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CHAPTER 4

The Developing Cancer Stem Cell Model: Clinical Challenges and Opportunities

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**Summary**

We have formally demonstrated in Chapter 2 that colon cancers harbor a fraction of tumor cells with self-renewal properties and multi-lineage differentiation potential. These cells, referred to as cancer stem cells (CSCs), are of particular interest because they are believed to be the clonogenic core of the tumor and therefore represent the cell population that drives growth and progression. Many efforts have been made to design therapies that specifically target the CSC population, since this was predicted to be the crucial population to eliminate. However, additional insights described in Chapter 3 have complicated the initial elegant model, by showing a dominant role for the tumor microenvironment in determining CSC characteristics, such as high Wnt activity, within a malignancy. This is particularly important since dedifferentiation of non-tumorigenic tumor cells towards CSCs can occur, and therefore the CSC population in a neoplasm is expected to vary over time. Moreover, evidence suggests that not all tumors are driven by rare CSCs, but might instead contain a large population of tumorigenic cells. Even though these results suggest that specific targeting of the CSC population might not be a useful therapeutic strategy, research into the hierarchical cellular organization of malignancies has provided many important new insights in the biology of tumors. In this review, we highlight how the CSC concept is developing and influences our thinking on future treatment for solid tumors. We primarily focus on the latest developments that have been achieved to inhibit the Wnt pathway in the context of colon CSCs, discuss some of the pitfalls that can be anticipated and present new opportunities for therapeutic intervention. Finally we recommend ways to design clinical trials to assess drugs that target malignant disease in a rational fashion.
INTRODUCTION

In recent years, the cancer stem-cell (CSC) theory of malignancies has received much attention. Although the idea that malignancies depend on a small population of stem-like cells for proliferation has been around for more than a century, technical developments only in the past few decades made it possible to strengthen these speculations with experimental data \[1\]. An important reason for the widespread interest in the CSC model is that it can comprehensively explain essential, poorly understood clinical events, such as therapy resistance, minimal residual disease, and tumor recurrence. In many cases, however, the initial explanatory power of the CSC model has waned as novel data challenge and redefine the CSC concept. The original, somewhat rigid interpretation of the model presents malignancy as a hierarchically organised tissue with a CSC population at the top that generates the more differentiated bulk of the tumor cells (Fig. 1A) \[2\]. In this model, the differentiated tumor cells have lost their clonogenic capacity and only the CSCs contribute to the expansion and long-term progression of the malignancy. This model suggests that CSCs should be the target for successful therapeutic intervention. Unfortunately, CSCs seem to be more resistant than differentiated tumor cells to most of the common therapies \[3-8\] which could explain therapeutic failure; the applied drug effectively kills most of the differentiated tumor cells, resulting in tumor shrinkage, yet the CSCs are relatively unharmed and reside in the fibrotic tissue that remains from the initial tumor bulk. After therapy is discontinued, the highly tumorigenic CSCs resume growth, which clinically manifests itself as a relapse. With this in mind, many researchers were convinced that specific and effective targeting of the CSC population could cure the patient. Crucially, this assumption relies on the idea that the CSC population is stable over time, and that CSC features are intrinsic qualities that cannot be attained by differentiated tumor cells. However, novel data, from our group and several others, suggest that this is not the case \[9-12\]. The CSC phenotype is much more fluid than anticipated and is strongly regulated by the tumor-cell environment. We refer to this concept as the dynamic CSC model (Fig. 1B); this nuanced view of the nature of CSCs might settle much of the dispute between those who view CSCs as a factual entity and those who consider them an illusion. Additionally, this notion directly affects the design of novel therapies aimed at targeting the CSC population. In any case, research into the CSC concept has substantially expanded our knowledge of the biology of malignancies, including response to therapeutic interventions. These insights will have an effect on clinical oncology in the near future. In this review, we highlight the latest developments in CSC research, with particular emphasis on colon CSCs and the transduction cascades implicated in their maintenance, and discuss the implications for clinical oncology; these mainly relate to identification of novel targets to overcome therapy resistance, and improved setups for clinical trials that take into account the efficacy of interventions on the CSC compartment.

