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Review

Heterogeneity of Cancer Stem Cells: Rationale for Targeting the Stem Cell Niche

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ABSTRACT

Malignancy is fuelled by distinct subsets of stem-like cells which persist under treatment and provoke drug-resistant recurrence. Eradication of these *cancer stem cells* has therefore become a prime objective for the development and design of novel classes of anti-cancer therapeutics with improved clinical efficacy. Here, we portray potentially clinically-relevant hallmarks of cancer stem cells and focus on their recently appreciated properties of cell variability and plasticity, both of which make them elusive targets for cancer therapies. We reason that this 'disguise in heterogeneity' has fundamental implications for clinical management and elaborate on rational strategies to combat this diversity and target a broad range of tumorigenic cells. We propose exploitation of cancer stem cell niche dependence as a promising approach to interfere with various, rather than few, cancer stem cell subsets and suggest cancer-associated fibroblasts as a prime microenvironmental target for tumor stemness-depleting intervention.

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Contents

Abbreviations: ABC, ATP-binding cassette; ALDH, aldehyde dehydrogenase; CAF, cancer-associated fibroblast; CSC, cancer stem cell; EMT, epithelial-to-mesenchymal transition; FACS, fluorescence-activated cell sorting; LSC, leukemic stem cell; mAb, monoclonal antibody; MGMT, O(6)-methylguanine-DNA-methyltransferase; MRD, minimal residual disease; SP, side population; TKI, tyrosine kinase inhibitor; TME, tumor microenvironment; TSC, tissue stem cell.

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1. Introduction

The perception on how tumors develop and are propagated *in vivo* has changed dramatically over the past decade. In particular, the original clonal models of cancer evolution have been abandoned and tumors are now appreciated to be tremendously complex comprising genetic and epigenetic heterogeneity within single site lesions. Moreover, comparative investigations of primary- *versus* secondary site tumor beds have revealed strong subclonal diversification of clinical metastases that might at least in part be responsible for the failure of many systemic therapies to control or eradicate metastatic disease.

One aspect of intratumoral heterogeneity is reflected by the pyramid-like structure of tumors with functionally-defined cancer stem cells (CSCs) at the apex of the malignant hierarchy. Conserved in most tumor entities, CSCs, or cancer-initiating cells, are endowed with unique functional properties and dictate the whole course of tumor evolution including cancer initiation, metastatic progression, and disease recurrence after clinical remission. Thus, these cells have emerged as a highly attractive target population for anti-cancer treatment, and strategies to eliminate these cells are being heavily explored. However, recent evidence has suggested that aside from dormancy and detoxification, CSC targeting approaches are faced with additional challenges including low immunogenicity of CSCs, cellular heterogeneity of CSC pools, and a general plasticity of stemness phenotypes. In this review, we summarize the latest advances in our understanding of CSC biology and function, and highlight potential implications of tumor cell variability for the conceptual design of CSC-directed therapies. We propose CSC heterogeneity as yet another example for Darwinian selection during tumor progression and suggest that microenvironment-targeted strategies will guide the development of anti-CSC treatments in the future, based on the inherent niche dependence of CSC populations.

2. The Cancer Stem Cell Concept

Organ development –and homeostasis depends on small populations of dedicated stem cells, which maintain tissues by continuous replacement and also secure demand-adapted regeneration in case of emergencies, such as injury [1]. Functionally, stem cells are characterized by their selective ability for self-renewal and differentiation, which allows them to generate all cell lineages within a given tissue [1]. Furthermore, stem cells exhibit a high degree of evolutionary fitness conferred, amongst others, by sophisticated mechanisms of detoxification [2,3] and residence in protective microenvironments (i.e., stem cell niches) [4,5].

Starting with the seminal article of Al-Hajj and co-workers in 2003 [6], the principles of stem cell biology have been increasingly used to explain basic biological and clinico-pathological features of cancer, even though the first connection between stem cells and malignancies were already proposed in the mid-20th century [7,8]. In particular, it is now appreciated that cancer arises from the malignant transformation of a stem/progenitor cell or, alternatively, from a non-stem cell that has regained stemness potential by a dedifferentiation process [9–11]. This paradigm is corroborated by the remarkable convergence of stem cells and CSCs in terms of preferentially activated signalling cascades, as well as their overlapping expression of certain markers. As an example, both stem cells and CSCs show activation of the self-renewal-associated pathways Wnt/β-catenin, Bmi-1, sonic hedgehog, Notch and PTEN [12], and both populations express tissue-specific stem cell markers, such as CD34 (blood) [13,14] and Lgr5 (colon) [15,16]. Importantly, this concordant molecular profile is reflected in several key aspects of CSC biology including longevity, dormancy/quiescence, niche dependence, and the potential for asymmetric cell division [17–20]. Accordingly, CSCs are selectively required for cancer initiation and subsequent propagation, properties that have led to the designation of CSCs as the 'beating heart' of malignant growth [18], and to their declaration as prime therapeutic targets [21]. Methodologically, CSCs can be purified from biological samples using flow cytometry/FACS employing phenotypic markers such as CD44 and CD133, or functional characteristics such as dye extrusion and enzymatic activity [22]. On the functional level, *bona fide* CSCs show tumor-initiating potential *in vivo*, are capable of anchorage-independent growth *in vitro* and are notably resistant to cytotoxic and targeted anti-cancer drugs as well as radiotherapy [18–20]. However, it has to be stressed that the frequency and identity as well as other hallmarks of CSCs vary substantially among tumor entities (Table 1). In addition, methodological factors such as the particular experimental conditions used can impact the detection of CSCs. As an example, tumor engraftment in more severely immune-compromised mice increases the detectable frequency of tumorigenic cells by several orders of magnitude [23], demonstrating the challenges in implementing a universal definition of CSCs.

Several landmark studies have established that the transition from single site tumor growth to life-threatening metastatic disease is mechanistically enabled by CSCs, which seem to have particular resistance to the rate-limiting steps of the metastasis cascade including anoikis, extravasation, and re-settlement/survival in 'unnatural' environments [24–26]. Accordingly, metastatic cancer cells are enriched in stemness-associated gene signatures and also show functional stem cell properties [27]. One missing link between stem cell traits and metastasis could be the recent appreciation that CSCs exhibit a distinct transcriptional program otherwise found during developmental tissue remodelling and commonly referred to as epithelial-to-mesenchymal transition (EMT) [28,29]. This could at least in part explain the increased migratory potential of these cells, as well as their poor response to treatment. Importantly, the relationship between EMT and CSCs seems to be causal, because if cells are forced to undergo an EMT (e.g., by treatment with TGF-β, knockdown of E-cadherin, or ectopic expression of the EMT transcription factors TWIST or Snail), they simultaneously acquire phenotypic and functional properties of stem cells [30,31].

