors, particularly in basal cell carcinoma [214]. GDC-0449 also demonstrated an effective antitumorigenic activity in lung adenocarcinoma and SCLC and was able to increase the cytotoxic effects of cisplatin by affecting the side population [133]. It is currently under evaluation in a phase II clinical trial in SCLC in combination with cisplatin and etoposide (ECOG-1508, NCT00887159).

Concerning the Wnt pathway, the evaluation of pharmacological activity of DKN-01, a neutralizing mAb targeting extracellular dickkopf-1 (Dkk-1) and inhibiting the canonical Wnt/β-catenin signaling pathway, has recently been completed in a phase I trial in patients with relapsed or refractory NSCLC, multiple myeloma and advanced solid tumors (NCT01457417). Results from the trial indicated a safe pharmacological profile and potential clinical activity suggesting potential for future development in combination with other agents [215]. The small molecule FJ9, an antagonist of Disheveled (Dvl) protein, has been demonstrated to significantly downregulate canonical Wnt signaling and to possess promising anticancer activity. Preclinical studies showed that treatment with FJ9 was able to induce apoptosis in several lung cancer cell lines and to inhibit tumor growth in murine xenograft models [216].

Targeting the "side population" may also represent another approach to overcome resistance to therapy by increasing drug retention within CSCs. Xia et al. developed an image-based high-content screening (HCS) to specifically identify and analyze the high drug-efflux cancer cells (HDECC) in lung cancer cells lines. They screened 1.280 pharmacologically active compounds and identified 12 effective HDECC inhibitors. In vitro testing demonstrated that these inhibitors were able to overcome multidrug resistance and sensitize HDECCs to chemotherapeutic drugs; in addition they were able to significantly decrease in vivo tumorigenic activity of tumor cells, possibly by affecting CSCs' content [217].

Inhibition of activity of ABC efflux transporters has long been investigated as a possible way to overcome multidrug resistance (MDR), but compounds developed so far have shown limited efficacy and generalized toxicity [101]. The possibility that selective inhibition of drug efflux could also help in overcoming CSC-mediated drug resistance might however open the way for investigation of new treatment schedules or novel compounds. In this respect the calcium-channel blocker verapamil is also known to inhibit ABC transporter P-glycoprotein (P-gp), one of the major determinants of the MDR phenotype [78]*:* clinical trials in NSCLC comparing verapamil plus chemotherapy vs. chemotherapy alone demonstrated an improved outcome with a median survival significantly improved in the verapamil arm ($p = 0.02$) [218]. Tariquidar, another inhibitor of P-gp, has been investigated in combination with docetaxel for the treatment of recurrent metastatic solid tumors in a phase II trial (NCT00072202), and the results have indicated some anticancer efficacy particularly in lung cancer patients [219]. Several other compounds, including cyclosporine A, biricodar, PK11195, and curcumin, have been found to inhibit the ABC transporter family and counteract multidrug resistance, but none of these has been exhaustively tested in clinical trials [220].

Targeting the ALDH family of enzymes, highly expressed in CSCs (see Sect. 3.1.2), represents another strategy to potentially overcome drug resistance induced by CSCs. Disulfiram (Antabuse), an FDA-approved pan-ALDH1 inhibitor originally used in the treatment of chronic alcoholism, has demonstrated its efficacy in targeting CSCs in several solid tumors including lung cancer [221]. In particular disulfiram was able to re-sensitize cancer cells to standard therapies or enhance the cytotoxic effects of chemotherapy [222]. In a small phase II clinical trial, disulfiram in combination with cisplatin and vinorelbine was well-tolerated and significantly prolonged overall survival in patients with metastatic NSCLC [223]. Salinomycin, traditionally used as an antibacterial drug, has also demonstrated anticancer activity by directly targeting $ALDH^+$ CSCs. In in vivo preclinical models of NSCLC, salinomycin in combination with paclitaxel was able to drastically decrease metastasis formation compared to chemotherapy alone by targeting $ALDH^+$ lung CSCs [107]. The natural compound silibinin, a bioactive flavonoid agent, was proven to target $ALDH1^+$ CSCs and to sensitize them to the EGFR-TKI erlotinib thus decreasing the ability of $ALDH^+$ cells to escape targeted therapy and to sustain tumor recurrence [224].

Other strategies have been reported to sensitize CSCs to standard chemotherapy in particular by acting on mechanisms sustaining CSCs' resistance to DNA damage or apoptosis (see Sect. 3.2.2.1). Combination therapy with an inhibitor of DNA damage checkpoint protein kinase-1 (Chk1), particularly activated in CSCs compared to differentiated cells counterpart, was able to drastically reduce tumor growth and CSC subset compared to chemotherapy alone [97]. Furthermore inhibition of the anti-apoptotic protein Bcl-XL (consistently expressed at high levels in lung CSCs) using the small molecule inhibitor ABT-737 showed a specific cytotoxic activity toward quiescent/slow-proliferating CSCs [100]. Finally a differentiation strategy using all-trans retinoic acid (ATRA) in combination with cisplatin was proven to sensitize the subset of chemoresistant and metastatic $CD133^+CXCR4^+$ CSCs to cisplatin treatment strongly reducing tumor dissemination [150].

It must be considered however that the intriguing possibility to target CSCs through inhibition of stemness-related signaling pathways or exploiting specific properties of CSCs such as high expression of ABC transporters or ALDH enzymes, ability to escape apoptosis, and relative cellular quiescence also raises serious concerns because similar pathways/functional activities are shared with normal stem cells: anti-CSCs' therapies should therefore potentially be designed to preserve normal stem cells and to specifically target only molecules uniquely expressed or functionally activated in CSCs.

3.2 Targeting Tumor Microenvironment Cross Talk

Strategies aimed at interfering with microenvironment stimuli able to regulate the stemness phenotype and/or CSCs functional activities could offer an innovative way to potentially bypass CSC-mediated chemoresistance.

As described in Sect. 3.2.3, EMT is a crucial process mediating chemoresistance also through the generation of the CSC subset; thus therapeutic strategies able to reverse or inhibit EMT could sensitize tumor cells to conventional drugs and impair CSCs' formation [154]. Metformin, one of the first-line medications for the treatment of type 2 diabetes, has been recently shown to possess anticancer activity and to inhibit EMT process [225, 226]. In lung cancer Li et al. demonstrated that metformin increases the sensitivity of TKI-resistant lung cancer cells to erlotinib or gefitinib by reversing EMT [227]. EMT inhibition was linked to decrease of IL-6 signaling activation in TKI-resistant cells induced by metformin treatment. Combinatorial therapy with TKI and metformin effectively inhibited tumor growth in xenografts derived from resistant cancer cells, which was associated with EMT reversal and decreased IL-6 signaling activation, thus potentially representing an effective treatment to overcome TKI resistance and prolong survival of EGFR-mutated NSCLC [227]. Similarly, another group showed in lung adenocarcinoma that metformin was able to inhibit EMT by blocking the IL-6/STAT3 axis. Enhanced IL-6 expression could promote EMT in lung cancer cells via STAT3 phosphorylation, and metformin was able to reverse such a mechanism by blocking STAT3 phosphorylation. Importantly, metformin inhibited tumor growth and distant metastases in xenograft-bearing mice due to inhibition of EMT [178]. Interestingly a recently identified inhibitor of the stemness phenotype, napabucasin (Boston Biochemicals), also acts through inhibition of STAT3 signaling [228], and preliminary clinical investigation of this compound in advanced NSCLC has provided promising results [229]. IL-6 is abundantly released by stroma cells in tumor microenvironments; thus, as suggested by these studies, metformin or other drugs interfering with stromal signals may effectively impair tumor-stroma cross talk preventing EMT activation in tumor cells and acquisition of drug resistance.

CXCR4/CXCL12 axis contributes to NSCLC progression, and targeting this axis has been considered a potential therapeutic approach for lung cancer treatment in particular to counteract metastatic disease [230]. CXCR4/CXCL12 pathway is able to guide tumor dissemination to distant site and also to activate pro-survival and self-renewal pathways in tumor cells [231]. In particular we have observed CXCR4 coexpression in a defined subset of $CD133^+$ CSCs was able to survive chemotherapy and endowed with high dissemination potential and ability to initiate metastasis [32, 46]. In several PDX models of lung cancer, we have observed that cisplatin treatment, although effective in reducing tumor size, induces a relative enrichment of chemoresistant $CD133^+CXCR4^+$ cells in the residual tumor and that this enrichment correlated to an increased metastasis formation. Combination treatment with CTCE-9908, a small molecule inhibitor of CXCR4, was able to counteract the relative increase of $CD133^+CXCR4^+$ cells induced by cisplatin and drastically reduce metastatic dissemination, suggesting that CXCR4 blockade could specifically impair dissemination of chemoresistant and metastatic CSCs [46]. Moreover since stromal CXCL12 was demonstrated to induce EMT and acquisition of stem-like properties in NSCLC cells, inhibition of CXCR4 can

also impair the microenvironment-derived modulation of CSCs and chemoresistance [46].

Altogether these evidences highlight the crucial role of tumor-stroma cross talk in mediating chemoresistance and tumor progression induced by CSCs and indicate the potential of novel strategies aimed at interfering with this interaction to sensitize CSCs to standard chemotherapy and impair their fostering by microenvironment stimuli.

4 Conclusions and Future Perspectives

The cancer stem cell hypothesis has provided a new perspective in the understanding of mechanisms subtending drug resistance and for the development of novel strategies that may increase the efficacy of current therapies for cancer [81]. In fact, despite increased knowledge of the molecular basis of cancer development, evaluation of novel early diagnosis methods and employment of targeted therapies, lung cancer remains the most lethal cancer worldwide with an overall 5-year survival rate of approximately 15% [2]. This clinical evidence strongly supports therefore the urgent need to identify novel strategies to overcome drug resistance and tumor progression. CSC research has been a field in great expansion in the last decade with the achievement of several milestones including the demonstration of their existence in solid tumors, their characterization, and the understanding of drug resistance properties that may allow the design of new anticancer strategies to potentially improve effectiveness of current treatments.

The first evidence of CSCs in primary lung cancers was independently provided by two groups that identified lung CSCs on the basis of their surface expression of the CD133 marker [31, 32]. However many other groups have reported the existence of different cellular subsets with stem-like properties and ability to initiate tumor that were identified using different markers. It must be stressed however that many studies used lung cancer cell lines that, even if informative, may not faithfully recapitulate the biology of primary tumors; therefore validation in clinical sample represents the best way to validate potential markers used for the selection of lung CSCs. Lack of consensus regarding optimal CSC markers and the possibility that indeed different lung CSC subsets may exist further complicate our understanding of such populations and consequently our ability to efficiently target these cells. These controversies also arise from the poor knowledge of normal stem cells counterpart in lung tissue: some evidence indicates the possibility of the existence of distinct stem/progenitor cell subsets deputed to the maintenance of anatomically defined regions of the respiratory tracts from which different lung cancer histological subtype may be generated with implications for the phenotype of corresponding CSCs. Although this notion is well proven in murine models, knowledge about lung stem cell biology in humans is still in its infancy.

Despite difficulties in optimal markers selection for lung CSC isolation, one of the common traits of these cell subsets is the ability to resist current therapy used for lung cancer treatment, both conventional chemotherapy and targeted therapy. While current treatments may shrink tumors by eradicating actively dividing and

differentiated tumor cells, CSCs can survive these insults due to their unique properties and lead to drug resistance and subsequent tumor relapse. Self-renewal and pro-survival pathways activated in CSC as well as expression of drug transporters concur in conferring drug resistance to CSCs and represent the ideal targets for development of novel treatment strategies to improve patient response and prolong their overall survival. Signaling pathways associated with stem cell properties, such as differentiation and self-renewal capacities, have all been found often hyper-activated in CSC, and specific inhibitors blocking signaling activation are under investigation in preclinical studies and clinical trials showing some promising results. Major concerns arise from the evidence that the same signaling pathways are shared by CSCs and normal stem cells; thus further studies are necessary to identify more precise therapies which can selectively target CSCs but avoid toxicity to normal stem cells.

Besides intrinsic properties of CSC, others extrinsic factor derived from the tumor microenvironment can mediate CSC-induced drug resistance. Several cytokines released from stromal cells may trigger the activation of EMT in cancer cells resulting in acquisition of stemness properties together with other capabilities such as increased invasion/dissemination, resistance to anoikis and resistance to apoptosis/chemotherapy. In lung cancer EMT is associated with metastatic progression, resistance to EGFR inhibitors, chemotherapy, and generation of CSCs. The documented plasticity of differentiated tumor cells able to convert to stem-like phenotype under microenvironmental signaling represents another layer of complexity in CSC targeting. Compounds able to impair tumor-stroma cross talk could prevent de novo generation of CSCs and acquisition of drug resistance through the induction of EMT thus possibly improving the effectiveness of current therapies.

A very significant aspect to be considered regarding CSC targeting is that current parameters used in clinical evaluation of treatment efficacy in particular in terms of local tumor shrinkage may not be appropriate for the evaluation of CSC subset depletion. Indeed, strategies that effectively target CSCs are not expected to have an immediate impact on tumor shrinkage but rather on long-term end points such as tumor recurrence or metastatic progression. For these reasons novel biomarkers are needed to evaluate the efficacy of innovative therapies in CSC targeting. Circulating tumor cells shed from primary tumor into blood stream represent a non-invasive liquid biopsy of tumors and may offer the unique possibility to monitor the modulation of CSC populations during treatment to ascertain therapy efficacy.

Compelling evidences have been provided in preclinical models for the existence of lung cancer stem cells and their drug resistance phenotypes although the inherent complexity of lung cancer and the difficulties related to establishment of primary cultures of lung-derived cells have restrained advancements in lung CSC characterization if compared to other solid tumors. Novel therapeutic strategies targeting CSCs have been tested in experimental models and already evaluated in clinical studies in advanced NSCLC, but further efforts are needed to translate current lung CSC knowledge into clinical practice and fulfill the expectation to provide innovative ways to overcome drug resistance in lung cancer.

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Conflicts of Interest No potential conflicts of interest were disclosed.

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Overexpression of YY1 Regulates the Resistance of Cancer Stem Cells: Targeting YY1

Benjamin Bonavida

Abstract Most human cancers respond poorly to conventional therapeutics, and those that respond develop resistance to subsequent treatments. It has been reported that in many, but not all, studied cancers, there exists a mini population of cancer stem cells (CSCs) that is highly drug resistant and that its survival leads to recurrences and metastases. Hence, new targeted therapies directed at CSCs have been the subject of many investigations, and several agents are currently being investigated clinically. Several transcription factors are overexpressed in CSCs (e.g., SOX2, OCT4, NANOG, BMI1) that regulate stemness such as pluripotency and also regulate drug resistance. The transcription factor Yin Yang 1 (YY1) has been reported to be overexpressed in many cancers and is associated with cell proliferation, viability, EMT, metastasis, and chemo-immune resistance. Due to many common features of YY1 activities and cancer stem cell transcription factors, it was hypothesized that a crosstalk may exist between YY1 and CSCs transcription factors. Proteomic analysis was performed for the expression of YY1, SOX2, OCT4, and BM1 and delineated the presence of four groups of cancers with different molecular signatures consisting of different levels of expression of the above four transcription factors. These findings supported the hypothesis that YY1 is involved in the regulation of cancer stem cell transcription factors and their roles in resistance. Thus, targeting YY1 alone may be considered as a new therapeutic approach when used alone or in combination with various conventional or novel drugs to reverse cancer resistance in patients.

Keywords Yin Yang 1 (YY1) · Cancer stem cells (CSCs) · Resistance · YY1 inhibition · Therapy

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Abbreviations

BMI-1	B cell-specific Moloney murine leukemia virus integration site 1
CSC	Cancer stem cell
EMT	Epithelial–mesenchymal transition
HDAC	Histone deacetylases
Nanog	Nanog homeobox
OCT 4	Octamer-binding transcription factor 4
RKIP	Raf kinase inhibitor protein
SOX2	SRY (sex determining region Y)-box 2
YY1	Yin Yang 1

1 Introduction

Current therapeutic modalities against cancers consist mainly of chemotherapy, radiotherapy, and immunotherapy. While these modalities result in significant clinical responses, in a sub-population of cancer patients, however, a large proportion does not respond initially and the responding sub-population develops resistance to subsequent treatments. Hence, such unresponsive patients suffer from metastasis, a poor quality of life, and poor survival. A major underlying mechanism of resistance is the presence of a small sub-population of self-renewal tumor cells, cancer stem cells (CSCs), that are endowed with mechanisms that enable them to be highly resistant to most cytotoxic therapies. The CSCs, similar to normal stem cells, are differentiated by their ability to be pluripotent, self-renewal, and driving tumorigenesis, and are highly resistant [1]. They also are involved in EMT and metastatic spread [2]. The origin of CSCs has been proposed to develop from two different possibilities, namely, (1) transcription of normal stem cells or progenitor to CSCs through various gene mutations as well as epigenetics modifications [3] or (2) the cells acquire CSC prosperities through oncogenic-induced plasticity [4]. Thus, for a good therapeutic response, the CSCs have to be targeted specifically and to respond to the targeted therapy.

1.1 Properties of Cancer Stem Cells

CSCs are able to proliferate and differentiate through symmetrical and asymmetrical divisions with tumorigenic properties. They also acquire various phenotypic properties such as the formation of spheres in serum-free medium, overexpression of drug-efflux pumps, enzymatic activity of aldehyde dehydrogenase-1, and tumorigenicity in animal models that is maintained after serial transplantations [5]. CSCs acquire significant markers according to the tumor type [5]. Due to the complexity

and diversity of surface markers in different cancers, one must be cautious in the isolation of CSCs in pure form. Both surface markers and functional assays are recommended for the identification of CSCs.

1.2 Regulation of Cancer Stem Cells

Tumor cells are heterogeneous and consist of several distinct subsets with different activities of different phenotypes [6]. Hence, such subsets respond differently to various therapeutic interventions. Noteworthy, CSCs express transcription factors, all of which participate in the development and phenotypic properties of CSCs and are responsible for the regulation of stem cell self-renewal and pluripotency. Such transcription factors consist of the Sex-detaining Region Y-box2 (SOX2), POU class 5 homeobox X1 (POU5F1), also known as Octamer-6 binding transcription factor (OCT4), B-cell specific Moloney Murine-Looking Virus Insertion Site 1 (BMI1), and Nanog homeobox (NANOG) [7]. Various properties of these factors have been recently described (see review) [8]. Briefly, SOX2 overexpression correlates with tumor recurrence, poor prognosis, and chemoresistance in certain cancers [9]. In glioma, SOX2, OCT4, and NANOG positively correlated with tumor grade [10]. OCT4 synergizes with SOX2 to regulate transcription. In addition, OCT4 and SOX2 heterodimers bind to the NANOG proximal promoter region to induce transcription [11, 12]. High OCT4 expression is associated with higher histological grade in esophageal squamous cancer [13]. NANOG maintains embryonic stem cells' pluripotency independently of the LIF-STAT3 pathway [14]. Interestingly, SOX2, OCT4, and NANOG co-occupy the promoter region of at least 353 genes [15]. Overexpression of NANOG is associated with the high-grade subtype of ovarian cancer and correlates with poor rates of disease survival [16]. BMI1 is involved in the maintenance and/or self-renewal of many stem cell types. BMI1 regulates the tumor suppressors p161NK4A and p14ARF [17, 18]. Noteworthy, BMI1 binds directly to the Phosphatase and tensin homolog (PTEN) promoter and results in the activation of the PI3K/AKT pathway and EMT induction [19]. BMI1 expression correlates with the grade of various tumors and chemoresistance [20].

2 Resistance of Cancer Stem Cells: Role of Cancer Stem Cell Transcription Factors in Resistance

CSCs exhibit intrinsic resistance to various cytotoxic agents [21–23]. The growth and function of neoplastic stem cells (NSCs) depend on a complex network of signaling cascades and molecules. The oncogenic signaling is considered to derive from three distinct classes of molecules, namely, (1) the driver legions (primary oncogenic kinases) that are often disease specific and disease related, (2) broadly

spread mutated oncogenic kinases, and (3) cytokine-activated stem cell kinases that regulate cell growth and survival [24, 25]. These three classes of molecules contribute to resistance and are considered as therapeutic targets [26–28].

1. Role of NANOG in resistance

   Wang et al. [29] reported that there was an abnormal elevation of stemness factors like NANOG, OCT4, and SOX2 in the preferential enrichment of several types of CSCs. The activation of NANOG at a specific time results in CSCs rather than normal pluripotent cells or differentiated somatic cells. The upregulation of NANOG correlates with poor survival outcomes in patients with various cancers. NANOG mediates the regulation of a pathway involved in cancer development, such as proliferation, self-renewal, motility, EMT, immune invasion, and drug resistance. Experimentally, inhibitors of NANOG have been considered as potential therapeutic targets. Qin et al. [30] have reported in ovarian cancer that NANOG regulates resistance to chemotherapy. Also, NANOG regulates EMT that is also clearly involved in resistance. NANOG expression in ovarian cancer cell lines correlated with the high expression of mesenchymal cell markers and inversely correlated with the low expression of epithelial markers. Silencing of NANOG with RNAi reversed EMT and restored the expression of E-cadherin. There was no effect on proliferation or colony formation by modulating NANOG. The downregulation of NANOG increased the sensitivity of resistant cancer cells to cisplatin. Li et al. [31] reported that the DNA-binding protein inhibitor, ID-1, is involved in tumor progression, self-renewal, and pluripotency of stem cells. Using gastric cancer cells, they reported that the knockdown of ID-1 suppressed colony formation, tumor spheroid formation, cell proliferation, and cell migration. It also suppressed the expression of NANOG and OCT4. ID-1 knockdown sensitized the tumor cells to cisplatin-mediated cytotoxicity. Thus, it appears that ID-1 features are the result of targeting NANOG and OCT4 that are responsible for the proliferation, invasion, and resistance. Song et al. [32] reported that immune editing of tumor cells regulates the response of the immune system, particularly by the antigen-specific T cells. Such an immune-editing results in the enrichment of NANOG expression in tumor cells and results in a stemlike phenotype and immune resistance. The underlying mechanism was identified as HDAC1 and was being upregulated by NANOG. The NANOG-deficient HDAC1 drives epigenetic silencing of the cell cycle inhibitors, CDKN2D- and CDKN1B-induced stemlike features. Inhibition of HDAC1 significantly sensitized refractory cancer cells to activation with antigen-specific T cells. Thus, NANOG regulates immune resistance by upregulating HDAC1 and the epigenetic state of tumor cells.

2. Role of OCT4 in resistance

   Kobayashi et al. [33] reported that small cell lung cancer cells are resistant to gefitinib. OCT4 and the putative cancer stem cell marker CD133 are highly expressed in the gefitinib-resistant persisters (GRPs) in NSCLC cells and GRPs

exhibit many features of CSCs. Overexpression of OCT4 in tumor cells increased resistance to the chemotherapeutic drug gefitinib. Therefore, OCT4 maintains the resistance of tumor cells to gefitinib. Lu et al. [34] reported that studies in bladder cancer revealed a positive correlation between OCT4 expression and tumor recurrence in 122 bladder cancer specimens. Chemotherapy induced OCT4 expression in bladder cancer cells. The forced expression of OCT4 reduced drug sensitivity, whereas knockdown of OCT4 enhanced the sensitivity to cisplatin. Inhibition of OCT4 by alltransretinoic acid (ATRA) synergistically sensitized the cells to cisplatin. Villodre et al. [35] reported that in the majority of cancers, there is a negative correlation between the expression of OCT4 and prognosis and it negatively correlated with the survival of pancreatic cancer. Gwak et al. [36] reported in breast cancer that OCT4 was associated with ALDH1 expression but not with EMT. OCT4 was also independently associated with poor prognosis in the whole group and in the hormonal receptor-positive subgroup, but not in the hormonal receptor-negative subgroup. OCT4 expression was associated with poor clinical outcome in patients with hormonal receptor-positive breast cancer who were treated with tamoxifen.

3. Role of SOX2 in resistance

Wuebben et al. [37] reported that the progression of pancreatic ductal carcinoma (PDAC) correlated with the expression of SOX2. The authors generated four cell lines stably overexpressing SOX2 or knockdown of SOX2. Interestingly, overexpression of SOX2 inhibited tumor cell growth in vitro and inhibited tumorigenicity. Also, SOX2 inhibited the response of PDAC cells to drugs that are used in PDAC in clinical trials. Noteworthy, these findings are unique regarding the contrasting effect of SOX2 in PDAC compared to other cancers. Song et al. [38] reported on glioblastoma multiform (GBM), a lethal type of adult brain cancer and is highly resistant to adjuvant chemotherapy. GBM CSCs expressed CD133 (prominin-1), which is used to isolate CSCs from patients' cancers. Their findings demonstrated that CD133-positive cells are highly tumorigenic and drug-resistant. Microarray analysis identified SOX2 as the most enriched gene among the stemness in CD133-positive cells. The overexpression of SOX2 enhanced stemness in GBM cell lines, and silencing SOX2 inhibited tumor initiation and drug resistance. Garros-Regulez et al. [39] reported that SOX2 is overexpressed in patients with GBM and is associated with poor outcomes. In addition, they reported that SOX2 regulates tumor cell resistance to the drug temozolomide. Mu et al. [40] have used human prostate cancer models for investigation. The tumors develop resistance to the anti-androgen drug, enzalutamide, through lineage plasticity and the loss of TP53 and RB1 functions through the overexpression of SOX2. Das et al. [41] reported the development of a 3D cell culture system of drug-resistant breast CSC enrichment and that they can be generated under hypoxic conditions. Among a variety of drugs, actinomycin D was shown to downregulate SOX2 expression and resulted in the depletion of stem cell population and a decrease in resistance. Wuebben and Rizzino [42], in their review,

addressed the role of SOX2 and its association with tumor grade and patient survival. They also addressed its role in the regulation of drug resistance. Tripathi et al. [43] investigated the mechanism of drug resistance in small cell lung cancer (SCLC) using patient-derived tumor xenografts. They identified MCAM, an upregulated surface marker, in chemoresistant SCLC lines and xenografts. Depletion of MCAM reduced cell proliferation and reduced the IC50 inhibitory concentrations of chemotherapeutic drugs. This sensitization was mediated by SOX2-dependent upregulation of mitochondrial 37 s ribosomal protein 1 (ATP-binding cassette) subfamily C member 1 (MRP1/ABCC1), and the PI3/AKT pathway.

4. Role of BMI1 in resistance

   Bartucci et al. [44] reported in hepatic carcinoma that the expression of BMI1 correlated with poor patient survival. Several BMI1 inhibitors were synthesized and tested. One of the inhibitors, RU-A1, downregulated the expression of BMI1, impaired cell viability, reduced cell migration, and sensitized the tumor cells to 5-FU. The inhibitor also was effective on CSCs. Chen et al. [45] reported in head and neck squamous cell carcinoma (HNSCC), a highly resistant cancer, the identification of CSCs in which the expression of BMI1 was linked to the stem cell phenotype. The inhibition of AP-1 or BMI1 sensitized tumor cells to cisplatin cytotoxicity and eliminated lymph node metastasis. Yin et al. [46] reported that treatment of pancreatic cell lines with gemcitabine at a certain concentration induced the expression of BMI1. Knockdown of BMI1 enhanced ROS production and promoted the cytotoxicity of gemcitabine. BMI1 inhibition suppressed the activation of NF-κB and its downstream targets. Siddique and Saleem [47] reported that the chemoresistance of CSCs is due, in part, to the activation of BMI1.

3 Targeting Cancer Stem Cells

Multiple strategies have been devised to target CSCs. Those consist of (1) targeting cell-specific surface markers, (2) interfering with signaling pathways, (3) alteration of microenvironment signals, (4) inhibition of drug-efflux pumps, (5) manipulation of miRNA expression, (6) induction of cell death by apoptosis, and (7) cell differentiation.

1. Targeting cell-specific surface markers

   (a) Monoclonal antibodies directed against CSC markers, in combination with conventional therapies, have been used [48]; for example, the use of anti-CD133 (prominin 1) mAb. This surface marker is expressed in many cancers and shown to correlate with poor prognosis. Polymeric nanoparticles loaded with paclitaxel targeting CD133 have been used in experimental tumors [49].

In ovarian cancer, elimination of CD133-expressing cells resulted in long-term disease-free time survival [50]. Other cancers were also affected by anti-CD133 monoclonal antibodies [5].

(b) The use of anti-CD44 mAb. CD44 is a transmembrane protein that mediates cell-cell interactions with the receptors hyaluronic acid, selectin, collagen, osteopontin, fibronectin, and laminin [51]. CD44 is also overexpressed in many cancers [5].

(c) Anti-CD47 mAb CD47 is a transmembrane protein receptor for the thrombospondin family members and for the signal regulatory protein α (SIRP α) [48]. Two monoclonal antibodies were developed, namely, BCH12.2 and BCH12 [52]. CD47 is expressed on AML cancer stem cells and on the majority of human solid tumor cells. These two antibodies were tested in various experimental models [5].

2. Targeting signal cascades

(a) Many CSCs deliver signals to the TME [53]. There are aberrant signaling pathways in CSCs such as Notch, Hedgehog, WNT/β-catenin, NF-κB, PI3K/AKT, and PTEN [54]. Notch inhibitors tested in clinical trials included an inhibitor of the γ-secretase complex involved in Notch activation and antibodies against DLL4 and Notch 1, 2, and 3 receptors [55].

(b) The Hedgehog pathway contributes to CSC development and maintenance, as well as the acquisition of EMT. In preclinical studies, inhibitors of Hedgehog signaling have led to the inhibition of drug resistance, relapse, and metastasis [56]. Inhibition of Hedgehog and mTOR signaling pathways in biliary tract cancer, with rapamycin and vismodigib, resulted in a decrease in NANOG and OCT4 and a decrease in CSCs, ALDH-positive cell proliferation [57].

(c) The WNT/β-catenin signaling pathway is dysregulated in many cancers. OMP-54F28 is an inhibitor used in clinical trials. It is an antibody that experimentally inhibits CSC resistance and tumor-initiating capability [58, 59].

(d) The PI3K/AKT mTOR signaling pathway has been reported to maintain the CSC phenotypic features. For example, inhibitors of PI3K/AKT activity inhibited the formation of mammospheres of breast cancer cells and reversed the EMT phenotype [60]. Another example is shown in the radio-resistant prostate cancer cells that were treated with a combination of the PI3K/AKT mTOR inhibitor (BEZ 235) and radiotherapy resulted in an increased radiosensitivity, apoptosis, and reduction of cancer cell markers [61].

(e) The loss of PTEN, a tumor suppressor, in cancer cells has been linked to the development of CSCs [62].

(f) Hyperactivation of NF-κB is linked to the regulation of proliferation, resistance, and regulation of the CSC phenotype. Inhibition of NF-κB results in the inhibition of proliferation, EMT, migration, self-renewal activity, and stem cell-related signaling [63].

3. Targeting the TME

   The TME provides signals for CSC's maintenance, self-renewal, regulation, and homeostatic processes (e.g., angiogenesis, hypoxia, and weakly acidic pH). The interaction between CSCs and tumor stroma is mediated by the CXCL12-CXCR4 axis [64]. This contact also regulates cell growth, metastasis, and resistance. Several inhibitors for CXCR4 have been developed (e.g., AMD3100, CTCE-9908) and tested experimentally and have demonstrated inhibition of tumor growth and metastasis in murine models [65, 66]. An inhibitor of CXCL12, NOX-A12, suppressed CXCL12-induced chemotaxis of CLL and also induced chemosensitivity [67]. In addition, tumor angiogenesis promoted the TME and CSC survival. Targeting VEGF disrupted the CSC niche [61, 68]. Tumor hypoxia, a feature of TME, is associated with tumor growth, progression, metastasis, and resistance to both chemotherapy and radiotherapy. Thus, targeting hypoxia reversed these various activities [69]. The acidic tumor environment can be managed by, for example, inhibitors for pH regulating pathways like carbonic anhydrase 9 [70].

4. Targeting by ATP-binding cassette transporters

   The aberrant expression of ABC transporters is responsible for the chemoresistance in cancer cells, including CSCs [71].

5. Manipulation of miRNA expression

   miRNAs consist of small, noncoding RNAs (20–24 nucleotides in length) that negatively regulate post-transcription by binding with the 3' UTR of target mRNAs. They have a broad effect over self-renewal, differentiation, and cell division [72]. miRNAs also regulate key properties of CSCs [73]. Targeting miRNAs can be achieved by anti-sense oligonucleotides. For example, miR-21 is upregulated in different CSCs and its knockdown inhibits cell proliferation, migration, and tumor growth in many cancers [74].

