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Cancer stem cell targeted therapies

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Abstract

In normal dividing tissues, cell homeostasis is maintained by rare cellular elements, the stem cells, that have the unique property of self-renewal and differentiation to generate a population of functionally mature tissue elements. Recent studies carried out in the last three decades support the existence of stem cells also in tumors, the so-called cancer stem cells. Cancer stem cells have the property of initiating and maintaining tumor growth, are able to self-renew and to differentiate, are the main drivers of intra- and inter-tumoral heterogeneity and the main cellular mediators of drug resistance, leading to tumor recurrence and metastasis. Cancer stem cells can be identified in many tumors according to specific immunophenotypic features, but cancer stemness cannot be defined as a fixed property, due to cancer plasticity.

For these properties, cancer stem cells represent attractive targets for developing new anti-cancer therapies and there is supporting evidence that the combination of conventional anticancer therapies with drugs targeting cancer stem cells could lead to cancer eradication. Ongoing studies in some tumors strongly support the clinical utility of developing efficient strategies of cancer stem cell targeting.

Key words

- cancer
- cancer stem cells
- targeted therapy
- stem cells
- leukemia
- solid tumors

INTRODUCTION: THE CANCER STEM CELL THEORY

Growing evidences indicate that tumor cells display stem cell-like properties, including the capacity to initiate and maintain tumors, long-term self-renewal and repopulation capacities and typical gene expression profiles. The cancer stem cell (CSC) theory, known also as the hierarchical model, was proposed several years ago and suggested that cancers, as well as normal tissues, are maintained through the differentiation of few cells with stem cell features, distinct from other subpopulations; like normal stem cells, cancer stem cells are capable of self-renewal and long-term maintenance of tumors, through their capacity to differentiate into a progeny that is non-tumorigenic [1]. The cancer stem cell theory explains many clinical observations made through the study of cancer patients, such as that the large majority of tumors relapse after an initial therapy based on chemical drugs or on radiations and the phenomenon of tumor dormancy [1]. At variance with this theory, the stochastic model proposes that all cells have the ability to be tumorigenic and capable of self-renewal or differentiation, thus generating a condition of tumor heterogeneity; the potential of each tumor cell can be influenced in a variable way by genetic and epigenetic factors related to the tumor microenvironment, resulting in the generation of phenotypically heterogeneous tumor cells [2]. The clonal evolution model describes tumor evolution that may occur both in the context of the CSC model and stochastic model, proposes that mutant tumor cells with a proliferative advantage outgrow other cells and progressively undergo an expansive process [2]. The current view of CSCs bypasses these divergent models and takes into account the cancer stem cell plasticity, related to the peculiar property of these cells to translate between non-CSC and CSC properties, and the identity of CSCs, considered non as a distinct subpopulation of tumor cells, but as a state or process by which cancer cells gain some peculiar malignant characteristics, including tumorigenic capacity, chemoresistance and metastatic potential [2]. Thus, according to this new view, there is coexistence of multiple cellular states within the cells that functionally display properties of CSCs [2].

LEUKEMIC STEM CELLS

The assay currently used to assess CSC activity was based on xenotransplantation assays performed into immunodeficient mice. This assay was derived from historical studies on hematopoietic stem cells (HSCs) and based on the evaluation of the capacity of a given cell to reconstitute hematopoiesis following transplantation into lethally irradiated recipient immunodeficient mice and currently considered as an assay of stemness [3]. This assay was initially applied to the characterization of CSCs in acute myeloid leukemia (AMLs), providing evidence that these cells display stem cell membrane markers ($CD34^+CD38^-$) identical to those observed in normal HSCs [4].

The development of the studies on the characterization of leukemic stem cells was of fundamental importance because it was propaedeutic for the study of CSCs

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of other tumors. Particularly, these studies have contributed to define the main determinants and parameters essential for leukemic stem cell detection. Thus, it was shown the essential role of the xenotransplantation assay for leukemic stem cell identification, related to the immunodeficient mouse strains (NOD, NOD/SCID or NOD/SCID/IL2R-γ mice, with the more immunodeficient mice being more recipient for engraftment and growth of leukemic stem cells) [reviewed in 5] or to the type of leukemic samples assayed for leukemic stem cell content [6] or to the procedure of the xenotransplantation assay (thus, using a humanized bone marrow ossicle xenotransplantation model 87% of AML samples engrafted to high levels of bone marrow chimerism [7]. Thus, these studies have highlighted that the frequency of leukemic stem cells (LSCs) varied consistently among various leukemic samples and xenotransplantation models, thus indicating that the evaluation of LSCs is highly dependent on the model used [8].

Furthermore, the studies carried out on the characterization of LSCs have contributed to show that these cells are heterogeneous and can be identified according to a membrane phenotype. Particularly, according to the expression of CD34, an antigen expressed on normal HSCs and hematopoietic progenitor cells (HPCs), AMLs can be subdivided into $CD34^+$ (about 75% of cases) and $CD34^-$ (about 25%). Basically, these studies showed that LSCs are rare cellular elements accounting for about 1 per 1×10^6 leukemic blasts and characterized for their property to give rise to leukemic engraftment that could be propagated for multiple serial transplants, thus supporting that these cells have self-renewal properties and produce a non-LSC cell progeny [9].

LSCs have been explored in detail in $CDF34^+$ AMLs, showing that these cells were predominantly resident in the $CD38^+/CD38^-$ fraction, but in 50% of cases were present also at the level of $CD34^+/CD38^+$ fraction and, more rarely, at the level of $CD34^-$ cells [9]; in the majority of these patients it was observed the coexistence of $CD34^+/CD38^-$ cells resembling stem/multipotent progenitor cells and of $CD34^+/CD38^+$ fraction, resembling granulocyte-macrophage progenitors [10]; in line with these observations, the $CD34^+/CD38^-$ fraction possesses a higher LSC content and $CD34^+/CD38^-$ cells have a gene expression profile different from $CD34^+CD38^+$ cells [10]. These findings have promoted parallel studies aiming to define the immunophenotypic features of $CD34^+/CD38^-$ LSCs, providing evidence that several cell membrane markers are upregulated on these cells, including CD123, CD47, TIM3, CD96, CLL-1, GPR56 and CD93 [11, 12]. CD123 was extensively explored in AMLs, showing that its expression increases at relapse and is particularly overexpressed in some AML subtypes, such as AMLs bearing FLT3-ITD (internal tandem duplication) mutations [13-15]. More recently, it was shown that CD99 is a cell surface protein frequently overexpressed on AML stem cells: high CD99 expression on AML blasts enriches for functional leukemic stem cells; furthermore, anti-CD99 mAbs exhibit anti-leukemic activity in AML xenografts [16].