2.1. Cancer Stem Cells are Therapy-Resistant and Mediate Disease Recurrence

Clinically, the relevance of CSCs is largely seen in their intrinsic resistance to various cytotoxic and targeted anti-cancer drugs, which secures their persistence during treatment and predisposes the patient to relapse [2]. This is in line with studies showing that states of remission or minimal residual disease (MRD), which often escape clinical detection, are established and sustained specifically by CSCs [32–35]. Indeed, CSCs are selected during *in vivo* chemotherapy, and recurrent tumors are enriched in CSCs or CSC-related gene signatures [36,37]. Along similar lines, expression of surrogate CSC markers correlates with reduced survival in different tumor entities and also predicts poor response to therapeutic intervention [38–40].

Several non-overlapping mechanisms of protection contribute to the treatment refractoriness of CSCs. For instance, their inherent tendency to remain quiescent over extended periods of time substantially reduces their sensitivity to anti-proliferative drugs such as classical cytostatics

and tyrosine kinase inhibitors (TKIs) [20,41]. To overcome this limitation, a two-step strategy with cell cycle-triggering, dormancy-breaking agents (e.g., arsenic trioxide, G-CSF, IFN-α) was proposed to activate CSCs for re-sensitization to subsequent treatment [42]. Another possibility is the use of compounds whose mode of action is independent of cell cycle progression and in fact, the feasibility of this strategy has been demonstrated for hematological cancers where an epigenetic-modulating agent (i.e., HDACi) could synergize with classical TKI-based therapy to eradicate dormant leukemic stem cells (LSCs) [43]. These data also argue for a prominent role of epigenetic cues in mediating drug resistance of CSCs.

CSCs have also evolved – or adopted from their cell of origin – several mechanisms of detoxification, which protect them from both xenobiotics and harmful metabolic by-products. For instance, members of the ATP-binding cassette (ABC) family of drug transporters efflux a broad spectrum of cytotoxic or targeted anti-cancer drugs [2] and aldehyde dehydrogenase (ALDH) protects the CSCs from naturally occurring or therapy-induced reactive aldehydes [44,45]. Moreover, CSCs, particularly those found in brain tumors, utilize O(6)-methylguanine-DNA-methyltransferase (MGMT) to counteract the cytotoxic effects of DNA-alkylating agents such as temozolomide [46], and in fact, MGMT promoter methylation is an established predictive marker for temozolomide treatment response [47]. Altogether, neutralization of CSC detoxification mechanisms is considered an attractive therapeutic concept reinforced by proof-of-concept studies [48,49]; however, clinical benefit including prevention of disease recurrence remains to be shown in large trials.

Specific compounds may not be neutralized by either mechanism and manage to damage CSCs. Similarly, CSCs are not principally shielded from radiotherapy. However, even in the case of significant DNA damage, CSCs are able to evade cell death due to marked proficiency in DNA repair mediated at least in part by prompt and sustained activation of DNA damage response pathways [50] including the MRE11-RAD51-NBS1 [51] and ATR/Chk1 hubs [52].

Table 1

Phenotypic Identity and Estimated Frequency of CSCs in Various Tumor Entities. Generally, CSCs from hematological malignancies are better characterized which is likely the result of the decade-long research in the field of hematopoiesis as a paradigm for stem cell biology. Hence, the molecular signature of these cells is well-characterized and strategies to re-sensitize them to treatment are already being explored [186,187]. Conversely, CSCs from solid tumors remain much more elusive, entailing ambiguous phenotypic identities and significant challenges for drug development in most tumor types. Nevertheless, CSCs from both hematological malignancies and solid tumors share many characteristic properties including clonogenicity, tumor-initiating potential, asymmetric cell division, activation of (embryonic) stem cell pathways, detoxification/drug resistance, niche dependence, and their implication in disease recurrence. Note that this table is intended to exemplify the differences and similarities between various CSC populations and also depict their elusive nature and the difficulties in defining them. Accordingly, this table does not claim completeness. 'Very low', 'low' and 'int to high' represent CSC frequencies of <1%, 1-10% and >10%, respectively. CSC frequencies are not directly comparable between studies owing to differences in methodology and/or sampled material. ALDH, aldehyde dehydrogenase; CSC, cancer stem cell; SP, side population.

Tumor Entity	Marker Signature of CSCs	Frequency	Reference
Blood Cancers			
Acute lymphoid leukemia	CD34+/CD38-/CD19+	low	[188]
Acute myeloid leukemia	CD34+/CD38-	low	[107]
Chronic myeloid leukemia	CD34+	––	[14]
	CD34+/CD38-/CD90+	––	[189]
	CD34+/CD38-/CD26+	very low	[190]
Hodgkin lymphoma	CD27+/ALDH+/(CD19+/CD20+)	very low	[191]
Multiple myeloma	CD138-/CD34-/(CD19+/CD20+)	low	[192]
Myelodysplastic syndrome	CD34+/CD38-/CD90+	very low	[193]
Solid Tumors			
Bone sarcoma	Stro-1+/CD105+/CD44+	––	[194]
	CD133+	low	[195]
	SP+	low	[196]
Breast cancer	CD44+/CD24-	low	[6]
Colorectal cancer	CD133+	low	[133]
	Lgr5+	low	[135]
	Wnt+	––	[134]
	CD44+	low	[132]
	Krt19+/Lgr5-	––	[131]
	ALDH+	low	[44]
Glioblastoma	CD133+	int to high	[197]
Hepatocellular carcinoma	CD133+/CD44+	low	[198]
	CD90+/CD44+	low	[199]
	EpCAM+	low	[200]
Lung cancer	CD133+	int to high	[125]
	ALDH+	low	[127]
	CD44+	––	[128]
	CD117+	––	[129]
	CD90+	very low	[130]
	SP+	low	[126]
Medulloblastoma	CD133+	int to high	[197]
Melanoma	ABCB5+	int to high	[139]
	CD271+	int to high	[201]
	CD20+	low	[202]
Ovarian cancer	CD44+/CD24+	very low	[118]
	CD44+/CD24-	––	[119]
	CD44+/CD117+	very low	[120]
	CD24+	––	[121]
	ALDH+/CD133+	very low	[123]
	ALDH+	int to high	[124]
	SP+	low	[37]
Pancreatic cancer	CD44+/CD24+	very low	[203]
Prostate cancer	CD44+/CD24-	low	[204]
	SP+	very low	[205]
Renal cell carcinoma	CD105+	low	[206]

Cancer immunotherapy pursues the goal to target malignant cells exploiting the natural specificity and adaptability of the immune system [53], having fuelled high hopes for curative intervention especially after primary cytoreduction using surgical or systemic regimens. Moreover, the discovery that certain chemotherapeutic agents as well as radiotherapy induce a specific type of cell death perceptible by the immune system ('immunogenic cell death') [54] suggests that treatment combinations of classical and immunotherapeutic modalities are promising and might even achieve long-term protective immunity. However, it was shown that CSCs evade immune recognition due to low expression of MHC class I and down-modulation of tumor-specific (or -associated) antigens [55,56]. Furthermore, CSCs can express immune checkpoint molecules thereby preventing effective tumor antigen recognition and/or blocking cytotoxic anti-tumoral T cell responses [56,57]. Finally, CSC niches often constitute an immunosuppressive environment hence limiting immunological accessibility. Thus, it must be deduced that CSCs are less visible for the immune system and escape immune surveillance, even though a single report [58] found that vaccination with CSC-derived antigens results in substantial anti-tumor immunity superior to that achieved with unselected 'bulk' tumor antigens.