6. Induction of apoptosis of cancer stem cells

   Since the NF-κB regulates resistance to apoptosis, inhibitors of NF-κB small molecules, parthenolides, preferentially target breast cancer stem cells [75] and leukemia cells [76].

7. Induction of CSC differentiation

   Various differentiation agents like retinoid acid, histone deacetylase inhibitors, miRNAs, tyrosine kinase inhibitors, and signaling pathways inhibitors have been investigated to differentiate CSCs [5]. For example, Ginestier et al. [77] suggested that retinoic acid and its analogs might induce differentiation of breast cancer stem cells. The same was also found for HDAC inhibitors [78].

4 YY1 and Its Association with the Transcription Factors of Cancer Stem Cells

Yin Yang 1 (YY1) is a ubiquitously expressed zinc finger transcription factor that exerts various functions including transcriptional regulation (positively and negatively), cell proliferation, chromatin remodeling, and apoptosis [79, 80]. The enrichment of binding sites for YY1 on the NANOG promoter was identified in the interactomes of both SOX2 and OCT4 [81]. It has been reported that in various cancer tissues, a correlation among the four CSC transcription factors and YY1 existed [81–83] (Fig. 1).

We have recently reported findings derived from data mining from proteomic data sets of solid tumors (n = 16) and hematological malignancies (n = 1) [8, 84]. The analysis performed in our recent report by Kaufhold et al. [8] through the grouping of marker expression patterns, we identified four distinct tiers of cancers:

(a) Tier 1 cancer group (prostate, lung, cervical, endometrial, ovarian, glioma) showed low YY1 expression with the concomitant high expression of SOX2, OCT4, and BMI1 (Fig. 2). The comparison of SOX2 and OCT4 expressions yielded an R^2 value of 0.99, showing a strong direct correlation.
(b) Tier 2 cancer group was characterized by high YY1 and low SOX2 (skin, testes, and breast cancers). SOX2 and BMI1 had a strong inverse correlation and YY1

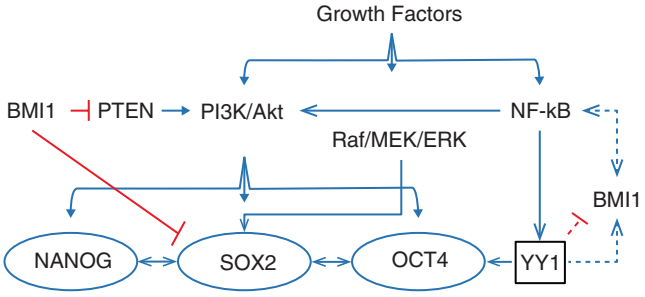

Fig. 1 Hypothesized cross-talk between YY1 and CSC transcription factors. This model reflects prior findings and proposed linkages between YY1 and CSC markers. (Reproduced with permission from the publisher [8])

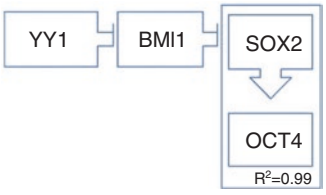

Fig. 2 Hypothetical functional dynamics of CSC-related transcription factors. SOX2 and OCT4 have a strong direct correlation (R2 = 0.99). (Reproduced with permission from the publisher [8])

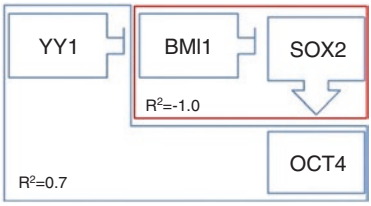

Fig. 3 Hypothetical functional dynamics of CSC-related transcription factors. YY1 is positively associated with OCT4 (R2 = 0.7), while SOX2 is negatively associated with BMI1 (R2 = −1.0). (Reproduced with permission from the publisher [8])

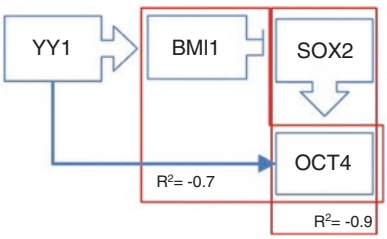

Fig. 4 Hypothetical functional dynamics of CSC-related transcription factors. There are significant negative correlations between SOX2 and OCT4 (R2 = −0.9) as well as between BMI1 and OCT4 (R2 = −0.7). (Reproduced with permission from the publisher [8])

had a direct correlation with OCT4 expression. Overall, the pattern was high YY1, low SOX2, high BMI1, and high OCT4 (Fig. 3).

(c) Tier 3 cancer group (liver, stomach, renal, pancreatic, urothelial cancers) had low YY1 and low SOX2 expressions. Both SOX2 and OCT4 had a strong inverse correlation and BMI1 and OCT4 also had an inverse correlation. This group, overall, has a molecular signature of low YY1 and SOX2 with high BMI1 and OCT4 (Fig. 4).

(d) Tier 4 cancer group is characterized by high YY1 and high SOX2 and consists of colorectal, lymphoma, and melanoma cancers. YY1 and SOX2 showed a strong correlation with OCT4. Overall, the signature was YY1, SOX2, and OCT4 were high, whereas BMI1 was low (Fig. 5).

Overall, the above findings demonstrated that YY1 expression correlated strongly and differentially with CSC transcription factors' expression in different tiers.

YY1 has been reported to induce EMT in cancer cells [85, 86]. The induction of EMT by YY1 facilitates the acquisition of a stem cell-like phenotype in cancer cells. In gastric cancer, Wang et al. [83] reported that YY1 promotes stemness through increasing the expression of several stem cell markers including CD44, NANOG, OCT4, and SOX2, which also play a role in metastasis. Katsushima et al. [87] reported the Notch1/LncRNA/TUG1/YY1 axis is an important pathway for self-renewal of glioma stem cells.

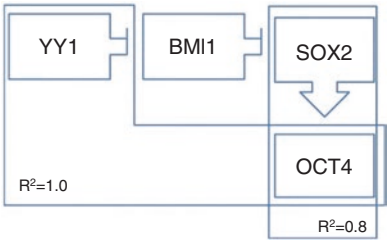

Fig. 5 Hypothetical functional dynamics of CSC-related transcription factors. There are strong positive associations between YY1 and OCT4 (R2 = 1.0) as well as between SOX2 and OCT4 (R2 = 0.8). (Reproduced with permission from the publisher [8])

The dual nature of YY1 activities, as both an activator and a suppressor, may be responsible for the differential patterns observed in the above four tier groups. Analysis of the putative promoters of SOX2, OCT4, NANOG, BMI1, and YY1 for the presence of putative YY1 binding sites is done using the SABiosciences Text. Many applications (SABiosciences' Text-Mining Application) demonstrated the presence of YY1 binding sites in all of the regulatory regions of the four CSC transcription factors, as well as on YY1 itself. Interestingly, none of the putative transcription binding sites for BMI1, SOX2, and OCT4 were found on the YY1 or on each other's regulatory regions.

The above analyses were performed on the expression patterns of whole tumor tissues, and not on the CSC subsets. Clearly, the above findings need to be validated with purified CSCs and delineate whether different patterns could emerge.

5 YY1 Inhibitors and Reversal of Resistance [88]

Reported studies in many cancer types showed that the inhibition of YY1 in drug-resistant cancer cell lines sensitized the cells to drug-induced apoptosis. Thus, YY1 regulates drug resistance via multiple mechanisms. It was suggested that the development of YY1-specific inhibitors may be considered a new therapeutic modality to inhibit YY1 activities, namely, cell proliferation, viability, EMT, metastasis, and chemoimmune resistance. However, all of the following inhibitors that have been recently reported, including siRNA YY1, NO donors, proteasome inhibitors, and inhibitors of survival pathways (e.g., NF-κB), reversed the above YY1-mediated effects. However, such inhibitors were not directly specific for YY1 but have acted through different pathways that led to YY1 inhibition. Among the various inhibitors of YY1 that have been reported in the literature are briefly described below.

1. miRNA,

   Aguilera et al. [89] reported overexpression of YY1-inhibited miRNA-193a-5P in cancer cell lines and concluded that there is an axis miR-193a-5P/YY1/APC

in the development of endometrioid endometrial carcinoma. Zhang et al. [90] reported the association of miR-7 and YY1 in colon cancer and that miR-7 was downregulated in colon cancer. The binding of miR-7 to YY1 3' UTR leads to downregulation of YY1 expression in colorectal cancer. Knockdown of YY1 resulted in the inhibition of proliferation and induction of apoptosis. In cervical cancer, Zhou et al. [91] demonstrated the association of miR-181 and YY1. YY1 is negatively correlated with miR-181 expression. The overexpressions of miR-181 inhibited cell proliferation, induced apoptosis, and arrested tumor cell cycles at G1. In nasopharyngeal squamous cell carcinoma, miR-34a is downregulated and YY1 is a direct target of miR-34a [92]. Overexpression of miR-34a resulted in apoptosis, inhibition of migration, and invasion. Other associations of miRNA and YY1 have been recently summarized [88].

2. Betulinic acid

   Betulinic acid is a small triperpenoid found in bark extract, and, along with its analogs, it exerts many antitumor activities [93, 94]. Betulinic acid inhibits YY1 [95].

3. NO donors

   We have reported the treatment of cancer cell lines with NO donors at relatively high levels inhibited cell proliferation, EMT, metastasis, and sensitized drug-resistant tumor cells to both chemo- and immuno-mediated apoptosis [96]. We have also identified a dysregulated NF-κB/Snail/YY1/RKIP/PTEN loop in many cancer cell lines of different origin and that was responsible, in large part, for cell proliferation, EMT, metastasis, and drug resistance. Treatment with NO donors disrupts the loop by inhibiting NF-κB, YY1, and Snail and upregulating the repressed RKIP and PTEN, resulting in the inhibition of proliferation, EMT, and sensitization to drug-induced apoptosis.

4. Proteasome inhibitors

   Proteasome inhibitors have been used successfully for the treatment of multiple myeloma and they have also been used in other cancers alone, or in combination, with other drugs. Initially, proteasome inhibitors were shown to inhibit NF-κB activity by inhibiting the degradation of phospho-IκBα and, therefore, preventing the translocation of NF-κB into the nucleus to mediate its activity. The inhibition of NF-κB results in the inhibition downstream of its various targets, including YY1. Below are a few recent examples of the use of proteasome inhibitors in cancer:

   (a) Zhang et al. [97] reported that the treatment of mantle cell lymphoma (MCL) with the proteasome inhibitor carfilzomib inhibited cell proliferation and induced apoptosis in MCL cell lines as well as freshly derived MCL cells. The induction of apoptosis was the result of both the extrinsic and intrinsic caspase pathways.

(b) Ettari et al. [98] reviewed the selective and nonselective immunoproteasome inhibitors that have been reported and showed promising results. They also reviewed their mechanisms of action, their relationship between their activities, and their potential therapeutic applications.
(c) In another recent review by Manasanch and Orlowski [99], they reported the FDA approved proteasome inhibitors in MM and MCL cells and clinical responses as well as relapses and resistance. Noteworthy, where there have been pre-clinical data observed in some inhibitors in solid tumors, however, such findings were not confirmed in the clinical trials. Several means to reduce resistance by NF-κB inhibitors should reveal new applications in the clinic.
(d) Citrin et al. [100] reviewed the application of proteasome inhibitors in the treatment of malignant and nonmalignant hematological diseases.
(e) Potts et al. [101] reviewed the proteasome inhibitors marizomib (NPI-0052; salinosporamide A) as a novel proteasome inhibitor with activities not shared by the FDA approved bortezomib. The inhibitor marizomib was tested in a variety of cancers and was also tested in combination with the immunomodulating agent lenalidomide.
(f) Sanchez et al. [102] reported preclinical findings demonstrating that the proteasome inhibitor CEP-18770 enhanced the activity of the anti-myeloma drugs bortezomib and melphalan, both in vitro cell lines and in vivo xenografts.

5. NF-κB iInhibitors

Since YY1 is regulated by NF-κB, inhibitors for NF-κB will also inhibit downstream YY1 and consequently inhibit cancer stem cells resistant to cancer therapies. Below are a few examples of NF-κB inhibitors that have been reported by us and others in the literature:

(a) Vaisitti et al. [103] reported a novel NF-κB inhibitor, IT-901, that was tested preclinically on CLL cells and demonstrated that this inhibitor inhibits the transcriptional activity of NF-κB as well as it activated the intrinsic pathway of apoptosis. In addition, a synergistic activity was shown by the combination treatment of IT-901 and ibrutinib. The antitumor activity of IT-901 was demonstrated in a tumor xenografts model of CLL.
(b) Qian et al. [104] reported that sporamin, a Kunitz-type inhibitor, inhibited cell viability, cell proliferation, and induced apoptosis in pancreatic cancer cell lines. These effects were mediated by the inhibition of the NF-κB pathway.
(c) Ukaji et al. [105] reported that the NF-κB inhibitor, DHMEQ, inhibits cancer progression and metastases in animal studies. These investigators also reported that the treatment of breast carcinoma cells with DHMEQ inhibited the metalloprotease (MPP-)2. In addition, we have also reported the treatment of cancer cell lines with DHMEQ-sensitized tumor cells to apoptosis for both chemo and immunotherapies [106].

(d) Lee et al. [107] reported that sodium butyrate inhibited NF-κB activity. In this study, the oral administration of sodium butyrate in an experimental murine colitis model inhibited NF-κB signaling and reversed histone acetylation.
(e) Wang et al. [108] reported that celecoxib, a cox inhibitor, inhibited NF-κB (p52 and p65) transcriptional activities and inhibited breast cancer cell lines proliferation and induced apoptosis.
(f) Nunes et al. [109] investigated whether artesunate, an artemisinin derivative, for its cancer activities as well as its ability to decrease the resistance of prostate cancer cells to androgen receptor antagonists. The combination of artesunate and bicalutamide inhibited NF-κB activity and induced apoptosis in prostate cancer cell lines. Both in vivo and ex vivo xenograft studies showed the antitumor activity by the combination treatment.
(g) Yang et al. [110] reported the effect of resveratrol in inhibiting proliferation and acetylation of p65, c-jun, and phos in a model of inflammatory arthritis.
(h) Durand and Baldwin [111] reviewed the effect of NF-κB activity in cancer and strategies to inhibit NF-κB signaling event and consequences. In another event, de Castro Barbosa et al. [112] described several promising anticancer drugs induced by NF-κB inhibitors.

6 Concluding Remarks

This brief review has described the proposed role YY1 plays in the regulation of several activities associated with cancer cells, both in the bulk population and the CSC subset. In addition, YY1 appears to regulate the expression of several transcription factors associated with the CSC phenotype, though YY1 association is differentiated in four subsets of cancer. These various YY1-associated signatures represent pathways that can be relevant in their roles in the pathogenesis of CSCs and their resistance to cytotoxic therapies. While the proposed findings were primarily derived through the combination of the researched literature and the bioinformatic analyses, clearly they were not necessarily focused on the CSC subset, but nevertheless, suggested several hypotheses that can be tested and validated. Since YY1 is a central factor with pleiotropic activities that are associated with the development of cancer, tumor progression, metastases, regulation of CSCs, and drug immune resistance, therefore, it was proposed that further studies are important to develop specific chemical inhibitors targeting YY1 that can be used both direct and in combination with conventional therapeutics. It is also hypothesized that such inhibitors targeting YY1 may be "one for all" therapeutic intervention for the majority of cancers.

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Cancer Stem Cell Challenges in Melanoma Characterization and Treatment

Alessandra Tuccitto, Chiara Castelli, Malcolm Ronald Alison, and Michela Perego

Abstract Melanoma is the most aggressive and lethal form of skin cancer. Many challenges remain despite great progress achieved with recently developed targeted therapies. Melanoma genetic and functional heterogeneity is the major cause of therapy failure, with different subpopulations that can be spared by treatments and which then support tumor regrowth. Many studies have suggested different approaches to isolate and characterize cancer stem cells (CSCs) from melanoma, also known as melanoma-initiating cells (MICs). MICs isolated by different groups can have different phenotypes, and MICs can also show plasticity, switching between MIC and more mature melanoma cell features, further underlying the complexity of this cancer. This chapter describes our current definition of MICs and their identification, with particular attention to their relevance in the therapy resistance. Moreover, we will discuss some of the controversial issues in the field and the possible therapeutic approaches to successfully target melanoma CSCs.

Keywords Melanoma · Cancer stem cells · Tumor initiation · Heterogeneity Therapy · Resistance

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Abbreviations

ABCB1	ATP-binding cassette subfamily B member 1
ABCB5	ATP-binding cassette subfamily B member 5
ABCG2	ATP-binding cassette subfamily G member 2
ALDH1	Aldehyde dehydrogenase 1
BRAF	V-raf murine sarcoma viral oncogene homolog B1
BRAFi	V-raf murine sarcoma viral oncogene homolog B1 inhibitor
$BRAF^{V600E}$	Amino acid substitution at position 600 in BRAF, from a valine (V) to a glutamic acid (E)
CSC	Cancer stem cell
CD	Cluster differentiation
CIK	Cytokine-induced killer
CTLA-4	Cytotoxic T-lymphocyte antigen-4
CXCL16	C-X-C motif chemokine ligand 16
CXCR4	Stromal cell-derived factor-1
CXCR6	C-X-C motif chemokine receptor 6
EMT	Epithelial-mesenchymal transition
FDA	Food and drug administration
JARID1B	Histone demethylase Jumonji/Arid1b
KIT	C-Kit tyrosine kinase receptor
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
MDR1	Multidrug-resistance gene product 1
MDSC	Myeloid-derived suppressor cells
MEK	Mitogen-activated protein kinase kinase
MHC	Major histocompatibility complex
MIC	Melanoma-initiating cell
MITF	Microphthalmia-associated transcription factor
NF-κB	Nuclear factor kappa b
NGFR	Nerve growth factor receptor
Notch	Translocation-associated Notch protein
PD-1	Programmed cell death-1
RGP	Radial growth phase
SP	Side population
TME	Tumor microenvironment
Treg	T regulatory cell
UV	Ultraviolet
VEGFR1	Vascular endothelial growth factor receptor 1
VGP	Vertical growth phase
WNT	Wingless-type mmtv integration site family

1 Introduction

Cutaneous melanoma is one of the most common skin cancers, and it has been reported an increasing incidence rate worldwide in 2012 with 232,000 new cases [1]. This is mainly due to an increased burden of environmental factors, familial causes, immune suppression, and augmented exposure to sunlight and tanning beds [2]. Although melanoma represents only 4% of skin cancers, it is responsible for 80% of skin-cancer related deaths [3].

The normal counterpart of melanoma is represented by melanocytes; these are neural crest-derived cells that reside in epidermis where they are responsible for melanin synthesis and distribution to adjacent keratinocytes. Melanin can absorb UV radiation, protecting cells from ultraviolet (UV)-induced DNA damage [4]. Because of their peculiar function and location, it is likely that melanocytes are subjected to UV-induced mutations. These initiating events such as *BRAF* (V-raf murine sarcoma viral oncogene homolog B1) mutations probably occur early in life, favoring the subsequent process of transformation characterized by the accumulation of additional, different mutations, activating oncogenic pathways responsible for the molecular and functional heterogeneity that is an intrinsic feature of malignant melanoma. Whether initiating mutations arise in fully differentiated melanocytes or in particular subsets of melanocytes, such as stem or progenitor cells, is still an open question. Nevertheless, cells endowed with transformation potential, intrinsic plasticity, and stem features persist in the malignant melanoma and constitute the melanoma-initiating cells (MICs).

Under normal conditions, melanocytes are non-proliferating cells, but genetic mutation occurring in melanocytes or in melanocyte precursors as discussed above could drive their proliferation. Hyper-proliferating melanocytes give rise to benign nevi that can be further transformed, firstly to dysplastic nevi and finally to melanoma [5].

The model of melanoma progression proposed by Clark [6–7] identifies six phases, each of which is characterized by specific histological and clinical features, summarized in Fig. 1. Small symmetrical benign nevi proliferate and gain the atypical morphology of hyperplastic and dysplastic nevi (phase 1), and subsequently these altered nevi progress to a melanoma lesion confined to the epidermis (melanoma in situ, or radial growth phase melanoma, RGP, 4). Later, RGP melanomas start to grow vertically deep into the dermis (vertical growth phase melanoma, VGP, 5), finally reaching the lymphatic system and/or blood vessels, so spreading to distant organs (metastatic melanoma, 6).

However, it is also known that not all melanomas adhere to this progression scheme, with malignant lesions developing directly from mature melanocytes, melanocyte precursor cells, and potentially stem cells in the hair bulge or dermal stem cells. Several studies [8–10], including the cancer stem cell (CSC) discovery, contribute to shedding light on other possible drivers of melanoma generation and progression. Here, we report evidence of CSCs in melanoma, their isolation, and

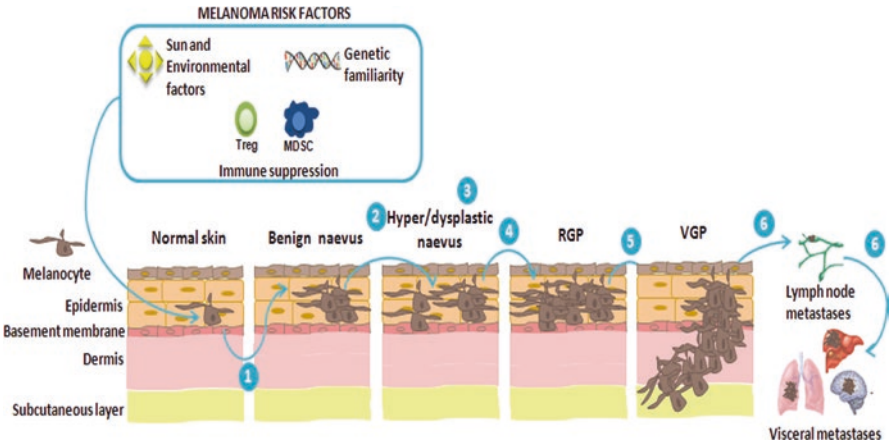

Fig. 1 A schematic overview of melanoma initiation and progression. This figure shows the six phases of melanoma progression described by Clark. Melanocytes resident in the basement membrane of the epidermis start proliferating after UV exposure and/or genetic mutational events forming a benign nevus (1). In the second phase, melanocyte aberrant proliferation leads to the formation of a hyperplastic and dysplastic nevus (2–3). The dysplastic nevus then forms a melanoma in situ which grows horizontally in the epidermis (RGP, radial growth phase, 4) and then progresses by invading the basement membrane and growing deep in the dermis (VGP, vertical growth phase, 5). Finally, in the metastatic form, melanoma cells colonize the lymphatic and vascular systems spreading to lymph nodes and distant organs (6)

characterization. We will discuss how melanoma stem cells influence melanoma heterogeneity and resistance to therapy and the implication of CSCs for the design of successful melanoma therapy.

2 Cancer Stem Cells and Melanoma: Definition and Identification

Melanoma stem cells, defined as melanoma-initiating cells (MICs), have been isolated by several research groups [11–17] applying mainly three complementary strategies. One methodological approach is based on the direct sorting (from short-term melanoma cell lines or from melanoma cell suspensions obtained from surgical specimens) of melanoma subpopulations expressing a given cell surface marker followed by testing for their tumor-initiating capacity in vivo [11–13, 15, 18]. In the second experimental approach, tumor cells isolated from specimens or established cell lines are subjected to in vitro culture conditions permissive only for CSCs. The selected putative CSCs generate "spheroids" (melanospheres in the case of melanoma), hopefully recapitulating the heterogeneity of the original tumor, and are then verified for their tumor initiating capacity in vivo [14, 19–24]. Finally, a third approach to identify MICs is based on the use of the side population (SP) assay that

relies on the intrinsic feature of stem cells to extrude cytotoxic dyes as Hoechst 33342. SP cells can be identified by flow cytometry, because they extrude Hoechst 33342 more rapidly, thus being fluorescent-dull. In melanoma, several groups have used SP to identify MICs [17, 25–26].

In 2005, CD20, a transmembrane protein expressed on the surface of B cells, was found expressed on a small subset of melanoma cells endowed with tumor-initiating capacity, self-renewal, and multipotency [11]. This subpopulation was enriched in metastatic ability compared to the primary lesions [27–29].

Other surface antigens, such as CD133, were reported to identify MICs [30–31]. CD133 expression was positively associated with poorly differentiated melanoma [32] and with an invasive phenotype associated with stromal cell-derived factor-1/CXCR4 [33]. Recently, CD133 in melanoma was associated with distinct melanoma lineages [34] with a role in angiogenesis promotion [35].

The low affinity nerve growth factor receptor (NGFR or CD271 or p75) was used to isolate MICs [15] and subsequently confirmed as crucial for CSC maintenance in melanoma [36]. Furthermore, CD271 expression correlates with poor prognosis in melanoma patients [37–38] and the ability to disseminate [39]. CD271 expression was shown to be heterogeneous in melanoma cell culture [40] and doubts on its validity as an MIC marker have been cast by some authors [41–42]. Other surface markers found correlated to MIC subpopulations are members of the ATP-binding cassette (ABC) transporters. Among these, ATP-binding cassette subfamily B member 5 (ABCB5) was reported as a useful prognostic marker on sentinel lymph nodes of melanoma patients [43] and could select for melanoma disseminating and invasive cells [44–45]. Moreover, ABCB5 and ATP-binding cassette subfamily G member 2 (ABCG2) were shown to be associated with MICs [12–13]. In addition, a role of the multidrug-resistance gene product 1 (MDR1 also known as ABCB1) was reported as a marker for highly self-renewing melanoma cells when co-expressed with ABCB5 and ABCG2 [46].

In 2010, the same group that proposed CD20 as an MIC marker found that the histone demethylase Jumonji/Arid1b (JARID1B or KMD5) was expressed by 1–5% of melanoma cells that were also slow proliferating and responsible for tumor maintenance [18]. Some limitations of JARID1B studies come from the lack of assays able to isolate live cells based on its activity.

Among all the detoxification enzymes, aldehyde dehydrogenase 1 (ALDH1) has been associated with CSCs in many tumors with conflicting results in melanoma [16–17, 47]. Indeed, some groups successfully used ALDH1 enzymatic activity to select MICs [16–17] but were challenged by Praskmickaite and colleagues [47], showing that there were no differences in in vivo tumor-initiating capacity between ALDH1-positive and ALDH1-negative cells.

Another important marker for the identification of MICs is C-X-C motif chemokine receptor 6 (CXCR6), the C-X-C motif chemokine receptor 6 that binds the chemokine C-X-C motif chemokine ligand 16 (CXCL16). Taghizadeh and colleagues [48] demonstrated that the CXCR6-positive cells are able to self-renew and to generate highly aggressive melanomas.

Table 1 Melanoma-initiating cell (MIC) markers

MIC markers	Description	References
CD20	Transmembrane protein expressed on B cell surface	[11]
CD133[a]	Transmembrane glycoprotein which contains five transmembrane domains	[12, 30, 31]
CD271	Low-affinity nerve growth factor receptor	[15]
MDR1[b]	Multidrug-resistance protein 1 is a plasma membrane-associated protein also known as ATP-binding cassette subfamily B member 1 (ABCB1)	[46]
ABCG2[c]	ATP-binding cassette subfamily G member 2 is a plasma membrane-associated protein	[12]
ABCB5[d]	ATP-binding cassette subfamily B member 5 is a plasma membrane-associated protein	[13]
ALDH1	Aldehyde dehydrogenase 1 is an enzyme that catalyzes the oxidation of aldehydes	[16, 17]
JARID1B	Histone demethylase Jumonji/Arid1b	[18]
CXCR6	C-X-C Motif chemokine receptor 6 that binds the chemokine CXCL16	[48]

Note: [a]CD133 was also reported to be co-expressed with ABCG2 [12], CD44 [50], and CD166 [49]
[b]MDR1 was also co-expressed with ABCG2 and ABCB5 [46]
[c]ABCG2 was also found to be co-expressed with CD133 [12]
[d]ABCB5 was also co-expressed with VEGFR-1 [51]

Notably, almost all the markers used to isolate MICs show some overlap between one and another, further underlying melanoma complexity. CD133, one of the most discussed MIC markers, was reported to be co-expressed with ABCG2 and ABCB5 [12, 49], or CD44 [50]. ABCB5 was reported to be co-expressed with CD166 in association to cellular adhesion and primary tumor growth [49] or with VEGFR1 (vascular endothelial growth factor receptor 1or Flt-1) being required to maintain tumor vascularization [51]. In addition, ABCB5-positive MICs were shown to be enriched in SP cells [52] as well as ABCB1, while JARID1B-positive MICs are rich in SP cells [26]. In Table 1 we summarize the MIC markers.

3 Melanoma Cancer Stem Cells: Open Questions

Comparing the large set of data currently available on melanoma cells identified as MICs, it is clear that there is still not a consensus on the phenotype of MICs. These discrepancies might be in part due to experimental differences between laboratories (mouse model or different injection method used to assess putative MIC tumorigenicity) which could explain differences in the abilities to detect stem cell properties [53]. Additionally, some enzymes used to process tissues before MIC isolation have been shown to affect surface antigen expression [38, 53]. Another point of discussion is the frequency of MICs, considered as a rare population by

most authors. Conversely, some data have suggested that almost all cells in aggressive melanoma could be potential MICs [54–55]. This challenges one of the main dogmas of stem cell and CSC theory, i.e., their rarity, and, more in general, casts doubt on the hierarchical organization of human melanoma that is central to the stem cell model.

Overall, the most reliable scenario is that different MICs co-exist in melanoma [56]. It is also plausible that MICs are not a static compartment, but some cells could acquire a different phenotype in response to signals coming from the tumor itself or the tumor microenvironment [13, 18, 57–61]. Concerning the origin of MICs, it is still not known whether MICs derive from melanocyte progenitors or stem cells located in the hair bulge [62–63], dermal stem cells [64], or more mature melanocytes that dedifferentiate or mutate gaining CSC features. However, histological studies suggest that the majority of melanomas arise from the epidermis, not from the dermis or hair follicles [65]. However, during embryogenesis, dermal cells can migrate in to the epidermal layers and thus likely constitute a long-term reservoir of melanocyte stem cell precursors [66], being the possible target of oncogenic transformation [67].

Although the whole field is still debated, it is nevertheless well accepted that MIC presence is related to melanoma progression, drug resistance, and relapse [13, 15, 38]. However, whether this is a cause or a consequence remains an open question. Notwithstanding, targeted strategies to eliminate MICs are clearly the consensus view.

4 Melanoma Heterogeneity and Plasticity: Cause or Result of Therapy Resistance?

While primary and early melanoma stages can be successfully eliminated by surgery, aggressive melanoma remained almost untreatable until 2011. In that year, the Food and Drug Administration (FDA) approved the use of antibodies targeting the immune checkpoint regulator CTLA-4 (cytotoxic T-lymphocyte antigen-4) and the introduction of targeted therapy against the $BRAF^{V600E}$ mutation, the most common genetic alteration found in nearly half of melanoma patients. Following CTLA-4, other immune checkpoint therapies became available from 2014 for advanced melanoma patients, always aiming at preventing tumor-reactive T cell exhaustion or suppression. The most promising one is targeting the PD-1 (programmed cell death-1) receptor expressed on tumor infiltrating T cells via monoclonal antibodies. However, about 30–40% of patients are resistant to immune checkpoint-based therapy [68–69].