Other studies have characterized LSCs in $CD34^-$ AMLs: these AMLs are characterized by a low CD34 expression (<2% of $CD34^+$ cells), <10% of undifferentiated leukemic blasts and are enriched in nucleophosmin1 (NPM1)-mutated AMLs. In $CD34^-$ AMLs, both $CD34^+$ and $CD34^-$ cells act as leukemic stem cells and display similar molecular properties, thus suggesting that $CD34^+$ and $CD34^-$ LSCs in these AMLs represent the same cells displaying plasticity in CD34 expression [17]. In line with these findings, in NPM1-mutated AMLs both some $CD34^-$ and $CD34^+$ cells are able to have the function of LSCs [18, 19].

The study of LSCs in NPM1-mutated AMLs allowed also to evaluate their prognostic impact (the presence of an increased level of $CD34^+/CD38^-$ cells together with the positivity for leukemic molecular-specific markers, such as mutated-NPM1 in minimal residual disease was a negative prognostic factor) [19]; furthermore, the study of LSC subpopulations in NPM1-mutated AMLs allowed to perform a reconstruction of the clonal architecture and dynamics of these leukemias with identification of a putative pre-leukemic subclone [20]. These studies unequivocally showed that LSCs responsible for leukemia development in mice bear NPM1-mutations, in line with the observation that these LSC subclones are responsible for leukemia relapse [21].

The studies on the characterization of LSCs in primary AML samples showed that their number is highly heterogeneous, ranging from 1 in 10 to 1 in 10^6 bulk AML cells and is significantly higher in poor and intermediate-risk AML patient samples at diagnosis than in the good-risk AML group [22].

Thus, these studies have shown the consistent heterogeneity in the phenotypes of engrafting AML stem cells; furthermore, it is evident that the AML LSCs engrafting immunocompromised mice do not necessarily represent the founder AML clones responsible for leukemia relapse. The heterogeneity of LSCs is further supported by a recent study showing that $CD34^+/CD38^-$ cells present in various $CD34^+$ AMLs are heterogeneous and correlates with genetic risk groups and outcomes [23]. Importantly, these authors showed that the AMLs bearing immature LSCs defined as $CD34^+/CD38^-/ALDH\ (aldheyde\ dehydrogenase)^{high}$ showed a shorter overall survival, event-free survival and complete remission rates [23]. In contrast, AML cells in which immature LSCs ($CD34^+CD38^-$) displayed more mature phenotypes were associated with better outcomes [23]. Therefore, the most immature phenotype of LSCs represents a clinically relevant biomarker of negative clinical outcome [23]. In line with these observations, AMLs displaying $CD34^+/CD38^-/ALDH^{high}$ LSCs have increased expression of LSC and HSC signatures, while AMLs with $CD34^+/CD38^-/ALDH^{low}$ cells are enriched for a progenitor signature [24]. These observations support the initial studies by Cheung *et al.* showing that high ALDH activity in AML blasts defines a subgroup of leukemias with adverse prognosis and high NOD/SCID engrafting potential [25]. The enrichment in stemness-related transcriptional programs in AMLs bearing $ALDH^{high}$ LSCs suggests that these leukemias derive from the malignant transformation of stem cells or immature progenitors and provides an explanation for the therapy resistance and

poor prognosis of these leukemias. The clinical significance of this immature LSC population in AML is strongly supported by a recent clinical study based on the treatment of older AML patients with the B cell lymphoma 2 (BCL-2) inhibitor Venetoclax in combination with the hypomethylating agent Azacitidine: this treatment resulted in deep and durable remissions, due to the targeting of LSCs through metabolic damage of these cells caused by disruption of the tricarboxylic acid (TCA) cycle [26].

CANCER STEM CELLS IN SOLID TUMORS

The studies carried out on AMLs provide a clear example of the evolution of the cancer stem cell model. The use of equivalent cell sorting and xenografting techniques, has led to the identification of cancer stem cells in various solid tumors, starting from breast cancer, where it was shown that as few as 100 $CD44^{+}CD24^{-/low}$ breast cancer cells initiate tumor growth when transplanted into immunodeficient mice [27], to colon cancer [28-30], brain tumors [31], prostate cancer [32], pancreatic cancer [33], ovarian cancer [34], lung cancer [35]. Basically, these studies have shown that CSCs isolated from various solid tumors are able to generate tumors in xenograft assays and generate in vivo in the immunodeficient animals non-tumor initiating cells, thus suggesting a hierarchical organization of these tumors [27-35]. The capacity to initiate and propagate tumor development varies considerably between different cells within a solid tumor and this variation is due to a hierarchical relationship between tumorigenic and non-tumorigenic cells [27-35]. These studies have also shown that there are many markers that enrich for cancer-initiating cells in solid tumors, including CD133 in various tumors, CD44 in breast cancer, LGR5 in colon cancer, ALDH1 in breast and gastrointestinal cancers, SOX2 in glioblastoma, but the identification of markers selective and sensitive for CSCs remains largely elusive.

The discordant findings in the phenotype and properties of CSCs in solid tumors may be also related to the experimental conditions and xenograft assays used to assess the tumorigenic potential. In this context, particularly challenging was the debate originated by the study and characterization of CSCs in human melanomas. In melanoma, cells separated according to the expression of the neural crest stem cell marker CD271/p75/NGFR displayed differing capacities for tumor formation in patient-derived xenografts (PDX) assays, CD271+ cells being more tumorigenic than CD271- cells [36, 37]. However, Quintana *et al.* have shown that using highly efficient PDX assays [38], 16 cell surface markers, including CD271, were found unable to identify melanoma cells with enriched tumorigenic potential [39]. Proposed reasons for the discordant results include differences in the PDX assays used and in other experimental procedures [39]. However, a more recent study by Boyle et al definitely supported the view that regardless of the assays used, melanoma does not follow a CSC model associated with CD271 expression [40].

The findings observed in melanoma, as well as the difficulties in defining reproducible phenotypes for these cells in the large majority of solid tumors supported the progressive shift from a model of cancer stem cells based on the normal tissue developmental hierarchies to a more nuanced model of stemness taking into account both the genetic and nongenetic influences contributing to the functional acquisition of stem-like properties by tumor cells and to generation of tumor heterogeneity [8] and the role of microenvironment which is a critical driver of heterogeneity, plasticity and evolution within the CSC population [41].

Given the limitations of marker-based selection and the plasticity of CSC state, it is evident that development of optimal assays to measure stem cell function in specimens of human tumors is strictly required. In spite these important limitations, a large number of studies, basically based on measuring tumor stemness through surface markers, have supported the view that high expression of CSC biomarkers in the large majority of solid tumors is a property associated with poor overall survival and/or disease-free survival compared with low or no expression of CSC biomarkers [42, 43]. Furthermore, elevated stemness biomarkers levels are associated with decreased tumor differentiation, increased metastasis and advanced tumor stage [42, 43].

CANCER STEM CELLS AND TUMOR INITIATION

The cancer stem cell model implies that human tumors can derive from the malignant transformation of tissue stem cells and that these disorders can be preceded by a stage of premalignant tumorigenesis occurring at the level of stem cells and generating a pre-malignant condition.