BIOLOGY OF CANCER STEM CELLS

Identification. Malignancies have been known for many decades to be highly heterogeneous tissues \[13-16\]. Cancer cells differ in morphology, marker expression, proliferative potential,
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and therapy resistance. Crucially, they also differ in their capacity for long-term replication and tumorigenicity. This is shown by isolating various tumor-cell populations, based on cell-surface marker expression, and injecting them into immune-deficient mice. In several instances, tumor induction was most successful by a small population of cells exhibiting expression of cell-surface molecules associated with immature cell types. For example, in colorectal cancer, the CD133$^+$ population of cells, which comprises around 0.1–10% of the cancer-cell population, has the sole capacity to induce xenografts upon transplantation, whereas the CD133$^-$ fraction of cells fails to generate tumors [8, 17, 18]. Therefore, CD133$^+$ colorectal cancer cells are assumed to contain CSCs, and CD133 is referred to as a CSC marker. CSC markers differ between different types of malignancies, and even within a particular type of cancer various marker combinations have been reported that can enrich for CSCs—e.g., human breast CSCs can be isolated based on CD24$^-$/CD44$^+$ expression, and also by high aldehyde dehydrogenase (ALDH) activity [19].

CSCs share two main features with normal stem cells: they can self-renew (generate more CSCs) and they display multilineage differentiation potential [20]. Single-cell transplantation studies

Figure 1. The developing cancer stem-cell model (A) The initial, strictly hierarchically organised cancer stem-cell (CSC) model. CSCs differentiate into progenitor-like cells that give rise to terminally differentiated cells, which have lost the capacity to self-renew and drive tumor growth. (B) The emerging dynamic CSC model; CSCs differentiate and give rise to the differentiated cell population within the tumor. CSCs are dependent on signals from the microenvironment shaped by stromal cells. Dedifferentiation of differentiated tumor cells occurs under the influence of the microenvironment. Stromal cells (green) represent myofibroblasts, endothelial cells, mesenchymal stem cells, or infiltrating immune cells.
have revealed these qualities in individual tumor cells isolated from many types of malignancies, by showing that single CSC can initiate phenocopies from the original human cancer upon injection into immunodeficient mice [21-23]. From these studies, it is apparent that stem-like cells and more differentiated cells are present and contribute to cellular diversity in malignancies.

An issue that is intensely debated is how many CSCs are present within a tumor. Initial reports on colon, breast, brain, and lung cancer suggest a frequency of less than one CSC in every 1000 cells [8, 17, 18, 24, 25], which makes the distinction between CSCs and differentiated cells highly relevant. However, more recent data from several malignancies indicate that the number of cells displaying tumorigenic capacity might be much higher [26-28]. The number of CSCs predicted to exist in a particular neoplasm depends on the experimental procedure used, particularly the type of immunodeficient mouse strain into which the CSC sample is transplanted [27]. So far, the effect of experimental procedure on prediction of the number of CSCs in a neoplasm has only been convincingly shown in melanoma, where studies show as many as one in every four cells functions as a CSC [27]. If this is the case, viewing malignancy as a hierarchically organised tissue becomes less relevant, since a large population of cells drives tumor growth, in accordance with the classical, non-hierarchical model of malignancies [29].

**Stem-cell-associated pathways: A focus on Wnt signalling.** In addition to expression of cell-surface molecules that are typically associated with stem-like cells (i.e, CD133 is expressed in intestinal and haematopoietic stem cells [30-32]), CSCs display many features classically attributed to stem cells. In a range of tissues, CSCs show high activity of signal transduction routes that define stem cells [33]. As a defining example, we describe below in more details the role of the Wnt signaling pathway in colon CSCs.