Despite the initial success, almost all patients under BRAFi therapies experienced relapse, with relapsed tumors being resistant to any further therapies. Studies on resistant melanomas have shown a feedback upregulation of other important pathways controlling melanoma proliferation or activation of alternative pathways

due to ex novo mutations [70–79]. Combination therapy with MEK (mitogen-activated protein kinase kinase) and BRAF inhibitors, further improves the duration of the response, even if not preventing relapse. Tumors resistant to combination therapies could present with ex novo mutations or amplification of other pathways to compensate BRAF-MEK inhibition such as KIT (C-Kit tyrosine kinase receptor), Notch (Translocation-associated Notch protein), or WNT (Wingless-type mmtv integration site family) [80–87]. Again, targeting these pathways can be a therapeutic strategy, but ultimately, always leads to tumor relapse. One study showed that some resistance mechanisms appear in pre-treatment biopsies, challenging the idea of sequential drug treatment based on profiling of a single melanoma lesion [88]. In addition, heterogeneity was found in tumors relapsed after initial therapy failure. Genetic intratumor heterogeneity within the same melanoma lesion or comparing temporally and/or distinct lesions has been well documented [89–95], as well as single cell studies showing heterogeneity comparing primary and metastatic lesions [91–92] or among different metastases found in the same patient [93–95]. Moreover, about 15% of melanomas do not show any initial response to target therapy, demonstrating so-called intrinsic resistance. Melanoma heterogeneity could be responsible for intrinsic resistance, with much evidence also associating MIC presence to drug resistance. For example, an increased frequency of JARID1B-positive cells has been found after BRAFi targeted therapy, driving tumor relapse [96] and the expression of ABC transporters could help MICs to resist treatments [26], indeed ABCB5-positive cells selectively survive after Dacarbazine exposure [97]. In addition, BRAFi therapy has shown to positively select for melanoma cells expressing JARD1B, CD271, and ABCG2 with increased metastatic potential [98–99]. We expect that biomarker analysis or genetic studies on patients will allow the selection of patients for optimal therapy, or to predict therapy outcome. Thus, tumor heterogeneity constitutes a big challenge to tumor therapy.

Melanoma is not only a highly heterogeneous tumor, but it also shows a striking plasticity allowing rapid switching between different phenotypes or transcriptional programs. Melanoma plasticity could be directly related to its neuroectodermal origin, involving signaling profiles reminiscent of epithelial-mesenchymal transition (EMT), which has been linked to metastatic potential and therapy resistance in many cancers [100]. Pathways normally triggered during embryonic development can be reactivated, enhancing tumor invasion potential [81–82]. Moreover, some transcription factors, such as MITF (microphthalmia-associated transcription factor), could also modulate melanoma antigen expression, contributing to escape from immune system controls [101–102]. Indeed, melanoma antigens are expressed with some specific patterns, with high expression levels in brain metastasis, but low in lymph nodes and visceral metastases [103].

Melanoma plasticity could be also the result of tumor microenvironmental signals that could expand the survival of a specific subpopulation or favor the differentiation or de-differentiation of MICs [13, 18, 57–60, 96, 104–110].

We provided clear evidence that MIC self-renewal is sustained by IL-10 through an autocrine and/or paracrine loop while interleukin 6 (IL-6) is involved in inducing

MIC differentiation. Moreover, in an in vivo model, interleukin 10 (IL-10) ablation reduced tumor-initiation capacity, and IL-6 inhibition prevented MIC differentiation, resulting in smaller tumor masses [61]. These data are in line with the observation by Wilson and colleagues [107] who indicated an active role of pro-inflammatory cytokine signaling circuits to maintain MICs and further stress that in melanoma a direct relationship between tumor functional heterogeneity and immune-related factors exists.

5 Targeting Melanoma Cancer Stem Cells

Tumor heterogeneity is the major challenge leading to relapse with subpopulations that show a high degree of plasticity, so being able to better survive and escape from therapies. Plasticity is strongly linked to the ability to differentiate into various cell types that is one of the main CSC characteristics. Thus, it is a reasonable assumption that the resistant subpopulations overlap with the MIC compartment. With this view, complete tumor eradication can only be achieved if the MICs are eliminated, at the same time or after tumor bulk targeting.

Specific elimination of MICs, in single or combinatory therapy, could be achieved by directly targeting the surface antigens that select for MICs or stimulating specific immune responses against MIC-specific antigens. Other approaches could aim at blocking molecular pathways sustaining MIC self-renewal, leading to MIC exhaustion or at stimulating MIC differentiation into mature cells. Once MICs differentiate into mature cells, they could also become sensitive to therapies that are effective against the majority of tumor cells.

Targeting CD20-positive melanoma subpopulations should be possible using monoclonal antibodies such as rituximab that it is already an FDA-approved therapy for some leukemia. In the adjuvant therapeutic setting, the majority of advanced melanoma patients receiving anti-CD20-blocking antibody remained disease-free after 3 years' follow-up [29]; it was also reported a regression of a metastatic melanoma lesion in one patient treated in the non-adjuvant setting [111]. Another proposed strategy was to engineer T cells to recognize CD20-positive melanoma cells, leading to tumor regression in mice [28]. More recently, the CD20 antibody was conjugated to vincristine, a common chemotherapy used in melanoma, achieving specific killing of melanoma CD20-positive cells and empowering the chemotherapy effect in vitro and in vivo [112]. Likewise, targeting ABCB5-positive cells by monoclonal antibodies [13], or by silencing its gene expression, was shown to successfully reduce tumor burden in mice [113–114].

CD133 expression blockade has also been successful in limiting tumor growth [35, 50]. However, CD133 antibody design could be a challenge since normal and tumor tissues show differential glycosylation of CD133 epitopes that are not recognized by the same antibodies [115]. Antibody-mediated blockade of CD271 strongly inhibits tumor metastasis in melanoma xenografts [116]. Additional strategies

potentially successful in controlling tumor growth and progression might rely on targeting specific pathways linked/associated with the expression/function of the MIC markers. Several examples in the literature reported the efficacy of these approaches in an experimental setting in vitro and in animal models. The pharmacological or genetic downregulation of the Notch1 pathway that regulate CD133 expression attenuated melanoma growth and metastasis [35, 50, 86, 117–118]. Moreover, since CD133-positive cells also highly expressed VEGFR2, treatment with etoposide and bevacizumab reduced MICs [119]. In the case of CD271, targeting the NF-kB signaling pathway, which is upregulated in melanoma CD271-positive cells resistant to BRAFi-targeted therapy, also led to tumor reduction [120]. Similarly, the simultaneous inhibition of mitochondrial respiration prevented the emergence of the JARID1B-positive resistant clones in the presence of targeted therapies [96].

Some natural agents are also able to inhibit melanoma growth leading to a reduction of MIC subpopulations. ABCB5-positive melanoma cells can be targeted by some natural compounds [60] including honokiol [121]. Honokiol, a biphenolic natural compound derived from *Magnolia officinalis*, reduces CD166, CD271, and JARID1B expression on melanoma cells, interfering with the Notch pathway [121]. Similarly, lunasin, a bioactive peptide present in soybean is able to selectively target ALDH-positive MICs [122].

Melanoma is a highly immunogenic tumor and thus has been always considered an optimal candidate for immunotherapy. The role of the immune system in controlling MIC expansion is now becoming an intense field of research. Recently, in an autologous setting, cytokine-induced killer (CIK) cells have been reported to kill MICs which were spared by previous chemotherapy or BRAF inhibitor treatment [123]. While CIKs exert their activity by a major histocompatibility complex (MHC)-unrestricted mechanism, it would be very interesting to also consider MICs as possible targets for T cell-mediated recognition, with the final aim of designing therapeutic vaccines, probably useful in the adjuvant setting. In an experimental model, in vivo treatment with an ALDH (high) dendritic cell vaccine showed efficacy, with development of T cells selectively targeting ALDH-positive cells reducing primary tumor growth and metastatic spread [124]. In addition, a dendritic cell vaccine against MICs showed better results than the same vaccine against melanoma antigens [125–126].

The MIC secretome has been recently described and associated with tumor malignancy [61, 107], suggesting that modulating IL-10 or interleukin 8 (IL-8) could be useful in blocking MIC self-renewal and reducing the reservoir of MICs in the tumor. Moreover, soluble factors such as IL-6 secreted by cells comprising the tumor mass of melanoma or presenting in the tumor microenvironment (TME) could be instrumental for inducing MIC differentiation. Indeed, a soluble IL-6 vaccine in clinical trials led to the long-term overall survival of advanced-stage melanoma patients [127–128].

In light of these findings, future melanoma therapy must take into consideration MIC markers and important pathways that can be detectable/operable and

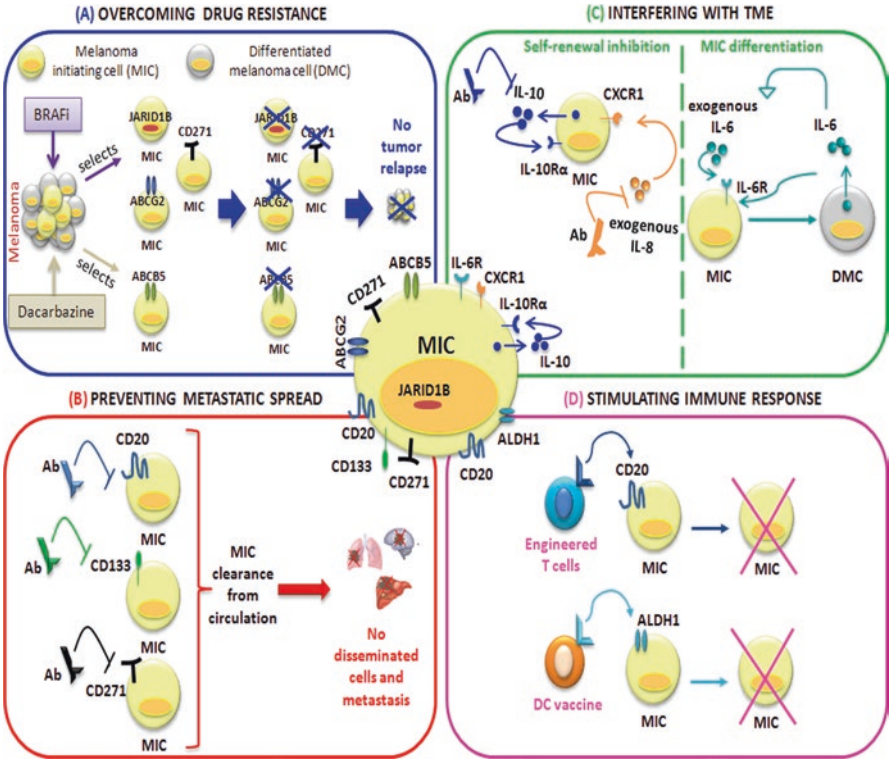

Fig. 2 Therapeutic strategies for targeting MICs and eradicating melanoma. Different strategies to targeting MICs are summarized in this figure. (**a**) Overcoming drug resistance. MICs specifically survive after first-line melanoma therapy; thus, their specific simultaneous targeting will avoid tumor relapse. (**b**) Preventing metastatic spread. MICs can migrate out of primary lesions and disseminate. Their elimination from the lymphatic and blood circulation will prevent metastasis formation. (**c**) Interfering with tumor microenvironment (TME). MICs can self-renew, expand, and/or differentiate in response of tumor microenvironmental signals. Targeting soluble factors sustaining self-renewal would lead to MIC exhaustion ultimately reducing tumor mass. Moreover, inducing MIC differentiation to mature melanoma cells would make them sensitive to targeted therapies, allowing melanoma eradication. (**d**) Stimulating the immune response. Using specific MIC markers to develop dendritic cell (DC) vaccines or to engineer T cells specifically targeting MICs could eliminate them from lesions before/during therapy and could contribute to the overall success of targeting therapies

targetable. Melanoma heterogeneity imposes the design of combinatory therapeutic approaches, targeting both the tumor bulk and MICs, to prevent tumor dissemination and relapse (Fig. 2). With this aim, it will be crucial to be able to assess therapeutic efficacy at early time points, characterizing the subpopulations eliminated or spared by the first-line therapy. Thus, many efforts are currently focused on understanding the biology of melanoma heterogeneity to minimize toxicities, for precise targeting and to further improve overall therapy responses.

6 Conclusions

Melanoma is extremely heterogeneous, with different subpopulations co-existing within the same tumor. This great heterogeneity is the major issue challenging the goal of melanoma complete eradication, since targeting one subpopulation is not sufficient to guarantee complete melanoma remission. The current knowledge about melanoma CSCs, functionally defined as melanoma-initiating cells (MICs), provides a possible interpretation for such heterogeneity. Important unanswered questions concerning the nature and phenotypic definition of MICs still exist, and studies are still needed to fully clarify the precise mechanisms by which MICs drive this melanoma heterogeneity. Certainly, the TME has an active role in shaping MICs. Indeed, only understanding the causes of MIC characteristics and plasticity and their interaction with the TME will give us the opportunity to develop a successful therapeutic strategy resulting in long-lasting cures, ultimately eliminating all melanoma cells from patients.

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Harnessing the Immune System to Target Cancer Cells

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Abstract The novel developments of cancer immunotherapy demonstrated that patients' immune system can fight cancer. However, immunotherapy strategies need optimization in order to provide benefit to a broader number of cancer patients. In addition, since cancer stem cells/cancer-initiating cells represent the component of tumor resistant to therapeutic interventions, targeting of these cells is mandatory to achieve complete eradication of tumors.

This chapter summarizes the most promising immune-based therapeutic approaches as propaedeutic introduction to the concept of cross-talk between cancer stem cells/cancer-initiating cells and tumor microenvironment and immune responses. The ability to optimize the targeting of these cells through immunotherapy will allow the successful harnessing of the immune system to beat cancer.

Keywords T cell-mediated immune responses · Tumor-associated antigens, MHC molecules · Immune checkpoint molecules · T cell receptor, Chimeric antigen receptor · Immunotherapy

Abbreviations

CEA	Carcinoembryonic antigen
CT	Cancer-testis
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen-4
DCs	Dendritic cells
EMA	European Medicines Agency
FDA	Food and Drug Administration
gp100	Glycoprotein 100

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HLA	Human leukocyte antigen
ICD	Immunological cell death
IFA	Incomplete Freund's adjuvant
LAG3	Lymphocyte-activation gene 3
LAGE	L antigen
MAGE	Melanoma associated antigen
MART-1/Melan-A	Melanoma antigen recognized by T cells-1; melanoma antigen-1
MHC	Major histocompatibility complex
MUC-1	Mucin-1
NKG2A	Natural killer C-type lectin-like receptor A
NY-ESO-1	New York esophageal antigen-1
OX40	Costimulatory molecule, member of the tumor necrosis factor receptor superfamily
PD-1/PD-L1	Programmed cell death-1/ligand-1
TAAs	Tumor-associated antigens
TIM3	T cell immunoglobulin and mucin-domain containing-3

1 Introduction

Immunotherapy can be considered a revolutionary approach for clinical management of tumors with different histological origins. Several efforts have been dedicated for decades to induce efficient and sustained anti-tumor immunosurveillance in cancer patients. Immunotherapy can include both active and passive approaches. Active immunotherapy is aimed at increasing the ability of patient's immune system, through vaccination with tumor antigens or their peptides, to recognize and kill tumor cells. Passive immunotherapy implies the administration of either immunomodulating agents or ex vivo-activated and -expanded immune cells. Active immunotherapy was pursued for several years based on the concept that T cells can recognize tumor-associated antigens presented by either tumor cells or by professional antigen-processing cells (APC), such as dendritic cells (DCs); however, the clinical efficacy was rather disappointing [1]. The observed limited clinical responses in cancer patients undergoing active immunotherapy were due to the usage, as source of antigens, of molecules expressed also by normal tissues and thus to the presence of tolerogenic T cells [1].

Moreover, tumor microenvironment (TME) can mediate immunosuppressive functions by the expression of immunomodulating factors and/or the presence of immune regulatory cells, such as myeloid-derived suppressor (MDSCs), T regulatory (Tregs) cells, or suppressive monocytes (M2) [1–5]. TME represents a critical player in regulating effective anti-tumor immune responses or in promoting tumor growth [2, 3, 5]. The targeting TME-associated immunosuppressive signaling represents a good option to overcome its immunosuppressive features and to enhance T cell-mediated immune responses. The clinical development of immune checkpoint blockade (CPB) agents led for the first time to improvement of overall

survival of cancer patients, demonstrating that the old paradigm came true and patients' immune system can indeed beat cancer [6–11]. However, a significant proportion of patients is unresponsive or develops resistance to these types of therapies [8, 9, 12]. The genomic and epigenetic makeup of tumor cells together with the phenotypic traits of TME can determine the variable responsiveness of cancer patients to immunotherapy.

The generation of engineered T cells, with either T cell receptor (TCR) or chimeric antigen receptor (CAR) for adoptive cell therapy, represents a promising approach for the treatments of both solid and hematological malignancies [13–18]. The latest has been approved by FDA and EMA for the treatment of adult and pediatric B cell malignancies refractory to standard therapies [13, 14]. Although these strategies showed unprecedented clinical success in tumors resistant to other therapeutic interventions, some patients can be unresponsive, and, moreover, their antitumor activity in solid tumors need to be optimized.

The identification of biomarkers represents the unmet need to predict patients who can benefit from a defined type of immunotherapy or to optimize a combination of different immune-based strategies or of immunotherapy plus standard interventions. Moreover, efforts are currently made to identify novel molecular targets specifically expressed by tumor cells to develop novel cancer vaccines or ACT treatments.

This chapter will briefly summarize the common immunotherapy approaches that have been clinically developed as introductory to the subsequent chapters that will address the interaction of cancer stem cells with TME and cell-mediated immune responses to target these cells.

2 Cancer Vaccines

2.1 Tumor-Associated Antigens (TAAs)

TAAs are recognized by T lymphocytes in the form of MHC/peptide (8–15 aa) complexes. The molecular characterization of different types of TAAs allowed the development of TAA-based cancer vaccines to induce antigen-specific T cell responses [1, 19, 20]. Different categories of TAAs have been identified:

1. *Self/differentiation antigens* that are specific for cellular lineages: these TAAs, which include carcinoembryonic antigen (CEA), epithelial cell adhesion molecule (Ep-CAM), mucin-1 (MUC-1), survivin-1, and, for melanoma, melanoma antigen recognized by T cells-1 (MART-1/Melan-A), glycoprotein 100 (gp100), and tyrosinase (Tyr) [20], are overexpressed by tumor cells but also detectable on normal cells.
2. *Cancer-testis (CT) antigens* (e.g., MAGE, GAGE, LAGE, NY-ESO-1) are expressed in tumors with different histological origins, while among normal tissues they are found only in testicular germ cells and placenta [21].

3. Neo-antigens generated from non-synonymous somatic mutations. These TAAs represent tumor-specific antigens that are not detectable on normal cells. These TAAs can display superior immunogenicity compared to the categories mentioned above [22].

Cancer vaccines based on the usage of self/shared or CT TAAs have been developed for both preclinical and clinical studies. Good manufacturing practice (GMP)-compliant peptides can be relatively easily synthesized, although with high costs, and can be safely manipulated for vaccination of cancer patients. Peptide cancer vaccines, including single or multiple epitopes, have been administered, emulsified in the Incomplete Freund's adjuvant (IFA) Montanide in the context of phase I/II clinical studies, to patients with melanoma or other solid tumors [23, 24]. However, limited clinical responses were registered (15–20%), although circulating TAA-specific T cell responses could be commonly detected in patients following the administration of vaccines. Changes at the level of aa sequence of peptides have been introduced to increase the affinity and binding to HLA molecules (e.g., gp100 209-2 M, MART-1 27 L or CEA CAP1-6D) [25–27]. However, durable clinical responses were not achieved due to the failure in generating antigen-specific T cells cross-reactive with native TAAs [28, 29]. In a Phase III clinical study, the combination of the modified gp100:209–217 epitope with high doses of IL-2 for the treatment of advanced melanoma patients showed improvement in overall clinical response in patients treated with vaccine plus interleukin-2 group than in the interleukin-2-only group [30]. However, the efficacy of this treatment should be assessed in larger cohort of patients.

The usage of long-peptide (up to 20 aa length)-based vaccines has also been investigated. These peptides can contain HLA class II-restricted epitopes eliciting $CD4^+$ T cell-mediated responses that can support anti-tumor CTL responses. Promising results have been shown in a model of HPV cancer vaccine [31]. Nevertheless, these long peptides showed limited success in inducing TAA-specific T cells even following the improvement of HLA binding through the introduction of modifications in the peptide sequences [32].

Of note, failure in clinical success of cancer vaccines can rely also on the lack or sub-optimal expression of HLA class I molecules by tumor cells, representing an obstacle for antigen-specific T cells to recognize and kill cancer cells [33–35].

The occurrence of non-synonymous mutations in tumor cells can lead to the generation of neo-antigens. These mutated TAAs are tumor-specific and are not detectable in normal cells, thus, they display higher immunogenicity as compared to self-antigens and do not induce immunotolerance [1, 36]. The advances in high throughput next-generation sequencing (NGS) allowed a deep characterization of cancer genomes. The isolation of neoepitopes can be performed through NGS data from matched tumor and normal cells and computational prediction of somatic mutations [36, 37]. The identification from neoantigens of mutated epitopes with high affinity for defined HLA molecules occurs in silico [36, 37], although this process is still challenging, since computational analysis cannot predict their efficacy in eliciting antigen-specific T cells [38].

Different proof of concept clinical studies have been performed to identify immunogenic epitopes from the mutational landscape of melanoma, glioblastoma, non-small-cell lung cancer (NSCLC), colorectal cancer (CRC) and pancreatic cancer [39–43]; however, immunogenic neoepitopes are rarely shared among patients [36, 40, 41, 43–45]. Clinical benefit has been observed for neoepitope vaccination [36, 40, 41, 43–45]. Moreover, tumor-infiltrating lymphocytes (TILs) recognizing mutated antigens have been isolated from solid tumors. These TILs are critical for tumor eradication, following their ex vivo expansion and administration in patients [46].

The application of neo-antigens for cancer vaccines represents a promising approach; however, their identification occurs at single patient's level and through a long process. The safety and efficacy of vaccination with neo-antigens need to be evaluated along with the development of clinical trials in large cohorts of patients.

2.2 DNA/RNA and Virus-Based Vaccines

Vaccines constituted of nucleic acids encoding for TAAs have also been exploited to induce anti-tumor immune responses in cancer patients. These nucleic acids are delivered in vivo in the forms of viral vectors, plasmids, and lipid nanoparticles [47].

DNA vaccines are bacterial plasmids containing genes encoding TAAs together with different immunostimulatory molecules [48, 49]. Nevertheless, DNA vaccines contain genes that stimulate innate immunity and through inflammatory milieu can induce the adaptive immune response. DNA vaccines have shown limited efficiency in vivo in terms of immunogenicity and success in eliciting TAA-specific T cell responses that are immunogenic as compared to other vaccines.

RNA vaccines display more versatility and can provide co-stimulatory signals through stimulation of Toll-like receptors [50]. However, they resulted in lack of efficient delivery and targeting. In order to prevent their degradation, "gene gun" delivery has been exploited to deliver mRNA coated on nano- and gold particles directly into cytoplasm [51]. However, limited induction of tumor-specific immune responses were observed [52, 53]. A personalized vaccination with cancer patients' mRNA mutanome has been shown to induce both $CD4^+$ and $CD8^+$ T cell responses against neo-antigens [54].

Although DNA and RNA vaccines are relatively easy to synthesize and to deliver, the immunosuppressive TME and tumor-associated immune evasion strategies can affect their therapeutic efficacy.

Virus-based strategies can mimic natural cellular infection, and through APC cross-priming, they can induce T cell responses against the TAAs encoded by the vector. They have been shown to be safe and immunogenic. Two human papillomavirus vaccines have been approved for the prevention of HPV-mediated cervical cancers.

2.3 Dendritic Cells and Whole-Cell Vaccines

DCs are professional antigen-presenting cells that can mediate TAAs' delivery in vivo and the induction of anti-tumor T cell responses. DCs are generated ex vivo from either monocytes or $CD34^+$ progenitor cells with granulocyte colony-stimulating factor (GM-CSF) and cytokines [55, 56]. They can be isolated in vitro and loaded with TAAs, and subsequently inoculated into cancer patients as fully matured DCs [57]. Clinical benefit and safety of administration of DCs and DC-based cancer vaccines to cancer patients have been reported [57–59].

Other strategies for cancer vaccines are represented by whole-tumor cells [1, 24, 60]. These vaccines either can directly engage TCR on T lymphocytes or can be up-taken by DCs and, then, stimulate T cells through cross-priming [61–64].

Phase I/II clinical studies for melanoma patients, using the combination of allogeneic whole-tumor cells with or without Bacillus Calmette-Guérin (BCG) as adjuvant, showed clinical responses [65–67], although with limited duration.

Tumor cells producing IL-2 or Il-4 were shown to be promising vaccination tools for melanoma patients [68–71]. Tumor cell-secreting GM-CSF have led to the induction of tumor-specific T cell responses and to the accumulation of DCs at the vaccination site [72, 73] and clinical benefit in both pancreatic and prostate cancer patients [21, 74]. However, the promising therapeutic treatments with these vaccines in mouse models were not confirmed in clinical studies [71].

Taken together, the vaccination approaches mentioned above showed quite disappointing clinical efficacy (10–20%) [1, 24] due to the low immunogenicity of self/shared TAAs and the immunosuppressive functions of the TME. The choice of TAA needs to be carefully evaluated to design strategies for cancer vaccines or further combination strategies.

3 Immune Checkpoint Blockade

The discovery of immune checkpoint molecules, such as cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed death-1/ligand 1 (PD-1/PD-L1) and the development of antagonist mAbs (CPB) represented a revolution for immunotherapy [75]. These agents release the physiological break of T cell-mediated immune responses enhancing their anti-tumor activity. The clinical development of these strategies contributed to change the paradigm of cancer treatment. The first impressive report of the clinical efficacy of anti-CTLA-4 mAb was in 2010 showing, for the first time in the context of immune-based treatment, improvement of overall survival of melanoma patients with advanced melanoma [7, 76]. During the last 8 years, six therapeutic agents including one targeting CTLA-4 (ipilimumab), two anti-PD-1 mAbs (nivolumab and pembrolizumab), and three blockers for PD-L1 (atezolizumab, avelumab, and durvalumab) have been approved by both FDA and EMA for the treatment of different types of cancers such as melanoma, lung, head and neck, bladder and Merkel cell cancer, and Hodgkin's lymphoma.

Despite this unprecedented clinical success, CPB is not effective in all type of tumors, as cancers with low mutational burden and/or low immunogenicity may be intrinsically resistant to these therapies. In addition, a significant proportion of patients develop resistance in the course of treatment in relation to the loss of antigenicity or changes in the immunological features of TME. Indeed, multiple studies showed a relationship between mutational load of tumors and clinical responsiveness to CPB [77–80]. Of note, it was recently reported that lack of clinical responses to CPB therapies correlated also with impaired expression of HLA class I molecules in tumors, that might result in the failure of antigen-specific T cell responses against cancer cells [81, 82].

The hallmark of CPB is the lack of predictive biomarkers that can contribute to optimize patients' stratification and the usage of these therapeutic interventions. The combination of different CPB (anti-CTLA-4 and anti-PD-1) has also been approved for the treatment of melanoma, based on the improvement of clinical responses (60%), despite high rate of toxicities were also observed [83, 84].

Preliminary results obtained from treatment of cancer patients with the combination of CPBs plus cancer vaccines indicated that this strategy can be promising [85]. Of note, the clinical development of other CPBs, such as LAG3, TIM3, CD27, CD137, GITR, OX40, and ICOS [86] opened the landscape for possible therapeutic interventions and their combinations [87]. Along this line, few classes of chemotherapeutic agents (e.g., doxorubicin, cyclophosphamide, doxorubicin, bortezomib, etc.) can induce immunological cell death (ICD). This phenomenon can lead to the release of danger signals and TAAs by damaged and dead tumor cells and, subsequently, induce anti-tumor immune responses [88, 89].

In addition, radiotherapy can also induce cell death and activate a cascade of immunological effects, a phenomenon known as the "abscopal effect" [90, 91]. These evidences represent the rationale for the combination of chemotherapy or radiotherapy with CPB; the clinical efficacy of these combinations is currently under evaluation in a number of clinical trials [89, 92–95]. Importantly, further investigations are warranted to optimize the combination of therapies mentioned above. Different types of cancer, bearing variable genomic profile and immunosuppressive features, will need a tailored therapeutic intervention.

4 Adoptive Cell Therapy

Advances in the available technologies to engineer T cells, through the usage of gamma retroviruses or lentiviruses that can stably integrate exogenous genes encoding for high affinity antigen-specific TCR, has allowed to generate tumor-reactive immune cells [15–17].

The treatment of hundreds of cancer patients in the context of clinical studies with advanced malignancies with TCR-engineered T cells showed successful tumor regressions of multiple lesions [15–17]. Nevertheless, these studies also highlighted

some risks associated with TAA selection. The usage of TCR specific for self/differentiation TAAs, such as MART-1/Melan-A, gp100, and CEA, was associated with toxicities that could also reach high grade of severity, due to cross-reactivity with normal tissues expressing these antigens [15–17]. On the other hand, when metastatic melanoma or synovial sarcoma patients were treated with T cells engineered with TCR specific for NY-ESO-1 antigen, 55% and 61%, respectively, of objective response were registered without any toxicities [96, 97]. Other TAAs are being studied for targeting with engineered T- cells, also in the context of clinical studies, in order to validate their usage for successful and safe clinical application. Importantly, targeting of neo-antigens with TCR-engineered T cells revealed to represent a promising approach without the risk of targeting normal tissues [15, 18, 98, 99]. Recently, tumor regression and improvement of overall survival were documented by the treatment of a patient with metastatic breast cancer with T cells, expressing high-affinity TCR-targeting neo-antigens [18].

Another ACT promising approach is represented by the injection into cancer patients of autologous tumor-infiltrating lymphocytes (TILs) isolated and expanded ex vivo recognizing neo-antigens. These TILs showed to be able to mediate tumor regression and to improve patients' survival [15, 17, 99].

Of note, another successful strategy is represented by T cells transduced with chimeric antigen receptor (CAR) constituted by single-chain variable fragment of a monoclonal antibody, conferring antigen specificity, and T cell-derived signaling molecules [100–103]. These T lymphocytes are activated through CAR engagement in MHC-independent manner and upon binding with membrane expressed TAAs [104, 105]. The administration of CAR-T cells targeting CD19 to patients with B-cell malignancies refractory to other treatments showed impressive and unprecedented clinical responses [101, 103, 106]. Although this type of treatment was associated with severe adverse events, such as cytokine release syndrome and neurotoxicity, with the appropriate surveillance and supportive care, these side effects were manageable. The risk-benefit ratio of treatment with CAR-T cells was considered acceptable, leading to recent approval by FDA and EMA of these biological drugs for the treatment of some pediatric and adult B-cell malignancies [13].

Investigations are currently ongoing to develop and optimize CAR-T cells for the treatment of solid tumors [13, 106–111]. The major limitation for the application of CAR-T cell therapy for solid tumor is represented by the need to select TAAs that are tumor specific, avoiding off-target reactivity and generation of severe side effects.

It needs to be considered also the hurdle of achieving large number of engineered T cells to be administered into cancer patients, although the optimization of clinical grade production and manufacturing of these immune cells is being addressed by different studies.

Taken together, ACT can provide clinical benefit to patients with advanced and refractory tumors; however, the dynamic genomic and immunogenic profiling of tumors as well as of TME should be considered in order to design interventions that could achieve successful tumor rejection.

5 Conclusions

Immunotherapy represents the breakthrough for treatment of cancer patients. Innovative strategies have been developed, leading to improvement of clinical responses and overall survival of cancer patients even with advanced diseases. Although limitations emerged with respect to significant proportion of patients unresponsive or developing resistance to these therapies, ongoing investigations are aimed at tailoring these therapeutic interventions based on genomic and immunological makeup of cancer patients. Nevertheless, the molecular identification of novel TAAs, either neo-antigens or "off-the-shelf," still represents the major challenge in relation with intra and inter-tumor molecular heterogeneity.

Moreover, novel combinatorial treatments represent the promising strategy to overcome immunosuppressive tumor milieu and inducing effective anti-tumor immune responses.

Of note, cancer stem cells/cancer-initiating cells represent a rare population within tumor lesions that is responsible of resistance to therapeutic interventions and can display low susceptibility to immunotherapy. Therefore, the design of immunotherapy successfully in targeting these cells will allow the achievement of complete eradication of tumors. The dissection of the mechanisms regulating the interplay between cancer stem cells/cancer-initiating cells and the immune system are warranted in order to optimize immunotherapy interventions. This critical point for the choice of immune-based therapies or their combination and patients' clinical outcome is discussed in Chaps. 7, 8, and 9 of this volume.

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Targeting Leukemia Stem Cells and the Immunological Bone Marrow Microenvironment

Cristina Maccalli, Sarah K. Tasian, and Sergio Rutella

Abstract The bone marrow (BM) niche encompasses multiple cells of mesenchymal and hematopoietic origin and represents a unique microenvironment that is poised to maintain hematopoietic stem cell quiescence. In addition to its role as a primary lymphoid organ through the support of lymphoid development, the BM hosts various mature lymphoid cell types, including naïve T cells, memory T cells, and plasma cells, as well as mature myeloid elements such as monocytes and neutrophils, all of which are crucially important to control leukemia initiation and progression.