Studies carried out in many tumors strongly support the view that these tumors are initiated through the malignant transformation of stem cells. Initial studies carried out in AML patients showed that normal HSCs display one or two of the mutations observed in leukemic blasts and can be considered as preleukemic HSCs [43]. These findings have suggested that AMLs develop through the acquisition of serial mutations at the level of self-renewing HSCs and at the genomic mutational level it is suggested that AML genomes clonally evolve from founder mutations [44]. Other studies have shown that preleukemic HSCs harbor some, but not all, of the mutations observed in the bulk leukemic blasts [45]. Particularly, mutations in landscaping genes, involved in chromatin changes, such as DNA methylation, histone modification and chromatin looping are early events during AML development, while mutations in genes involved in proliferation control are late events [45]. Furthermore, $CD34^{+}$ cells at remission harbor preleukemic mutations [45]. This conclusion is supported by the observation that AML patients where preleukemic mutations were detected in purified stem/progenitor cells at diagnosis, nearly all of these preleukemic mutations were also detected in CD34+ cells and also in mature hematopoietic cells [45]. The presence of these preleukemic mutations at the level of differentiated cells strongly supports the view that these preleukemic HSCs retain a normal differentiative function and, together with normal HSCs surviving to induction chemotherapy, contribute to bone marrow reconstitution

during the remission phase [45]. These initial observations were now confirmed through the study of many AML patients in remission: about 40-50% of these AMLs in remission retained mutations with a variant allelic frequency ≥1-2% and mutation persistence was most frequent in DNMT3A, SRSF2, TET2 and ASXL1, genes whose mutations are associated with preleukemic stem cells [46, 47]. Thus, these studies provide clear evidence about the persistence during chemotherapy-induced remission of preleukemic clones, carrying a subset of AML-related gene mutations; the presence of these mutations during remission is associated with an increased risk of leukemia relapse [45-47].

In line with these observation, a recent experimental model based on double mutant NPM1/DNMT3A knock-in mice, reproduced a condition of AML progression mimicking leukemia development in humans, where leukemia is preceded by a period of extended myeloid progenitor proliferation and self-renewal [48]. This self-renewal can be reversed by a small molecule acting as an inhibitor of Menin-MLL binding [48]. These observations give support to the idea that subjects at high-risk of developing AML might benefit from a preventive targeted epigenetic therapy [48].

The discovery of clonal hematopoiesis provided a strong support to the stem cell mutation model of leukemia development. Clonal hematopoiesis is a common, age-associated condition characterized by the expansion of HSC clones carrying recurrent somatic mutations and occurring in individuals without diagnosis of hematological malignancies [49, 50].

Clonal hematopoiesis is associated not only with aging, but also with some age-related conditions, such as high risk of hematological malignancies, cancer mortality, cardiovascular diseases and inflammatory conditions. Thus, clonal hematopoiesis varies according to age from <1% in individuals younger than 40 years to 15-20% in old individuals (80-90 years) [51]. Clonal hematopoiesis is associated with about 1% annual risk of developing a hematological malignant condition and a 2-fold to 4-fold higher risk of developing coronary artery disease, stroke and cardiovascular death, independently of traditional risk factors for cardiovascular diseases [51, 52]. It is particularly important to point out that the impact of clonal hematopoiesis in the risk of developing a hematologic malignancy is greatly influenced by the context in which it occurs: this risk is low when clonal hematopoiesis occurs in healthy subjects, whereas is clearly higher when clonal hematopoiesis is found in the cancer population and its presence in this last setting represents an independent, cancer-related mortality risk [53-56].

Very recent studies have explored more in detail the clinical significance of gene mutations occurring in preleukemic stem cells, suggesting a distinction within the clonal hematopoiesis of a benign condition not evolving to a hematological malignancy and a preleukemic condition [57, 58]. Thus, Abelson *et al.* showed that pre-AML cases were distinct from control clonal hematopoiesis cases because display more mutations per sample, higher variant allele frequencies and enrichment of mutations in specific genes [57]. Desai *et al.* provided evidence that mutations in IDH1, IDH1, TP53, DNMT3A, TET2 and spliceosome genes increase the odds of developing AML; importantly, all subjects with TP53 mutations and IDH1 and IDH2 mutations developed AML [58]. It is important to underline that in this study the median time of AML development from the initial detection of clonal hematopoiesis was 9.6 years [50]. Although individuals with clonal hematopoiesis do not have alterations in the number of peripheral blood elements under steady-state conditions, under stress conditions, such as anticancer chemotherapy, delay the hematopoietic reconstitution and determine a clinical condition requiring more patient's transfusional support [59, 60].

Similar observations were recently reported for other tumors. Thus, human glioblastoma mutations were observed also in normal neural stem cells present at the level of the subventricular zone, away from the primary tumor [61]. This study provides the first direct evidence that neural stem cells in human subventricular zone are the cells of origin that develop the driver mutations of glioblastoma [61]. Recent studies have shown that somatic mutations and clonal expansions are observed at the level of most of normal tissues. Thus, studies of immunostaining of normal sun-exposed human skin showed the existence of small clones, composed by few hundred cells contained TP53 mutations [62]. More recent studies of deep sequencing carried out on skin tumor biopsies showed that sun-exposed skin cells display many somatic mutations related to events of ultraviolet mutagenesis; furthermore, positive selection in some cancer genes occurs, leading to clonal expansions [63]. Thus, by middle age, sun-exposed skin is composed by thousand clones with one in every four cells carrying a positively selected cancer mutated gene [63]. Other recent studies carried out in normal esophageal epithelium showed a mutational burden lower than that detected bin ultraviolet-exposed skin, but positive selection was stronger, driving the development of clones carrying mutations in cancer genes (such as NOTCH1 and TP53) colonizing most of the esophagus by mid age [64, 65]. Other recent studies showed tissue-specific mutation accumulation at the level of adult stem cells of colon, small intestine and liver [66]; furthermore, RNA sequence analysis revealed macroscopic somatic clonal expansion occurring at the level of many normal tissues [67].

These observations support the stem cell origin of human cancers and indicate that mutations occurring at the level of the stem cells of normal tissues represent an in initial event in cancer development, inducing the formation of premalignant clones *(Table 1)*.

Cancer stem cells also essential cellular drivers of tumor progression, chemoresistance and tumor relapse. Thus, studies of characterization of the properties of glioma stem cells have strongly supported a model of cancer stem cell-related tumor progression in which slow cycling cancer stem cells give rise to a more cycling progenitor cell population with pronounced self-maintaining capacities, which in turn generates non-proliferative more differentiated cells [68, 69]. Chemotherapy facilitates the expression of pre-existing drug-resistant glioblastoma stem cells [68, 69].