The canonical Wnt pathway is mainly regulated at the level of β-catenin, a protein kept under low cytoplasmic concentration by the destruction complex. The latter contains the tumor suppressor protein adenomatous polyposis coli (APC); 2 kinases, casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK3-β); and Axin2, which scaffolds the complex together. In the absence of Wnt ligands, the membrane receptor complex formed by frizzled (Fzd) and low-density lipoprotein receptor–related protein 5/6 (LRP5/6) is not engaged, and CK1 and GSK3-β phosphorylate β-catenin at specific serine and threonine residues, priming its recognition by the U3 ubiquitin ligase β-transducin repeat-containing protein (β-TRCP). As a consequence, β-catenin is ubiquitinated and targeted for proteosomal degradation (Fig. 2A; [34]). Upon binding of Wnt ligands to the receptors, the destruction complex is dissolved by an ill-defined mechanism [35], and β-catenin is no longer degraded, which leads to its accumulation in the cytosol and, subsequently, translocation into the nucleus. There, it associates with the lymphoid enhancer factor/T-cell factor (LEF/TCF) family of transcription factors, converting them from repressors to activators of transcription. These nuclear events require, in a first step, displacement of the co-repressor Groucho [36] and, subsequently, recruitment of the histone acetylase CREB-binding protein (CBP)/p300 and co-activators, like pygopus (PYG) and BCL9 [37]. This step triggers a complex transcriptional program that directs cell fate, cell proliferation, and stem cell maintenance (Fig. 2B). Important Wnt target genes include c-MYC [38], Axin2 [39], and Ascl2 [40], which serve key functions in various stages during embryogenesis, but also
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during organ homeostasis and CRC development. Indeed, loss of function of Wnt components is critically involved in the pathogenesis of CRC [41]. Inactivation of the APC gene or activating mutations of β-catenin is reported in virtually all patients presenting with CRC [42] and is believed to be the critical initiating step in malignant transformation [43]. Although of various nature, those mutations ultimately result in stabilization of β-catenin and perpetual activation of the Wnt transcriptional program, even in the absence of any extracellular signals (Fig. 2C).

Interestingly, most patients contain constitutively activating mutations of the Wnt pathway, however such tumors often still retain a certain degree of regulation of the pathway. Several lines of evidence support this finding. The first example is the histopathologic observation that not all tumor cells deficient for APC display homogeneous nuclear β-catenin staining, a surrogate for Wnt signaling activity [12, 44]. This observation has been dubbed the “β-catenin paradox.” Second, the two-hit hypothesis that normally results in inactivation of a tumor suppressor gene is thought to be the consequence of two independent events. However, for APC this does not seem to be the case and it has been shown that the type of germline APC mutation that is present in familial adenomatous polyposis (FAP) patients influences the nature of the second, “somatic” hit in the APC gene [45]. Importantly, this never results in a complete loss of function of the protein and suggests a fine-tuned balance of Wnt activity that is required for optimal cell transformation [46], a principle often quoted as the “just-right” signaling model. Finally, recent observations from our laboratory have shown that Wnt signaling activity in colon cancer is also characterized by a gradient in which colon CSCs are functionally marked by a highly active Wnt signaling pathway, whereas the differentiated progeny of these cells show markedly lower levels of activity.

Reports also suggest that Notch and Hedgehog are crucial pathways in sustaining colon CSCs, which is another similarity between normal stem cells and malignant stem cells [47, 48]. Similarly, lung CSCs are characterised by high activity of the Notch and Wnt pathways, and express high levels of Oct4 [49-52]. In addition to their close association with CSCs, activation of these pathways is believed to induce a more general, immature phenotype in the tumor clone and to drive invasion and therapy resistance. Therefore, stem cell-associated pathways such as the Wnt pathway are promising new targets for anticancer drugs, much like the tyrosine-kinase inhibitors that are currently at the apex of novel cancer drugs in clinical testing.

CSC environment and dedifferentiation. Studies have shown widespread plasticity and dedifferentiation in normal somatic tissue cells that receive the right environmental input or, in an artificial setting, that are transduced with the right factors, as is the case in induced pluripotent stem cells [53-56]. Compared with normal cells, these processes are expected to occur more readily in cancer cells, which typically have an immature phenotype because of derailed signal transduction routes that guide normal differentiation. Indeed, we showed that differentiated colorectal cancer cells can reacquire a CSC phenotype, including the capacity to induce new tumors, upon exposure to factors secreted by myofibroblasts [12]. Myofibroblasts are prominent cells in the stroma of colorectal cancers and we identified hepatocyte growth factor (HGF) as the mediating factor [12]. Similar findings have been reported for mammary cancer [9-11], which suggests an important interplay in tumors between stroma and cancer cells,
to an extent that the stroma cells determine the CSC properties of nearby cells (Fig. 1B). CSCs are reported to shape their own environment by recruiting and activating specific cell types. For example, production of interleukin 6 by breast CSCs attracts and activates mesenchymal stem cells to produce the CSC supportive cytokine CXCL7 [57]. The notion that CSC fate is intimately linked with the microenvironment is substantiated by several reports that suggest the presence of a CSC niche in various tumor types, by showing a close association between CSCs and a specific subset of stromal cells [12, 57, 58]. Two recent studies report that glioblastoma stem...
cells have the potential to differentiate into the vascular endothelial lineage, a cell type that was previously shown to form the stem cell niche in this malignancy [59, 60].