The BM niche is an attractive milieu for tumor cell colonization because of its ability to provide signals which accelerate tumor cell proliferation and facilitate tumor cell survival. Cancer stem cells (CSCs) share phenotypic and functional features with normal counterparts from the tissue of origin of the tumor and can self-renew, differentiate into multiple cell lineages, and initiate tumor formation. CSCs possess a distinct immunological profile compared with the bulk of the tumor and have evolved complex strategies to suppress immune responses through multiple mechanisms, including the release of soluble factors and the overexpression of molecules implicated in cancer immune evasion. This chapter discusses the latest advancements in our understanding of the immunological BM niche and highlights current and future immunotherapeutic strategies to target leukemia CSCs and overcome therapeutic resistance in the clinic.

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Abbreviations

AML	Acute myeloid leukemia
BM	Bone marrow
CAR	Chimeric antigen receptor
CML	Chronic myeloid leukemia
CSC	Cancer stem cell
HSC	Hematopoietic stem cell
IDO1	Indoleamine 2,3-dioxygenase-1
LSC	Leukemia stem cell

1 Introduction

The bone marrow (BM) niche is a 3D structure situated in close proximity to trabecular bone [62]. The cellular components of the BM niche can be categorized into two functional types: essential cell types like endothelial cells, mesenchymal stromal cells (MSCs), and megakaryocytes, which provide close proximity signals to hematopoietic stem cells (HSCs) that are destined for differentiation and subsequent export into the circulation, and accessory cell types like osteoblasts, specialized tissue-resident macrophages, and nerve cells, which exert long-range and often indirect influences on HSCs [72]. Several other cellular elements with specialized functions, including immune cells, provide distinct chemical signals and physical interactions essential for HSC maintenance and regulation of blood production [72]. The niche also encompasses matrix elements and microvessels which shape the unique biochemical composition of the BM milieu. For instance, quiescent HSCs tend to reside in poorly perfused, relatively hypoxic areas which trigger metabolic adaptations that prevent differentiation [47].

The cancer stem cell (CSC) hypothesis has been initially documented in leukemia in 1994 and stipulates that cancer develops in a hierarchical manner from CSCs that self-renew and give rise to a differentiated cell progeny by asymmetric division [43, 48, 68]. Leukemia stem cells (LSCs) have been phenotypically and functionally characterized both in chronic myeloid leukemia (CML) [27] and in acute myeloid leukemia (AML) [30].

CML is a rare clonal disorder of HSCs, with an annual incidence of 1 to 2 cases per 100,000 individuals [31]. Following a latent period of approximately 7 years, CML presents in chronic phase in 85–90% of individuals and, if untreated, progresses to either myeloid or lymphoid blast crisis in a 5-year time frame. Although

overall survival has improved with the introduction of potent tyrosine kinase inhibitors (TKIs), the persistence of LSCs is a bottleneck to cure in CML [27].

AML is a molecularly heterogeneous malignancy characterized by infiltration of the BM with abnormally differentiated and proliferating cells of the hematopoietic lineage. AML is currently cured in 35–40% of adult patients who are 60 years of age or younger and in 5–15% of patients who are older than 60 years of age [17]. The outcome in older patients who are unfit for intensive chemotherapy remains dismal, with a median survival of only 5–10 months. AML has been shown to follow a CSC model by cell sorting of multiple populations from 16 primary human AML samples and by subsequently identifying in a xenograft assay which fractions contain LSCs [20]. The analysis of gene expression from functionally validated populations yielded an LSC-specific signature as well as an HSC gene signature and identified core transcriptional programs shared by LSCs and HSCs. Both stem cell programs significantly and independently predicted patient survival.

Seminal studies in the late 1990s first described the prevalence of LSCs in primary human AML specimens using limiting-dilution transplantation assays, reporting LSC frequencies varying over a 500-fold range (from 1 in 10,000 to 1 in five million) [11, 61]. The quiescence of both normal stem cells and LSCs is critically determined by interactions with the HSC niche, including endothelial cells, perivascular cells, adipocytes, macrophages, and cells of the adaptive immune system [67]. The majority of AML samples express CD34, and most studies of LSCs have focused on the $CD34^{+}CD38^{-}$ cell compartment, which has been associated with leukemia initiation and relapse [83]. However, transplantation studies have shown that LSCs are also present in at least one other subpopulation, usually the $CD34^{+}CD38^{+}$ fraction or sometimes the $CD34^{-}$ fraction. The analysis of LSC populations with a collection of antibodies associated with primitive cell types, such as CD123, CD33, CD117, CD90, or CD44, did not reveal any clear association between surface expression profile and a lower oxidative state which is indicative of LSC quiescence [41]. Cycling LSC populations have also been detected in AML with an Mixed lineage leukaemia (*MLL*) gene rearrangement and are characterized by CD93 expression [32]. In addition, functionally defined LSCs were detectable in populations from relapsed AML samples that contained all permutations of CD34 and CD38 expression, suggesting that LSCs are dynamic and unstable and can diverge and evolve with acquisition of different phenotypes at relapse [30].

Due to resistance to a variety of therapeutic modalities including radiotherapy, chemotherapy, immunotherapy, and molecularly targeted drugs, such as TKIs, LSCs could underpin treatment failure and leukemia recurrence. Recently, metabolic features and gene signatures consistent with high oxidative phosphorylation and increased mitochondrial mass, but not the persistence or quiescence of LSCs, have been correlated with resistance to cytarabine in a patient-derived xenograft (PDX) model [21].

A primary LSC gene signature has been identified in vivo in a mouse xenotransplantation model [70]. In approximately 50% of patients with AML, LSCs overexpressed either CD32 or CD25 or both antigens. $CD32^{+}$ or $CD25^{+}$ LSCs could

initiate AML development, were cell cycle-quiescent and chemotherapy-resistant in vivo, and also expressed the transcription factor Wilms' tumor 1 (WT1) and the kinase HCK. These molecules could represent valuable targets for LSC-specific therapy, as suggested by the maintenance of long-term multi-lineage hematopoietic reconstitution capacity by normal human hematopoietic stem cells depleted of CD32-/CD25-expressing cells.

A list of genes that are differentially expressed between $CD34^+CD38^-$ LSCs and their $CD34^+CD38^+$ non-LSC counterpart has recently been generated using BM samples from 78 patients with AML [55]. The prognostic accuracy of this 17-gene signature (LSC17 score) was suggested by its correlation with higher percentages of BM blast cells at diagnosis, with a higher incidence of FLT3-ITD mutation and adverse cytogenetic features, and with higher relapse rates and lower response rates to induction chemotherapy. Furthermore, a high LSC17 score was associated with shorter overall survival irrespective of whether or not patients received a subsequent allogeneic stem cell transplantation.

2 The BM Immune Microenvironment

The BM is conventionally viewed as a primary lymphoid organ containing various immune cell populations (Fig. 1). Billions of lymphocytes recirculate through the BM per day. Lymphocytes are distributed through the BM parenchyma and stroma, are condensed in follicle-like structures, and encompass 8–20% of BM mononuclear cells, with a T-cell to B-cell ratio of 5:1. Antibody-producing plasma cells account for 1% of the BM mononuclear population. Plasma cells are found in close proximity to CAR cells and are dependent on CXCL12 signaling through CXCR4 for BM homing [56]. Other mature cell types, such as megakaryocytes and eosinophils, have been shown to contribute to the plasma cell niche (Fig. 1) [52].

Early in lymphoid development, B-cell precursors remain in the BM, while T-cell progenitors migrate to the thymus. CXCL12-abundant reticular (CAR) cells, a subpopulation of MSCs identified in a genetic mouse model [78], are detected in close association with pre-pro-B cells, the earliest B-cell precursors [84]. CAR cells also maintain HSCs in an undifferentiated state, as shown by accelerated myeloid differentiation in response to CAR cell ablation [57]. Clusters of dendritic cells (DCs) co-localize with naïve T cells and B cells in the BM perisinusoidal space, as shown by multiphoton imaging [71]. BM-resident DCs deliver survival signals to recirculating B cells through the production of macrophage migration-inhibitory factor (MIF) and their conditional ablation leads to the specific loss of mature B cells [71]. The factors required for T-cell survival in the BM are less clearly defined. Perisinusoidal DCs can cross-present blood-borne antigens to BM-resident T cells, pointing to a protective role against pathogens [22].

Approximately one-third of BM $CD4^+$ T cells are regulatory T cells (Treg) in humans, including memory or "activated" Treg cells, the trafficking of which is regulated by CXCL12 under homeostatic conditions [93]. Naïve T cells contribute

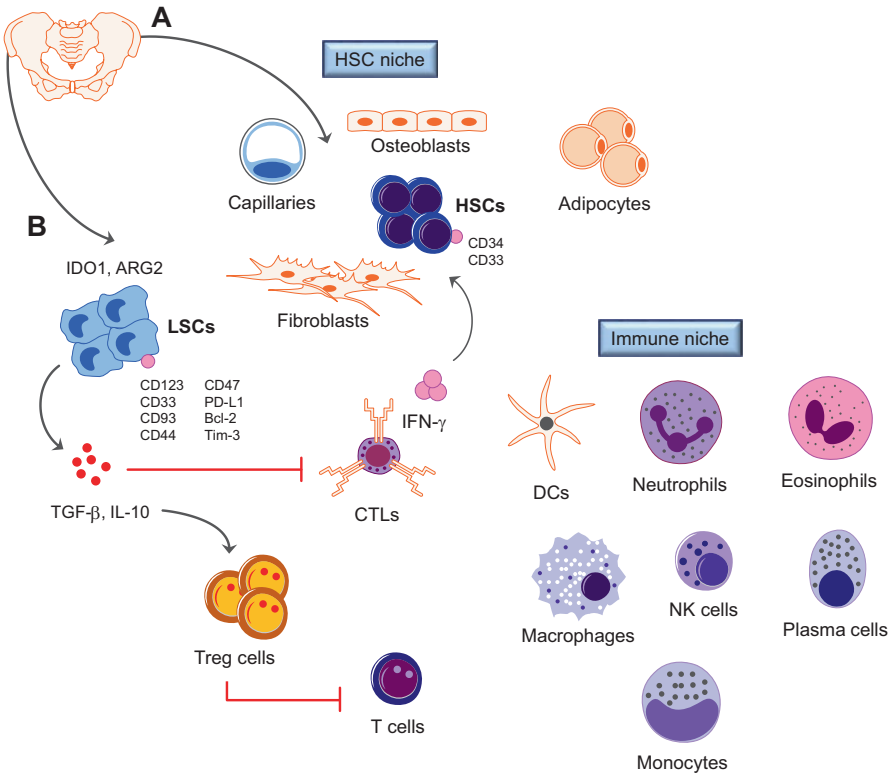

Fig. 1 The immune niche in normal (**a**) and leukemic (**b**) BM. The BM microenvironment hosts a variety of immune cell types, including T cells, B cells, plasma cells, dendritic cells, neutrophils, macrophages, eosinophils, and regulatory T cells. Immune cells support steady-state and emergency hematopoiesis, provide an immune-privileged niche that protects HSCs from immune destruction, and contribute to leukemia control. Candidate leukemia stem cell markers, including CD123 [19], CD44 [33], Bcl-2 [41], and Tim-3 [38], as well as markers of normal hematopoietic stem cells are shown. Microenvironmental soluble factors, such as IFN-γ produced by cytotoxic T cells, might promote leukemia cell proliferation [73]. IDO1 = indoleamine 2,3-dioxygenase-1, ARG2 = arginase-2, LSC = leukemia stem cell, HSC = hematopoietic stem cell, DC = dendritic cell, CTL = cytotoxic T lymphocyte

20% of BM-resident $CD8^+$ T cells, with the largest subsets (~30% each) being $CD45RA^-CCR7^+$ central memory T cells and $CD45RA^-CCR7^-$ effector memory T cells [51]. A smaller fraction is comprised of $CD45RA^+CCR7^-$ effector T cells. Long-lived memory $CD4^+$ T cells are localized in close contact with IL-7-secreting stromal cells [85]. IL-7 is responsible for maintaining T-cell quiescence in the absence of antigen receptor engagement and signaling. Experiments in mice have shown that central memory T cells adhere to BM microvessels more efficiently than effector T cells [51]. This interaction is mediated by PSGL-1 on circulating central memory T cells and selectins on endothelial cells. In addition, the α4 integrin VLA-4 and its vascular ligand VCAM-1 play a major role in central memory T-cell

arrest in BM microvessels [51]. Interestingly, markers indicative of antigen experience, such as CD44 and CD122, can be detected on two-thirds of BM T cells.

Finally, myeloid immune cells such as neutrophils and monocytes reside in specific niches within the BM. Under steady-state conditions, both cell types express CXCR4 and are retained into the BM through CXCL12-induced signaling [18]. During inflammation, neutrophils are released through interaction with CXCL1 and CXCL2, that is, CXCR2 ligands produced by megakaryocytes [39]. In contrast, monocytes are released through interaction of CCR2 with CCL2 produced by CAR cells, MSCs, and endothelial cells [92].

The BM also serves other functions, acting as a secondary lymphoid organ where T-cell and B-cell responses are initiated. Other features of a secondary lymphoid organ include the presence of follicle-like structures and the ability of the BM to control systemic diseases, such as inflammatory, infectious and autoimmune conditions. In mice, the BM contains 1–5% $CD3^+$ T cells and 1–2% $CD11c^+$ DCs in different stages of maturation and harbors DCs that capture, process, and present antigens to naïve $CD4^+$ and $CD8^+$ T cells, as revealed by the formation of large multicellular clusters with DCs, resulting in primary immune responses [22]. After intravenous antigen delivery, the first immune responses are documented in the BM and concomitantly in spleen, consistent with the accessibility of both sites to blood-borne antigens. Specifically, CD69 upregulation was measured 4 hours after challenge with ovalbumin (OVA), whereas the first cell division occurred 26 hours later [22]. T-cell responses initiated in the BM gave rise to long-term immunological memory in mice lacking secondary lymphoid organs.

3 Immunophenotypic and Functional Features of LSCs

Leukemia cells and LSCs express antigens which are immunogenic and can be recognized by immune cells, as well as Major histocompatibility complex (MHC) molecules and costimulatory ligands that allow the interaction with T cells [2].

Immune responses to leukemia have been clinically documented [53]. The in vivo immunogenicity of leukemia-associated antigens (LAAs) has also been confirmed in patients receiving allogeneic hematopoietic stem cell transplantation (HSCT) for AML and CML. Cytotoxic T lymphocyte (CTL) responses have been reported against a broad range of LAAs and CTAs, including HOXA9 [7], proteinase-3 [7], survivin [74], WT1 [8], and preferentially expressed antigen in melanoma (PRAME) [63].

Some leukemia antigens originate from the oncogenic event and are leukemia-specific, such as BCR/ABL1 in chronic myeloid leukemia (CML), PML/RAR-α, FLT3-ITD, and mutated nucleophosmin-1 in acute myeloid leukemia (AML). However, few leukemia-specific chromosomal rearrangements give rise to antigenic proteins, and these include the fusion proteins AML1-ETO, DEK-CAN, and PML/RAR-α, resulting from the t(8;21), t(6;9), and t(15;17) chromosomal translocations, respectively. The majority of antigens have been characterized as LAAs, that is,

molecules being expressed by both normal and leukemic cells. For example, WT1 is not a leukemia-specific molecule, being detected at low levels in various normal tissues, such as gonads, kidney, and the hematopoietic system, but is highly over-expressed by leukemia cells.

Other LAAs belong to the cancer testis antigen (CTA) family, a large group of immunogenic proteins that are normally expressed only in germ cells of the testes and, to a lesser extent, in ovaries and placental trophoblasts. Given the immune-privileged status of the above tissues, CTAs are considered to be de facto tumor-specific antigens and are promising targets for tumor immunotherapy approaches. Preferentially expressed antigen in melanoma (PRAME) has been broadly characterized as an AML-associated CTA, although its expression pattern in normal tissues, such as the adrenal glands, the endometrium, and the pancreas, is broader than that of "classical" CTAs. PRAME-specific T cells may also recognize normal kidney epithelial cells and dendritic cells [1].

Importantly, some LAAs might be downregulated in LSCs compared with more differentiated leukemia cells. The use of Affymetrix Hu133A microarrays with 5 AML samples allowed the identification of 261 DNA repair, signal transduction, and cell cycle genes, the expression of which was significantly lower in AML-derived LSCs compared with $CD34^+CD38^+$ leukemia cells [25]. These findings were consistent with the increasing chromosomal aberrations and mutations that are typical of AML. Interestingly, CD123 (the transmembrane α chain of the IL-3 receptor), a molecule identified in 2000 as an LSC-specific marker in AML [35] and found to be co-expressed with CD33 in 70% of adult AML cases [19], was detected on LSCs but not on mature leukemia cells.

The TNF superfamily ligand-receptor pair CD70/CD27 has been shown to be expressed on AML blasts and AML stem/progenitor cells, but not on HSCs from healthy BM donors [65]. CD70/CD27 signaling activates stem cell gene expression programs and promotes cell proliferation in AML cells, and mediates drug resistance in CML [66]. Soluble CD27, the levels of which might reflect the extent of CD70/CD27 interactions in vivo, was significantly elevated in the sera of newly diagnosed AML patients and was a strong independent negative predictor of overall survival. Antibody blocking of CD70/CD27 interactions induced asymmetric cell divisions and differentiation in AML blasts and AML stem/progenitor cells, inhibited cell growth and colony formation, and significantly prolonged survival in murine AML xenografts. Interestingly, TKIs downregulate micro-RNA miR-29 expression, leading to upregulation of CD70. Combining TKIs with CD27/CD70 blockade can effectively eliminate human $CD34^+$ CML stem/progenitor cells in xenografts and LSCs in a murine CML model, suggesting that CD70/CD27 interactions could be targeted to overcome treatment resistance in CML LSCs [66]. It has to be emphasized that TKI-resistant LSCs are extremely rare in the BM of patients with CML. In addition, LSCs cannot be selectively isolated form the normal HSCs that reconstitute the BM after TKI therapy.

IFN-γ is a major effector cytokine secreted by CTLs. Murine LSCs and human $CD34^+$ CML progenitor cells express receptors for IFN-γ [73]. Although CML LSCs express costimulatory molecules and MHC molecules and induce the

proliferation of effector T cells in vitro, IFN-γ-stimulated, PD-L1/PD-L2-over-expressing LSCs have been shown to accelerate CML progression after serial transplantation in mice [73]. Adoptively transferred CTLs enhanced the expansion of LSCs via IFN-γ only in mice with high leukemia antigen load. When recipient mice were analyzed 18 hours after transfer, an experimental setting where leukemia antigen load is low, neither LSC number nor IFN-γ serum levels were increased, and CTLs could successfully eradicate LSCs. Gene signatures indicative of IFN-γ responsiveness have recently been reported in human AML and CML cell lines [87]. Interestingly, higher expression levels of IFN-γ-related genes, including *STAT1*, *IRF1*, and *IFNGR1*, may correlate with shorter relapse-free and overall survival in children and adults with AML [87, 88]. IFN-γ is also a prototypical inducer of indoleamine 2,3-dioxygenase-1 (IDO1) [54], which catabolizes the essential amino acid tryptophan to immune-suppressive intermediates, collectively referred to as kynurenines, and is over-expressed by a variety of solid tumors and hematological malignancies, including AML [10, 13–15, 24]. Small molecule inhibitors of IDO1, such as indoximod and epacadostat, are being tested in the clinic and have shown to be well tolerated and safe [3, 4, 6, 75, 76]. Studies suggest that IDO1 is selectively elevated in cells with stemness properties also denominated tumor-initiating cells (TICs) from breast cancer, prostate cancer, and mesothelioma cell lines, as well as primary human glioblastoma cells [77]. TICs were serially transplanted, leading to IDO1 overexpression in recipient mice. All types of TICs also expressed higher levels of the tryptophan uptake machinery, including the LAT1 (SLC7A5)/CD98 (SLC3A2) heterodimeric amino acid transporter. It is presently unknown whether LSCs in AML and CML rely on IDO1 expression as an immune evasion strategy and whether patients with hematological malignancies may benefit from IDO1 targeting with small-molecule inhibitors. A phase 1b/randomized phase 2a clinical trial of indoximod (1-methyl-D-tryptophan) in combination with idarubicin and cytarabine (3 + 7) is actively recruiting patients with newly diagnosed AML aged >18 years in the USA, and should be completed in July 2018 (clinicaltrials.gov identifier: NCT02835729).

4 Targeting LSC-Associated Antigens to Overcome Therapeutic Resistance

The identification of "actionable" immunotherapy targets within the LSC compartment would be highly beneficial to implement innovative approaches to clinical translation. Strategies for targeting LSCs fall into two broad categories: therapies that eradicate LSCs (termed "LSC-specific") and therapies that eradicate both the bulk of AML and the LSC compartment (termed "LSC-active") [61]. The first defined LSC-specific immunophenotypic property was expression of the IL-3 receptor α chain (CD123) within the $CD34^{+}CD38^{-}$ compartment [35]. Some of the differentially expressed molecules are being targeted in preclinical models of

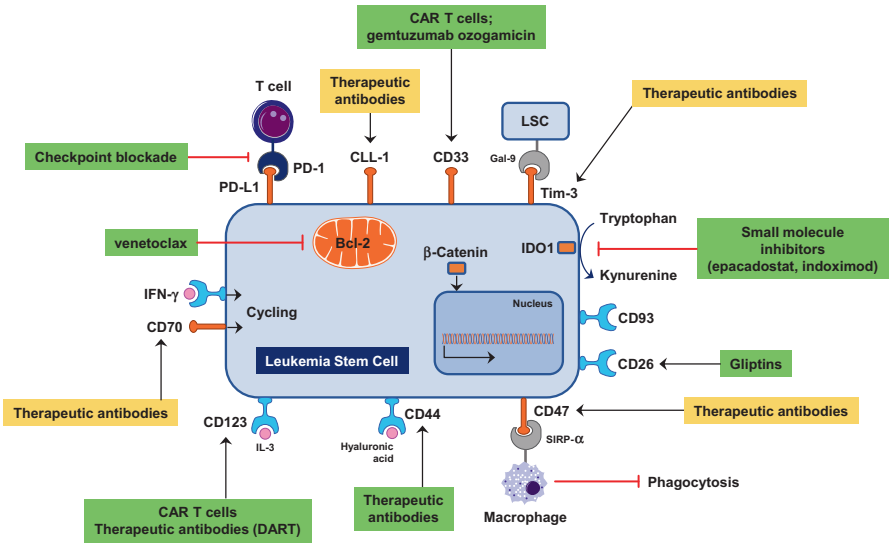

Fig. 2 Actionable targets expressed in leukemia stem cells. Therapeutic strategies currently being evaluated include the use of chimeric antigen receptor (CAR)-modified T cells and antibodies such as bispecific CD3 × CD123 DART® [86]. Green boxes highlight therapeutic strategies that are being investigated in clinical trials, such as CD33 [91] and CD44 targeting [90] and Bcl-2 antagonism [40], whereas yellow boxes denote therapeutic approaches that are being explored in murine models, such as TIM-3 blockade [38]

hematological malignancies and in clinical trials, mostly using antibody-based and cell-based therapeutic approaches (Fig. 2).

CD123 has been targeted with neutralizing monoclonal antibodies in Nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice [34] and in patients with relapsed/refractory AML [29]. 7G3 treatment has been shown to reduce the engraftment potential of AML-derived LSCs and improved mouse survival. 7G3 inhibited IL-3-mediated intracellular signaling of isolated AML $CD34^{+}CD38^{-}$ cells in vitro and reduced their survival.

SL-101 is a novel antibody conjugate comprising an anti-CD123 single-chain Fv fused to *Pseudomonas exotoxin-A* [28]. The antileukemia potency of SL-101 was initially measured using a panel of AML cell lines. Colony-forming assay indicated that SL-101 selectively suppressed the function of leukemic progenitors while sparing normal counterparts. Mechanisms underpinning the cytotoxic activity of SL-101 included rapid and efficient internalization of antibody, sustained inhibition of protein synthesis, induction of apoptosis, and blockade of IL-3-induced phosphorylation of STAT5 and AKT. In a patient-derived xenograft (PDX) model using NOD-scid $IL2rg^{null}$ (NSG) mice, in vitro pre-treatment of LSCs with SL-101 impaired their repopulating capacity.

SGN-CD123A is an antibody-drug conjugate utilizing the pyrrolobenzodiazepine dimer (PBD) linker and a humanized CD123 antibody with engineered

cysteines for site-specific conjugation [46]. Mechanistically, SGN-CD123A induces activation of DNA damage response pathways, cell cycle changes, and apoptosis in AML cells. In vitro, SGN-CD123A mediated potent cytotoxicity of $CD123^+$ AML cell lines and primary AML samples, including those from patients with unfavorable cytogenetic profiles or FLT3 mutations. In vivo, SGN-CD123A eradicated AML in a disseminated disease model, induced remission in a subcutaneous xenograft model, and significantly delayed growth in a multidrug-resistance xenograft model. Moreover, SGN-CD123A also resulted in durable complete remission of a patient-derived xenograft AML model. An ongoing clinical trial with SGN-CD123A will evaluate its safety and efficacy in AML patients (clinicaltrials.gov identifier: NCT02848248).

CD123 could be a viable target for AML-directed chimeric antigen receptor (CAR) therapy. Two CARs containing a CD123-specific single-chain variable fragment, in combination with a CD28 costimulatory domain and CD3-ζ signaling domain, targeting different epitopes on CD123 were recently developed [50]. CD123-CAR-redirected T cells mediated potent effector activity against $CD123^+$ cell lines and primary AML samples, without eliminating granulocyte-macrophage and erythroid colony formation in vitro. Importantly, CD123 CAR T cells exhibited antileukemia activity against a xenogeneic model of disseminated AML. Patient-derived T cells modified to express CD123 CARs could exert cytolytic activity against AML blasts in vitro.

Studies by the Children's Hospital of Philadelphia Group have shown that CD123 expression increases over time in vivo even in initially $CD123^{dim}$ populations and that human T cells transduced with the anti-CD123-41BB-CD3ζ construct (CART123) eradicate primary AML in immunodeficient mice, regardless of baseline CD123 expression [26, 81]. However, a single administration of CART123 also eradicated normal human hematopoiesis, as predicted from the expression of CD123 on normal circulating B cells and myeloid cells and on megakaryocytes. Also, phenotypically defined human stem/progenitor cells were undetectable in CART123-treated animals at 1 month posttreatment, correlating with the known expression of CD123 on progenitor cells.

Severe hematological toxicity of CD123-redirected CAR T cells could be obviated for by CAR T-cell depletion with optimal timing after AML eradication [81]. Three CAR T-cell termination strategies were recently evaluated, including the use of transiently active anti-CD123 mRNA CART (RNA-CART123), T-cell ablation with alemtuzumab after treatment with anti-CD123-41BB-CD3ζ T cells (CART123), and T-cell ablation with rituximab after treatment with CD20-co-expressing CART123 (CART123-CD20) [81]. Rapid and durable leukemia elimination in murine xenograft models of human AML could be consistently detected and required CAR T-cell persistence for 4 weeks prior to ablation. Importantly, subsequent antibody-mediated depletion of CART123 or CART123-CD20 did not impair leukemia remission. These studies will facilitate the clinical implementation of T-cell depletion strategies to augment the feasibility of CAR T-cell therapies for patients with AML.

Bcl-2 has been shown to be over-expressed in quiescent LSCs, which are characterized by a low rate of energy metabolism and a low cellular oxidative status, but not in HSCs [41]. Within minutes of in vitro treatment with Bcl-2 inhibitors, oxidative phosphorylation was severely impaired in primary unfractionated AML cells. Venetoclax, a Bcl-2 inhibitor, has been granted breakthrough therapy designation by the US FDA in 2017 for use in combination with low-dose cytarabine in treatment-naïve elderly patients with AML who are ineligible for intensive chemotherapy. The overall response rate to Venetoclax monotherapy was 19%; an additional 19% of patients demonstrated antileukemia activity not meeting International Working Group (IWG) criteria, that is, partial bone marrow response and incomplete hematologic recovery [40]. When administered in combination with azacitidine, decitabine, or null low-dose cytarabine to patients with multiply relapsed/refractory AML, Myelodysplastic syndrome (MDS), and blastic plasmacytoid DC neoplasm,venetoclax induced objective responses in 21% of cases, with an estimated 6-month survival of 24%. Importantly, responses were identified in patients with diploid/intermediate cytogenetics, RUNX1, and/or IDH1/2 mutations [16].

The myeloid differentiation antigen CD33 is expressed on leukemic blasts from 85% to 90% of AML patients [91]. Gemtuzumab ozogamicin (GO) utilizes an anti-CD33 antibody conjugated to the antitumor antibiotic calicheamicin. GO shows in vitro cytotoxicity against human AML cell lines [23, 44, 58, 69] and is being successfully employed to treat patients with $CD33^+$ AML [36]. GO has been approved in 2017 by the U.S. FDA for use in adults with newly diagnosed CD33-expressing AML and in patients aged 2 years and older with relapsed/refractory $CD33^+$ AML. CD33 expression levels have been associated with clinical responses to GO [60]. Interestingly, CD33 single nucleotide polymorphism rs12459419 C>T in the splice enhancer region eliminates the CD33 IgV domain, which is the antibody-binding site for GO. Results of a recent Children's Oncology Group randomized clinical trial of GO in children with newly diagnosed AML suggest that patients with the CC genotype for rs12459419 have a substantial response to GO, making this a potential biomarker for the selection of patients with a likelihood of significant response to GO [42].

Studies to date have not determined whether GO, besides acting on more mature $CD33^+$ progeny, can indeed directly kill $CD33^+$ LSCs in vivo, and whether long-term benefit from GO is related to successful targeting of LSCs, including AMLs that harbor $CD33^-$ LSCs. It has been proposed that $CD33^-$ LSCs remaining after chemotherapy-induced bulk reduction might enter cell cycle and acquire CD33 and thus become susceptible to CD33 targeting [91].

Flotetuzumab (MGD006/S80880), a novel T-cell redirecting (CD123 × CD3) bispecific DART® protein, has been tested in a phase 1 study in 45 patients with relapsed/refractory AML and MDS [86]. Toxicity was manageable, with drug-related adverse events ≥G3 being observed in 44% of patients. Antileukemia activity was documented in 57% of patients and the overall response rate was 43%. Markers of T-cell activation, such as CD25, CD69, and PD1, were detected in the peripheral blood of patients after treatment.

Patient-derived CML cells and LSCs in mouse models of CML express programmed death ligand-1 (PD-L1), the blockade of which triggers the loss of LSCs and prevents development of CML-like disease, if combined with T-cell immunotherapy [31, 64]. CML LSCs could evade immune surveillance through a variety of molecular mechanisms, including the cytokine-mediated downregulation of MHC class II molecules [79].

C-type lectin-like molecule 1 (CLL-1) is prevalent in AML, both at diagnosis and relapse, and is not expressed on HSCs in normal and regenerating BM samples [89]. The $CD34^{+}CLL\text{-}1^{+}$ population, containing the $CD34^{+}CD38^{-}CLL\text{-}1^{+}$ cells, does engraft in NOD/SCID mice with outgrowth to $CLL\text{-}1^{+}$ blasts. A high CLL-1(+) fraction was associated with quick relapse. Bispecific antibodies that redirect the cytotoxic activity of effector T cells by binding to CD3, the signaling component of the T-cell receptor, and a tumor target such as CD19 on acute lymphoblastic leukemia show encouraging clinical results [5, 9]. The safety and potency of target cell depletion of a CD3 T cell-dependent bispecific full-length human IgG1 therapeutic antibody targeting CLL-1 have been recently reported [45]. CLL-1 CAR T cells have also been engineered to express inducible capspase-9, a safety switch that could accelerate the clinical development of this immunotherapy strategy by allowing control of unwanted T-cell reactivity against normal myeloid cells [80].