Table 1
Mutations observed in the preneoplastic lesions observed at the level of various tissues; the most recurrent mutations observed in the corresponding developed tumors are also shown (last column)

Tumor type	Cells of origin	Preneoplastic lesions	Mutations in preneoplastic clones	Mutations in developed tumors
Acute myeloid leukemia	Hematopoietic stem cells	Clonal hematopoiesis	DNMT3A, TET2, ASXL1	DNMT3A, NPM1, FLT3, IDH1, IDH2,
Esophageal carcinoma	Basal esophageal stem cells	Barrett's esophagus squamous dysplasia	NOTCH1, TP53, NOTCH2, FAT1, NOTCH3	TP53, NOTCH1, KMT2D, NFE2L2, FAT1, EP300
Colorectal cancer	Intestinal stem cell	Aberrant crypt foci	AXIN2, ERBB2, PIK3CA, ATM, FBXW7, ERBB3, CDK12	
Endometrial cancer	Endometrial stem cell	Endometrial hyperplasia	PIK3CA, ARHGAP35, FBXW7, PIK3R1, FOXA2, ZFHX3, ERBB2, ERBB3	PTEN, PIK3CA, ARID1A, PIK3R1, CTNNB1, CTFC, KRAS, RNF43, ARID5B, TP53
Lung cancer	Brochioalveolar epithelial stem cells	Atypical adenomatous hyperplasia	TP53, NOTCH1, FAT1, CHEK2, PTEN, ARID1A, ARID2, IDH1	
Skin cancer	Basal stem cell		TP53, NOTCHG1, FAT1, NOTCH2, NOTCH3	Squamous: NOTCH1, TP53, FLNB, NOTCH2, CDKN2A Basaloid: TP53, PTCH1, TERT, CDKN2A
Melanoma	Mature melanocytes/ Melanocyte stem cell	Benign nevus	BRAF	BRAF, NRAS, NF1, TERT, CDKN2A
Glioblastomna	Subventricular zone stem cells	Unknown	TERT, EGFR, PTEN, TP53, PDGFR, IDH1, NF1, PIK3CA	TERT, TP53, PTEN, EGFR, PDGFR, IDH1
Hepatocarcinoma	Dedifferentiated adult hepatocytes	Cirrhotic liver dysplastic hepatic nodules	PKD1, KMT2D, STARD9, APOB, ARSM1, ALB, ARID1A	TP53, CTNNB1, ALB, PCLO, FLG, CSMD3, XIRP2

The studies carried out in various models have also supported a great plasticity of the cancer stem cell properties at the level of the cellular elements composing a tumor. Thus, in human, as well as in murine, colorectal cancers, $LGR5^+$ cells were identified as cancer stem cells [70]; depletion of $LGR5^+$ cells from tumor organoids established from human biopsies determines an initial tumor regression, followed by tumor regrowth due to generation of new $LGR5^+$ cells from differentiated tumor cells, thus supporting a consistent plasticity of the CSC properties [70]. Studies in experimental models of colorectal carcinogenesis support a distinct role for different CSCs during tumor evolution: thus, tumor growth of the bulk tumors is maintained by $LGR5^-$ cells that continuously replenish $LGR5^+$ CSCs (in fact, $LGR5^+$ cell ablation cannot induce tumor regression); liver tumor metastases are driven by $LGR5^+$ CSCs [71].

Cancer stem cells are involved in relapse events, as evidenced by the studies on AML patients. The majority of AML patients relapse through a cellular mechanism involving leukemic stem cells belonging to a leukemic clone already present at the time of initial diagnosis [72]. Particularly, two types of AML have been identified: i) the first AML type contains a rare population of LSCs, with a stem-early progenitor-like immunophenotype; ii) the second AML type relapses through the main $CD33^+$ leukemic blast cell population and displays growth factor dependency [72]. The study of gene expression profile supports that these two AML relapsing subtypes are originated from different primary AMLs [72]. In all instances, the relapsing clone was characterized by an increased number of LSCs [72].

Relapse is an event where CSCs play a key role not only in hematopoietic tumors, but also in several solid tumors. Several studies support a role for CSCs in relapse of many solid tumors. The peculiar properties of CSCs explain in large part their role in tumor recurrence: cancer stem cells largely survive to chemo-radiotherapy and after these treatments enter into a condition of quiescence and dormancy for different period of time; however, following changes in their genome due to their intrinsic genetic instability and in the tumor microenvironment these cells are reactivated, become proliferative and are responsible for tumor recurrence [73]. This model of CSC-mediated cancer recurrence is supported by experimental studies and by observations in cancer patients undergoing standard treatments. Thus, concerning experimental models, particularly interesting was the observation made by Li *et al.* in lung cancer suspensive tumor model, showing that in this tumor high levels of cancer stem cells undergoing asymmetric self-replicative cell divisions in latent tumor are the key issue to reactivate this dormant tumor [74]. Thus, a change in environmental conditions, represented by a high level of serum insulin Growth Fcator-1, can induce the quiescent-to-proliferative, progressive tumor transition through promotion of CSC symmetric divisions [74].

Several observations made in cancer patients supported a role for CSCs in disease relapse in various solid tumors. Thus, Merlos-Suarez *et al.* observed that a gene signature specific for adult intestinal stem cells predicts disease relapse in colorectal cancer patients [75]. Roy et al showed that disease relapse in head and neck squamous cell carcinoma patients is associated with increased p38 MAPK expression in CSCs and p38-inhibited tumor cells show significantly reduced expression of CSC markers [76]. Sun *et al.* showed that quiescent CD13+ CSCs are enriched after chemotherapy in hepatocellular carcinomas and serve as a reservoir for disease recurrence [77].

CANCER STEMNESS

As above discussed, considerable controversy remains as to how best to define CSCs and the level to which the various cancers are organized at cellular level according to a hierarchical structure [8]. Growing evidences support the view that stem cell-associated molecular features, defined as "stemness", are a key biological determinant in cancer [8]. The stemness phenotype may reflect either the presence of CSCs in a given tumor or, alternatively, the acquisition of stem-like properties by non-CSC tumor cells or both [8]. However, whatever is the mechanism operating in a tumor responsible for stemness acquisition, stemness was progressively considered as a key phenomenon in tumor development for its strong association with poor outcomes, as initially shown for acute leukemias [78]. In fact, this initial study, through the definition of a set of genes preferentially expressed in leukemic cell populations enriched in LSCs identified a 17 gene signature; this 17-gene leukemic signature allowed to attribute a stemness score to AML, highly predictive of initial therapy resistance [78].