Collectively, therapeutic CSC eradication is a highly attractive concept holding the potential to revolutionize cancer treatment through inhibition of metastasis and prevention of disease recurrence [59]. However, several lines of protection are operative in CSCs, which poses significant challenges especially for agents that directly act on the cancer cells. Tumor cell variability, including diversification within CSC subpopulations, further complicates the issue, hence asking for novel treatment concepts to target and eradicate these cells.

3. The Cancer Stem Cell – Heterogeneity Cycle

The traditional view on cancer development bases on a non-hierarchical model in which a malignant ancestor cell clonally expands to finally constitute the whole tumor (disregarding *de novo* mutations provoked by genomic instability). Generally, hematological malignancies have retained at least some aspects of mono- or oligoclonal evolution and a prime example here is Philadelphia + chronic myelogenous leukemia which can be efficiently and sustainably (but often not curatively) targeted by agents that inhibit the causal fusion protein BCR-Abl [60,61]. In contrast, other hematological cancers [62] and, in particular, solid tumors [63] have jettisoned more or less all features of oligoclonal growth, being composed of a plethora of phenotypically and functionally different populations that trigger further subclonal diversification, similarly as occurring in a viral quasi-species [64]. Most importantly, the extent of clonal diversity may even be an essential determinant in the pathogenesis of some tumors [65].

The description of tumor heterogeneity in the current detail only became possible owing to the compelling advances made in high-throughput and -content methods in recent years [66–70]. These techniques have allowed for in-depth characterization of the huge genetic, epigenetic and phenotypic variability of malignancies, thereby deepening our understanding of the basic principles of tumor evolution as well as the underlying mechanisms that drive metastasis, drug resistance and recurrence. Several landmark studies have applied multi-region sequencing to delineate spatial diversity within the tumor genome [71–73]. Using phylogenetic reconstruction, these studies found evidence for branched evolution among different regions of the tumor (including metastases), with only a limited number of overlapping mutations being detectable in all regions sampled. Importantly, this mutational variability was also reflected in differential DNA contents of the respective lesions ('ploidy heterogeneity') and furthermore involved functional aspects, depending on the type and location of the particular mutation. As an example, spatial heterogeneity of renal carcinoma for a missense mutation in an auto-inhibitory domain of the mTOR kinase was shown to entail constitutive activity of the enzyme *in vitro* as well as increased phosphorylation of downstream targets *in vivo* [72]. Thus, single biopsies are often not representative of the whole tumor mass, suggesting that clinical decisions are frequently based on incomplete information. Temporal genetic heterogeneity is also a characteristic hallmark of cancer that depicts the longitudinal evolution of tumor cells along the Darwinian selection line [71,74]. However, in contrast to spatial heterogeneity where different cancer clones eventually co-exist, temporal heterogeneity can at least in part be explained by out-competition of weaker clones by populations that have increased proliferative capacity and/or a lowered apoptotic threshold. Moreover, provided that representative biopsies are repeatedly taken, temporal genetic heterogeneity can principally inform rational treatment decisions based on a precision medicine approach [75,76], and it is expected that technical advances in minimally-invasive diagnostic procedures such as liquid biopsies of circulating tumor cells and/or circulating cell-free tumor DNA [77] will provide data for more precise decisions. Liquid biopsies are also promising because tumor-derived circulating material might comprise the fingerprints of several anatomical sites, hence such samples hold the potential to be representative of targetable traits shared by the whole tumor mass. Although not finally proven, it is likely that the principles of spatio-temporal tumor heterogeneity also apply to the epigenome [78]. However, whether genetic and epigenetic tumor heterogeneity are jointly regulated based on co-dependencies [79] or whether they exhibit distinct patterns and dynamics during tumor evolution [80] remains to be determined.

3.1. Crossroads of Metastasis, Cancer Stem Cells and Heterogeneity

A prime focus in oncology has been to understand and dissect the deadliest aspect of cancer, metastasis, and the mechanisms underlying the poor treatment response of metastatic lesions are now being uncovered. Even though specific microenvironments contribute to chemoprotection of metastatic cells [81], heterogeneity and dedifferentiation are the major reasons why metastases are hard to treat and, in fact, often exhibit therapy-refractoriness [27,82–84]. In humans, the heterogeneous nature of metastasis was first comprehensively demonstrated by Campbell and co-workers who applied paired-end sequencing of multiple cancer lesions to show a different genetic make-up of secondary sites as well as ongoing, partially convergent, clonal evolution among metastases [82]. Importantly, they found that clonal rearrangements within the primary tumor occur early in development and often propagate into secondary sites, implying that metastasis-initiating cells are heterogeneous from the start. Formally, this concept was corroborated in a recent study making use of a genetically-engineered, tamoxifen-inducible pancreatic cancer model combined with the cell labelling system 'Confetti' [85]. Cre expression in PDX1-positive (pancreatic) cells activates oncogenic $KRAS^{G12D}$ and deletes one allele of p53, and further leads to stochastic expression of one of four fluorescent proteins in the transformed cells. Using this elegant system, the authors modelled subclonal diversification and demonstrated polyclonality of both primary tumors and secondary sites including peritoneal, lung and diaphragm metastases. Thus, strong evidence suggests that cancer growth involves subclonal evolution processes early on, leading to intratumoral heterogeneity and ongoing diversification particularly in metastatic sites.

It is also well-established that dedifferentiation and (embryonic) stem cell signatures both correlate with poor prognosis in cancer patients [86]. This would argue for a stem cell program operative in metastasis-initiating cells, which would also produce cellular heterogeneity based on asymmetric cell division [87]. Hence, it is not surprising that a recent study, employing a highly sensitive flow cytometric capture assay to purify and analyze single metastatic breast cancer cells from patient-derived xenografts, found enrichment of stem cell-linked signatures in these cells, which furthermore correlated with a lower proliferative- but a superior tumor-initiating capacity [27]. Importantly, the authors also detected stem cell programs in rare cells of the primary tumor (~1%) that overlapped significantly with those of the metastatic

cells. Thus, CSCs, or cancer cells that exhibit stemness, are clearly over-represented in metastatic sites, and one could assume a stem cell origin of cancer metastasis [87]. In turn, this implies that stem cell properties are not primarily adopted in the new microenvironment of a respective secondary site, but that rare leader cells at the invasive front of the primary tumor (the metastasis-initiating cells) already possess these characteristics.