CD47 is a broadly expressed transmembrane protein that serves as the ligand for signal regulatory protein α (SIRPα), which is expressed on phagocytic cells including macrophages and DCs. When activated, SIRPα initiates a signal transduction cascade resulting in inhibition of phagocytosis. CD47 is preferentially expressed on AML-derived LSCs cells compared to their normal counterpart and inhibits their phagocytosis through the interaction with an inhibitory receptor on phagocytes [49]. CD47 expression levels were lower in AML patients with t(8;21) compared with patients with unfavorable cytogenetic features such as FLT3-ITD. Moreover, CD47 expression predicted worse overall survival in three independent cohorts of adult AML patients dichotomously stratified into $CD47^{low}$ and $CD47^{high}$ expression groups. Treatment of mice engrafted with human LSCs with therapeutic anti-CD47 antibodies resulted into AML depletion and targeting of LSCs.

Studies that used SIRPα-Fc fusion protein to disrupt SIRPα-CD47 engagement have suggested that macrophage-mediated phagocytosis and clearance of AML stem cells depend on absent SIRPα signaling [82]. Importantly, SIRPα-Fc treatment did not significantly enhance phagocytosis of normal hematopoietic targets by activated human macrophages.

CD44 is a type I transmembrane protein and functions as the major cellular adhesion molecule for hyaluronic acid, a component of the extracellular matrix. CD44 is expressed in most human cell types and has been implicated in myeloid leukemia pathogenesis. A naturally occurring leukemogenic splice variant of t(8;21), AML1-ETO9a, significantly increases the expression of CD44 at both RNA and protein levels [59]. Furthermore, the CD44 promoter is bound by AML1-ETO9a and AML1-ETO at the chromatin level, indicating that CD44 expression links the 8;21 translocation to the regulation of a cell adhesion molecule that controls AML growth.

Ligation of CD44 with activating antibodies (H90) eradicates AML LSCs in NOD/SCID mice by blocking LSC trafficking to supportive microenvironments and by altering their stem cell fate [33]. In vitro H90 treatment led to multiple changes indicative of terminal differentiation, such as increased expression of lineage antigens, ability to reduce nitroblue tetrazolium, and acquisition of mature morphology. The number of $CD34^{+}CD38^{-}$ cells within the AML graft in both BM and peripheral blood was considerably reduced in H90-treated mice as compared with control mice [33].

RG7356, a recombinant antiCD44 IgG1 humanized monoclonal antibody, has been administered to 44 patients with refractory/relapsed AML or patients not eligible for intensive chemotherapy in a phase I dose-escalation study [90]. Two patients achieved complete response with incomplete platelet recovery or partial response, respectively. One patient had stable disease with hematologic improvement. Overall, RG7356 was safe and well tolerated, with one dose-limiting toxicity (grade 3 hemolysis exacerbation) occurring after one 1200 mg dose. Whereas the majority of adverse events were mild or moderate, infusion-related reactions occurred in approximately 60% of AML patients, mainly during cycle 1. Two patients experienced grade 3 drug-induced aseptic meningitis. Based on the results of this study, the recommended dose for future AML evaluations will be 2400 mg every other week.

Other approaches to target CD44-expressing LSCs include the manufacturing of CAR T cells redirected against CD44 isoform variant 6 (CD44v6) and containing a CD28 signaling domain [12]. CD44v6 CAR T cells required in vitro activation with cytokines such as IL-7 and IL-15 for antitumor efficacy in vivo and spared normal HSCs and CD44v6-expressing normal keratinocytes when administered to AML-bearing mice. The co-expression of a suicide gene allowed fast and efficient ablation of CD44v6 CAR T cells and rescued mice from acute graft-versus-host disease.

TIM-3 is a type 1 cell-surface glycoprotein originally identified in murine $CD4^{+}$ Th1 cells. In humans, TIM-3 is expressed also in a fraction of T cells, NK cells, monocytes, and DCs. TIM-3 is broadly expressed in human AML, with the only exception of acute promyelocytic leukemia and is not detected in normal HSCs [38]. $TIM\text{-}3^{+}$ but not $TIM\text{-}3^{-}$ AML cells were shown to reconstitute human AML in immunodeficient mice, suggesting that the $TIM\text{-}3^{+}$ leukemic population contains most functional LSCs. Moreover, antihuman TIM-3 mouse IgG2a antibodies with complement-dependent and antibody-dependent cellular cytotoxic activities inhibited the engraftment of AML after xenotransplantation, and, when administered directly to mice grafted with human AML, they eliminated LSCs capable of reconstituting human AML in secondary recipients [38].

Galectin-9 (Gal-9), the ligand for TIM-3, is elevated in AML patients and in mice receiving human AML xenografts. Ligation of TIM-3 by Gal-9 activates both NF-κB and β-catenin pathways, stimulating self-renewal of LSCs [37]. These studies thus suggest that the TIM-3/Gla-9 autocrine loop could potentially be targeted to treat myeloid leukemia [37, 38].

5 Conclusions

LSCs rely on a number of signaling pathways that are associated with the ability to self-renew and that are shared with normal HSCs, such as WNT/β-catenin and Hedgehog. The original conceptual framework that AML development recapitulates the normal hematopoietic hierarchy might represent an oversimplification of the complex biology of AML. LSCs may in fact reside in more than one population, and their functional heterogeneity and remarkable plasticity are increasingly being recognized.

Inflammatory cytokines such as IFN-γ and signaling via CD27 might induce the expansion of LSCs. A deeper understanding of the immune BM niche will further support and inform the development of immunotherapies targeting LSCs. Monoclonal antibodies and T-cell-based approaches targeting candidate LSC-specific molecules are being developed in the clinic with encouraging results. It is conceivable that LSC-directed therapies will have to be offered in combination with conventional treatments, either before or concurrent with chemotherapy, in order to avoid chemotherapy-induced evolution and increased complexities of the LSC population [30, 61]. Recent studies have shown a 10- to 100-fold increase in LSC frequency at relapse using paired specimens from AML patients at diagnosis and following relapse after conventional chemotherapy [30]. New endpoints to evaluate response are likely to be required to assess LSC-directed therapies, that is, patient survival rather than response rates, which might not be increased by therapies that target a tiny proportion of the bulk disease [61].

Although identifying and pursuing antigenic targets to eradicate LSCs is an active area of research, the efficacy of this approach is still unknown and may be limited by the relative plasticity of LSC phenotypes.

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Crosstalk Between Prostate Cancer Stem Cells and Immune Cells: Implications for Tumor Progression and Resistance to Immunotherapy

Matteo Bellone and Sara Caputo

Abstract Prostate cancer is a prototypical example of cancer as a disease of stem cells. Indeed, both normal and malignant prostate epithelia contain an androgen-independent, self-renewing stem cell population that survives in androgen deprivation conditions and can regenerate the complexity and heterogeneity of the tissue when androgens are either replaced, as for normal murine prostate in androgen-cycling experiments, or not replaced as it occurs in castrated prostate cancer patients. Thus, identification of prostate cancer stem-like cells (PCSC) and comprehension of the mechanisms regulating their function and interactions with the tumor microenvironment are of paramount importance in designing effective therapeutic strategies for prostate cancer patients. Here, we will focus on known and potential interactions between PCSCs and the immune system that may either block or favor cancer progression, depending on PCSC-intrinsic and PCSC-extrinsic mechanisms. We will also underline the clinical and biological needs to be addressed in the near future to increase efficacy of prostate cancer immunotherapies.

Keywords Prostate cancer · Metastasis · Circulating tumor cells · Cancer stem cells · Cancer-propagating cells · Immune cells · Immunotherapy · Hormone · Immunosuppression · Tenascin-C

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Abbreviations

AR	Androgen receptor
CAR	Chimeric antigen receptor
CK	Cytokeratin
CSC	Cancer stem cell
CTC	Circulating tumor cell
CTLA-4	Cytotoxic T-lymphocyte antigen 4
DC	Dendritic cell
DHT	Dihydrotestosterone
DTC	Disseminated tumor cell
EMT	Epithelial-mesenchymal transition
ESC	Embryonic stem cell
GITR	Glucocorticoid-induced tumor necrosis factor receptor family-related gene
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HGPIN	High-grade prostatic intraepithelial neoplasia
HLA	Human leukocyte antigen
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-γ
IL-10	Interleukin-10
IL-6	Interleukin-6
iNKT	Invariant natural killer T cells
iNOS	Immunosuppressive NO synthase-2
LN	Lymph node
M-CSF	Macrophage colony-stimulating factor
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
MØ	Macrophage
mPIN	Mouse prostatic intraepithelial neoplasia
MSC	Mesenchymal stem cells
NE	Neuroendocrine
NO	Nitric oxide
PAP	Prostate acid phosphatase
PCSC	Prostate cancer stem-like cell
PROM1	Prominin-1
PSA	Prostate-specific antigen
PSCA	Prostate stem cell antigen
PSMA	Prostate-specific membrane antigen
STAT	Transducers and activators of transcription
STEAP	Six-transmembrane epithelial antigen of the prostate
Syn	Synaptophysin
TAM	Tumor-associated macrophage
TCR	T cell receptor

TGF-β	Transforming growth factor β
Th17	T helper 17
TNC	Tenascin-C
TNE-SCs	PCSCs from mPIN lesions
TNF	Tumor necrosis factor
TPIN-SCs	PCSCs from NE tumors
TRAMP	Transgenic adenocarcinoma of the mouse prostate
Treg	Regulatory T cell

1 Introduction

Prostate cancer is among the most frequently diagnosed cancers [1], and the mean prevalence of the disease in men in their 80s reaches 60% [2]. Additionally, a relevant percentage of patients undergoing radical prostatectomy for prostate cancer will experience disease recurrence [3], likely because of early spread of metastatic cells [4]. Once prostate cancer has become castration-resistant, it is substantially incurable. Indeed, prostate cancer is among the leading causes of death by cancer [1]. Thus, prostate cancer is a frequently occurring disease, which leads to aggressive, expensive, and often disabling treatments and overtreatments, and may eventually become incurable.

The biology of prostate cancer has only partially been deciphered, and this lack of information limits our therapeutic approaches. Prostate cancer is often characterized by a slow rate of growth, and by an overall long natural history when compared to other solid tumors, with a wide spectrum of biological behaviors, ranging from indolent to highly malignant stages [5]. Acinar-type adenocarcinoma comprises more than 90% of prostate malignant lesions and usually originates from the peripheral zone of the gland [6]. Prostate adenocarcinoma may be associated with high-grade prostatic intraepithelial neoplasia (HGPIN), which is currently its only recognized premalignant precursor [6]. Interestingly, PIN lesions are often preceded by proliferative inflammatory atrophy, which is characterized by atrophic lesions with either acute or chronic inflammatory infiltrates and proliferating epithelial cells. Proliferative inflammatory atrophy is rather common in aging prostate, and infectious agents, urine reflux, dietary regimen, and estrogens are all factors contributing to its establishment [7]. Thus, a connection appears to exist between chronic inflammation and prostate cancer development.

While prostate adenocarcinoma frequently invades the seminal vesicles and the bladder neck [8], its metastatic spread occurs by exploiting both the lymphatic and the circulatory system and along the nerve fibers as perineural invasion [9]. Metastases can be retrieved primarily in the pelvic lymph nodes (LN), bone, and marrow and less frequently in the lungs and liver [8]. Various other subtypes of prostate cancer may occur in association with acinar-type adenocarcinoma or in their pure forms and include small cell neuroendocrine (NE), adenoid cystic and

basal cell, squamous cell, and urothelial and sarcomatoid carcinomas [6]. Small cell NE carcinoma is a distinct clinical-pathological entity that accounts for 0,5–5% of malignant prostate cancer. NE differentiation is one of the unique features of prostate cancer, often occurs after androgen deprivation therapy [10, 11], and correlates with poor prognosis and resistance to current therapies, such as androgen ablation, radiotherapy, and chemotherapy. Sarcomatoid carcinoma of the prostate is rare, and a history of prostate cancer treated by radiation and/or hormone therapy is present in over one half of the cases. The latter is also associated with poor prognosis [6].

The characteristic intra-patient and inter-patient heterogeneity and the initial dependence on androgens make prostate cancer a prototypical example of cancer as a disease of stem cells. The idea of stem cell involvement in the biology of normal and neoplastic prostate dates back to the 1980s, when Isaacs and colleagues [12, 13] elegantly showed that hormone deprivation in rodents causes prostate atrophies, as differentiated epithelial cells depend on androgens for viability. However, the gland regenerates when androgens are replaced, thereby making a strong case for the existence of a stem cell population with capacity of self-renewal and differentiation. Similarly, prostate cancer is highly sensitive to castration and almost invariably recurs into a castration-independent variant, thus suggesting that stem cells are also present in prostate cancer. Indeed, a strong support to the existence of prostate cancer stem-like cells (PCSC) comes from experimental evidences both in humans [14–38] and mouse models of prostate cancer [31, 39–54]. We refer the interested reader to excellent reviews on PCSCs [55–60].

The prostate epithelium is composed of three primary cell types, namely the most abundant luminal secretory cells, the underlying basal cells, and the rare NE cells. PCSCs might reside within any of the three subpopulations. High and variable sensitivity of the three prostate epithelial cell populations to tissue dissociation and in vitro survival has limited the research field for decades. Recent advances in 3D organoid cultures [30, 31, 52] have rapidly expanded our knowledge on lineage relationships in the prostate epithelium and have also underlined the relevant crosstalk between cancer cells and their surrounding stroma.

PCSCs and more differentiated prostate cancer cells strictly depend on specialized supportive microenvironments [61], which are composed of collagen fibers, matrix proteins, and normal cells, among which endothelial cells, fibroblasts, and immune cells are the major components. Continuous crosstalk among these cell populations supports cancer cell survival, resistance to therapies, and eventual selection of invasive clones. The immune system should also protect the host from cancer development and progression. Thus, cancer cells must develop strategies to dodge cancer immune surveillance. Similar mechanisms apply to cancer stem-like cells (CSCs) in general and to PCSCs in particular.

Herein, we summarize current knowledge on the known interactions between PCSCs and the immune system, and we underline the clinical and biological needs to be addressed in the near future to increase therapeutic efficacy of immunotherapy in prostate cancer.

2 CSCs: An Operational Definition

Prostate cancer is a multifocal disease, and each focus may harbor diverse genetic alterations [62]. To explain the complex process of tumor development, two fundamental models have been proposed. The clonal progression model posits that any of the cancer cells within a tumor is potentially capable of promoting tumor growth. The tumorigenic capability depends on genetic and epigenetic hints that induce self-renewal ability in a cell that then gives rise to tumor clones able to propagate the tumor. This process is stochastic. Conversely, the hierarchical evolution model predicts that only a small subpopulation of cancer cells among the tumor bulk is endowed with tumorigenic potential, thus suggesting a hierarchical organization of the tumor, as it occurs in normal tissues. According to the hierarchical evolution model, CSCs while proliferating give rise to heterogeneous and more differentiated prostate cancer cells that progressively lose tumorigenic potential. Thus, only CSCs can drive tumor growth and metastasis. Theoretically, the clonal progression and the hierarchical evolution models need not be mutually exclusive. Indeed, it has been shown that some tumors can originate from differentiated cells that reacquire properties of adult stem cells [63].

For the sake of clarity, it is important to define what we mean by CSCs. According to several experts in the field (e.g., [58, 64–67]), the CSC is the cellular subset that uniquely sustains growth of a malignant tumor. Thus, CSCs or cancer-propagating cells do not necessarily correspond to the cell of origin or the tumor-initiating cell. The latter is the cell within a tissue that receives the first genetic hit promoting an oncogenic mutation. Because adult tissue stem cells possess self-renewal (i.e., the capacity to undergo undefined cycle of mitotic divisions into either an identical or a more differentiated cell) and pluripotency properties (i.e., the capacity to differentiate into a more specialized cell type), and are thought to survive for long in a rather quiescent condition, usually at the basal layer of the tissue, these cells are more likely hit by mutations and, thus, may more likely become the cell of origin of a malignancy. As these cells are considered the subset of cells within a tumor with the capacity to self-renew and give rise to the heterogeneity of the tumor, they have been termed CSCs. However, it is well known that several genetic alterations are required within a cell to drive malignancy [68], and it is more likely that additional mutations will accumulate in rapidly proliferating and more specialized progenitor or transit-amplifying cells along the differentiation pathway of that particular tissue than in tissue stem cells. If these transformed cells keep or reacquire self-renewal and multipotency properties (e.g., [63]), they can indeed be defined as cancer-propagating cells or CSCs. Thus, the cell of origin may be distinct from the CSC.

Based on these considerations, we prefer to call cancer-propagating cells as stem-like cells and make ours the operational definition posited by Clarke et al. [64]: “CSCs can only be defined experimentally by their ability to recapitulate the generation of a continuously growing tumor.” Thus, CSCs are those cells within a tumor that possess the capacity to self-renew and cause the heterogeneous lineages of cancer cells that comprise the tumor [64]. Additionally, because of the predominant

quiescent state, CSCs are insensitive to conventional cancer therapies that mostly target the rapidly proliferating tumor bulk and therefore represent a source of tumor relapse [69]. Clearly, failure to eliminate CSCs together with more differentiated cancer cells sets the stage for cancer recurrence after any traditional or experimental therapy.

3 Role of CSCs in the Evolution of Prostate Cancer

Several technical strategies have been implemented to prospectively isolate and characterize CSCs. Among these, serial orthotopic transplantation of selected cellular populations in animal models is the most convincing means to demonstrate the existence and the identity of CSCs [70]. Indeed, injected CSCs should re-establish the characteristic phenotypic heterogeneity of the primary tumor in the host. Additionally, CSCs should be isolated from the developed tumor and possess self-renewing capability on serial in vivo passaging. The application of this technique to prostate cancer is rather cumbersome. First, there is a relevant issue related to the amount of biological material available for research investigation. Prostate cancer is often multifocal, and pathologists ask for most of the removed prostate for disease scoring. Thus, the material lent to the lab is often represented by minute amounts of tissue from biopsies or a large tumor burden, which also adds a substantial bias, as large prostate cancer lesions in the post-prostate-specific antigen (PSA) era are rather unusual. Because prostate cancer metastases are not surgically removed in most of the cases, fresh biological material from metastases is also rarely available. Additionally, the prostate cancer epithelium is composed of three cell subtypes with different sensitivities to tissue dissociation and low ex vivo rate survival even for a few hours. Also implantation of prostate cancer cells into the prostate is a complex surgical procedure when compared with subcutaneous injection and allows infusion of only small volumes. Finally, even in syngeneic models, implantation of cells devoid of their original stroma may substantially reduce the likelihood of tumor growth and/or induce growth of a tumor that does not recapitulate the morphologic characteristics of the primary lesion.

Notwithstanding that, several groups have succeeded in isolating bona fide PCSCs (reviewed in [55, 60]). Maitland and collaborators have pioneered the work wherein they identified a population of putative PCSCs from human specimens, which were defined as $CD44^{+}/\alpha2\beta1^{hi}/CD133^{+}$ [14]. These cells were sorted by magnetic beads, based on data obtained from normal prostates [71, 72], and showed high in vitro colony-forming and Matrigel invasion capabilities. However, their in vivo tumorigenic potential was not assessed [14].

Guzmàn-Ramìrez and coauthors applied the neurosphere assay [73] to surgical specimens [19] and identified PCSCs in a population not only phenotypically similar to the one identified by the Maitland's group [14] but also resembling the basal compartment of the prostate. These authors did not investigate the in vivo tumorigenicity of the identified PCSCs.

Others chose to combine the neurosphere and the xenotransplant techniques to identify PCSCs from surgical specimens [24]. These PCSCs did not express AR and PSA, expressed the human pluripotent stem cell marker TRA-1-60 together with CD151 and CD166, and were reminiscent of basal cells. Similar triple-positive PCSCs were directly isolated from surgical specimens and recapitulated the original tumor in serial transplantation experiments [24].

Several other groups within the last decade have isolated putative PCSCs (reviewed in [55–60]). As a result, a plethora of PCSC subpopulations have been identified, which are characterized by quite different cell surface markers (Table 1).

More limited is the information regarding PCSCs isolated from genetically modified mouse models of prostate cancer. One of the first reports came from Smith and collaborators [39], who investigated the existence of PCSCs in transgenic adenocarcinoma of the mouse prostate (TRAMP) mice, which developed autochthonous prostate lesions evolving from mouse prostate intraepithelial neoplasia (mPIN) to NE differentiation [74]. They identified a population of AR^- and breast cancer-resistant protein-positive cells capable of regenerating the prostate cancer with NE features in castrated mice. In human samples, these rare epithelial cells were found in the basal cell compartment of the transformed epithelium and expressed neither p63 nor high molecular weight cytokeratins. Interestingly, these cells survived androgen deprivation and proliferated under hypoxic conditions [39].

Table 1 Known cell surface markers for human PCSCs

Origin	Phenotype	In vivo tumorigenicity	References
Prostatic tissue	$CD44^+/a2b1^{hi}/CD133^+$	Not assessed	Collins 2005 [14]
Xenograft from cell lines	$CD44^+/a2b1^{+/hi}$	Increased but not unique tumorigenicity	Patrawala 2007 [15]
Cell lines	$CD44^+/CD29^-/CD133^+$	Increased but not unique tumorigenicity	Hurt 2008 [18]
Cell lines	$CD133^+$	Not assessed	Vander Griend 2008 [17]
AKT/ERG-transformed primary basal cells	$CD49f(a6)^{hi}/Trop^{hi}/p63^+$	Unique in NSG	Goldstein 2010 [20]
Cell lines	$E\text{-cadherin}^+$ $CD44^+/a2b1^{+/hi}$	Unique in SCID	Bae 2010 [22]
Cell lines	$ALDH^{hi}$ $CD44^+/a2b1^{hi}/CD133^-$	Increased but not unique tumorigenicity	van den Hoogen 2010 [23]
Xenograft from cell lines	$TRA\text{-}1\text{-}60^+$ $/CD151^+/CD166^+$	Increased but not unique tumorigenicity	Rajasekhar 2011 [24]
Benign cell line and CAF	$a2b1^{hi}/CD133^-$	Increased but not unique tumorigenicity	Taylor 2012 [26]
Xenograft from cell line in castrated mice	$NKX3\text{-}1^+/CK18^+/AR^+/$ $Syn^+/ALDH^+/CD44^+$	Reinitiated by androgen replacement	Germann 2012 [28]
Cell lines	$ALDH^{hi}/CD44^+/\alpha2\beta1^+$	Assessed in castrated and non-castrated mice	Chen 2016 [38]

Owing to the high frequency of NE tumors, the TRAMP model is particularly amenable to investigate the origin of NE prostate cancer. Indeed, whether acquisition of a NE phenotype in prostate cancer is either due to trans-differentiation of exocrine epithelial lesions or to selection of already existing NE cells is yet to be defined. In vitro data, mostly obtained with human androgen-sensitive LNCaP prostate cancer cells, support a process of transdifferentiation to the NE phenotype [75]. Additionally, genetic analyses of prostate cancer samples found that NE prostate cancer cells shared almost identical genetic markers with exocrine prostate cancer cells from the same patient [76], and basal epithelial cells were enriched in genes normally associated with neurogenesis [37]. Indeed, expression of N-Myc and activated AKT1 was sufficient to transform human prostate epithelial cells into a common progenitor of adenocarcinoma and NE cancer [77]. Also, Beltran and colleagues identified N-MYC as a driver of NE prostate cancer [78] and found common genetic alterations in epithelial and NE lesions from the same patients, thus suggesting a divergent evolution of NE prostate cancer from epithelial adenocarcinoma [79]. These data, however, can rule out neither metastasis-to-metastasis seeding as a mechanism of tumor progression [80] nor clonal selection operated by androgen deprivation therapy. Additionally, these studies [37, 76, 77, 79] did not identify PCSCs generating in vivo both epithelial and neuroendocrine prostate cancer.

In vitro data obtained in TRAMP mice also lend weight to the hypothesis that NE tumors arise from precursors that initially maintain a transitional epithelial/NE phenotype [81]. However, the same authors showed that in subcapsular renal grafting of microdissected prostate ducts, epithelial and NE cells most invariably gave rise to tumors of their lineages [81], suggesting a distinct lineage of origin for NE carcinomas.

Organoid in vitro cultures and genetic lineage-tracing experiments have been of some help in identifying PCSCs [82]. Based on these experiments, it has been concluded that while post-natal prostate development involves basal multipotent stem cells differentiating into basal, luminal, and NE cells, basal and luminal unipotent progenitor cells mediate prostate regeneration in adult mice [31, 83]. In prostate cancer, more often luminal but also basal CSCs contribute to cancer development and progression [31, 84]. However, the identified multipotent progenitor cells in the adult human and mouse prostate epithelium did not give rise to NE cells [31], thus supporting the existence of specialized CSCs for exocrine and NE prostate cancer, respectively. The authors also underlined that the culture conditions used might have not been permissive to NE differentiation [31].

Utilizing the sphere assay, we established triplicates of long-term PCSC lines from unsorted prostate cells obtained from different stages of TRAMP progression [51]. Notwithstanding the caveats and limitations of the TRAMP model, in which oncogenic transformation and cell immortalization are initiated by the SV40 early genes (small and large T antigens; Tag) mostly targeting *p53* and *Rb*, transcriptome analysis showed that genes upregulated in each stage-specific PCSC line were significantly associated with distinct clinical subgroups of prostate cancer patients, thus indicating that mouse PCSCs define to some extent human prostate cancer progression signatures. Indeed, the first genetic hits in TRAMP mice are followed

by additional genetic and epigenetic alterations, among which the loss of expression of Nkx3.1 already at the stage of preneoplastic lesions and *AR* mutations in more advanced disease, as it may occur in humans. In addition, gene signatures of PCSCs from NE tumors (TNE-SCs), which also in humans were characterized by loss of *p53* and *Rb* [85], were the most malignant, and those from mPIN lesions (TPIN-SCs) were the less malignant [51]. TNE-SCs expressed stem cell markers, such as Prominin (PROM), CD49f, Sca-1, p63, and CD117, the NE marker Synaptophysin (Syn), and also cytokeratins, therefore suggesting that these CSCs are pluripotent. Indeed, in the presence of dihydrotestosterone (DHT), TNE-SCs downregulated the expression of stemness-related genes, like CD49f, and upregulated basal cell markers, such as p63 and CK14, as well as Syn. While we cannot exclude that TNE-SCs originated from TPIN-SCs, thus adhering to the common precursor hypothesis, the relevant genetic diversities between the two PCSC populations [51] make this possibility unlikely. Additionally, in transplantation experiments, TNE-SCs or TPIN-SCs gave rise to tumors of their lineages [51], thus suggesting that in vivo TNE-SCs are less susceptible to hormones and that NE tumors originate from a distinct cell lineage.

Moreover, in the Pten-null mouse model, CSCs were enriched by the Sca-1 and CD49f surface markers [42, 45]. Using the aggressive Pten/Tp53-null model, Kelly and collaborators obtained organoids from both basal and luminal cells and showed that luminal cells generated either multilineage or luminal-only organoids. While basal cells did not generate tumors in vivo, the organoids from PROM1$^+$ luminal cells contained at least two populations of luminal PCSCs, multipotent progenitors and a major population of committed progenitors [54], suggesting that PCSCs can be found in populations at different stages of differentiation, which however maintain self-renewal capacity.

Altogether, these findings strongly support the existence of CSCs as drivers of prostate cancer development and progression. As experimental evidence exists in favor of both basal and luminal PCSCs, and consensus has been reached neither on definitive markers of PCSCs nor on the CSC that give rise to NE prostate cancer, these data are also in support of the existence of multiple PCSCs at different stages of differentiation yet retaining stem-self renewal and pluripotent properties.

4 Role of CSCs in the Development of Prostate Cancer Metastases

Metastatic disease is a fearful complication of prostate cancer and the main cause of cancer-related death. Most patients develop bone with LN metastases, approximately 20% have visceral metastases, and only 6% develop lymph node-only disease recurrence [86]. The site of metastasis has a relevant impact on patient survival, and liver and lung metastases are associated with dismal prognosis [86]. Thus, a better understanding of the metastatic process in prostate cancer is essential for treatment decisions and the design of more effective therapies.

Two general models have been proposed to explain the process of systemic cancer progression [87]. The linear progression paradigm establishes that tumor ontogeny fully occurs in the primary tumor and identifies the metastatic spread as a late event following the establishment of a large tumor mass. Thus, metastases are genetically similar to the primary tumor, and chances of metastatic disease increase with time and aggressiveness, which in prostate cancer is defined by the Gleason grading system [88, 89]. Conversely, the parallel progression model predicts that tumor cells leave the primary lesion before the acquisition of full malignant phenotype and deploy to secondary growths where disseminated tumor cells (DTC) acquire additional mutagenic hits and morphological abnormalities. As a consequence, greater genetic and epigenetic disparities should be found between primary tumor cells and metastasis founders. Indeed, in several solid tumors, DTCs exhibit significantly fewer genetic abnormalities than primary tumor cells, and heterogeneous chromosomal rearrangements can be found in primary tumors and DTCs from different sites [87]. Whether a tumor disseminates through a parallel or progression model clearly has relevant clinical implications in prostate cancer. Indeed, if metastatic dissemination occurs early, then radical prostatectomy will not significantly reduce the risk of metastatic disease. Hence, an unmet clinical need is the identification of biological markers of early metastatic spreading.

In most recent years, the metastatic disease in prostate cancer has been the focus of numerous genomic investigations through either one or a combination of whole-exome sequencing, array comparative genomic hybridization, and RNA transcript profiling [90]. Kumar and colleagues [91] analyzed multiple tumors from men with disseminated prostate cancer and found substantial inter-individual and limited intra-individual genomic diversity. These findings suggest that molecular characterization of a single metastasis provides a reasonable assessment of the mutational burden present in disseminate tumors within an individual and that in most prostate cancers, metastatic dissemination can be attributed to a limited number of clones possessing the major oncogenic driver alterations. It should also be considered that the genetic analyses are made more difficult by the phenomenon of metastasis-to-metastasis seeding that is rather common in prostate cancer and occurs either through the transfer of different tumor clones between metastatic beds or through de novo monoclonal seeding of daughter metastases [80].

A comparative molecular analysis between single biopsies from primary prostate cancers [92] and an unrelated cohort of bone and soft tissue metastases from castration-resistant prostate cancer [93] has found a significantly higher mutational burden in the metastases than the primary lesions, with AR signaling more frequently altered in the former samples. Although the substantial genetic divergence between metastatic and primary tumor might be due to the limitation of the techniques in detecting minor subclones in the primary tumor, these data may suggest a late separation of the metastatic clone from the primary lesion. In another comparative analysis on longitudinally collected primary and metastatic prostate tumors, the authors showed that while in one case the metastatic subclone was detected in the primary tumor and showed additional exclusive genetic alterations, in another patient, the metastasis and the primary tumor were largely genetically unrelated,

strongly supporting the parallel progression model of metastasis [94]. Hong and colleagues also found cross-metastatic seeding and a case in which clones from a distant metastasis had reseeded the surgical bed of radical prostatectomy, the latter suggesting that the surgical bed provided a niche for metastatic clones [94]. Others have investigated copy-number alterations of 2 LN metastases and 34 morphologically distinct prostate areas from the same individual and found that the metastatic clone most likely originated from areas of intraductal carcinoma of the prostate [95], which should not be misinterpreted with the less aggressive HGPIN [96]. Based on the finding that one area of carcinoma with perineural invasion was distant from but genetically highly related to the intraductal carcinoma, the authors hypothesized that the metastatic clones acquired the capability of migrating through the ducts and along the nerves to colonize both other parts of the prostate and the LNs. Thus, the behavior of prostate cancer metastatic clones is rather heterogeneous and takes advantage of several anatomical and morphological characteristics of the primary lesion.

Moreover, Haffner and colleagues tracked the clonal origin of metastatic prostate cancer in one patient and showed that the lethal clone arose from a small, relatively low-grade cancer focus in the primary tumor [97]. Similarly, it has been reported that in one patient multiple late metastases (i.e., 17 years after radical prostatectomy) appeared to be originated not from the bulk of the tumor but from a 2-mm low-grade region of it [95]. Thus, only selected and not necessarily aggressive foci within the primary tumor might harbor genetic and microenvironmental features allowing cancer cells to migrate to pelvic LN and eventually to other organs.