The Cancer Genome Atlas (TCGA) network performed a detailed molecular analysis of various cancer types integrating various molecular analyses involving study of tumor DNA (exome sequencing; genome sequencing; DNA methylation and copy number evaluation) RNA (mRNA and microRNA sequencing) and proteins/phosphoproteins. This analysis allowed to discover molecular signatures supporting a taxonomy differing from the current organ- and tissue-histology based classification [79]. The identification of integrated cancer subtypes sharing mutations, copy number alterations, signaling pathway similarities that influence the appurtenance of a tumor to a molecular subtype, independently of the tissue of origin or tumor stage [79]. This approach showed that at least 10% of patients can be differently classified and, in some instances treated, on the basis of molecular taxonomy [79]. More recently, the PanCancer study englobed the multiplatform molecular analysis of 11 286 tumors from 33 cancer types [80]. This study provided evidence of clustering primarily organized by histology, tissue type or anatomic origin; integrative clustering emphasized the dominant role of cell-of-origin patterns [80]. Similarities among histologically or anatomically related cancers allowed to perform pan-cancer analyses, involving gastrointestinal, gynecological, squamous cancers; interestingly, this analysis allowed to define also a group of cancers related by stemness features [80]. The stemness features revealed the aggregation of high stemness tumors across distinct tumor types [80].

By multiplatform analysis of transcriptome, methylome, and transcription factors performed in the large majority of tumors above described, two cancer stemness indices were discovered: one was relative of epigenetic features (mDNAsi); one was relative of gene expression (mRNAsi) [81]. These two indices allowed to evaluate the stemness features of the various tumors associated with oncogenic dedifferentiation [81]. The oncogenic dedifferentiation is associated with mutations in genes encoding oncogenes and epigenetic modifiers, perturbations in mRNA/miRNA transcriptional network, deregulation of signaling pathways and expression of genes involved in the control of self-renewal of normal and cancer stem cells, such as MYC, OCT4, SOX2 [81]. Analysis of tumor microenvironment showed a correlation between cancer stemness and immune checkpoint expression and infiltrating immune cells [81]. The dedifferentiated oncogenic phenotype was more prominent among metastatic tumors [81].

Smith et al have developed gene signature for normal human stem cells and have used these signatures to better elucidate the relationship between epithelial cancers and stem cell transcriptional programs: this approach showed that the adult stem cell signature selected cancers with poor overall survival and genetic alterations of oncogenic drivers, such as small neuroendocrine lung cancer, prostate and bladder cancers [82]. At the level of gene expression, DNA methyltransferase expression correlated with adult stem cell gene expression signature status [82].

Miranda *et al.* explored in 22 cancers the relationship between cancer stemness, intra-tumoral heterogeneity and immune response [83]. The results of this study showed that the stemness phenotype confers immunosuppressive properties on tumors, resulting in microenvironments scarcely reactive to immunological challenge that foster and maintain intra-tumoral heterogeneity were observed [83]. Finally, studies on cancer cells with high stemness features showed that these cells have intrinsic immunosuppressive properties [83].

CANCER STEM CELL TARGETED THERAPIES

Given the important role played by CSCs in tumor initiation, progression, relapse and drug resistance, it is quite obvious that they represent an attractive target in clinical studies *(Table 2)*.

Thus, Dalerba *et al.* investigating the properties of colon cancer stem cells have identified caudal-type homeobox transcription factor 2 (CDX2) as a biomarker that could be used to quantify the number of undifferentiated colon cancer cells, displaying properties of cancer stem cells [84]. Particularly, these authors identified biomarkers not expressed in ALCAM/CD166-positive tumors (with stem-like properties), but present in ALCAM/CD166-negative tumors and identified the CDX2 protein [84]. Only 4% of colon cancers had lost CDX2 protein expression and these patients displayed reduced 5-year disease-free survival; stage II colorectal cancer patients with CDX2 loss treated

Table 2
Agents targeting CSC-associated surface markers, altered signaling pathways or mutated molecules in ongoing clinical trials

Drug name	Target	Disease	Clinical phase	Study Identification	Current status
Vismodegib	Hedgehog pathway	R/R Medulloblatoma	II	NCT00939484	Completed
		Basal cell carcinoma	II	NCT01700049	Completed
		Sarcoma	II	NCT01700049	Completed
		SCLC	II	NCT01700049	Completed
		Pancreatic cancer	II	NCT01088815	Completed
		Ovarian cancer	II	NCT00739661	Completed
		Colorectal cancer	II	NCT00636610	Completed
Venetoclax	BCL2				
Venetoclax+Azacitidine		AML not eligible for standard therapy	III	NCT02993523	Active, completed
Venetoclax+Azacitidine		After allogenic stem cell transplantation	III	NCT04161885	Ongoing
Venetoclax+Decitabine		AML high-risk	I	NCT03844815	Not yet recruiting
Venertoclax+Azartidine Or Decitabine		AML not eligicle for standard therapy	III	NCT03941964	Ongoing
Venertoclax+Chemotherapy		Refractory/relapsing pediatric AML	I	NCT03194932	Completed
Venetoclax+low-dose cytarabine		AML not eligible for standard therapy	III	NCT03069352	Completed
Venetoclax+Dinaciclib		Refractory/relapsing AML	I	NCT034844815	Ongoing
Daratumumab (HuMax®-CD38)	CD38				
Daratumumab		Refractory/relapsing multiple myeloma	II	NCT00574288	Completed
Daratumumab		Refractory/relapsing multiple myeloma	II	NCT02944565	Completed
Daratumumab		Refractory/relapsing multiple myeloma	II	NCT03871829	Ongoing
Daratumumab+Lenalidomide+Dex		Multiple myeloma transplant-inelegible	III	NCT02252172	Ongoing
Daratumumab+Lenalidomide		Multiple myeloma transplant-inelegible	III	NCT02195479	Ongoing
Daratumumab+Borteziomib+Dex +Thalidomide		Multiple myeloma transplant-eligible	III	NCT02541383	Ongoing
Magrolimab (mAb Hu5F9-G4)	CD47	Solid tumors	I	NCT02216409	Completed
TTI-621		Solid tumors	I	NCT02663518	Recruiting
IBI 188		Advanced malignancies	I	NCT03763149	Recruiting
CC-90002		Hematologic neoplasms	I	NCT02641002	Completed
AO-176		Solid tumors	I	NCT03834948	Recruiting
SRF231		Solid tumors	I	NCT03512340	Recruiting
Bivatuzumab mertansine		Metastatic breast cancer	I	NCT02254005	Completed
Tagraxofusp (SL-401)	CD123	AML, BPDCN	I	NCT03113643	Recruiting
		BPDCN after SCT	II	NCT04317781	Recruiting
		$CD123^+$ AML, BPDCN	II	NCT043342962	Not yet recruiting
KHK283		AML	I	NCT02181699	Completed
Talacotuzumab		AML	III	NCT02472145	Completed
SGN-CD123A		AML	I	NCT02848248	Terminated
IMGN632		AML	II	NCT03386513	Recruiting
XmAb 14045	CD123/CD4	AML	II	NCT02152956	Recruiting
Flotetuzumab (MGD006)	CD123/CD3	Refractory/relapsing AML	II	NCT02152956	Recruiting
JNJ-63709178	CD123/CD3	AML	III	NCT02472145	Completed
Mylotarg (gemtuzumab ozogamicin)	CD33	$CD33^+$ refractory/relapsing AML	IV	NCT03727750	Recruiting
Vadastuximab talirine (SGN-CD33A)		AML	I	NCT01902329	Completed
IMGN779		AML	I	NCT02674763	Recruiting
Napabucasin	STAT3				
Napabucasin+FOLFIRI		Metastatic colo-rectal cancer	III	NCT03522649	Recruiting
Napabucasin+low-dose gemcitabinbe		Metastatic pancreatic adenocarcinoma	III	NCT03721744	Recruiting
Napabucasin+nab-paclitaxel+ gemcitabinbe		Metastatic pancreatic adenocarcinoma	III	NCT02993731	Recruiting
Ivosidenib	IDH1				
Ivosidenib		Refractory/relapsing AML, MDS	I	NCT02074839	Recruiting
Ivosidenib		Myeloid neoplasms	I	NCT03564821	Recruiting
Ivosidenib+chemotherapy		Refractory/relapsing AML, MDS	I	NCT04250051	Recruiting
Ivosidenib or enasidenib+chemotherapy		Refractory/relapsing AML, MDS	III	NCT03839771	Recruiting
Ivosidenib or enasidenib+chemotherapy		Newly diagnosed AML	I	NCT02632708	Recruiting
Ivosidenib or enasidenib+azacytidine		AML, elderly patients	I/II	NCT02677922	Active, not recruiting
Ivosidenib+nivolumab		Refractory/relapsing AML, MDS	II	NCT04056910	Recruiting
Ivosidenib+venetoclax ± azacytidine		Refractory/relapsing AML, MDS	I/II	NCT03471260	Recruiting
Olutasidenib + azacitidine or cytarabine		Refractory/relapsing AML, MDS	I/II	NCT02719574	Recruiting
Olutasidenib + ASTX 727		Refractory/relapsing AML, MDS	I/II	NCT04013880	Recruiting
Enasidenib	IDH2				
Enasidenib		Advanced AML	I/II	NCT01915498	Completed
Enasidenib		IDH2-mutant myeloid neoplasms	I	NCT03515512	Recruiting
Enasidenib		AML post stem cell transplantation	I	NCT03728335	Recruiting
Enasidenib		Refractory/relapsing, high-risk MDS	II	NCT03744390	Recruiting
Enasidenib		Pediatric AML	II	NCT04203316	Recruiting
Enasidenib+chemotherapy		Refractory/relapsing AML	II	NCT03881735	Recruiting
Enasidenib+azacytidine		Refractory/relapsing AML	II	NCT03683433	Recruiting
Enasidenib+azacytidine		Refractory/relapsing, high-risk MDS	II	NCT03383575	Recruiting
Enasidenib+azacitidine or AraC		AML ≥ 60 years	II	NCT02577406	Not yet recruiting
Enasidenib + CPX-351		Relapsed AML	II	NCT03825796	Recruiting