**Cancer stem cells and therapy response**

**Therapy resistance.** Therapy resistance after an initial seemingly successful treatment is common and is usually explained by the presence of a resistant subpopulation of cells. In most cases, it is assumed, this resistance is a clonal trait and mediated by an acquired mutation. For example, imatinib resistance in chronic myeloid leukaemia (CML) results from additional mutations in the *BCR-ABL* fusion gene that frequently underlies this disease [61]. These resistance-conveying mutations are located within the genetic region that encodes for the binding pocket of the drug, thereby providing evidence for direct genetic-mediated resistance. Research into the CSC concept has revealed another layer of complexity; it seems that CSCs, which might have the same genetic background as the bulk tumor, can have a highly different response to therapeutic interventions according to their degree of differentiation (Fig. 3A, B) [3]. Studies in patients with CML show that CML stem cells are resistant to imatinib treatment, independent of the presence of resistance-associated mutations in the *BCR-ABL* gene [7, 62]. Stem-like cells in gastrointestinal stromal tumors (GIST; a malignancy driven by activated c-Kit that is also targeted by imatinib) are also resistant to imatinib [63]. The clinical relevance of this observation is apparent from a study of patients with GIST in whom long-term imatinib treatment was discontinued [64]. In most patients, the result was rapid relapse of the malignancy, suggesting that the clonogenic core of the cancer was not effectively targeted [64, 65]. A similar scenario occurs for chemotoxic drugs; in colorectal cancer, CSCs are more resistant than non-CSCs to conventional chemotherapeutics. This is concluded from experiments in which human primary tumors transplanted into mice after treatment with oxaliplatin or irinotecan showed an increased fraction of cells with a CSC phenotype, compared with tumors before treatment or untreated tumors, and increased tumorigenicity [6, 8]. Additionally, a recent study suggests enrichment of CSC-like cells in patients with colorectal cancer given fluorouracil [66]. Several molecular mechanisms have been suggested to underlie the differential sensitivity among tumor cells, including upregulation of antiapoptotic molecules and high expression of drug efflux pumps in CSCs compared with differentiated cells [67]. An increase in CSCs was also detected in human breast cancer samples after therapy, suggesting that this occurs in several cancer types [5, 68]. However, caution is warranted since treatment-mediated enrichment for CSC-like cells is common but not the general rule, and could be dependent on the CSC marker analysed [69]; for example, in testicular cancer the immature cells that drive tumor growth are more sensitive to cisplatin treatment [70].

In addition to supporting CSCs, the tumor microenvironment has a direct role in mediating therapy response; a study in patients with breast cancer reported a strong association between ALDH expression in the stromal compartment and a favourable clinical outcome to neoadjuvant treatment. In this study, ALDH expression by cancer cells was not prognostic or affected by therapy; therefore, the researchers proposed a function for the stroma in determining the efficacy of therapy on the CSC compartment [71]. Radiotherapy is also reported to enrich for
CSCs. In patients with glioblastoma multiforme exposed to high-dose irradiation, an increased fraction of CD133⁺ CSCs is observed in the residual tissue [72].

The question remains whether it is sufficient for an effective therapy to specifically eradicate the CSC population or if the non-CSCs also need to be targeted. The potential for differentiated cells to reacquire CSC features suggests that differentiated cells also need to be targeted (Fig. 3C). Moreover, the debate over the exact number of CSCs present in a malignancy, which depends on the experimental protocol used to demonstrate stemness of cancer-cell populations, needs to be resolved to realistically pursue CSC-specific therapies. However, it is clear that at least the CSC population needs to be targeted efficiently. Computational simulations have been performed of CSC-driven malignancies exposed to drugs that selectively kill the more differentiated cells; these simulations result in relapsing tumors that are, in many ways, more

**Figure 3.** Therapy resistance in the cancer stem-cell model (A) Cancer stem-cells (CSCs, red) are more resistant than differentiated cells (blue) to most conventional therapeutic interventions. Therefore, after treatment, tumors are enriched with highly clonogenic cells that induce a rapid relapse. (B) In theory, in the strictly hierarchic model, CSC ablation results in loss of proliferative capacity and decline of the malignancy. (C) Novel data show that differentiated cells can reacquire CSC features through signals from the environment and stromal cells (green); specific CSC eradication results in rapid restoration of CSC features in a subset of cells, and a subsequent relapse when therapy is discontinued.
malignant than the initial tumor, since they exhibit more invasive behaviour, are enriched with CSCs, and show increased heterogeneity [73, 74]. These theoretical studies might provide an explanation for the modest relation between (partial) radiological tumor response and patient prognosis in many malignancies [75]. In any case, these observations urge the development of drugs that are effective against the clonogenic core of the malignancy, since CSCs might not be solely responsible for fuelling tumor growth and long-term recurrent disease.