3.2. Dissecting the Heterogeneity of Ovarian Cancer Stem Cells

A hallmark of CSCs is their potential to undergo asymmetric cell division and produce differentiated progeny, resulting in phenotypic and/or functional tumor heterogeneity [9,19,88–91]. Certainly, this is one reason why CSC-rich metastases are highly heterogeneous and clinically hard to treat. In contrast, the possibility that a given CSC pool is heterogeneous *itself*, has only been considered recently, potentially because it is more intuitive to think of a rare cell subset as a homogeneous population. Ovarian cancer is the most lethal gynecological tumor entity and is characterized by high rates of recurrence (>60%) as well as acquisition of drug resistance in the relapsed setting [92,93]. Moreover, the semi-solid metastatic colonization of peritoneal surfaces by hundreds of independent tumor nodules further highlights the stem cell-driven nature of this tumor type [94]. To investigate the degree of cell variability present in ovarian CSC populations, we recently employed multicolour flow cytometry to sub-characterize the *side population* (SP), an established marker of ovarian [95] and other CSCs [96,97] molecularly based on functional ABC drug transporter activity [98–100]. Interestingly, we found that the stem-like SP accounting for typically less than 1% of cells was highly heterogeneous, with a 7-marker panel identifying at least 5 but up to 19 subsets in various cell lines (7 markers theoretically define $2^7 = 128$ cell populations, but only populations exceeding the cut-off of 0.1% are referred to here) [89]. As such, the cellular heterogeneity was comparable between the CSC subset and the bulk of non-CSC (non-SP), which was a surprising and novel finding, even though the link between CSCs and heterogeneity was drawn before [90,101]. Moreover, we found that the CSC subpopulations were stable because at least under *in vitro* conditions and in the absence of selective pressure they could be resolved repeatedly over a period of 9 weeks [89]. We also believe that the degree of observed heterogeneity in CSC populations heavily depends on the number and quality of the investigated markers, with additional biphasic expressions increasing the number of subsets exponentially. Thus, novel single cell-based technologies, such as single cell DNA/RNA sequencing [69,70,102], mass cytometry (CyTOF) [67], next generation fluorescence flow cytometry [103] and the recent 'imaging mass cytometry' platform adding spatial resolution within tissue context [68], will dissect the heterogeneity of CSC communities in ever more detail [66], and their combination with single cell functional analyses will reveal corresponding functional correlates [104]. The question remains what will be the translational relevance of these high-content data, i.e., which aspects of them can be utilized to improve cancer therapies? At least from what is known now, the possibility must be considered that, if enough parameters are measured, every single CSC is unique. In addition, even genetically-defined cancer subclones can exhibit functional heterogeneity [105] and likewise, different subclones can functionally converge to cooperate for tumor maintenance [106].

3.3. Three (Non-Mutually-Exclusive) Models for Cancer Stem Cell Heterogeneity

Conceptually, the heterogeneity of CSC populations might be depicted in three different, but complementary, models (Fig. 1A). In the hierarchical model that largely reflects the hematopoietic paradigm of stem cell biology, a multi-potent long-term CSC sits at the apex and gives rise to all the more committed and differentiated cell types of the tumor system, including short-term CSCs, committed progenitor

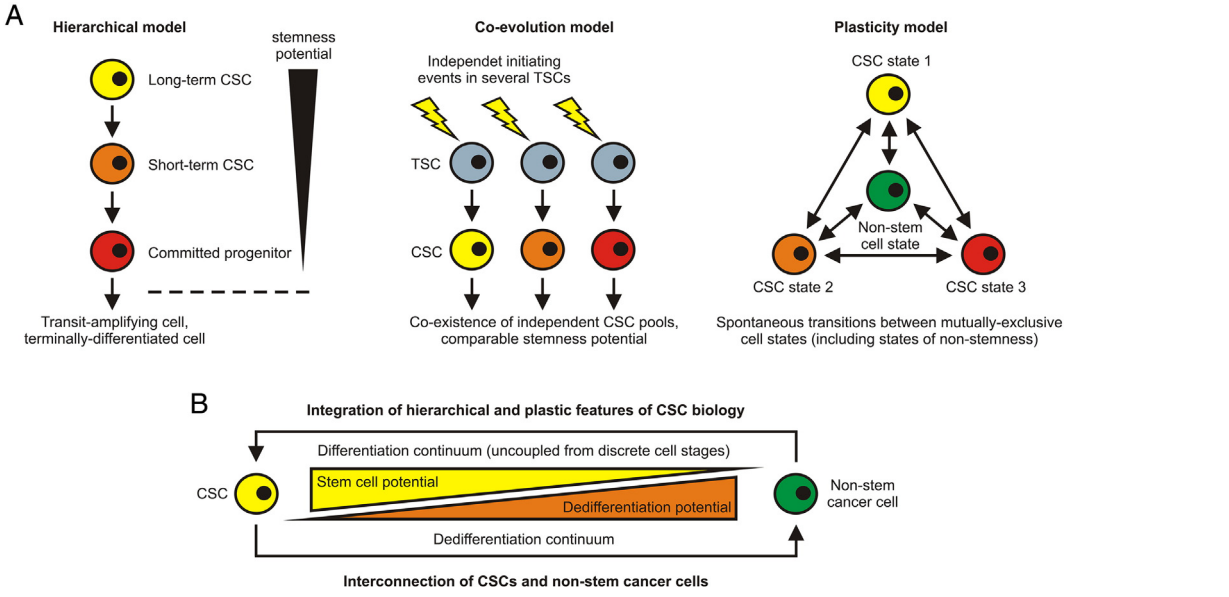

Fig. 1. Routes to Cancer Stem Cell Heterogeneity. (A) In the hierarchical model of CSC biology, a long-term CSC gives rise to a short-term CSC, which in turn produces a committed progenitor cell. The stemness potential is thereby progressively reduced, but it is not completely lost until more downstream levels of the hierarchy are reached, such as the transit-amplifying- and the terminally-differentiated cell stages. This model is largely reflective of established paradigms of stem cell biology and adult tissue organization (e.g., hematopoiesis and colonic crypt homeostasis). In the co-evolution model, different CSC populations are produced simultaneously or successively by unique initiating events in independent TSCs. Alternatively, they emerge as a result of additional genetic or epigenetic hits that occur in pre-existing CSCs (scenario not depicted). Finally, the plasticity model foretells that CSCs, due to spontaneous and stochastic transitions, can switch between distinct cell states including states of stemness and states of non-stemness. This model would thus comply with the possibility that CSCs can arise *de novo* from non-stem cells and are dynamically regulated in the TME. **(B)** Model integrating hierarchical and plastic features of CSC biology. Both differentiation and dedifferentiation define a continuum of cell stages, and the potential to differentiate is inversely proportional to the potential to dedifferentiate. Thus, the most primitive (*the* CSC) and the most mature (terminally-differentiated) cell stages are directly connected. CSC, cancer stem cell; TME, tumor microenvironment; TSC, tissue stem cell.