with adjuvant chemotherapy displayed 5-year improved overall survival compared to those with CDX2 loss not-treated with chemotherapy [84]. These findings were confirmed in a more recent study carried out in a large set of colon cancer patients [85]. Particularly, this study showed that both patients with microsatellite instability-positive (corresponding to patients with mutations in DNA-repair pathway genes involved in mismatch repair) and microsatellite stability CDX2-negative type II colon cancers display a negative prognosis [85] Studies in animal models of colon carcinogenesis have shown that CDX2 acts as a suppressor of intestinal tumorigenesis, thus explaining why its loss is associated with poor-prognosis colorectal cancer [86].

The study of the role of Hedgehog signaling pathway in basal cell carcinoma has led to the clinical development of Vismodegib, a specific inhibitor of this pathway, that targets cancer stem cells. Alterations in the Hedgehog signaling pathway have been involved in the pathogenesis of basal-cell carcinoma; most basal cell carcinomas are treated surgically, but local-advanced or metastatic basal-cell carcinomas require a medical treatment. Thus, a phase I clinical study using Vismodegib in patients with basal cell carcinoma locally advanced or in metastatic stage showed a 43% response rate, with 21% of complete response [87, 88]. A long-term evaluation of this study on 100 patients showed 48.5% of responses in the metastatic group and 60.3% of responses in the locally-advanced group; median overall survival was 33.4 months for metastatic patients and not estimable for those with locally-advanced tumors [89]. These studies have supported the approval of this drug by FDA for treatment of basal cell cancers at advanced stage. Recent studies have explored the mechanisms through which Vismogedib could exert its inhibitory effect on cancer stem cells of basal cell carcinomas. Despite the consistent efficacy of Vismodegib in the treatment of basal cell carcinoma, residual disease persists in some patients; thus, Biehs *et al*., using a model of basal cell carcinoma have shown that Vismodegib treatment did not result in complete eradication and, quiescent, residual tumor progenitor cells undergo a switch to a stem-like transcriptional program, resembling that of interfollicular epidermis and isthmus, whereas untreated tumors resemble hair follicle bulge [90]. This differentiation switch was related to the activation of Wnt pathway: thus, the combined treatment with both Vismodegib and a Wnt inhibitor reduced the residual tumor burden and enhanced tumor differentiation [90]. These findings were supported by a parallel study that characterized the slow-cycling population residual after Vismodegib treatment: these residual cells correspond to $LGR5^{+}$ cancer stem-like cells, exhibiting high Wnt activity [91].

The Hedgehog signaling pathway is upregulated in pancreatic adenocarcinoma cancer stem cells; however, the administration of Hedegehog inhibitors (Vismodegib or GDC-0449) in combination with Gemcitabine to pancreatic cancer patients did not lead to a decrease of CSCs and to tumor inhibition [92].

Another drug, Venetoclax, a BCL2 inhibitor, was recently approved for the treatment of elderly AML patients [26]. Particularly, Venetoclax, increasing ROS (reactive oxygen species) production targets leukemia stem cells in older AML patients [26]. A clinical study involving elderly AML patients with a poor prognosis and very limited response to standard therapy showed that about 67% of patients receiving combined therapy with Venetoclax and Azacitidine had complete remissions, some of the remissions being prolonged [93]. This drug represents an important step towards LSC-targeted therapy. A third drug targeting CSCs, recently approved for the treatment of an AML subtype is represented by IDH inhibitors. These drugs were developed with the assumption that targeting leukemic mutations might be an effective strategy to eradicate AML malignant clone. IDH1 and IDH2 mutations are pre-leukemic mutations and the eradication of the leukemic stem cell clone bearing these mutations could represent an effective therapy in AMLs characterized by IDH mutations. Targeting IDSH1 with Ivosidenib, a specific inhibitor, elicited 30% of complete remissions among IDH1 mutated/relapsed AML patients, with a median response of 8 months [94]. Targeting IDH2 with Enasidenib, a specific inhibitor, resulted in an overall response rate of 40.3% and a median response duration of 5.8 months [95].