A process of precocious systemic prostate cancer progression might also be inferred from epidemiological analyses. Welch and colleagues recently compared cancer dynamics in breast and prostate cancers, two conditions for which screening has been particularly prominent [98]. They noticed that while the incidence of metastatic breast cancer has been stable since 1975, the incidence of metastatic prostate cancer dropped by half in the last 30 years. Possible and not mutually exclusive explanations for the stable incidence of metastatic breast cancer are that mammography has been unable to identify it at an early stage, and that breast cancer is a systemic disease by the time it is detected. In support of the latter hypothesis, shortly after expression of the oncogenic transgene, transformed epithelial cells from atypical ductal hyperplasia disseminate to the metastatic site in a mouse model of breast cancer. Interestingly, also in humans, bone marrow dissemination can occur at the stage of ductal carcinoma in situ [99]. The decline of metastatic prostate cancer can most likely be attributed to PSA screening, which has anticipated the diagnosis of cancers destined to become metastatic. However, PSA screening, while substantially reducing the risk of metastatic prostate cancer presentation, has reduced the risk of prostate cancer death for metastatic disease only by about one fifth [100]. This suggests that as for melanoma [101] and breast and [99] pancreatic cancers [102], microscopic, clinically undetectable metastases may develop very early in the course of disease, even at pre-neoplastic stages such as high-grade PIN.

Indirect evidence of early metastatic spread in prostate cancer came from a clinical study showing that 13.3% of patients undergoing radical prostatectomy, and

found affected by a tumor with extensions beyond the prostate (i.e., pT3) but LN negative by routine histologic evaluation, were instead affected by occult LN metastases, which correlated with disease recurrence and death by prostate cancer [103]. Inspired by this publication and reasoning that PCSCs were the most likely candidate for early metastatic spread, we looked for PCSCs in TRAMP mice already at the mPIN stage [104]. We started by flow cytometry analyses of prostate-draining LN from age-matched TRAMP and wild-type (WT) mice and found that both 6-week-old TRAMP (age at which mice are healthy) and WT mice did not contain any bona fide PCSC (i.e., $CD45^{-}/CD31^{-}/CD44^{+}/CD166^{+}/Sca1^{+}$ cells; Ref. [46, 105]). These cells were instead enriched in prostate-draining LNs of 16-week-old TRAMP mice, age at which mice are usually affected by well-differentiated adenocarcinoma [74]. $CD45^{-}/CD31^{-}/CD44^{+}/CD166^{+}/Sca1^{+}$ cells were also found in prostate-draining LNs from wild-type mice, thus suggesting this phenotype is not restricted to PCSCs. To overcome this technical matter, we applied the neurosphere assay to prostate- and non-prostate-draining LN of 10–12-week-old wild-type and TRAMP mice, age at which the latter are predominantly affected by high-grade mPIN [74] and in our colony never developed LN metastasis before week 17 [106]. Interestingly, prostaspheres were generated exclusively from prostate-draining LNs of TRAMP mice and were morphologically, phenotypically, and functionally identical to TPIN-SCs and markedly different from ex vivo prostate epithelial cells obtained from age-matched TRAMP mice [104]. Thus, in TRAMP mice, the migration of PCSCs from mPIN lesions into LNs can occur several weeks before metastasis is clinically evident.

The complex process of metastasis implies the generation from the primary tumor bulk of circulating tumor cells (CTC) that are able to invade secondary organs [107, 108]. As early as in 1997, reverse transcription-PCR assay for PSA mRNA allowed the identification of CTCs in the peripheral blood and DTCs in the bone marrow of prostate cancer patients. These analyses showed that while control subjects were negative both in the blood and the bone marrow, the frequency of CTCs was 16% and 27% in the blood of pT2 and pT3 patients, respectively. When the bone marrow was analyzed, the frequency of DTCs increased to 56% and 73%, respectively, demonstrating a substantial enrichment of potentially metastatic cancer cells in the bone marrow already at the time of radical prostatectomy. The technique for isolating CTCs has substantially improved in the last two decades, and benefits of several sophisticated methods (e.g., [109–111]). As an example, by taking advantage of a microfluidic device, Storey and colleagues [112] showed that CTCs often circulate as clusters that traverse capillaries. CTCs also resist more to apoptosis and more likely give rise to a metastatic deposit than single CTCs [113]. Although extremely rare, CTCs have also been identified in early stages of cancer [109] and shown to predict progression-free survival and overall survival in breast [114] and prostate cancers [115–117]. From a genetic standpoint, CTCs also represent an advantage when the metastatic site is inaccessible. By comparing the landscape of mutations of CTCs, one metastatic sample, and multiple foci in the prostate, Lohr and colleagues [118] reported that the primary tumor foci were markedly heterogeneous. Additionally, one focus resembled the CTCs and the metastasis,

suggesting the metastasis originated from this focus and was genetically well represented by CTCs. Thus, investigations on CTCs may also shed light on the metastatic process.

CTCs represent a heterogeneous population also containing subsets of cells endowed with epithelial-mesenchymal transition (EMT) [119] and/or CSC characteristics [120]. As an example, Baccelli and colleagues [121] identified a metastatic CSC population in CTCs from breast cancer patients that was $EPCAM^+/CD44^+/CD47^+/MET^+$ and generated bone, lung and liver metastases in xenograft models. Similarly, in human pancreatic adenocarcinoma, a population of circulating CSCs has been identified that expresses CD133 and CXCR4 and determines the metastatic phenotype of individual tumors [122]. In a mouse model of metastasis, it has been reported that CTCs reached the bone marrow and directly competed with hematopoietic stem cells for occupancy of the stem cell niche through the CXCR4-CXCL12 axis [123]. These DTCs were $CD44^+/CD133^+$, possessed CSC properties [124], downregulated CXCR4, and could be mobilized again by treating mice with the CXCR4 competitor AMD3100 [123].

All together, these findings support the parallel progression model in prostate cancer, and the existence of circulating PCSCs precociously detaching form the primary focus in the prostate gland, and endowed with the capability of migrating to and surviving at the site of metastasis.

5 Interactions Between PCSCs and the Immune System

PCSCs both at the primary site and in the newly colonized tissue need to adapt to the surrounding environment and to be protected from the aggressiveness of the immune system. Because of the lack of sufficient information on the lymphatic drainage and its mostly fibromuscular structure, the prostate has for long been considered an immune-privileged site [125]. The prostate is a walnut-sized fibromuscular gland located below the bladder and around the posterior urethra. It is composed of a fibrous capsule under which smooth muscle fibers and collagenous tissues that surround the urethra are circularly positioned. Deep to this layer is located the prostatic stroma that is composed of connective, elastic, and smooth muscle tissues surrounding the tubuloalveolar structure. Two different cell layers support the gland: a basal layer of low cuboidal epithelium expressing cytokeratin (CK) 5, CK14, and p63 and a luminal layer of columnar secretory cells positive for AR, CK8, and CK18. Additionally, the prostate gland contains a small population of NE cells expressing synaptophysin and chromogranin A. Small papillary epithelium inbuddings are frequently present in the prostate gland. The urethra and the ejaculatory ducts that pass through the organ can subdivide the prostate into either lobes (inferoposterior, inferolateral, superomedial, and anteromedial) or zones, which are differently affected by neoplastic transformation. Seventy percent of prostate adenocarcinomas develop from the peripheral zone, which account for approximately 75% of the glandular tissue in the normal gland. The prostate is also composed of central, transitional, and periurethral zones [126].

Nowadays it is quite well established that the prostate has lymphatic vessels that drain extracellular fluids from the prostate to the pelvic lymph nodes [127]. Additionally, prostate cancer is the site of lymphangiogenesis, also promoted by myeloid-derived cells [128], and expression of vascular endothelial growth factor 3 by lymphatic endothelial cells in the primary tumor is associated with lymph node metastasis, Gleason grade, extracapsular extension, and surgical margin status [129]. It is interesting to note that in xenotransplant models, metastasis to lymph nodes was independent of lymphangiogenesis [130].

The prostate is also no longer considered as an immune-privileged site, and prostate cancer is known to be antigenic and, in some instances, immunogenic. Indeed, the transformed gland contains several prostate cancer-associated antigens, such as PSA, prostate acid phosphatase (PAP), prostate-specific membrane antigen (PSMA), prostate stem cell antigen (PSCA), and six-transmembrane epithelial antigen of the prostate (STEAP), exploitable for active immunotherapy strategies. Additionally, prostate cancer can stimulate the immune response, as shown by the presence of tumor-infiltrating $CD4^+$ and $CD8^+$ T lymphocytes [131].

5.1 The Immune Infiltrate in Prostate Cancer

The Cancer Genome Atlas (TCGA), which includes genome-wide DNA sequencing, RNA sequencing, and copy number profiles, has been exploited to investigate the characteristics of the immune infiltrate in several cancer histotypes. Rooney and colleagues [132] devised an RNA-based metric of immune cytolytic activity and took advantage of TCGA data set to identify and characterize the correlates of antitumor immunity in thousands of TCGA solid tumor samples. Cytolytic activity positively correlated with recurrent mutations on beta-2-microglobulin; human leukocyte antigen (HLA)-A, -B, and -C; and Caspase 8 (CASP8) genes. These recurrently mutated genes identified the loss of antigen presentation and the blockade of extrinsic apoptosis as two main tumor-resistant strategies to cytolytic activity. Additionally, high cytolytic activity was associated with amplifications occurring in genes involved in immunosuppression, such as PD-L1/2. Interestingly, different types of tumors showed different immune cytolytic activities, and prostate cancer belonged to the ones with the least cytolytic activity. These findings are in line with the evidence that immunogenicity and response to immunotherapy associate with a high tumor mutational burden that generates neoantigens [133–135], and prostate cancer, at difference with melanoma, which is highly sensitive to immunotherapies, has a rather low prevalence of somatic mutations [136]. Additionally, prostate cancer appears to belong to the group of the so-called "non-inflamed" or "not T cell-infiltrated" tumors, as to differentiate them from the "inflamed" or "T cell-infiltrated" tumors [137, 138]. The former are usually characterized by a dense stroma, mostly infiltrated by macrophages, poor expression of chemokines, and lack of type I interferon (IFN) signaling. Likely, poor effector cell trafficking is the main reason for tumor escape in this subset of tumors. Conversely, inflamed tumors are rich in

chemokines, type I interferon signaling, and T cells, which are often functionally inhibited by immunosuppressive mechanisms. Prostate cancer is indeed poorly infiltrated by T cells, which are mostly nonfunctional [139].

In addition, early studies found an infiltrate, composed mainly of $CD3^+$ T cells and $CD11c^+$macrophages [140]. Recently, it has been reported that the density of $CD8^+$ T cells was higher in the normal prostate epithelium adjacent to the tumor than in the tumor, whereas $CD8^+$ cells were enriched in the tumor than in the normal stroma, thus suggesting mechanisms adopted by the transformed prostate epithelium to block T-cell infiltration [141]. Indeed, reactive nitrogen species-dependent nitration of chemokines in prostate cancer hinders T-cell infiltration [142].

Moreover, $CD4^+$ T cells have been found infiltrating prostate cancer, especially in the weeks following castration [131]. Interestingly, in TRAMP mice castration induced $CD4^+$ T cells to expand and develop effector function after vaccination [143], suggesting that also $CD4^+$ T cells are impaired in prostate cancer, and that androgen deprivation therapy can increase efficacy of immunotherapy. It has also been reported that on both univariate and multivariate analyses, increased $CD4^+$ T-cell counts in prostate cancer patients were associated with reduced cancer-specific survival independently of disease stage [144]. Indeed, many of these $CD4^+$ cells are regulatory T cells (Tregs; Ref. [145]).

Infiltration by B cells in prostate cancer is usually modest, but it has been shown to be more relevant in more advanced lesions [146]. Interestingly, a tumor-driving role by prostate-infiltrating B cells has been found in TRAMP mice following castration [147–149]. Indeed, in transplantation models, androgen ablation caused infiltration of prostate tumors with B lymphocytes that induced IKK-α nuclear translocation and transducer and activation of transcription 3 (STAT3) activation in surviving cancer cells, thereby enhancing androgen-independent growth and metastatic progression [148]. On the same line, genetic inactivation of IKK-α in castrated TRAMP mice completely prevented the appearance of androgen-independent NE tumors [147]. Moreover, macrophages (MØs) [150] favor NE differentiation by releasing IL-6, while mast cells promote well-differentiated adenocarcinoma and hamper the occurrence of NE cancers [151].

Macrophages are the most abundant infiltrating immune cells in prostate cancer. While few macrophages characteristically infiltrate the stroma surrounding the normal prostate epithelium, prostate cancer is infiltrated by a higher number of $CD68^+$ tumor-associated macrophages (TAMs), which localize mostly in the tumor stroma but also in the cancer cell region and the lumen composed by cancer cells [152, 153]. Interestingly, the frequency of TAMs is inversely correlated with histopathological grade [152, 153]. This is likely due to the fact that cancers of lower Gleason grade have a larger proportion of stroma than cancers of higher Gleason grade. Additionally, a reduced number of TAMs within the tumor mass associates with the presence of positive lymph nodes and are independent predictors for time to disease progression [152]. It has also been reported that prostate cancer cells and cancer-associated fibroblasts, by releasing several factors, among which MCP1, interleukin (IL)-6 and SDF1, attract monocytes and promote their differentiation to "alternatively" activated M2 macrophages [154], which favor angiogenesis and

tumor progression also by inhibiting immune surveillance. Interestingly, polarization to M2 macrophages is also induced by phagocytosis of apoptotic prostate cancer cells (i.e., efferocytosis; Ref. [155]). From a clinical perspective, while M1 macrophages are more frequent in organ-confined prostate cancer, $CD163^+$ M2 macrophages are more represented in and statistically associated with prostate cancer with capsular extension [154], increased incidence of metastases at diagnosis, and poor patient prognosis [156].

Altogether, these findings support the hypothesis that prostate cancer is a non-inflamed tumor and establish a relevant and variable role for the immune system in modulating prostate cancer progression.

5.2 Prostate Cancer Generates an Immunosuppressive Microenvironment

The growth of cancer in the prostate associates with mechanisms of immunosuppression that reshape the tumor in a tissue of acquired immune privilege [157–159]. Interestingly, prostate cancer cells convert inflammation in a tumor-promoting condition, also exerting several immunosuppressive activities [160]. The prostate cancer microenvironment is rich in transforming growth factor (TGF)-β and IL-6 [160], both factors promoting the induction of Tregs, which modulate the immune response either by releasing TGF-β and IL-10 or through cell-cell contact mechanisms involving cytotoxic T-lymphocyte antigen 4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR), and cytotoxicity. Indeed, bona fide $CD4^+/CD25^+$ Tregs have been originally described by Miller and colleagues as enriched both in the peripheral blood and the tumor bed of prostate cancer patients [145]. Others reported a significantly higher frequency of circulating $CD4^+/CD25^+/Foxp3^+$ Tregs in hormone-resistant non-bone metastatic prostate cancer patients than in age-matched healthy control subjects [161]. Another study showed that the frequency of prostate-infiltrating T helper 17 (Th17) cells and not of Tregs inversely correlated with Gleason grade [162], thus giving more relevance to the former cells and landing weight to the hypothesis that Th17 cells have an antitumor activity especially in the early phase of the disease [163]. Moreover, tumors and tumor-draining lymph nodes of aging TRAMP males are enriched with $CD4^+/CD25^+/Foxp3^+$ Treg cells [164]. Additionally, Malchow and colleagues elegantly showed that Tregs of a single specificity were selectively enriched in the prostate tumor of TRAMP mice [165]. These Tregs developed in the thymus and were specific for a prostate-specific antigen [165]. Tregs are immunosuppressive in prostate cancer patients [145], and in vitro depletion of Tregs leads to a significant boost in effector T-cell responses against prostate-specific antigens [166]. Drake and collaborators observed that antigen-specific $CD4^+$ T cells transfer into TRAMP mice results in the induction of Tregs [167]. Thus, Tregs may either come from the thymus [165] or be induced from the periphery [167]. Additionally, androgen

deprivation therapy in prostate cancer patients favors tumor infiltration by both $CD8^+$ T cells and Tregs, thus leaving their ratio unaltered and not impacting on disease-free survival [168]. We have also reported that Treg cells accumulate in the tumors of TRAMP mice, but in vivo antibody-mediated depletion of Treg cells or impairment in their function by cyclophosphamide followed by repeated tumor-specific vaccinations neither overcame tolerance nor impacted on tumor progression [164]. Thus, the role of Tregs in prostate cancer remains a subject of debate, likely because it depends on the characteristics of the tumor and its microenvironment, which in prostate cancer are highly heterogeneous.

Myeloid-derived suppressor cells (MDSC) are another relevant population of immunosuppressive cells in prostate cancer and consist of heterogeneous populations of immature myeloid cells expressing both Gr-1 and CD11b markers in mice. Based on the intensity of expression of Ly6G and Ly6C, they can be subdivided into PMN-MDSCs ($CD11b^+/Ly6G^+/Ly6C^{low}$) and M-MDSC ($CD11b^+/Ly6G^-/Ly6C^{high}$). In humans, the equivalent population of PMN-MDSCs expresses CD11b, CD14, and CD15 or CD11b, CD14, and CD66b. Human M-MDSCs are instead $CD11b^+/CD14^+/HLA\text{-}DR^{-/lo}/CD15^-$ [169]. Tumor-induced emergency myelopoiesis [170] gives rise to MDSCs that exert several pro-tumor activities, spanning from regulation of tumor angiogenesis, invasion, arrest and niche formation, to the suppression of immune surveillance [171]. MDSCs suppress the activity of T cells through multiple mechanisms, including inducible nitric oxide (NO) synthase and arginase 1, key enzymes in arginine metabolism [172]. As a consequence, arginine depletion and NO production contribute to the accumulation in the tumor of lymphocytes impaired in their functions. In vitro cultures of prostatic samples from patients affected by prostate carcinoma showed high levels of nitrotyrosines in tumor-infiltrating lymphocytes, suggesting that both tumor cells and MDSCs locally produce reactive oxygen species like peroxynitrites [139]. Both PMN-MDSCs [173] and M-MDSCs [174] are indeed enriched in the peripheral blood of prostate cancer patients and return to normal levels after radical prostatectomy [175, 176].

Moreover, in mouse models of prostate cancer, MDSCs are among the major infiltrating immune cell types [177]. We investigated MDSC dynamics both in the TRAMP-C1 transplantable model and in TRAMP mice, and we reported that in both models, tumor growth associated with emergency myelopoiesis. While in the TRAMP-C1 model there was an expansion of $CD11b^+/Gr1^{high}$ MDSCs especially in the peripheral blood and the spleen, which also accumulated in the tumor bed, the expansion of $CD11b^+/Gr1^{high}$ MDSCs in tumor-bearing TRAMP mice was modest. Indeed, in TRAMP tumors, $CD11b^+/Gr1^{int}$ and more mature $CD11b^+/Gr1^-$ cells dominated. Interestingly, modulators of the arginine metabolism (i.e., L-NAME and sildenafil) restrained the immunosuppressive function of MDSC cells in both models and limited TRAMP-C1 growth, but they neither impacted on tumor-specific immune tolerance nor blocked tumor progression in TRAMP mice [178].

Invariant natural killer T cells (iNKT) that display features of both innate and adaptive immunity appear to play an important role in prostate cancer immune surveillance. Prostate cancer patients display impaired number of circulating iNKT cells and interferon (IFN)-γ production [179]. Prostate tumor cells, through cell-cell

contact, inhibit IL-12-induced STAT4 phosphorylation and IFN-γ production in iNKT cells [180]. Additionally, we observed that TRAMP mice lacking iNKT cells developed a more aggressive disease and displayed a reduced overall survival [181], likely by favoring tumor infiltration by pro-angiogenic macrophages (Cortesi F. et al., manuscript in preparation).

Prostate cancer cells and tolerogenic DC may also exhaust effector T cells and induce Treg cells through the PD-1/PD-L1 axis. Indeed, prostate cancer cells and infiltrating immune cells variably express PD-L1 [182–186], and immune cells that overexpress PD-1 surround prostate cancer lesions [187, 188].

Finally, both tumor cells and immune cells locally release indoleamine 2,3-dioxygenase (IDO) that controls the immune response by promoting tryptophan catabolism and inducing T-cell tolerance [189].

5.3 PCSC-Mediated Mechanisms of Immunosuppression in Prostate Cancer

While our knowledge about specific immunosuppressive properties of diverse CSC populations is still limited, more is known about stem cells. Human embryonic stem cells (ESC) poorly express HLA class I molecules and do not express HLA class II molecules. ESCs also express no ligands for NKp30, NKp46, and CD16 and low levels of ligands for the activating NK cell receptor NKp44 [190]. Both ESC and mesenchymal stem cells (MSC) highly express the inhibitory molecule HLA-G [191]. MSCs do not express either HLA class II or costimulatory molecules, including CD40, B7-1 (CD80), and B7-2 (CD86) [192–194]. Thus, both stem cells and MSCs hide from immune surveillance because of the lack of molecules for both T- and NK cell recognition.

Additionally, MSCs actively suppress T-cell proliferation, macrophage activation, and Th1 responses by secreting prostaglandin E2 [195, 196], TGF-β, and hepatocyte growth factor [197]. Upon IFN-γ stimulation, MSCs also produce IDO, which metabolizes tryptophan to immunosuppressive kynurenines. Additionally, tryptophan depletion per se negatively impacts on T cell functions [198, 199]. By secreting hepatocyte growth factor [200], MSCs can also induce Tregs and tolerogenic dendritic cells (DCs; Ref. [201]). Tregs are also induced by MSC-secreted HLA-G molecules [202]. Given the intrinsic immunomodulatory features and the capacity to home to injured tissues, MSCs have been used to dampen autoimmunity and graft versus host reactions [203–205].

In analogy with normal SC, CSCs are also endowed with immunomodulatory activities. Early studies showed an activated TGF-β signaling pathway in human breast CSCs [206]. Additionally, CSCs in melanoma and brain, breast, colon, and prostate cancer express the immunosuppressive molecule CD200 [207], thus suggesting these cells are immunosuppressive.

In human glioblastoma, CSCs lack expression of HLA class I and II molecules, and NKG2D ligands, and can inhibit T-cell responses in vitro [208]. Interestingly, the STAT3 pathway is constitutively activated in CSCs from glioblastoma, and block of STAT3 markedly diminishes CSC immunosuppressive activity [209].

In human melanoma, a population of ABCB5$^+$ CSCs [210] preferentially expresses the inhibitory molecule PD-1, and this correlates with their tumorigenic potential qui [210]. Both bulk melanoma cells and ABCB5$^+$ melanoma CSCs were shown to inhibit the in vitro proliferation of T cells and their IL-2 production, while favored Tregs accumulation and IL-10 production with a mechanism partly mediated by CD86 [210].

More recently, Yamashina and colleagues reported that CSCs from chemoresistant tumors released proinflammatory cytokines favoring the induction of M2-like immunomodulatory myeloid cells from CD14$^+$ monocytes. In addition, the IFN-regulated transcription factor IRF5 has been recognized in these CSCs as the transcription factor promoting the production of macrophage colony-stimulating factor (M-CSF) and the generation of the M2-like myeloid cells [211]. Interestingly, MDSCs in ovarian carcinoma inhibited T-cell activation and triggered expression of miRNA101 in CSCs, thus inducing gene expression, sphere formation, and metastasis [212]. Myeloid cells and CSCs can also release the immunosuppressive NO synthase-2 (iNOS) [172, 213], which promotes CSC proliferation and glioma growth [213]. The latter are two additional examples of how the immune system can be manipulated by CSCs to their own advantage.

Moreover, human colon CSCs appear to be weakly immunogenic because of the downmodulation of HLA class I molecules on their cell surface and the expression of membrane-bound IL-4 [214]. Indeed, co-culture of human leukocytes with IL-4$^+$ CSCs inhibits proliferation of the latter, and neutralization of CSC-associated IL-4 rescues T-cell proliferation [214]. Several experimental evidences support the direct and indirect pro-tumorigenic effect of IL-4 in colorectal cancer. As an example, the IL-4 receptor alpha, a component of the receptor complex for both IL-4 and IL-13, has frequently been found expressed in human colon adenocarcinomas, IL-4 induces proliferation of tumors cells, and mice null for the IL-4 receptor alpha show fewer and smaller colorectal cancer tumors than the wild-type counterpart [215]. More importantly IL-4 contributes to the acquisition of a chemotherapy-resistant phenotype to colon CSCs [216]. IL-4 is also an inducer of arginase 1 in MDSCs, thus favoring immune suppression [217].

IL-4 is a pleiotropic cytokine, and experimental evidences also exist in favor of its antitumor activity. Indeed, immunotherapy with IL-4-producing tumor cells prolonged survival of mice affected by colon cancer [218] and melanoma [219], and this strategy has been investigated in clinical trials [220, 221]. As for many other cytokines, the effects of IL-4 on tumor cells likely depend on the tumor histotype, the amount of cytokine locally released at any given time, whether it targets CSCs of more differentiated cells, and the cells comprising the tumor microenvironment.

In addition to IL-4, CSCs can release other immunomodulatory cytokines and chemokines, including IL-6, IL-8, and CCL-2 [222, 223], and several others will likely be identified in the near future.

CSCs also produce and release extracellular matrix proteins, such as fibronectin, collagen, and integrins, which are essential for niche formation and regulate dormancy/proliferation of CSCs and their metastatic potential [224–227]. The extracellular matrix also modulates cancer immune surveillance [226]. We have recently investigated the immunomodulatory role of prostate CSCs in TRAMP mice, and we have found that CSCs obtained both from mPIN lesions and prostate-draining LNs scored as histopatologically negative by the pathologist, and not those obtained from more advanced prostate tumors, used the extracellular matrix protein Tenascin-C (TNC) to inhibit T-cell receptor–dependent T-cell activation, actin cytoskeleton polymerization, proliferation and cytokine production. We have also found that prostate CSCs migrated to prostate-draining LNs through the CXCR4/CXCL12 axis and contributed in generating a local immunosuppressive environment. Indeed, when TRAMP mice were precociously treated with the CXCR4 inhibitor AMD3100, T-cell proliferation in their prostate draining LNs returned comparable to wild-type mice either treated or not with the drug [104]. TNC has long been known for its in vitro immunosuppressive activity [228] and as a relevant molecule in physical and signaling support of CSCs and metastasis-initiating cells in their niche ([229]; also revised in [230]). Our data [104] add to these notions and support TNC as a major mechanism by which CSCs inhibit T-cell responses in TRAMP mice. In particular, TNC appears to exert a relevant role in the early phases of disease progression, likely favoring the generation of the metastatic niche. Thus, TNC tunes the local immune response to establish equilibrium between precociously disseminated nodal CSCs and the immune system [104]. We have also found that TNC is mostly expressed in both the epithelium and stroma of normal human prostate and low-grade PIN, its expression increases in high-grade PIN and returns to basal levels in prostate adenocarcinoma, and it is again overexpressed in LN metastases [104]. Hence, TNC also appears to be relevant in human prostate cancer and might be a therapeutic target especially in low-risk patients (i.e., Gleason scores $\leq$6, PSA concentrations <10 ng/mL, or T1-T2a) to prevent early metastases.

In conclusion, CSCs display several mechanisms of immune escape that allow them to survive in the primary tumor lesion and at the sites of colonization. All these molecular mechanisms should be considered when novel immunotherapies are designed to selectively target CSCs.

6 Immunotherapy Applied to PCSCs

An increased incidence of prostate adenocarcinoma in organ-transplanted patients during immunosuppressive therapies [231, 232] makes a strong case for prostate cancer immune surveillance. Prostate cancer is also the site of inflammation, which may progress into proliferative inflammatory atrophy, and anticipate prostate cancer [7]. Additionally, prostate cancer cells possess several tumor-associated antigens and can elicit tumor-specific T-cell responses [160]. Thus, prostate cancer has been the subject of intense investigation in the field of immunotherapy, which culminated in

2010 with the FDA approval of the first cancer vaccine [233]. Sipuleucel-T (Provenge; Dendreon Inc.) is a personalized cell-based vaccine made of autologous antigen-presenting cells pulsed with a chimeric protein that contains PAP and granulocyte-macrophage colony-stimulating factor (GM-CSF). Sipuleucel-T is beneficial to castration-resistant prostate cancer patients for their survival, and its use is associated with mild side effects [234]. A reduction in circulating T-cell receptor (TCR) sequences and an increase in TCR commonality between blood and prostate have been reported in Sipuleucel-T-treated patients, suggesting that the treatment favors recruitment of tumor-specific T-cell clones into the tumor [235]. Additionally, it has also been reported that the vaccine induces in treated patients an antibody response specific for non-targeted tumor-associated antigens, thus supporting the induction of epitope spreading [236]. Sipuleucel-T is also being investigated in association with standard therapies, including abiraterone and prednisone [237].

Another promising vaccine for prostate cancer patients is GVAX, which is made of whole allogeneic tumor cells engineered to express GM-CSF [238]. GVAX is proposed in association with cyclophosphamide and androgen deprivation for localized prostate cancer. The history of this vaccine is more troubled. After the first promising phase I/II clinical trials, two phase III clinical trials in castration-resistant prostate cancer patients were terminated because of the impossibility to meet the primary endpoint [160]. Ongoing clinical trials will clarify the therapeutic potential of this vaccine in prostate cancer patients.

PROSTVAC-VF is a third therapeutic vaccine designed on two engineered pox viruses delivering both PSA and three co-stimulatory molecules (LFA-3, ICAM-1, and CD80) directly to neoplastic cells [239]. PROSTVAC-VF showed encouraging results in a randomized phase II trial in metastatic castration-resistant prostate cancer patients and is currently tested on a large phase III trial (NCT01322490). Many more vaccines have been designed for prostate cancer patients [240], and several clinical trials in castration-resistant prostate cancer patients are ongoing (http://www.cancerresearch.org).

As prostate cancer is considered a non-inflamed tumor, several strategies have been attempted to induce local inflammation, thus favoring infiltration of T cells induced by the vaccine. Thus, vaccines are combined with either radiation, chemotherapy, and androgen deprivation therapy or other immunotherapies, including adoptive T-cell therapy (ACT) and immune checkpoint blockade [240].

ACT is based on the in vitro engineering and/or expansion of patient-derived immune cells, which are then reinfused into the patient. While this therapeutic strategy is extensively investigated in hematological malignancies and some solid tumors [241], only two clinical trials are ongoing in castration-resistant prostate cancer patients. A phase I clinical trial is based on the use of natural killer cells (NCT00720785). A phase II clinical trial of engineered T cells specific for the NY-ESO-1 antigen is proposed in combination with a DC-based vaccine using the same cancer-specific antigen (NCT01697527).

Vaccines can also be combined with immune checkpoint inhibitors with the aim to reduce immunosuppression in tumor-draining LNs and in tumors [242]. Ipilimumab has been the first checkpoint inhibitor entering the clinical arena. It is a

CTLA-4-blocking human IgG1 monoclonal antibody. CTLA-4 down-modulates T-cell responses by inhibiting co-stimulation by CD28, with which it shares the ligands CD80 and CD86. As CD80 and CD86 are mostly expressed on professional antigen-presenting cells in LNs, it is believed that the main activity of CTLA-4 is exerted in secondary lymphoid organs in which most of the tumor-specific T cells are activated and proliferate [243]. Indeed, CTLA-4 is constitutively expressed by Tregs, which use it to dampen the induction of antigen-specific T-cell responses [244]. Prostate cancer has been one of the first tumors in which anti-CTLA-4 has been experimentally tested either alone or in combination with GVAX [245]. A recent phase III trial comparing ipilimumab with placebo after radiotherapy in castration-resistant prostate cancer patients did not reach the primary endpoint (i.e., survival benefit; Ref. [246]). New trials with ipilimumab alone or in combination with other treatments are ongoing and include a phase III study in chemotherapy-naïve prostate cancer and neoadjuvant settings [247].

PD-1 is a T-cell co-inhibitory receptor expressed on activated $CD4^+$ and $CD8^+$ T cells and physiologically mediates immunosuppression by binding to its ligands PD-L1 and PD-L2 and inhibiting TCR-mediated effector functions. PD-1 interaction with is ligands occurs during the effector phase, and thus more likely in the tumor microenvironment [248], where both cancer cells and myeloid cells may express PD-L1 [242]. Interestingly, prostate cancer cells and infiltrating immune cells may express PD-L1 [182–186], and immune cells that overexpress PD-1 are found surrounding prostate cancer lesions [187, 188]. Prostate cancer is the subject of intense investigation on the therapeutic efficacy of anti-PD-1 (i.e., nivolumab, lambrolizumab, pembrolizumab) and anti-PD-L1 antibodies (i.e., darvalumab, atezolizumab; Ref. [247]). We invite the interested reader to visit specialized sites for more details (e.g., http://www.cancerresearch.org/prostate-cancer).

Taken together, these findings show that prostate cancer is a potential target for immunotherapy. Results of clinical trials have been so far below expectations and strongly suggest combining therapies and to focus on less advanced disease.