cells, transit-amplifying cells, and terminally-differentiated cells [107,108]. The heterogeneity of the CSC pool would thereby originate from the fact that both short-term CSCs and committed progenitor cells have retained certain characteristics of long-term CSCs that grant them with remaining stemness, while the phenotypic identities differ. In the co-evolution model, different CSC populations have independently, or successively, arisen due to unique initiating events in individual stem cells [20,109]. This model thus poses that independent CSC pools co-exist *in vivo*, which implies that the fitness of the individually transformed stem cells is roughly the same and/or that interclonal cooperation prevails over out-competition. However, the rarity of stem cells as well as the rather low frequency of initiator mutations precludes a substantial contribution of co-evolution to CSC heterogeneity *in vivo*. Finally, the plasticity model predicts that stochastic transitions between cell states [90,110] can generate dynamic populations of CSCs with mixed phenotype and/or function. Hence, this model complies with the possibilities that (i) CSCs arise *de novo* from non-CSCs and that (ii) they reversibly adopt states of stemness that are biologically dissimilar. Most likely, the hierarchical and the plasticity model are predominantly governed by epigenetic regulation, whereas genetic initiating events may play the major role in the co-evolution model. However, in either case, microenvironmental cues are operative decisive for CSC behaviour and fate, and all models are subject to Darwinian selection processes occurring within the tumoral ecosystem.

It is hard to tell which model applies best to the actual situation in a patient. Contribution from all of them is conceivable [90], even though the co-evolution model is less likely to play a dominant role due to probability issues. In contrast, the cell transition model has been fuelled by several recent studies demonstrating considerable plasticity of CSC populations. As an example, Chaffer and colleagues, in two independent studies on breast cancer [111,112], found that $CD44^{lo}$ non-CSCs can spontaneously convert to a state of stemness ($CD44^{hi}$), a process that is at least partially dependent on the EMT transcription factor ZEB1 and inducible by TGF-β. Similarly, Roesch and co-workers identified a subpopulation of label-retaining JARID1B-positive melanoma stem cells, which were required for continuous tumor growth indeed, but dynamically regulated and partially replenished from JARID1B-negative cells [113]. Finally, Chen and colleagues established that lung cancer cells can re-acquire stem cell properties through IGF-II-induced Nanog expression and dedifferentiation, challenging as well a strictly hierarchical organization of tumor stemness [114]. Overall, CSC plasticity by reversible cancer cell (de-)differentiation seems to play a major role in tumor maintenance and progression, suggesting that a tumor's proclivity to generate *de novo* CSCs, rather than its existing CSC content, determines the malignant potential ('aggressiveness'). Importantly, therapeutic intervention by adoptive cell transfer can also induce reversible cancer cell dedifferentiation, demonstrating the plasticity of the phenotype and fate of cancer cells under treatment [115].

Of note, a plastic model of tumor stemness is in line with the recent concept that CSC characteristics [69] and differentiation [67] define a continuum of cellular states and are uncoupled from discrete developmental stages. Thus, the states exhibiting the most and the least stemness (e.g., *the* CSC and a terminally-differentiated cancer cell, respectively) are directly connected, or interdependent, suggesting relative ease to cross the continuum and lose or re-acquire stem cell properties (Fig. 1B). In turn, such a model could also explain why the CSC phenotype is unstable (or reversible) [116] and underlies tremendous variation across and within tumor types, as well as in individual patients [117]. Treatment modalities that target a broad range of CSC populations are therefore of critical importance to allow an increasing rate of long-term cures. However, it is important to note that despite significant plasticity, not all cells of a tumor will adopt CSC properties at a given time, which is a prerequisite for the concept of targeting CSCs as such. Amongst others, this may be secured by a declining dedifferentiation potential as progenitor characteristics are re-acquired, which makes it extremely unlikely that a significant proportion of cells concurrently present as CSCs (Fig. 1B).

4. Therapeutic Implications of Cancer Stem Cell Heterogeneity

The lack of a consensus marker (set) for CSC populations even within defined tumor entities is one consequence of the plasticity and variability of this cell pool. As an example, ovarian CSCs were reported to reside in a variety of cell fractions including CD44+/CD24+ [118], CD44+/CD24- [119], CD44+/CD117+ [120], CD24+ [121], ALDH+/CD133+ [122,123], ALDH+ [124] and SP+ [89,95]. Similarly, at least six different marker combinations have been used to define lung [125–130] and colon [44,131–135] CSCs, respectively, and this ambiguity extends to most tumor entities (Table 1) [136]. Phenotypic instability also makes it possible that tumor-initiating capacity can stem from mutually-exclusive cell subsets [137] and in line with this notion, we observed in our studies that the designated non-CSC fraction (non-SP) still bore residual stem cell activity that supported low-level clonogenicity and engraftment [89]. We deduce from these data that direct targeting of CSCs based on exploitation of 'specific' (surface) markers is inefficient and probably foredoomed, even though the general feasibility of this approach has been proven in conceptual studies [138,139]. It is likely that for similar reasons, other strategies for CSC eradication such as vaccination [58], administration of 'CSC-selective' [30] or cell cycle-activating agents [140], and epigenetic modulation [43], will also lack efficiency and/or broad applicability. As an example, we recently demonstrated that the ionophore antibiotics salinomycin and nigericin, both shown to bear CSC-selective toxicity in various tumor models and settings [30,141–143], did not eliminate SP+ ovarian CSC due to drug transporter-mediated detoxification [48,144]. Obviously, the differential expression/activity of the protective transporter among the different CSC populations (= CSC heterogeneity) was responsible for this discrepancy. Nonetheless, several CSC-targeting drugs, including inhibitors of the Notch, Wnt and hedgehog pathways, are currently evaluated in clinical trials that cover a broad spectrum of tumor entities [145]. Some of these agents showed promising clinical activity and/or preliminary signs of efficacy (e.g., the γ–secretase inhibitor PF-03084014 and the anti-DLL4 mAb enoticumab/REGN421); however, adverse effects have also been noted [145]. Common side effects of γ–secretase inhibitors included secretory diarrhoea as well as cutaneous rash, both of which will need to be limited. In contrast, enoticumab showed a more favorable safety profile, with nausea, fatigue, headache and hypertension being the most common severe adverse effects. It is also important to note that the definition of suitable endpoints is a particular challenge in clinical trials involving CSC-targeting drugs. This is because the used agent is directed against a minority population of cells whose eradication may not instantly translate into detectable clinical benefit but rather protect in the long run (i.e., prevention of disease recurrence) [145]. Hence, adequate surrogate markers for CSC treatment response are required in such trials, even though final conclusions can only be drawn from long-term follow-up studies. Moreover, many compounds have not been tested in an adjuvant clinical setting where persisting CSCs may most adequately be targeted, which also limits interpretation of the results available so far.