The study of the effects of Enasidenib on IDH2-mutant AML is important because provides a number of important indications on a drug targeting leukemia-specific alteration present in LSCs. Thus, Enasidenib induced complete remissions with persistence of mutant IDH2 and normalization of HSC and progenitor compartments, with emergence of functional neutrophils bearing the mutant IDH2 allele [96]. The massive induction of neutrophil differentiation may induce a clinically relevant syndrome, the differentiation syndrome that needs careful medical monitoring and treatment [97]. Using sequential patient samples, the clonal structure of hematopoietic cell populations at different stages of differentiation was determined, showing that Enasidenib promoted cell differentiation from terminal or ancestral mutant clones; relapse arose by clonal evolution or selection of terminal or ancestral clones [98]. The resistance to Enasidenib may be related in some instances to the acquisition of new IDH2 mutations represented by trans or cis dimer-interface mutations [99]. The analysis of the molecular abnormalities of IDH2 mutant AMLs showed that both IDH2-R140 and IDH2-R172 mutations are equally responsive to Enasidenib [100]. Furthermore, response and survival were comparable among patients who, at study entry, were in relapse, or were refractory to intensive or non-intensive therapies [100]. Finally, a very recent study reported the first results of a trial carried out in older patients with newly diagnosed IDH2-mutant AML and showing durable responses among responsive (about 31%) patients [101].

A promising approach for treatment consists in the targeting of membrane antigens selectively or preferentially expressed on tumor cells, including CSCs. Particularly, targeted therapies involving CD38, CD47 and CD123 are under progress in some hematological malignancies, with promising results.

CD38 is highly expressed on myeloma cells, including a population of myeloma-initiating cells, characterized by high expression of CD47, positivity for CD138 expression and negativity for CD19 and CD45 expression [102]. Myeloma stem cells are also characterized by high expression of CD24 [103]. Despite deepening responses to frontline therapy, most of multiple myeloma patients never become minimal residual disease-negative and relapse with a drug-resistant disease whose development is mediated by drug-resistant cancer stem cells. Bortezomib, thalidomide and dexamethasone plus autologous stem cell transplantation is standard treatment for transplant-eligible patients with newly diagnosed multiple myeloma; lenalidomide plus dexamethasone or the combination of bortezomib, melphalan and prednisone are standard treatments for patients with multiple myeloma not eligible for autologous stem cell transplantation. Three recent clinical studies have shown that the addition of Daratumumab, a monoclonal antibody anti-CD38, improved the therapeutic efficacy of these three standard therapeutic regimens: a) among patients with newly diagnosed multiple myeloma who were ineligible for autologous SCT, the risk of disease progression or death was significantly lower among those receiving Daratumumab plus lenalidomide and dexamethasone than among those treated with lenalidomide and dexamethasone [104]; b) among patients with newly diagnosed multiple myeloma who were ineligible for autologous SCT, Daratumumab combined with bortezomib, melphalan and prednisone elicited a lower risk of disease progression or death than the same regimen without Daratumumab [105]; c) Daratumumab administration before or after the standard regimen plus SCT improved depth of response and progression-free survival compared to standard regimen plus SCT [106].

CD47 and its inhibitory receptor SIRPα form an innate immune checkpoint that can be targeted using anti-CD47 mAb; this system is formed by CD47 expressed on tumor cells and the inhibitory receptor SIRPα, which is selectively expressed on myeloid cells, particularly macrophages. CD47 is a "don't eat me" signal because it inhibits the phagocytosis of nonmalignant cells, such as red blood cells; inhibition of CD47-SIRPα promotes the lysis of opsonized cancer cells, often overexpressing CD47, by macrophages and granulocytes [107]. CD47 up-regulation is an important mechanism providing protection to normal HSCs during inflammation-mediated mobilization; importantly, leukemic stem/progenitor cells co-opt this capacity constitutively over-expressing CD47 and thus exhibiting the ability to evade macrophage killing [108]. Furthermore, chemoresistant leukemic cells overexpress CD47 and CD123 [109].

High CD47 expression was observed in non-Hodgkin lymphomas (NHLs) and correlates with negative prognosis [110]. Blocking anti-CD47 antibodies enabled phagocytosis of NHL cells and synergize with anti-CD20 monoclonal antibody rituximab [110]. In mouse lymphoma models, the combined administration of anti-CD47 and rituximab led to elimination of lymphoma and to a curative effect [110]. Hu 5F9, a humanized anti-CD47 blocking antibody is currently being studied in four different phase I clinical studies. The first results observed on 22 pre-treated chemoresistant lymphoma patients were recently reported: the anti-CD47 inhibitory mAb Hu5F9-G4, combined with rituximab, showed significant anti-tumor activity, with 33% complete responses in diffuse large B-cell lymphomas and 43% complete responses in follicular lymphomas [111]. A recent study, presented at the last ASCO Meeting, reported the results of a phase Ib clinical trial involving the administration of Hu5F9-G4 alone (10 pre-treated AML or MDS patients) or in combination with azacytidine (22 untreated AML or MDS patients ineligible for induction chemotherapy) to AML or MDS patients, showing that this treatment was well tolerated with robust anti-leukemia activity and induction of complete responses and minimal residual disease negativity [112].

Other recent studies have supported the targeting of CD123, the interleukin-3 receptor α (IL-3Rα), as a new therapeutic tool to target leukemic stem cells. CD123 is widely overexpressed in various hematological malignancies, including AML, B-ALL, CML, blasticplasmocytoid dendritic neoplasm (BPDCN) [113]. Importantly, CD123 is expressed both at the level of LSCs and more differentiated leukemic blasts and, consequently, is an attractive therapeutic target [14-16, 114]. Various agents have been developed as drugs targeting CD123 on malignant leukemic cells and on the normal counterpart. Tagraxofusp (SL-401, Stemline Therapeutics), a recombinant protein composed of a truncated diphteria toxin payload fused to IL-3, was introduced in therapy and was approved for use in patients with BPDCN, a rare clinical condition characterized by high CD123 expression at the level of leukemic blast cells, including LSCs [114]. This compound is under investigation for the treatment of other hematological malignancies. Various monoclonal antibodies anti-CD123, including bispecific monoclonal antibodies, are under evaluation for the treatment of AML minimal residual disease or of relapsing/refractory AML. Finally, recent studies are exploring the potential therapeutic impact of T cell expressing CD123 chimeric antigen receptors (CART) as a new immunotherapy for the treatment of relapsing/refractory AML and BPDCN. The most consistent clinical experience was performed with the CART 123 reported by Mardiros *et al.* in 2013 [115] and developed as a clinical drug by the Mustang Bio Inc. and called MB-102. Using MB-102, 7 AML and 2 BPDCN relapsing patients were treated, with some patients achieving a complete response [116, 117]. No major toxicities were observed in these 9 patients [116, 117]. In 2018, the Food and Drug Administration has granted Orphan and Drug Designation to MB-102 for the treatment of BPDCN.