6.1 How to Target PCSCs with Immunotherapy

As CSCs are resistant to chemo- and radiotherapy, it is very interesting to know if they can be targeted by the immune system and if immunotherapeutic approaches specifically designed to target CSCs can be effective in eradicating tumors. This is a new and very challenging field, and increasing evidences are accumulating on the immunogenicity of CSCs.

Proposing immunotherapy as a therapeutic strategy against CSCs implies that they must be recognized by the immune system. Nonconventional T cells, such as γδ T cells, NK cells, and iNKT cells can mediate antitumor immunity. Indeed, different papers reported that CSCs from brain, colon, or melanoma tumors express NKG2D ligands and can be efficiently targeted by NK and γδ T cells [249–252]. As already mentioned, CSCs may downregulate major histocompatibility complex

(MHC) molecules, thus preventing T-cell recognition. This has been reported for glioblastoma CSCs, which showed weak or no positivity for MHC molecules and for molecules of the antigen processing machinery [208]. However, IFN-γ treatment restored MHC expression and allowed in vitro recognition of CSCs by autologous T cells [208]. Thus, any therapeutic strategy aimed at increasing the intra-tumor release of IFN-γ might synergize with immunotherapies.

Several other CSCs isolated from brain and colon tumors resulted positive for MHC-I and MHC-II molecules [249–253], and brain CSCs engineered to express the CMV pp65 antigen were effectively killed both in vitro and in vivo by antigen-specific $CD8^+$ T cells [253].

In the TRAMP model, we have found that PCSCs express prostate cancer-associated antigens, ligands for NK cell receptors, and MHC class I molecules. While MHC class II were not expressed in steady-state conditions, both MHC class I and II molecules were upregulated in PCSCs upon IFN-γ stimulation. Both CSCs from exocrine and NE prostate tumors were recognized and killed by tumor-specific T cells and NK cells. We also showed that the infusion of DCs pulsed with irradiated PCSCs elicited a tumor-specific immune response that was stronger than that elicited by DCs pulsed with irradiated tumor cells, delayed tumor growth in mice challenged with CSCs, and induced tumor regression in TRAMP mice bearing autochthonous prostate tumors [105]. Thus, PCSCs in TRAMP mice can also be targeted by the immune system.

Active immunization strategies against CSCs have also been exploited in brain tumors by loading DCs with apoptotic CSCs or CSC-lysates. Finocchiaro and collaborators generated neurospheres enriched with CSCs from the murine glioma cell line GL261 (called GL261-NS) and showed that DCs pulsed with GL261-NS lysates elicited a strong in vivo and in vitro antitumor T cell immunity and cured both tumors generated by GL261-NS and by the differentiated tumor cell line GL261 [254]. Interestingly, vaccination with DCs pulsed with GL261 cells was not effective against CSC-generated tumors, thus suggesting the GL261-NS CSCs expressed unique antigens [254]. Yu and colleagues applied a similar approach and showed that human DCs loaded with apoptotic glioblastoma CSCs elicited antigen-specific T-cell responses against CSCs and were effective in prolonging survival of rats bearing brain tumors [255].

CSCs may express antigens common to more differentiated tumor cells as well as unique antigens often related to their stemness [256]. Targeting the latter antigens that are relevant for the function and survival of CSCs will have more chances to eradicate the tumor mass [257]. Thus, several groups have been focusing their research in identifying tumor antigens expressed by CSCs [258]. As few examples, CSCs from exocrine and neuroendocrine TRAMP tumors express STEAP and PSCA antigens at variable levels that can be targeted by antigen-specific T cells [105]. T cells can also target 13Ra2, SOX2, and CD133 on glioma CSCs [253, 259, 260], EpCAM in retinoblastoma [261], and CEP55 and COA-1 in colorectal CSCs [262].

Some of the surface markers expressed by CSCs have been exploited for antibody-based immunotherapeutic approaches. CD133 is a surface glycoprotein

expressed on CSCs from multiple solid malignancies, including prostate cancer (Table 1). Treatment with anti-CD133 antibodies reduced the proliferation of sarcoma [263] pancreatic and hepatic CSCs [264, 265]. Alternatively, CD133 has been used to selectively target drugs to CSCs, as for paclitaxel loaded polymeric nanoparticle [266]. Moreover, anti-CD133 antibody fused with pseudomonas exotoxin 38 inhibited tumor growth progression in a mouse model of $CD133^+$ ovarian cancer [267].

Moreover, CD44 has been frequently found expressed by CSCs in general and PCSCs in particular (Table 1). Interestingly, a murine IgG1 anti-human CD44 receptor, targeting the CD44-STAT3 pathway, decreased pancreatic CSC sphere formation in vitro and inhibited tumor growth, metastasis, and recurrence in xenotransplantation experiments [268]. In prostate cancer, CD44 is particularly relevant, as it has been implicated in cancer development and metastatic dissemination [269, 270]. More recently, CD44 has been proposed as a driver for prostate CSC formation through the Wnt pathway [271]. Thus, CD44 has been exploited in prostate cancer as a direct target of therapy [272] or to deliver drug-containing nanoparticles [273]. CD44 splice variants [274] might also be targets for ACT [275].

PSCA is a prostate cell surface antigen that is overexpressed during neoplastic transformation of the prostate gland [276]. Initially considered as specific for stem cells, PSCA is also expressed by differentiated cancer cells. Anti-PSCA monoclonal antibodies induced inhibition of tumor growth and metastasis and prolonged survival in human prostate cancer xenografts [277]. A phase I/II trial in hormone and chemotherapy-refractory prostate cancer patients vaccinated with DCs pulsed with PSCA showed that the responding patients who developed PSCA immunity had a significantly prolonged overall survival [278]. Moreover, DCs pulsed with a mixture of four different prostate-specific antigens (PSCA, PSA, PSMA, PAP) induced a strong cytotoxic T-cell response against the four antigens in castration-resistant patients. Additionally, long-term vaccination associated with increase in PSA doubling time [279]. Finally, T cell with a transgenic TCR obtained by fusing the β-chain of TCR to an anti-PSCA antibody single-chain fragment was cytotoxic against tumor cells expressing PSCA [280], thus suggesting that PSCA can also be used as target of ACT [281, 282].

EpCAM is a CSC surface marker associated with prostate cancer cell proliferation, tumorigenesis, metastasis, and resistance to chemotherapy and radiotherapy [283, 284]. Adoptive transfer of human T cells engineered with a chimeric antigen receptor (CAR) specific for EpCAM displayed cancer cell killing activities both in vitro and in vivo in xenograft models of human prostate cancer [285].

MUC1 is a transmembrane glycoprotein upregulated in prostate cancer and associated with prostate cancer metastasis and castration-resistant prostate cancer development [286]. MUC1 is also specifically upregulated in PCSCs, and vaccination with DCs loaded with MUC1 in patients affected by castration-resistant prostate cancer showed a delayed doubling time of PSA in a phase I/II clinical trials [287].

Thus, both active and adoptive immunotherapies can be exploited to selectively target PCSCs. Altogether, these reports also highlighted the current limitations in fully exploiting immunotherapies against PCSCs, namely, the paucity of well-defined antigens selectively expressed by PCSCs, the difficulty in having tumor-specific T cells in close contact with PCSCs, and the immunosuppressive niche that protects CSCs from the immune attack.

7 Conclusion and Future Directions

Clear evidences support the concept that CSCs drive prostate cancer development and progression. According to the CSC theory, eradication of the bulk of the tumor often results in remission but, if CSCs are not deleted, relapse and metastasis will likely occur. Thus, targeting CSCs could open novel therapeutic perspectives. As CSCs have been reported to be chemo- and radio-resistant, immunotherapy represents a valuable alternative approach. Indeed, strong in vitro data and in vivo experiments in mouse models of cancer suggest that CSCs are targets of the effectors of both innate and adaptive immunities. Nonetheless, immunotherapy has produced limited clinical results in prostate cancer patients. One consideration that applies to most if not all trials of immunotherapy in prostate cancer is that, as for many other tumors, immunotherapy has been tested so far in advanced and heavily pre-treated patients. Indeed, metastatic and castration-resistant patients have been the preferred targets. At that stage, disease is widespread and often bulky, and the immune system has already been crippled both by the tumor and the treatments. It is not even clear if at that stage CSCs still exert any relevant role in disease progression. Active immunotherapy, even if associated with other conventional therapies or checkpoint blockade, will hardly be efficacious in those patients. Thus, the first suggestion is to reserve ACT to advanced patients, either alone or in combination with strategies aimed at modifying the tumor microenvironment (see below).

In general, the ideal candidate for cancer immunotherapy is the patient in the early stages of cancer or with a minimal residual disease [288]. In prostate cancer, the ideal patient would be the one who at biopsy shows risk of early metastatic dissemination and the one with low tumor load. These patients might benefit from vaccination even in neoadjuvant settings. Experimental evidences support this hypothesis (e.g., [289]). Another ideal patient is the one who at radical prostatectomy shows culprits of high-risk disease (i.e., clinical T stage $\geq$ cT2c, a Gleason score ≥ 8, or a PSA > 20 ng/ml; [290]). Moreover, these patients should be offered either active immunotherapy or checkpoint blockade. In all these cases, immunotherapy should preferentially target CSCs.

As underlined in the previous sections of this manuscript, three major issues need to be addressed to significantly improve the efficacy of immunotherapy against prostate CSCs.

7.1 Identification of PCSC-Specific Cell Surface Markers

As highlighted in Table 1, prostate cancer lacks widely accepted markers of CSCs. CD44, which is frequently used to identify prostate CSCs, is also expressed by more differentiated prostate cancer cells [291, 292]. Nonetheless, CD44 is an interesting therapeutic target in prostate cancer, as targeting CD44 in prostate CSCs by Wnt inhibitors enhanced the therapeutic efficacy of docetaxel in mouse xenografts [271]. Additionally, differential expression and splicing of mRNA may generate CD44 variants that appear to be more selectively expressed by prostate cancer cells with mesenchymal characteristics [293]. CD44 variant 6, which has been implemented to build CAR T cells [275], may predict disease recurrence although with conflicting results [292, 294–298] and is associated with cancer metastasis and chemo- and radio-resistance [299]. Moreover, EpCAM is being investigated as potential target of CAR T cells [285]. Thus, clinical trials on the use of CAR T cells in castration-resistant prostate cancer patients are expected.

Additional and more selective markers of prostate CSCs might also be implemented for active immunotherapy in high-risk prostate cancer patients in the early phases of the disease.

It would also be interesting to investigate the expression of immune checkpoint inhibitors in PCSCs, as it has been reported that antibodies against checkpoint inhibitors may exert direct anti-tumor activities [300, 301]. We collected preliminary evidence that in vitro TRAMP prostate CSCs upregulate expression of PD-L1 upon IFN-γ stimulation (unpublished results), thus suggesting that also in prostate cancer, checkpoint inhibitory blockade might target CSCs both directly and indirectly .

7.2 Strategies to Favor Tumor Infiltration by Activated T Cells

A fundamental prerequisite for successful immunotherapy is that activated T cells, either endogenous or adoptively transferred, reach the tumor bed and get in close contact with CSCs. Tumor-specific T cells need to travel from the blood stream into prostate cancer-associated vessels and extravasate into a stiff stroma composed of fibroblasts, smooth muscle cells, extracellular matrix, and associated proteins. The stroma microenvironment is a dynamic tissue that promotes prostate cancer growth and metastasis [302]. Thus the first obstacle activated T cells need to overcome is high interstitial fluid pressure caused by increased vessel permeability, lymphatic impairment, and uncontrolled growth of tumor cells in a confined space [303]. Extravasation is an additional hurdle for T cells, as endothelial cells lining tumor vessel are poor in adhesion molecules, a phenomenon named endothelial anergy [304]. We originally proposed that delivery of vasoactive inflammatory tumor necrosis factor α (TNF) to tumor neovessels could represent an efficient means to

enhance T-cell extravasation and tumor infiltration [305]. In order to selectively deliver TNF to tumor neovessels, the cytokine was fused with a tumor-vasculature-homing peptide containing the Cys-Asn-Gly-Arg-Cys (NGR) sequence, a ligand of a CD13 isoform expressed by neo-angiogenic vessels [306, 307]. The new moiety named NGR-TNF transiently enhanced tumor vessel permeability [308], thus favoring the penetration of chemotherapeutic drugs in murine models of lymphoma, melanoma, and spontaneous prostate cancer without TNF-related systemic toxicity [309, 310]. NGR-TNF is currently tested in various clinical trials in cancer patients [311]. We have also observed that extremely low doses of NGR-TNF (5 ng/kg) were sufficient to induce VCAM-1 and ICAM-2 upregulation on endothelial cells together with the release, in the tumor microenvironment, of chemokines that promoted T-cell trafficking. Thus, in transplantable melanoma and autochthonous prostate cancer, the infiltration of either fully activated endogenous or adoptively transferred T cells was enhanced through rapid and transient modification of the tumor microenvironment consequent to NGR-TNF treatment [312]. We also demonstrated that the treatment increased the therapeutic efficacy of tumor vaccines and ACT with no evidence of toxic reactions [312, 313]. The effects of NGR-TNF were not confined to the transient activation of tumor-associated endothelial cells [314]. Indeed, the molecule also favored T-cell extravasation by loosening VE-cadherin-dependent adherence junctions [315]. Additionally, NGR-TNF transiently reduces tumor hypoxic areas [316] and favors survival and proliferation of tumor-infiltrating lymphocytes [314]. Several other strategies have been tested to enhance lymphocyte trafficking into tumors, as reviewed in [317].

The tumor stroma might also be metabolically hostile to activated T cells. Indeed, altered perfusion of the tumor mass favors hypoxia and reprograming of tumor cell energy metabolism [318], which also constitutes an emerging cancer hallmark [319]. As an example, we reported that lowering the pH in vitro to values similar to the ones detected in tumors (pH 6–6.5) impaired proliferation, cytolytic activity, and cytokine secretion of tumor-specific CTLs both in humans and in mice [320]. Importantly, in vitro buffering of pH to physiologic values completely rescued T-cell functions. However, longer exposure or lower pH values induced permanent damage and T-cell death [320], thus suggesting that part of T-cell immunity might be lost at tumor site when extreme metabolic alterations occur. TCR triggering at low pH is associated with IL-2Rα (CD25) and TCR reduced expression, reduced activation of STAT5 and extracellular signal-regulated kinase [320], and molecular alterations frequently displayed by anergic T cells [321, 322]. Analogous features were observed in tumor-specific CTLs in melanoma lesions, whose pH was 6.5 [320]. Hence, acidity represents a tumor-cell extrinsic mechanism of immune escape [323]. Several other metabolic alterations within the tumor microenvironment may act as immunosuppressors [159] and will be touched upon in the following section.

7.3 Strategies to Overcome Immune Suppression

Evidences are rapidly accumulating on the existence of immunosuppressive mechanisms within the tumor microenvironment that are directly operated by tumor cells, or in place as indirect consequences of tumor growth [159, 317, 324, 325]. Very recent is the evidence that even oxygen tension [326] or extracellular potassium concentration [327] alter T-cell functions in tumors. Several more immunosuppressive mechanisms have already been described in the previous sections of this text. The tumor is a dynamic tissue, and immunosuppressive mechanisms are likely modulated, depending on the phase of the disease and the treatments the patient has been subjected to. Advanced tumors are generally more immunosuppressive than early lesions. However, CSCs may already generate an immunosuppressive niche in preneoplastic lesions and at the stage of dormancy in the neo-colonized tissue. Thus, strategies counteracting immunosuppressive mechanisms should be tailored to the specific clinical condition. As summarized in Fig. 1, several strategies can be implemented, either alone or in combination, to increase the chances that activated T cells specific for antigens expressed by CSCs get in contact and kill CSCs. As an example, Imatinib mesylate (Imatinib), a selective inhibitor of PDGFR tyrosine

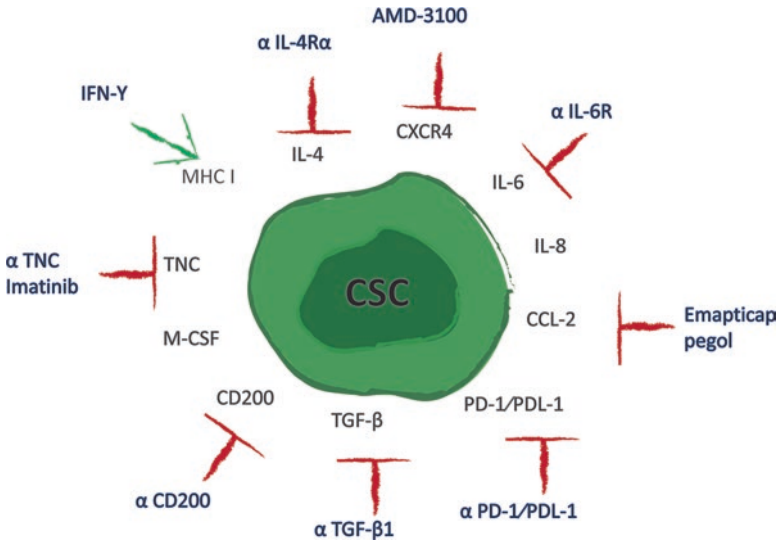

Fig. 1 Immunosuppressive mechanisms exerted by CSCs and suggested inhibition strategies. This figure schematically shows the main immunosuppressive mechanisms employed by CSCs and the potential therapeutic strategies to counteract them. In dark gray are depicted the inhibitory molecules expressed by CSCs, while in light gray are the downregulated molecules. Red arrows represent inhibitory drugs for potential therapeutic intervention. The green arrow represents a stimulatory drug favoring the expression of downregulated molecules. The immunosuppressive molecules include CXCR4, IL-6, IL-8, IL-4, CCL-2, TGF-β, M-CSF, NO, PD-1/PD-L1, CD200, and TNC. MHC I molecules are downregulated on the cell surface of CSCs

kinases, inhibits expression of TNC [328]. Interestingly, mRNA of PDGFβ is upregulated in TPIN-SCs and PCSCs from LNs [51], thus suggesting that treatment with Imatinib may inhibit PDGFR activity and TNC production by these CSCs. We have also found that TPIN-SCs express CXCR4 and migrate in vitro in response to the CXCR4/CXCL12 axis and CXCL12 is upregulated in prostate-draining LNs of TRAMP mice when compared with other LNs of the same mice or prostate-draining LNs of age-matched WT mice. More importantly, CXCR4 appeared to be critical for the development of LN-derived CSCs, as in vivo administration of the CXCR4 inhibitor AMD3100 prevented establishment of an immunosuppressive microenvironment in prostate-draining LNs [104].

PCSCs might also be targeted in vivo by monoclonal antibodies specific for checkpoint inhibitors and CD200 [329].

As CSCs produce a plethora of cytokines and chemokines, for which blocking antibodies or inhibitory molecules might exist (e.g., the anti-IL4Ralpha antibody Dupilimumab [330], the CCL2 inhibitor Emapticap pegol [331], or the anti-IL6 receptor antibody Tocilizumab [332]), an additional strategy would be to combine immunotherapy with antibodies or other molecules inhibiting interactions between the soluble factor and its receptor.

Finally, the enzyme iNOS is upregulated in CSCs as well as MDSCs and has potent immunosuppressive activities through the alteration of the arginine metabolism and the release of NO [333]. NO and iNOS have been the targets of several therapeutic attempts [334], several of which were terminated for relevant toxicities in humans. Phosphodiesterase-5 (PDE-5) inhibitors, like Sildenafil, interfere with the iNOS activity and might be effective in targeting the immunosuppressive activity of PCSCs [178] even in combination with chemotherapy [335]. Once again, all these strategies should be implemented in the early stages of prostate cancer or immediately after radical prostatectomy or radiotherapy.

Finally, even the smartest therapy targeting CSCs would not be effective if also more differentiated cancer cells are killed. Thus, effective cancer therapies should be aimed at killing all cancer cells. As a last example, Dubrovska and colleagues showed that the combination of the dual PI3K/mTOR inhibitor NVP-BEZ235, which eliminates PCSCs, and Taxotere, which preferentially kills more differentiated tumor cells, was significantly more effective in eliminating the tumor than the two drugs used as single agents in a mouse model of prostate [336].

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Cancer Stem Cells: The Players of Immune Evasion from Immunotherapy

Saad Rasool, Sergio Rutella, Soldano Ferrone, and Cristina Maccalli

Abstract Cancer stem cells or cancer-initiating cells (CSCs/CICs) represent a rare population within tumor lesions that are responsible for tumor formation, progression, and resistance to therapeutic interventions. These cells, due to their immunomodulating properties, can evade from immunosurveillance remaining as quiescent cells and might be responsible for resistance to immunotherapeutic treatments. Here the immunological properties of CSCs/CICs are summarized with special emphasis on the mechanisms underlying the impairment of T cell-mediated immune responses. A deep comprehensive genomic, molecular, and immunological characterization of CSCs/CICs needs to be further explored in order to design immunotherapy intervention that will allow the complete eradication of tumors, including the stem-like cellular components.

Keywords Cancer stem cells/cancer-initiating cells · Cell-mediated immune responses · MHC molecules · Immunotherapy

Abbreviations

APCs	Antigen-presenting cells
APM	Antigen-processing machinery
B7-H1, 3, 4	B7 homolog family members

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CIC	Cancer-initiating cell
COA-1	Colon antigen-1
CRC	Colorectal cancer
CSC	Cancer stem cells
CTL	Cytotoxic T lymphocyte
CXCR-4	C-X-C chemokine receptor type 4
EMT	Epithelial-to-mesenchymal transition
Ep-CAM	Epithelial cell adhesion molecule
GBM	Glioblastoma multiforme
GDF-15	Growth differentiation factor 15
Gp100	Glycoprotein 100
HLA	Human leukocyte antigen
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IL-10	Interleukin-10
IL-13α2	α2 Chain of interleukin-13 receptor
IL-4	Interleukin-4
MAGE	Melanoma-associated antigen
MART-1	Melanoma antigen recognized by T cells
MDSC	Myeloid-derived suppressor cell
Melan-A	Protein melan-A, *see also* MART-1
MHC	Major histocompatibility complex
MSC	Mesenchymal stem cell
MUC-1	Mucin 1
NKG2D	Natural killer group 2, member D
NY-ESO-1	New York esophagus 1 antigen
PD-1	Programmed cell death protein 1
PD-L1	Programmed death ligand 1
PGE2	Prostaglandin E2
STAT3	Signal transducer and activator of transcription 3
SVV-1	Survivin 1
TAA	Tumor-associated antigen
TGF-β1	Tumor growth factor beta 1
Treg	T regulatory cell

1 Introduction

Cancer stem cells (CSCs) or cancer-initiating cells (CICs) represent rare cells within tumor lesions that have been isolated from both hematological and different types of solid tumors [1–3]. These cells have been identified as a component of tumors responsible for tumor formation, resistance to therapies, progression and metastasization [1, 2, 4, 5]. CSCs/CICs share similarities with normal stem cells, including self-renewal, differentiation, and the ability to cycle between quiescence and

proliferation [6–9]. The failure of standard therapies to target CSCs/CICs can represent that principle reason of the occurrence of cancer relapse, even following signs of remission that can last several years [6–13]. The identification of CSCs/CICs within tumor tissues using markers that have been reported to be expressed or overexpressed by these cells has been exploited as a tool to assess their impact on patients' clinical outcome. Increase in the frequency of CSCs/CICs has been described upon progression of tumors with different histological origins [14–18]. Nevertheless, the lack of standardized reagents and methods for the ex vivo isolation of these cells hampers the accurate identification of CSCs/CICs within tumor tissues and determination of their prognostic role. Various mechanisms have been described as responsible for the resistance and survival of CSCs/CICs to therapies, including quiescence, enhanced DNA repair processes, aberrant activation of survival, and anti-apoptotic pathways [5]. These mechanisms have been discussed in the previous chapters of this volume. A deeper understanding of these mechanisms and the identification of novel CSC/CIC-associated molecular determinants will improve the efficacy of current therapeutic strategies and allow the identification of novel interventions targeting these types of cells. However, the heterogeneity of tumor tissues can also have an impact on the limited efficacy of the therapeutic intervention. Tumor cell heterogeneity is reflected by high plasticity that has been observed in CSCs/CICs, resulting in the modulation of their phenotype and cellular fate [1, 2, 19–22]. The use of transplantable immunodeficient mouse models showed clearly that modulation of markers, expressed by CSCs/CICs and used for their isolation and tumorigenic characterization, can occur even following serial in vivo transplantation [23–26]. It has to be noted that the lack of standardization for ex vivo isolation of CSCs/CICs and the different immunological background of mice used for in vivo studies are responsible for inconclusive results and complexity in terms of clear definition of cellular hierarchy and plasticity within tumor tissues.

The tumor microenvironment (TME) represents one of the principal factors influencing the properties of CSCs/CICs. The TME comprises the "niche" that maintains and allows the survival of CSCs/CICs [5, 27–32] and through the cross-talk with CSCs/CICs can directly modulate their phenotype and functional fate [5, 27–32].

A variety of "bona fide" CSC/CIC-associated markers, including CD34, CD38, CD133, CD44, CD24, CD166, ABCB5, ALDH-1, and Lgr5, have been identified depending on the histological origins of these cells (Table 1; Fig. 1). These markers are overexpressed by cells with "stemness" properties and generally shared with differentiated tumor cells [1, 2, 4, 9] (Table 1). Nevertheless, the plasticity and continuous evolution of these cells induced by the interaction with TME can lead to their phenotypic modulation [33, 34]. Notably, the mechanism of epithelial-to-mesenchymal transition (EMT), which leads epithelial cells to acquire mesenchymal properties and regulates normal physiological cellular programs such as embryogenesis, wound healing, and transdifferentiation, becomes reactivated in cancer cells and promotes the acquisition of "stemness" properties by malignant cells [35–40] [38–42]. The regulation and activation of EMT by immune cells have been reported in different types of tumors [39, 43]. Several findings have shown the

Table 1 CSC/CIC-associated markers

Molecule[a]	Functionb	Tumor type	Reference(s)
CD24	Sialo-glycoprotein presented on mature granulocytes and B cells and modulated growth and differentiation	Pancreatic and colorectal cancer	[153, 154]
CD34	This protein plays a role in attachment of stem cells to the extra cellular matrix	Leukemia	[155–158]
CD38	A transmembrane glycoprotein that regulates intracellular calcium ion signaling	Leukemia	[158]
CD44	Cell surface glycoprotein involved in cellular interaction, adhesion, and migration	Colon, pancreatic, breast, prostate, head and neck, and ovarian cancer	[1, 2, 4, 159, 160]
CD90	Cell surface glycoprotein involved in adhesion and communication in nervous and immune systems	Liver cancer	[158]
CD133	Transmembrane glycoprotein maintains stemness properties	Brain, colon, pancreatic, lung, ovarian, prostate, gastric, and liver cancer	[1, 2, 4, 161–165]
CD166	A cell adhesion molecule implicated in adhesion and differentiation of T cells	Colon, non-small cell lung cancer	[166, 167]
ALDH1A1	A cytosolic isozyme crucial for growth, differentiation, and maintenance of organs/tissues	Colon, AML, breast, gastric, and ovarian cancer and melanoma	[1, 2, 4, 15, 16]
EpCAM	Cell surface antigen functions as a cell adhesion molecule	Liver and colon cancer	[166, 168]
ESA	Enzyme with lactonase and ester hydrolase activity for breakdown of high-density lipoproteins	Pancreatic cancer	[153]
CBX3	Binds DNA and nuclear membrane proteins and is involved in DNA damage repair	Osteosarcoma	[169]
ABCA5	Membrane-associated proteins that transport various molecules across intracellular and extracellular membranes	Melanoma	[169]
LGR5	Plays a role in formation and maintenance of intestinal stem cells	Colon cancer	[170]
DNAJB8	Regulates chaperone activity and is also implicated as a cancer stem cell antigen	Renal cell carcinoma	[123]
DDX3X	ATPase activity and is involved in transcription, translation, and signaling	Multiple tumors	[171]
CD24	Sialoglycoprotein presented on mature granulocytes and B cells and modulated growth and differentiation	Pancreatic and colorectal cancer	[153, 154]

[a]Marker(s) expressed by CSCs/CICs and used for ex vivo selection of these cells
[b]Brief description of the biological function of the indicated proteins

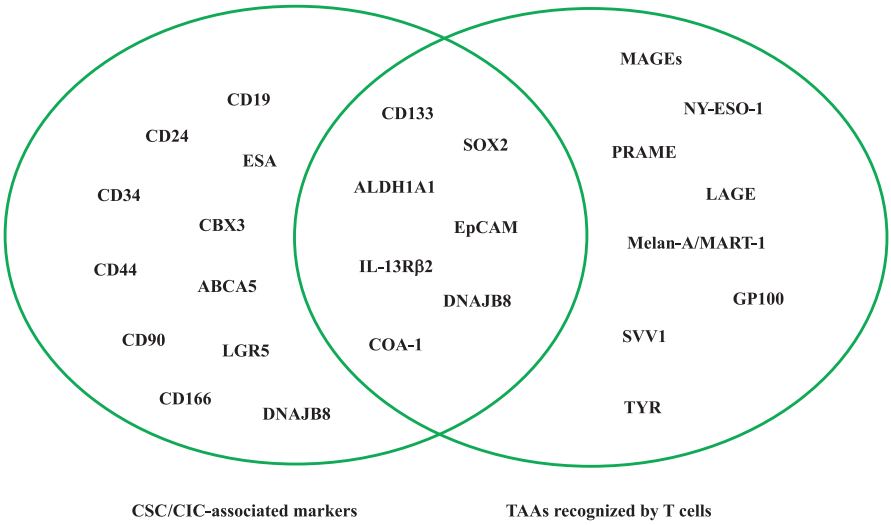

Fig. 1 Comparison of immune profiling of CSCs/CICs vs. differentiated tumor cells. Suboptimal immunogenicity of CSCs/CICs vs. differentiated cancer cells is determined by defective expression and functionality of HLA class I molecules, antigen-processing machinery (APM), NKG2D ligands, ligands of NK activatory receptors (e.g., NKp30 and NKp46), and suboptimal levels of TAAs. In addition, overexpression of immunomodulatory molecules has been shown to be associated with CSCs/CICs as compared to differentiated tumor cells. Taken together, these mechanisms lead to low immunogenicity of CSCs/CICs and escape, depending on the expression of specific immune-related molecules, from either adaptive or innate responses. CSC/CIC cancer stem cell/cancer-initiating cell; TME tumor microenvironment; mAb monoclonal antibody; MHC major histocompatibility complex; TAAs tumor-associated antigens; CT cancer testis; NKG2D ligands natural killer group 2D receptor ligands

relationship between the presence of inflammatory cells and the induction of EMT [35, 44]. These observations highlight the need to accurately consider the plasticity of CSCs/CICs and the possible EMT-driven de-differentiation of cancer cells into cells with "stemness" properties when isolating and tracking these cells. Importantly, the use of an immunodeficient xenograft model cannot provide a comprehensive picture of CSC/CIC properties and behavior and cannot reproduce the interaction of the tumor of origin with TME.

The ability to combine the detection of "stemness" core gene signature and functional properties can allow to link the presence of CSCs/CICs within tumors and patients' clinical outcome [18, 45]. In this context, immune responses may also be a determinant in the fate of CSCs/CICs, since they can either eliminate these cells or orchestrate the immune selection upon immune-permissive TME.

Immunotherapy represents the "fifth" therapeutic pillar, after surgery, chemotherapy, radiotherapy, and targeted therapy, for cancer. Knowledge gained on genetic and immune profiling of cancers led to the clinical development of novel tools and strategies that, for the first time, showed the improvement of OS and long-term responses in patients with different cancer types (for an overview, see Chap. 6).

However, the possible role of CSCs/CICs in the resistance to immunotherapy has also been reported [46]. These cells, as well as differentiated tumor cells, have developed multiple and evolutionary mechanisms to circumvent the immune attack [1, 2, 19, 47]. These cells display immunomodulating features allowing them to remain in quiescence, even following initial immune-mediated tumor recognition and regression, which drives their escape from immunosurveillance. This represents one of the mechanisms promoting tumor dormancy. Rare CSCs/CICs persisting from immune selection and upon interaction with tumor-permissive TME can exit from quiescence even years after initial tumor remission and activate the self-renewal program determining tumor recurrence, progression, and metastasization [5, 22, 47].