A link between tumor heterogeneity and drug resistance has been proposed since almost 40 years now [146,147]. In a broader sense, this Darwinian selection process is very much reflective of what happens with bacterial cell cultures exposed to antibiotics: Even though (long-term) selective pressure can produce *de novo* resistance mutations, most of the resistance is thought to stem from rare pre-existing subclones, especially in case of short-term exposure to very high doses of antibiotics [148,149]. Molecularly, the spontaneous emergence of drug-resistant subclones can be explained by copying errors occurring during DNA synthesis, which introduces mutations thereby generating new variants. In eukaryotes, elaborate proofreading and repair systems secure a low endogenous mutation rate [150], but cancer cells, including

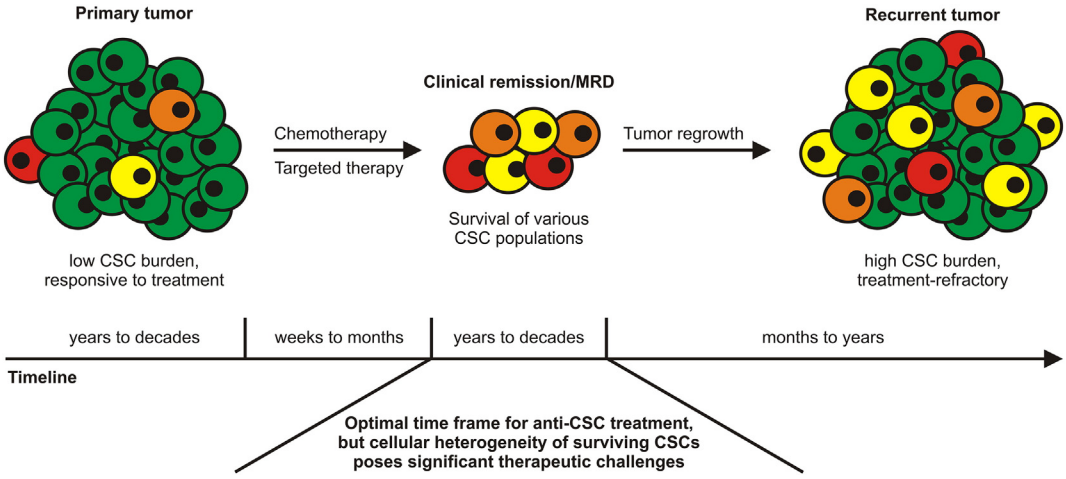

Fig. 2. Multiple Cancer Stem Cell Subsets Confer Tumor Sustenance during Remission and Provoke Treatment-Refractory Recurrence. Depicted is the typical clinical course of various malignancies with an overall timeline that covers decades. CSC-poor primary tumors show a favorable response to the initial therapeutic intervention and the patients enter clinical remission or MRD. However, heterogeneous CSC fractions persist which eventually causes treatment-refractory recurrence rich in CSCs and tumor heterogeneity. Conceptually, the cytoreduced setting of remission/MRD is very well-suited to initiate targeted anti-CSC therapy, but the heterogeneity of the surviving cells poses considerable therapeutic challenges. Finding vulnerabilities common to most CSC populations is therefore of critical importance to enable broad eradication of these cells and prevent life-threatening recurrence. CSC, cancer stem cell; MRD, minimal residual disease.

CSCs, are still prone to produce genetically dissimilar daughter cells because they innately exhibit genomic instability. Hence, it does not come as a surprise that longitudinal tracking of (medulloblastoma) cancer clones in mice and men revealed that the dominant clone at recurrence arose from a pre-existing clone that accounted only for a minor subset at the time of diagnosis [151].

Clinically, the heterogeneity of CSCs promotes treatment failure and relapse by providing an additional source of drug-resistant and/or immune-privileged disease clones [89]. Thus, remission and MRD not only represent states of low tumor but high CSC burden, but also states of significant heterogeneity of the surviving (stem) cell fraction (Fig. 2). Ultimately, this heterogeneity fuels the manifestation of clinically-apparent recurrent disease that is then stem cell-enriched and poorly responsive to both cytotoxic and targeted drugs. The question remains how can heterogeneous CSC populations be efficiently targeted *prior* to relapse? In the stem cell field, there is common sense that it is difficult and not necessarily constructive to focus primary therapies solely on CSC eradication. Clearly, this approach might not be feasible if high tumor loads need to be quickly reduced which is best accomplished using classical debulking regimens such as anti-proliferative chemotherapy, irradiation and surgery. Moreover, targeting CSCs in non-cytoreduced tumors might be a bit like looking for a needle in a haystack, e.g., because of 'physical' constraints such as drug accessibility. Based on these considerations, we deduce that the cytoreduced setting, including the different levels of remission and MRD, is extremely well-suited for CSC-directed treatment because the target cells, even though heterogeneous, are maximally exposed. However, some patients are also cured by the currently employed treatment modalities [61,152] and the fate of the CSCs in these individuals is particularly interesting. Since the tumor at one time was formed (or 'initiated'), it is unlikely that these patients' tumors never harboured CSCs. Instead, they might have been eradicated during primary treatment or they might have survived the therapeutic intervention but subsequently never managed to initiate secondary tumors. Indeed, CSCs can be principally eliminated by chemotherapeutic drugs, provided that the dosing is sufficiently high [48]. On the other hand, surviving CSCs in cured patients might be contained by immunological means, or they might lack proper support from their new sparse environment. In either case, they cannot provoke local or distant recurrence and may sooner or later undergo cell death.

4.1. Niche Dependence of Cancer Stem Cells

The search for broadly applicable and efficient CSC-targeting approaches asks for common vulnerabilities and leads to the very nature of this cell population. In order to be maintained in long-term, CSCs, similarly as physiological tissue stem cells (TSCs), must permanently prevent their differentiation into committed and terminal phenotypes [21,153]. Thus, therapeutic induction of differentiation should deplete, or exhaust, CSCs and in fact, the feasibility of this approach has been proven in elegant studies. For example, CD105-expressing renal CSCs could be triggered to enter the epithelial differentiation program using treatment with the kidney homeostatic regulator interleukin-15 [154]. The resulting cells were non-tumorigenic and further exhibited sensitivity to chemotherapeutic drugs. Similarly, Ordóñez-Morán and co-workers recently demonstrated that retinoid-induced expression of the Wnt antagonist HOXA5 interferes with the maintenance of colon CSCs by triggering their differentiation [155]. In genetically-determined colon cancer models, this switch of fate was associated with tumor regression and inhibition of metastasis. However, although providing fascinating proof-of-principle, these studies involved direct targeting of CSCs, therefore potentially sparing at least some of the heterogeneous CSC fractions. An alternative approach holding much potential especially for multi-CSC targeting purpose is to deprive them of their microenvironmental niche on which they critically depend [17,156]. Biologically, this niche represents a meshwork of highly specialized cells that concertedly ensure CSC maintenance by providing various factors that promote stemness while preventing differentiation, such as CXCL7 [157], PGE2 [158], HGF [134], and Jagged-1 [159]. Moreover, this niche might also indirectly protect CSCs based on local immune privilege, strictly regulated cell cycle entrance and progression, and favorable physico-chemical conditions such as limited accessibility for xenotoxins and low oxygen partial pressure. It might therefore not be coincidence that bone-metastatic prostate cancer cells, including CD133+/CD44+ putative prostate CSCs, target the endosteal hematopoietic stem cell niche to establish footholds in the marrow [160]. More importantly, in this study, experimental reduction of the niche size inhibited metastatic bone involvement whereas increasing it fostered the dissemination to this body site. In another elegant study, an activating monoclonal antibody against the adhesion molecule CD44 was demonstrated to eradicate LSCs, an effect that was at least in part