Current studies are attempting to target cancer stem cells present in different tumors through the targeting of signaling pathways that are activated in these cells and are essential for their survival and/or proliferation (such as PI3K/AKT, STAT, WNT/β-catenin, NOTCH) or transcription factors (such as YAP1) essential for tumorigenesis and maintenance of cancer stemness [118]. One example is given by the ongoing clinical

studies based on the use of STAT3 inhibitors. STAT3 is a potential target of anticancer therapy because this transcription factor promotes stem cell-like characteristics, survival, proliferation, metastatic potential and immune evasion of tumor cells [119]. STAT3 is hyperactivated in gastrointestinal tumors, where it represents a particularly attractive potential therapeutic target. Particularly, studies using Napabucasin (BB1608 or BB608), a small-molecule STAT3 inhibitor, have shown an inhibition of STAT3-induced gene transcription and of tumor spherogenesis [120]. In a mouse model of colon cancer tumorigenesis, Napabucasin inhibited spleen and liver metastases and inhibited cell signaling pathways, such as those implying NANOG, SOX2, MYC, β-catenin, and supporting cancer stemness [120]. Several clinical trials are investigating the safety and efficacy of Napabucasin in various gastrointestinal malignancies. Napabucasin monotherapy was investigated in a phase III clinical trial (CO.23 trial), comparing its efficacy to that of a placebo in refractory advanced colon cancer: the study failed to demonstrate a significant difference in the survival of the patients treated with Napabucasin compared to those treated with placebo [121]. In a biomarker-guided analysis, pSTAT3-positive colorectal cancer patients showed a significant gain in overall survival compared to those treated with placebo [121]. The CanStem303C trial (NCT02753127) is an ongoing randomized phase III clinical study evaluating Napabucasin in combination with 5-fluorouracile, leucovorin, irinotecan (FOLFIRI) in previously treated colorectal cancer patients [122].

In pancreatic cancer, an initial phase I/II study showed promising results derived from the administration of Napabucasin in association with paclitaxel and gemcitabine [123]. These promising results promoted the development of a randomized phase III trial, CanStem111P, using the same drug combinations in treatment-naïve pancreatic cancer patients with metastatic pancreatic adenocarcinoma (NCT02993731). However, very recently, this study was stopped for clinical futility. Similarly, no clinical benefit related to Napabucasin administration in patients with hepatocellular carcinoma or gastric cancer [124].

These studies are a paradigmatic example of the consistent difficulties encountered in translating into clinic, with patient's benefit, preclinical observations supporting the targeting of a signaling pathway involved in cancer stemness. This difficulty is seemingly related to the existence of compensatory mechanisms rendering cancer stem cells not strictly dependent for their survival from the inhibited pathway. Another difficulty encountered in this type of approach is related to the extent of inhibition of the pathway needed to be achieved to obtain a significant therapeutic effect and its compatibility with toxicity problems. Another element of additional complexity is related to the unexpected induction of secondary effects of some inhibitors: thus, clinically-used MEK1/2 inhibitors inadvertently increase Wnt activity and induce stem cell plasticity of colorectal cancer stem cells, thus revealing an important limiting side effect induced by RAS pathway inhibition [125].

The difficulties observed in the development of efficient strategies to obtain at clinical level the inhibition of cancer stem cells of frequent solid tumors is not surprising and is mainly related to the aggressive nature of these cells. Therefore, a better understanding of the biology of cancer stem cells is absolutely required for the development of possibly efficient therapeutic strategies. An example is given by the study of cancer stem cells in pancreatic cancer. This tumor is a prototype of drug resistance, with only about 30% of patients responding to current multidrug chemotherapy regimens: however, these responses are quickly followed by tumor resurgence and progression. Tumor relapse was related to the presence of chemoresistant tumor cells and, notably, of cells that can be identified by some markers, including $CD24^{+}/CD44^{+}/ESA^{+}$ [33], CD133 [126], c-Met [127], Nestin [128], DCLK1 [129] and Musashi [130]. Imaging studies directly support the role of cells identified as putative cancer stem cells in tumor progression and drug resistance [130]. A recent study based on a comprehensive molecular evaluation of the core dependencies of pancreatic cancer stem cells by integrating their transcriptomic, epigenetic and genomic landscape, allowed to identify dependence of these cancer stem cells on inflammatory and immune mediators [131]. However, many potential hurdles pose an obstacle in the development of pancreatic cancer stem cells-targeted therapy: i) consistent phenotypic and functional heterogeneity of cancer stem cells; ii) cancer stem cells plasticity, giving the opportunity to non-stem cancer cells to transdifferentiate into new cancer stem cells; iii) intensive desmoplastic stroma around tumor cells, reducing drug penetration [132].

The therapeutic targeting of CSCs in solid tumors is a very complex problem due their intrinsic plasticity and to the absence of reliable and stable markers for their identification. A remarkable example is given by the study of colon CSCs. Colon CSCs express a variety of markers, including CD133 [28, 29], CD44, CD166 [30], ALDH [133] and LGR5 [75]. Among these various markers particularly interesting are the properties of LGR5, a marker of Wnt/β-catenin-dependent adult stem cells of the colon and a regulator of the Wnt pathway [134]. Lineage-tracing experiments provided evidence about the existence of LGR5+CSCs in unperturbed colorectal cancers: these cells isolated from organoid-derived xenografts express a gene program typical of intestinal stem cells and propagate colon tumors to recipient mice with great efficiency; furthermore, these cells displayed both self-renewal and differentiation capacities [135]. However, studies carried out in mouse tumors engineered to recapitulate the clinical progression of human colorectal cancer showed that selective LGR5+ cell ablation markedly inhibits the growth of primary tumors, but does induce complete tumor regression and tumors restart to grow upon treatment cessation, due to LGR5-cells that replenish the LGR5+ pool [71]. These observations have modified the traditional view of a hierarchical organization of colon cancers which was replaced by a more dynamic model implying the existence of different cell types within the tumor that can act as sources of CSCs [136]. In line with this view, a recent study showed

that most colorectal cancer metastases are seeded by LGR5- cells, which possess the intrinsic capacity to differentiate into LGR5+CSCs independently of a specific microenvironment and restore epithelial hierarchies in metastatic tumors [137]. Thus, it is not surprising that several clinicopathologic studies have reached the conclusion that LGR5 expression in colorectal cancer is not associated with a poor prognosis, as might be expected for a CSC marker [138, 139]. These observations suggest that the best approach to eradicate colon CSCs would be based on targeting their function rather than their identity. In line with this idea, a very recent study by Morral *et al.* showed that the majority of ribosomal transcription and protein biosynthesis in colorectal cancers occurs in a limited subset of tumor cells, LGR5+and LGR5-, characterized by elevated levels of the RNA polymerase I subunit A; genetic ablation of these calls cause an irreversible growth arrest of colorectal cancer cells [140].

Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this paper.

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