Therefore, immunological properties of CSCs/CICs need to be deeply elucidated in order to identify efficient immunotherapy approaches to target these cells and possibly to assess their role as prognostic tools for patients' responsiveness to therapies.

In this chapter, an overview of immunological mechanistic regulatory properties of CSCs/CICs and potential immunotherapy approaches for efficient targeting of these cells will be provided.

2 Major Histocompatibility Complex (MHC), Tumor-Associated Antigen Expression in CSCs/CICs, and Cell-Mediated Immune Responses

Tumor-associated antigens (TAAs) are recognized in the form of peptides in association with MHC molecules by T cells (see Chap. 6). Multiple TAAs have been found to be expressed by CSC/CICs isolated from tumors with different histological origins (Fig. 2) [1, 22, 47]. TAAs belonging to the shared/overexpressed category, including ALDH1A1, CD133, CEP55, COA-1, EpCam, HEATR1 IL-13Rα2, SOX2, and DNAJB8, have been reported to be recognized by T cell-mediated responses (Fig. 2) [48–58]. However, these TAAs do not represent CSC/CIC-specific antigen. They represent antigens overexpressed by some epithelial tumors and, in some cases, shared with normal tissues. Upon wide investigation of these TAAs in Phase I/II vaccine clinical studies in cancer patients, their low immunogenicity has been revealed as the major cause for their limited clinical efficacy [59, 60]. It has been well demonstrated that the reason for the low immunogenic potency of these TAAs is the presence in vivo in cancer patients of tolerogenic T cells recognizing these self-antigens.

In addition, defective expression of MHC/peptide complexes has been described as a common mechanism occurring in tumors to escape from antigen-specific T cell responses [61]. Although this represents a key mechanism mediating the interactions between T cells and tumor cells, scant information is available regarding MHC molecule and antigen-processing machinery (APM) in CSCs/CICs (Fig. 2).

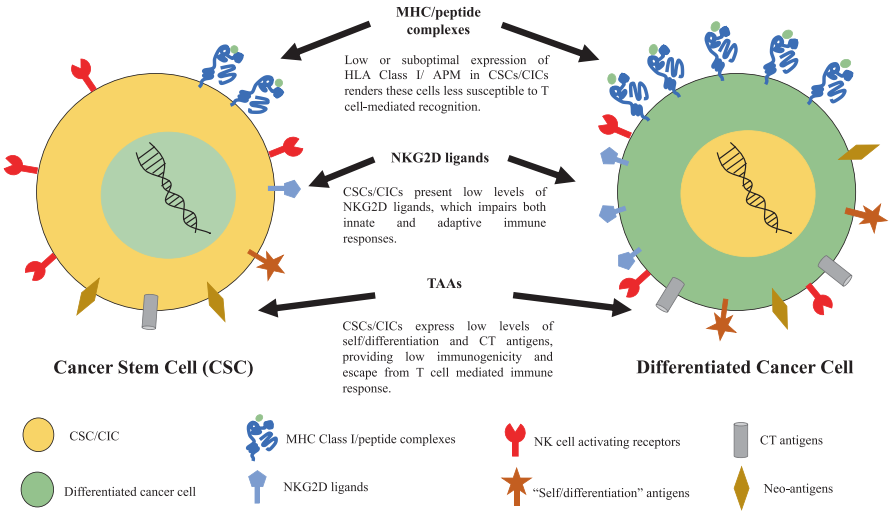

Fig. 2 CSC/CIC markers and antigens eliciting TAA-specific T cell-mediated responses. A variety of markers have been identified as expressed by CSCs/CICs, depending on their histological origin, and have been used for ex vivo isolation of these cells. In addition, multiple TAAs have been isolated as target molecules of T cell-mediated immune responses, representing either self/differentiated antigens (e.g., Melan-A/MART-1, Gp100, Tyr, SVV1, CD133, SOX2, ALDH-1, COA-1, EpCAM, and COA-1) that are expressed also by normal cells of the same tissues or CT antigens (e.g., MAGE, LAGE, PRAME, and DNAJB8) that are detectable only on normal cells of the testis and placenta. Few of these molecules are members of both categories and showed ability to elicit antigen-specific reactivity against CSCs/CICs, as indicated in the overlapping area of the two ellipses. CT cancer testis; TAAs tumor-associated antigens

Defective expression of the component of APM and HLA class I molecules has been reported in CSCs/CICs [47, 58, 62, 63] (see Fig. 2). Suboptimal or negative detection of APM components in "stem-like" cells isolated from glioma, melanoma and colorectal cancer (CRC) comprises proteasome and immunoproteasome subunits, peptide transporters and chaperone molecules that are involved in the assembly of β2-microglobulin (β2m) with HLA class I heavy chains, the loading of HLA molecules with TAA-derived peptides and their transportation to the cell surface [2, 58, 62–65]. Of note, it has been documented that cells resembling CSCs/CICs were found to be enriched in tumor lesions of patients failing to respond to chemotherapy and with reduced overall survival [63, 66], suggesting that CSCs/CICs resistant to chemotherapy can survive within tumor lesions [63, 66].

These findings can have relevance for the outcome of immunotherapy treatments for cancer patients, since defects in the expression of HLA class I and/or APM components could confer to CSCs/CICs the ability to escape from T cell-mediated responses, leading to resistance to these therapeutic interventions (Fig. 2). The mechanisms responsible for abnormal expression of MHC molecules by CSCs/CICs are mostly unknown; nevertheless, examples of modulation of these molecules upon IFN treatments of these cells have been shown [58, 62]. This finding is compatible with the possibility that the defective HLA class I APM component

expression is caused by abnormalities in the mechanism(s) which regulate their expression. Patients with this type of abnormality are likely to benefit from therapies with epigenetic strategies. On the other hand, in other cases, the HLA class I APM component expression and/or function in CSCs/CICs could not be restored by treating these cells with IFN-α or IFN-γ [58, 62] .

Along this line, preferential selection of TH2-type T cells was observed upon culturing in vitro of CSCs/CICs with autologous peripheral blood mononuclear cells (PBMCs) [58, 62]; this phenomenon was associated with suboptimal expression in these cells of HLA class I molecules [58, 62]. Therefore, MHC expression by CSCs/CICs should be evaluated for each patient in order to predict their eligibility for immunotherapy treatments aimed at eliciting TAA-specific T cell-mediated responses.

The defective expression of HLA class I and APM molecules is not a common feature of CSCs/CICs; these molecules have been detected in glioma- and melanoma-derived cells with "stemness" properties [64, 65, 67, 68], suggesting that the heterogeneity in tools and methods in isolating these cells as well as their plasticity can also affect their immunologic profile.

Low expression of HLA class I molecules on CICs/CSCs can render these cells optimal target for natural killer (NK) cells. Upfront of defective MHC and APM expression, the efficient detection of CSCs/CICs of ligands of NKG2D or activating receptors is required for the engagement of their receptor and eliciting of NK-mediated responses [64, 65, 67, 69] (Fig. 2), although downmodulation of NKG2D ligands on glioma-derived CSCs/CICs has been reported [62].

Antigens generated by tumor-specific somatic mutations, neoantigens, represent highly immunogenic TAAs that can elicit anti-tumor adaptive immune responses [70–73]. These results confirm previously evidences in mouse models of the relevance of private antigens for tumor rejection [74]. Of note, CSCs/CICs isolated from CRC, harboring somatic mutation in the SMAD4 gene, can efficiently induce, upon in vitro co-culturing with autologous PBMCs, T cell responses specifically recognizing the epitope derived from this neoantigen [75] (Fig. 2). Thus, further investigations are warranted to identify CSC/CIC-associated neoantigens in order to efficiently drive anti-tumor responses to efficiently targeting these cells. It needs to be highlighted that in order to achieve eradication of CSCs/CICs through this antigen-specific approach, optimal expression of HLA class I and APM is required. An integrative patient-specific analysis of antigenic and MHC molecule profiling should be developed with the goal to generate antigen-specific efficacious immunotherapy targeting both CSCs/CICs and differentiated tumor cells.

3 Antibody-Mediated Targeting of CSCs/CICs

The suboptimal expression of MHC/peptide complexes and APM components by CSCs/CICs, resulting in the impairment of antigen-specific T cell responses, can be overcome by targeting these cells with antibodies (Abs) that can recognize antigens

expressed on the membrane of cancer cells independently on MHC molecules and mediate tumor killing [76].

Abs have been developed to target antigens expressed by CSCs/CICs, although these TAAs are detectable also on differentiated tumor cells and, in some cases, on normal cells, including carbonic anhydrase IX (CAIX), CD133, chondroitin sulfate proteoglycan 4 (CSPG4), ERBB2, IL-13Rα2, and EGFRvIII [1, 2, 77].

CSPG4 is expressed on both differentiated cancer cells and CSCs/CIC, isolated based on the expression of ALDH-1^{bright} cells from glioma, head and neck cancer, triple-negative breast cancer (TNBC), and melanoma [60, 78]. This antigen can be upregulated on both CSCs/CICs and differentiated cancer cells upon culturing these cells under hypoxic conditions and, then, resulting in increased susceptibility to Ab treatment [60, 78]. Of note, CSPG4 is expressed by activated pericytes in TME [79], but is not detectable on pericytes at other anatomic sites. Therefore, the targeting of this antigen can lead to inhibition of tumor-associated angiogenesis and elimination of tumor cells, including cells displaying "stemness" properties [80] without causing side effects and toxicities [81–83].

4 CSCs/CICs Display Immunomodulating Properties

4.1 Soluble Factors, Cytokines, Chemokines, and Adhesion Molecules

Immunomodulating properties of CSCs/CICs have been documented in different studies, showing similarities with immune-privileged normal embryonic, hematopoietic, and mesenchymal stem cells [1, 84, 85]. Tumor cells with "stemness" properties can release multiple immunosuppressive cytokines or soluble factors, such as Galectin-3, GDF-15, IL-10, IL-13, PGE2, and TGF-β (Table 2), that can induce the TME resistance to immune responses [1, 2, 86]. Through the release of IL-10, IL-13, and TGF-β, CSCs/CICs can drive the differentiation of immune cells endowed with regulatory functions of T cells, such as T regulatory cells (Tregs) and myeloid suppressor cells (MDSCs) [87, 88]. Moreover, CSCs/CICs can secrete pro-inflammatory cytokines, e.g., IL-6, IL-8, and IL-13 (Table 2), thus contributing to the maintenance of the "niche" [89]. Among cells belonging to innate immunity, e.g., macrophages and dendritic cells (DCs), MDSCs can also promote tumor growth [90]. It has been shown that tumor-infiltrating macrophages (TAM) at tumor sites can regulate "stemness" properties of cells and, through activation of STAT3 (Table 2), contribute to the survival and proliferation of CSCs/CICs [91, 92]. CSCs/CICs can promote tumor-associated neo-angiogenesis by releasing VEGF [93]. This TME can actively cross-talk with CSCs/CICs representing the soil to recruit or to induce the differentiation of immunosuppressive cells [94]. TAMs can also modulate the phenotype and functional activity of antigen-specific T cells [95]. The presence and frequency of MDCS at tumor sites have been shown to correlate with patients' survival and responsiveness to immunotherapy [95].

Table 2 Immunomodulatory molecules associated with CSCs/CICs

Category[a]	Immunomodulating molecule	Function[b]	Expression in CSCs/CICs vs. differentiated tumor cells
Soluble factors/ enzymes	IL-4	TH2-type differentiation	Overexpressed
	IL-10/IL-13	Inflammatory cytokines	Overexpressed
	TGF-β	Regulating cell differentiation, development, and growth	Overexpressed
	PGE2	Lipid molecules regulating immunity and inflammation	Detected
	GDF-15	Contributes to proliferation and immune escape	Overexpressed
	STAT3	Regulates CSCs/CICs maintenance and proliferation	Overexpressed
	Galectin-3	Impairment of T cell responses	Overexpressed
	CD200	Involved in immunosuppression and regulation of anti-tumor activity.	Overexpressed
	IDO	Immunomodulatory enzyme produced by APC and tumor cells	Overexpressed
Immune checkpoint	PD-L1	Ligand of PD-1	Overexpressed
	B7-H3/B7-H4	Inhibition of T cell immune responses	Overexpressed
MicroRNA	Multiple types	Regulation of the expression of genes involved in "stemness" functions and immunological pathways	Differentially expressed

[a]Type of molecules
[b]Principal activity of the indicated molecules

The chemokine receptor CXCL12, which is detected on tumor and stromal cells, plays also an important role in maintaining self-renewal of CSCs/CICs and in recruiting immune-suppressive cells in TME [96].

In leukemia, the binding of CD47, which is overexpressed in "stem-like" cells, to signal regulatory protein alpha (SIRPα) leads to inhibition of phagocytosis representing an additional mechanism of the impairment of innate immune responses [97]. In addition, CSCs/CICs can exert immune-suppressive mechanisms through membrane-bound molecules, e.g., IL-4 and CD200, by inhibiting the proliferation and anti-tumor reactivity of cytotoxic T cells (Table 2) [58, 98]. The neutralization through the usage of mAb directed to IL-4 can restore in vitro anti-tumor immune responses [58].

4.2 Immune Checkpoint, Enzyme, and Signaling Molecules

Cells with "stemness" properties also express immune checkpoint molecules (e.g., CTLA-4, PD-L1, B7-H3, or B7-H4; *see* Table 2) [1, 22, 88]. Of note, these immunoregulatory molecules have been shown to be expressed by normal stem cells as a mechanism to protect themselves from immune attack [1, 99, 100]. Along this line, normal mesenchymal stem cells (MSC) can also induce through immunomodulating mechanisms the differentiation of both innate and adaptive responses into immune suppressive cells [94].

The enzyme indoleamine 2,3-dioxygenase (IDO), which mediates the catabolism of tryptophan and, through the deprivation of this amino acid, can impair T cell-mediated immune responses, has also been found to be expressed by CSCs/CICs (Table 2) [22].

An interesting relationship has been observed between STAT3 and MDSCs; in pancreatic tumor, this molecule can induce monocytes to differentiate into myeloid subset with immune suppressive functions, and these latest cells can also play a critical role in the induction of EMT program and CSC/CIC formation [101]. In a leukemia model, upregulation of the immunoglobulin mucin-3 (TIM-3)/Galectin 9 pathway in CSCs/CICs can induce the differentiation of MDSCs and TAMs (Table 2) [102, 103], and, on the other hand, these immune cells can also sustain and induce the generation and proliferation of "stem-like" cells [102, 103].

These evidences highlight the need to further explore the immune profile of CSCs/CICs and their interaction with immune cells and TME in order to better understand the mechanisms contributing to immune escape of these cells and to identify efficient tools to target these immune-resistant cells.

5 Micro-RNAs (miRNAs)

miRNAs are short single-stranded, non-coding RNAs that play a relevant role in the post-transcriptional regulation of genes that determine major cellular functions including proliferation, self-renewal, and differentiation [104]. mRNAs are degraded upon complementary binding of miRNAs, leading to prevention of translation and gene silencing [104].

miRNA expression is regulated by cellular transcription factors; however, the mechanisms involved in orchestrating miRNA and gene expression are still mostly unknown. Up- or downmodulation of miRNAs, either, has been reported in tumor cells [104]. miRNAs MiR34a, MiR31, and MiR205 regulating the expression of oncogenes (e.g., c-Met, Notch, and CDK6) or tumor suppressors are commonly found with altered levels in CSCs/CICs isolated from glioma and prostate or head and neck cancer, respectively [104] (Table 2). In addition, some miRNAs can influence the "stemness" behavior of CSCs/CICs; reduced levels of miRNAs 451 and

199b-5p in in vitro cultured cells isolated from GBM and medulloblastoma display low expression of the "stem-like" associated markers, such as CD133, OCT4, and Nanog [104, 105]. On the other hand, increased expression of MiR199b-5 in medulloblastoma cells was associated with improved patient survival [105]. Few miRNAs have also been linked to chemotherapy resistance of CRC-derived CSCs/CICs [104]. Of note, miRNAs play an important role as key regulators of immunological functions [106] (Table 2). miRNA-199a can inhibit interferon (IFN)-mediated responses by blocking the nuclear receptor corepressor LCOR and preventing cellular differentiation of breast cancer-derived CSCs/CICs [107]. Downmodulation of miRNA-124 that can regulate the expression of STAT3 was reported in glioma tissues [108]. The restoration of the expression of this miRNA in CSCs/CICs from glioma results in the inhibition of STAT3 and detection of anti-tumor T cell-mediated immune responses [108].

6 CSCs/CICs in Tumor Dormancy and Immune Evasion

The mechanisms that regulate tumor dormancy have not been fully dissected. Tumor cells can remain occult within tissues although clinical responses to therapeutic interventions are observed. These cells cannot be detected before the recurrence and/or progression of tumor, which can occur even years following the diagnosis and initial clinical responses. This phenomenon has been defined as tumor dormancy [5]. The quiescence of cells is one of the determinants of tumor dormancy, and the ability of CSCs/CICs to cycle between this status and quiescence indicates that these cells can be responsible for tumor dormancy. In addition, the interaction of tumor cells with TME and evasion mechanisms from immune responses are key factors leading to tumor dormancy [5]. In particular, the immunological profile and immunogenicity of cancer cells can shape the efficacy of anti-tumor immune responses; therefore, the prevalence of immune evasion mechanisms can promote tumor recurrence and progression. CSCs/CICs represent rare cells endowed with resistance to therapies and the ability to remain in quiescent state and thus are the major candidate to orchestrate tumor dormancy and recurrence [9]. Gene expression profiling of quiescent cells isolated from breast cancer revealed shared molecular traits with "stemness" [14]. Moreover, through their immunomodulatory ability and low immunogenicity, CSCs/CICs can evade from immune responses and remain dormant until changes in TME provide signaling to re-enter in self-renewal, causing neo-formation of tumor and metastasization [1, 2, 4, 109–111]. AML represents a good example of coexistence within tumors of "stem-like" cells that display differential relapse patterns [112]. Therefore, further characterization of the immunological profile of CSCs/CICs and their cross-talk with TME are warranted to better understand their role in tumor dormancy.

7 Targeting CSCs/CICs with Immunotherapy

Immunotherapy strategies to target CSCs/CICs and the differentiated components of tumors are aimed at eliciting TAA-specific CTL and to alter immunosuppressive TME.

A brief introduction to different immunotherapy approaches that have been developed over the last two decades is provided in Chap. 6 of this volume. Different immunotherapy interventions have been explored to target CSCs/CICs (Fig. 3), although they are still in preliminary evaluation. The most promising immunotherapies targeting CSC/CIC that have been developed at least in experimental settings are summarized below.

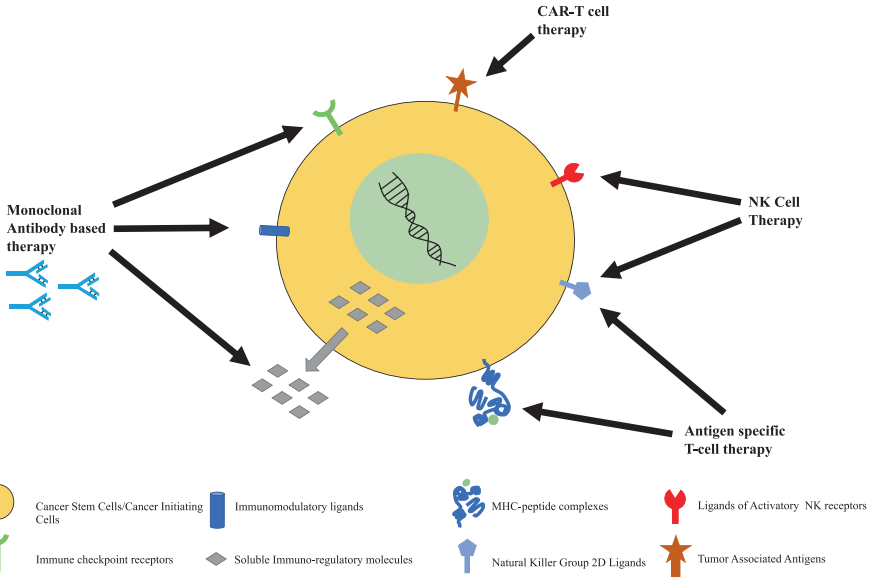

Fig. 3 Immunotherapeutic strategies to eradicate CSCs/CICs. Immune-based strategies can be exploited to target CSCs/CICs which are aimed at (i) eliciting or enhancing innate or adaptive cell-mediated immune responses; (ii) subverting the immunosuppressive TME through either antagonist or agonist mAbs specific for immune checkpoint molecules; (iii) inhibiting immunomodulating molecules through blocking mAbs or small inhibitors; (iv) ACT with T cells engineered with either high-affinity TCR or CAR specific for TAAs expressed by CSCs/CICs and differentiated tumor cells. CSC/CIC cancer stem cell/cancer-initiating cell; TME tumor microenvironment; mAb monoclonal antibody; MHC major histocompatibility complex; TAAs tumor-associated antigens; ACT adoptive cell therapy; TCR T cell receptor; CAR chimeric antigen receptor; NKG2D natural killer group 2D receptor ligands

8 Cancer Vaccines

T cell responses against self/shared TAAs, including ALDH1A1, CD133, CEP55, COA-1, EpCam, HEATR1 IL-13Rα2, and SOX, have been reported (Fig. 1) to target either in vitro or in mouse models CSCs/CICs. Nevertheless, these types of therapeutic strategies have been extensively investigated in Phase I/II clinical trials based on vaccination with TAAs of cancer patients [59]. Limited clinical benefit from these therapeutic strategies has been observed (10–20%) [59], although circulating antigen-specific T cell responses were detectable in these cancer patients [59]. The usage of either antigens or peptides derived from self/differentiation-protein for cancer vaccines represents the major cause of their low immunogenicity, since tolerogenic T cells recognizing this category of TAAs are present either in the circulation or at the tumor site, resulting in less efficient anti-tumor T cell-mediated immune responses. In addition, these TAAs have been shown to be expressed also at suboptimal levels by CSCs/CICs; therefore, cancer vaccines based on the usage of self/differentiation antigens do not represent an efficient strategy to target tumor cells with stemness properties [1].

Dendritic cells, which represent professional antigen-processing cells (APCs), have been used as a promising tool to elicit in vivo antigen-specific T cell responses [113–116]. CSCs/CICs have been used as sources of antigens upon cell lysates and to be loaded on DCs in syngeneic immunocompetent mice of melanoma or prostate TRAMP tumors leading to inhibition or delaying of tumor growth or tumor destruction and generation of antigen-specific T cells [117–120].

Additionally, a Phase I/II clinical study of vaccination of GBM patients with DCs loaded with CSC/CIC-lysates showed improvement of patients' progression-free survival [121, 122]. Of note, clinical responses of these patients were associated with increase in the circulation of the frequency of NK cells [121, 122]. Therefore, these data warrant that vaccination with multiple antigens loaded on DCs can represent a promising tool to achieve efficient targeting of CSCs/CICs. Strong anti-tumor activity was registered by vaccination of mice with DCs transfected with plasmids encoding for DNAJB8 antigen [123]. Nevertheless, these types of cancer vaccines can elicit anti-tumor T cell-mediated responses only when both CSCs/CICs and differentiated tumor cells express efficient levels of HLA class I and APM molecule. Therefore, this assessment at the level of tumor lesions should be performed as one of the criteria to select cancer vaccines as a therapeutic strategy for cancer patients.

9 Innate Immune Responses

NK cells represent the principal innate anti-tumor immune responses and are able to recognize and kill tumor cells independently of MHC expression. The absence or low levels of MHC molecules, when efficient levels of ligands for NK activator receptors are expressed, should render CSCs/CICs susceptible to NK cell-mediated

reactivity, as shown in melanoma, glioma, and CRC [64, 65, 67, 69, 124] (Figs. 2 and 3). Nevertheless, CSCs/CICs derived from glioblastoma can display suboptimal levels of these ligands as an additional mechanism of escape from immune responses [62]. These evidences highlight also that the immunological profile of CSCs/CICs can vary depending on the tissue of origin and their plasticity; therefore, the role of NK cell-mediated immune responses toward cancer cells with "stemness" properties should be better assessed along with their phenotypic characterization of MHC molecules and of ligands of NK receptors.

10 Immune Checkpoint Blockade and TME Targeting

Immune checkpoints represent molecules that play an important role in either promoting or inhibiting innate and adaptive immune responses [125–128]. The clinical development of immune checkpoint blockade agents, such as mAbs targeting CTLA-4 and PD-1/PD-L1 signaling, represented a breakthrough of immunotherapy, registering for the first-time improvement of survival for cancer patients with different advanced and aggressive types of tumors [125–128]. Nevertheless, a significant proportion of cancer patients either are unresponsive or develop resistance to these therapies [125–128].

Interestingly, expression of immune checkpoint molecules has been reported in CSCs/CICs isolated from different types of tumors (e.g., glioblastoma, CRC, breast and gastric cancer) [58, 62, 129, 130] (Table 2 and Fig. 3), suggesting that the blockade of these molecules could represent a strategy to efficiently targeting these cells. However, it has been shown that clinical success of immune checkpoint blockade is associated with mutational burden of tumors [71, 131, 132], and in addition, lack of clinical responses to these therapies is dependent on the absence of expression of HLA class I molecules by tumor cells [133, 134]. Thus, based on the low immunogenicity of CSCs/CICs, the choice of this therapeutic option could fail to cause destruction of these cells.

In a mouse model of vaccination of bladder cancer with a vaccine based on streptavidin-conjugated granulocyte-macrophage colony-stimulating factor (SA-GM-CSF) surface-modified CSCs, the upregulation of PD-L1 on tumor cells along with the induction of CTL-mediated immune responses was observed [135]. The combination of this vaccine with PD-1 checkpoint blockade improved the T cell-mediated elimination of tumors and the survival of mice [135].

These results suggest that the combination of cancer vaccines targeting CSCs/CICs with immunomodulating agents can lead to efficient eradication of tumors and enhancement of cell-mediated immune responses. Of note, it has been demonstrated that a connection exists between PD-L1 expression and maintenance of "stemness" properties in breast cancer cells, through the regulation of PI3K/AKT signaling that induces expression of stem cell-related protein (e.g., OCT4 and NANOG) [136]. Therefore, future studies are needed to better dissect the mechanisms of expression of immune checkpoint molecules by CSCs/CICs and their exploitation in therapeutic regimens targeting these cells.

As previously described, CSCs/CICs can secrete IL-10, IL-13, and TGF-β (Table 2 and Fig. 3) leading to the differentiation of T cells and myeloid cells in immunosuppressive subsets such as T regulatory (Tregs), myeloid-derived suppressor cells (MDSCs), or DCs with suppressive functions [1, 2, 86, 87, 89, 137, 138]. These cells and the presence of inflammatory cytokine and immunoregulatory factors contribute also to the formation of CSC/CIC niche. In addition, the infiltration of macrophages with suppressive functions, tumor-associated macrophages (TAM), at the tumor site can promote and sustain "stemness" properties within tumor cell subpopulations [139]. STAT3 represents a crucial molecule for both the regulation of proliferation and survival pathways of CSCs/CICs [91, 92] and for the cross-talk between TAM and CSCs/CICs, resulting in the TAM-mediated impairment of anti-tumor immune responses [95, 140].

The chemokine receptor CXCL12, which is expressed by different cellular components of TME, can mediate the recruitment at tumor site of Tregs, MDSCs, and suppressive DCs [96] as well as the maintenance of CSC/CIC self-renewal [96]. Indeed, the inhibition of the CXCL12/CXCR4 pathway resulted in the blocking of tumor growth and in the activation of T cell-mediated immune responses [96]. The blockade in vitro or in xenograft models of IL-8 receptor CXCR1 using mAbs or small-molecule inhibitors showed a selective depletion of CSCs/CICs within human breast cancer cell lines and, subsequently, induction of apoptosis [141].

Breast cancer cells upon development of chemotherapy resistance can activate both Wnt/β-catenin and NF-κB pathways, leading to the production by cancer cells of pro-inflammatory cytokines and to the enrichment of cells with "stemness" properties [142]. Blocking with small inhibitors targeting Wnt/β-catenin and NF-κB and anti-IL-8 mAb reduced the detection of CSCs/CICs within tumor lesions [142].

Taken together, these evidences suggest that the targeting of either suppressive immune cells or immunosuppressive or pro-inflammatory factors could result, on the one hand, in modulating "stemness" properties of cancer cells and, on the other hand, in restoring anti-tumor T cell-mediated immune responses.

The inhibition of IDO production by CSCs can represent an additional strategy to target CSCs/CICs, as recently demonstrated by one of the authors (*Maccalli* et al.*, manuscript in preparation*). Nevertheless, melanoma patients treated in the context of a Phase III clinical study with the combination of immune checkpoint blockade (anti-PD-1 mAb) with the IDO inhibitor epacadostat developed high-grade toxicities without improvement of progression-free survival and overall survival. This study has been terminated in advance. Further investigations are warranted to better understand the mechanisms of action of IDO and to optimize the regimens of administration of IDO inhibitors or their combination with other agents.

11 Adoptive Cell Therapy

Neoantigens represent strong immunogenic antigens for immunotherapy [72, 143] and promising TAAs for eliciting T cell-mediated responses to target CSCs/CICs [144]. These tools could also be exploited to generate ex vivo T cells engineered

with high-avidity T cell receptor (TCR) to target neo-antigen expressing CSCs/CICs [145] (Fig. 3). However, the clinical efficacy of this adoptive cell therapy (ACT) strategy could be limited by the evidence of suboptimal antigen processing and presentation by CSCs/CICs leading to the failure in recognition by these CTLs of cells with "stemness" properties. The combination of ACT with immunomodulating agents (e.g., IFNs or demethylating agents) [146] could represent a valid strategy to upregulate HLA class I APM components.

The better characterization of CSCs/CICs immune profiling could represent a tool for patients' stratification and decision of therapeutic options. Assessment of the efficacy of the combination of ACT with immunogenic cell death-mediated chemotherapy, which can upregulate the expression of HLA and APM molecules on CSCs/CICs, should be performed through in vitro and in vivo models [147, 148].

T cells engineered with chimeric antigen receptors (CARs) are recombinant receptors combining epitope-specific components derived from an antibody with intracellular signaling domain from T cell activation and costimulatory domains [145]. The unprecedented clinical success of CAR-T cell-based therapies leads to FDA and EMA approval of these tools for therapeutic treatments of advanced and therapy refractory $CD19^+$ pediatric and adult B cell malignancies [149]. The advantage of CAR-T cells is the isolation of highly activated T cells that recognize tumor antigen expressed on the membrane of tumor cells in HLA-independent manner. The use of CAR-T cells to target solid tumors is still under development, due to the induction, in some cases of severe toxicities. This was mainly due to the usage as source of antigens of TAAs shared with normal cells, such as the carbonic anhydrase IX (CAIX) or *ERBB2* antigens [145]. Promising results have been obtained by the administration of CAR-T cells specific for mesothelin to patients with malignant mesothelioma and pancreatic cancer [150]. IL-13Rα2-, CD133-, CSPG4-, and HER-2-specific CAR-T cells have been developed to target stem-like cells from different types of tumors overexpressing these antigens [34, 54, 55, 145, 151, 152] (Fig. 3). Neoantigens could represent good candidates to generate CAR-T cells targeting both CSC/CIC and non-stemness component of tumors without cross-reactivity with normal cells.

Therefore, further investigation to achieve a comprehensive characterization of genomic and immunological profiling of CSCs/CICs is needed to identify tools to efficiently target tumor cells with "stemness" properties and to subvert their immunogenic profile.

12 Conclusions and Perspectives

The advances in the identification of altered molecular pathways regulating "stemness" properties of cancer cells have contributed to understand the mechanisms regulating maintenance and survival of CSCs/CICs as well as their immune evasion properties and interactions with TME. The heterogeneity of tumors and their evolution and plasticity mediated by the cross-talk with TME are responsible for

immune responsiveness and patients' clinical outcome. The efficient targeting of CSCs/CICs will lead to complete eradication of tumors; however, none of the available therapeutic options have shown to be able to selectively target these cells. Further efforts need to be driven to fully understand genomics, epigenetics, and immunological mechanisms of CSCs/CICs in order to design therapeutic interventions aimed at reverting immune suppressive cancer cells and TME into efficient immunosurveillance.

The combination of cancer vaccines or ACT targeting multiple CSC/CIC antigens with either immunomodulating agents (Fig. 3), epigenetic drugs, or standard therapies represents promising approaches, although further preclinical and clinical investigations are required.

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Conflict of Interest Declaration All the authors have no conflict of interest to disclose.

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