mediated by interference with the mandatory interaction with stem cell-supportive niches [138]. Finally, it was also shown that disseminating CD90+/CD24+ breast CSCs depend on stromal cell help to be maintained in secondary sites and subsequently be able to engraft metastases [161]. Thus, accumulating evidence suggests that CSC maintenance, and therefore ultimately tumor growth and progression, critically rely on a supportive microenvironment, rendering tumor stromal cells a rational therapeutic target for a new form of cancer treatment. Importantly, because tumors of different origin, genotype and histology share common microenvironmental elements, stroma-directed therapies also promise to be broadly applicable [162]. In addition, mutational adaptation and acquisition of drug resistance should be preventable to great extent, owing to the genetically stable nature of the targeted cells [163].

5. Stromal Cells as Prime Target for Cancer Stem Cell-Directed Therapy

The tumor microenvironment (TME) is a complex cellular meshwork composed of lymphocytes, myeloid cells, vascular and lymphatic endothelial cells, and fibroblasts [163,164]. In addition, mesothelial cells and adipocytes are sometimes present, for instance when ovarian cancer cells colonize the omentum during peritoneal dissemination [94,165]. While the importance of lymphocytes and vascular endothelial cells has long been appreciated and is already therapeutically harnessed (e.g., by checkpoint inhibition and anti-angiogenesis), the great impact of fibroblasts on tumorigenesis has only been recently uncovered. This is paradoxical because fibroblasts constitute a major component of the tumor stroma, particularly in breast cancer, lung cancer, melanoma, and hematological malignancies (bone marrow niche). Moreover, because cancer-associated fibroblasts (CAFs) represent non-transformed cell types exhibiting genomic integrity, their targeting holds the potential for long-term responses, based on prevention of quick mutational adaptation [163].

5.1. Targeting Cancer-Associated Fibroblasts to Deplete Tumoral Stemness

Although the origin and phenotype of CAFs might be diverse, they functionally converge to accelerate malignant growth in various ways. For instance, CAFs secrete a battery of growth and survival factors (e.g., IGF, HGF, VEGF) that act on neighbouring tumor- and endothelial cells to foster tumor cell proliferation and angiogenesis, respectively [166,167]. They also lay into the extracellular space a dedicated matrix rich in collagens, fibronectin and other proteins such as tenascin C, thereby supplying the tumor cells with nutrients and structural support and further providing oncogenic signals [166,167]. Other prominent CAF-secreted factors include those that prime the tumor cells to undergo an EMT and adopt migratory potential (e.g., TGF-β) [166], and those that exert their effects indirectly through modulation of the anti-cancer immune response (e.g., IL-6, IL-10, IDO) [168]. Apart from that, the tumor-promoting activity of CAFs is thought to arise mainly from their expression of stem cell ligands and –factors, which they provide to the tumor cells in both juxtacrine and paracrine manner.

For example, it was shown that CAF-secreted CCL2 induces expression of NOTCH1 in breast cancer cells, thereby stimulating their CSC phenotype and granting them with self-renewal potential [169]. Likewise, CD90+ CAFs were shown to express IGF-II and engage with lung tumor cells in a paracrine network. Ligation of the IGFR1 receptor

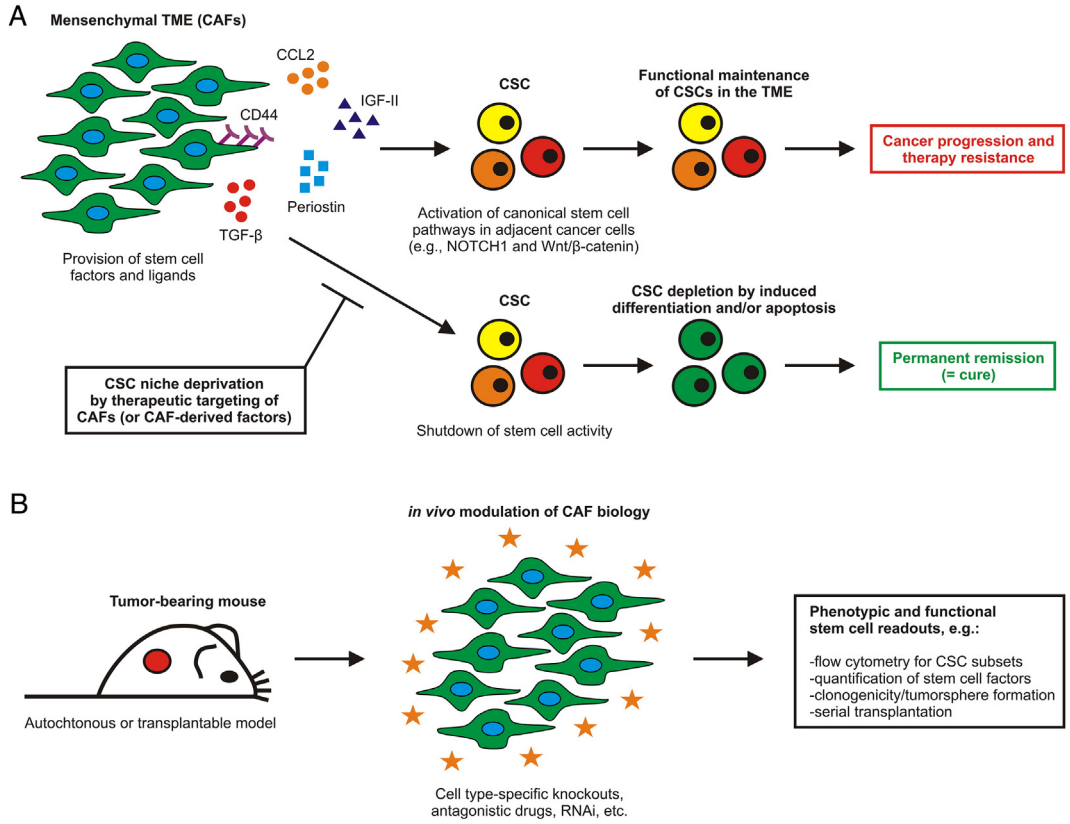

Fig. 3. Exploiting Niche Dependence to Broadly Target Cancer Stem Cells. (A) The mesenchymal TME consisting mainly of CAFs forms a specialized niche allowing CSC maintenance and fuelling tumor progression. This is accomplished by provision of various paracrine and juxtacrine factors that concertedly activate and sustain canonical stem cell pathways in the adjacent CSCs. Hence, targeting this niche represents an attractive therapeutic concept promising to be effective against a broad range of heterogeneous CSC populations. **(B)** Experimental approach to identify novel stem cell regulators on tumor stromal cells for improved cancer therapies in the future. Murine cancer models allow in-depth characterization of CAFs and further facilitate mechanistic target dissection using cell- and molecular-biological techniques. Established stem cell readouts can reveal whether the mode of action of a particular intervention is indeed based on exhaustion of the tumoral stemness potential, or whether other mechanisms are also involved. CAF, cancer-associated fibroblast; CSC, cancer stem cell; TME, tumor microenvironment.

on the tumor cells caused Nanog induction and led to dedifferentiation and re-acquisition of stem cell properties [114]. CAF-specific expression of the extracellular matrix protein periostin [161] and the cell surface glycoprotein CD44 [170] were also shown to be required for functional sustenance of CSC populations in the TME. In the former case, the effect mechanistically based on periostin-mediated recruitment of Wnt ligands, which subsequently activated Wnt/β-catenin signalling in the adjacent tumor cells. Other CAF-produced factors that induce cancer cell dedifferentiation and/or facilitate CSC maintenance include TGF-β [171], annexin A1 [172] and, possibly, matrix metalloproteinases [173]. Altogether, there is a body of evidence suggesting that CSCs rely on particular signals provided by the fibroblastic tumor stroma, rendering CAFs a highly interesting target for therapeutic interference with cancer stemness (Fig. 3A). However, the phenotypic identity of CAFs is still elusive and functional targeting strategies need to be elaborated.

Several markers are used to define CAFs, including αSMA and FAPα (both intracellular), and podoplanin and PDGFR-β (both on the cell surface) [164,167,174]. Using these markers, CAFs can be molecularly characterized both on the single cell level (flow cytometry, CyTOF) and within tissue context (e.g., confocal microscopy, imaging mass cytometry). From what is known now, CAFs generally express a panel of canonical mesenchymal markers (e.g., VCAM1, CD90, CD105, CD29, vimentin, desmin, endosialin, CXCL12) [164,174], share properties with smooth-muscle/myofibroblastic cells [175], and show a characteristic localization in the peri-tumoral stroma [176–178], even though they can also be found in more central areas of the tumor, albeit at lower frequency. It is also clear that global gene expression profiling approaches, such as gene array and the more recent RNA-seq technology, will be key to identifying novel stem cell regulators on CAFs that might provide a leverage for therapeutic intervention. Ultimately, the identified targets will need to be mechanistically investigated using suitable *in vivo* models, to separate cause from correlation. This can be accomplished, amongst others, by CAF-specific target gene knockout using Cre/loxP recombination [179] and target-selective interference with antibodies, small compounds or RNAi. In either experimental setup possible readouts include, but or not limited to, flow cytometry for CSC subsets, quantification of stemness-associated genes/transcription factors, and functional assays such as clonogenicity and serial transplantation (both performed on *ex vivo* purified tumor cells) (Fig. 3B) [20,89]. Finally, time-controlled gene expression using tetracycline- [180] or tamoxifen-regulated [181] systems will be necessary to reveal whether CSCs depend on continuous provision of stem cell factors by CAFs, or whether short-term interruptions of these paracrine and juxtacrine signalling axes are already sufficient to gain therapeutic benefit.

Once promising targets on CAFs have been identified, rational drug design should be implemented. These efforts should focus on therapeutic/neutralizing antibodies (surface and secreted targets) as well as small molecule inhibitors (surface and intracellular targets). Moreover, therapeutic selectivity for CAFs needs to be established so that normal fibroblasts and mesenchymal cells, which form the niche for physiological TSCs, remain largely unaffected. It is proposed that concomitant profiling of healthy mesenchyme (e.g., dermal fibroblasts) can disclose factors that are preferentially expressed by CAFs. Along similar lines, basic research on the pathways that govern tumor-induced activation and reprogramming of fibroblasts into tumor-promoting CAFs [182–184] will pave the way for more selective anti-CAF treatments. Ultimately, the use of CSC interference by CAF modulation will have to be demonstrated in clinical trials involving recurrence-prone tumor entities (e.g., ovarian cancer) and long-term follow-ups (several years), but until here this new paradigm of cancer treatment seems absolutely promising.

6. Concluding Remarks

CSCs represent the apex population of the intratumoral hierarchy whose elimination is believed to permanently eliminate the disease. However, these cells are hard to be therapeutically targeted, because they are inherently resistant to cytotoxic and targeted drugs, and furthermore evade radiotherapy and immune surveillance. On top of that, recent data show that CSC populations underlie significant diversification and plasticity, so that direct targeting approaches with monotherapies are unlikely to succeed ('disguise in heterogeneity'). We here propose another strategy for CSC targeting that exploits the intrinsic dependence of CSCs on productive interactions with microenvironmental cell types. CAFs express several stem cell factors and are highly abundant in the CSC niche. Blocking the relevant CAF-CSC signalling axes should thus deplete CSCs based on induced differentiation and/or apoptosis, leading to tumor regression.

It is important to consider the possibility of context-dependent CAF function and that specific CAF subpopulations might act to suppress, rather than support, tumor progression [185]. CAF-directed treatments must therefore be cleverly devised and specific for a population with clear and proven tumor-promoting activity. Lastly, the degree of niche dependence might vary among heterogeneous CSC fractions. Thus, on the path towards clinical application, combination therapies of microenvironment-targeted drugs with CSC-selective compounds should be envisaged to further limit potential persistence of CSCs during treatment.

Author Contributions

Wrote the first draft of the manuscript: Maximilian Boesch, Sieghart Sopper, Dominik Wolf. Designed and assembled the figures: Maximilian Boesch. Wrote the final version of the manuscript: Maximilian Boesch, Sieghart Sopper, Alain G. Zeimet, Daniel Reimer, Guenther Gastl, Burkhard Ludewig, Dominik Wolf. Approved the manuscript for submission and publication: Maximilian Boesch, Sieghart Sopper, Alain G. Zeimet, Daniel Reimer, Guenther Gastl, Burkhard Ludewig, Dominik Wolf.

Disclosure of Potential Conflicts of Interest

There are no conflicts of interest to declare. There is also no non-author involvement in the preparation of the manuscript.

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