Targeting CSCs: Wnt components. CSCs are greatly affected by signals from the microenvironment and also rely on signal transduction pathways intimately linked with stem-cell biology, both of which are, in principle, druggable targets [76]. Direct targeting of CSC-related signal transduction routes might be problematic since normal stem cells also heavily rely on these pathways. However, the aberrant activation of these pathways in cancer might provide a therapeutic window for drugs that interfere with these signals. As an example we will present some of the efforts that have been made to interfere with the Wnt transduction cascade by either targeting the pathway components or alternatively, the different routes that converge to activate the Wnt pathway.

In 2009, Chen and colleagues screened diverse chemical libraries and identified 2 classes of molecules with Wnt inhibitory features [77]. The first class acts primarily at the level of Wnt ligand production by specifically targeting porcupine (PORCN), an acyltransferase that adds a palmitoyl group to Wnt proteins, an essential step for their secretion. The second class regulates Axin2 stability and, importantly, also targets β-catenin degradation in the presence of APC mutations [77]. Additionally, another recent study has highlighted the role of the poly-ADP-ribosylating enzymes tankyrase 1 and 2 (TNKS) in promoting Axin2 degradation. Enzymatic inhibition of TNKS by XAV-939 is able to stabilize Axin2 and promotes degradation of β-catenin [78]. Although of potential interest for various Wnt signaling–dependent malignancies, the benefit for CRC is questionable as the first class of inhibitors will, in theory, be inefficient when APC mutations render the tumor Wnt-ligand independent [79]. However, as mentioned, APC mutations rarely represent complete null mutations. In agreement, Wnt ligands are expressed in various CRC cell lines, and blockade of Wnt1 with monoclonal antibodies can trigger apoptosis in cell lines bearing APC as well as β-catenin mutations [80, 81]. Conversely, Wnt natural inhibitors such as secreted Fzd-related proteins (SFRP) are often methylated and silenced in primary tumors [82]. These proteins share similarities with Wnt cell-surface Fzd receptors and can prevent their binding with Wnt ligands and subsequent activation of the pathway [83]. Similar to inhibition of Wnt1, re-expression of SFRP in CRC cell lines or their epigenetic reactivation results in decreased Wnt activity as well as cell death [82]. These insights clearly support a rationale for targeting the extracellular machinery upstream of the destruction complex. Therefore, an antibody-targeting approach against Wnt ligands and/or blockade of the Frz receptor signaling might provide an interesting therapeutic avenue to explore [84]. From a more fundamental biological perspective, it also supports the notion that full activation of Wnt signaling cannot be explained by APC mutations alone, or alternatively, that Wnt ligands activate crucial non-canonical Wnt signaling routes.

The transcriptional program that initiates malignant transformation requires nuclear localization of TCF/β-catenin, in which abrogation of this complex can block the target gene expression and cell growth in vitro [85]. Therefore, targeting the TCF/β-catenin nuclear complex
also holds great promise for successful therapy. The recruitment of transcriptional co-activators such as PYG, BCL9, and CBP/p300 is well documented, and their induced absence is expected to prevent proper Wnt activation. As a proof of principle, Emami and colleagues screened for TCF/β-catenin inhibitors and found the leading compound ICG-001, which specifically targets and inhibits the co-activator CBP [86]. Treatment of CRC cell lines bearing APC or β-catenin mutations with this compound induces dose-dependent cell death, whereas normal colonic epithelial cells are resistant. The effect is also seen in the APCmin mouse model and in tumor xenografts. As a result, ICG-001 is expected to shortly enter in clinical phase I trials.

**Indirect targeting of the Wnt signaling cascade.** Although of great potential, most Wnt inhibitors are still in preclinical testing or in the developmental stage. Additionally, given the fact that Wnt signaling is such an important pathway involved in regulation of tissue homeostasis, interference with crucial components of this cascade is predicted to be associated with serious adverse events. For example, imbalance of intestinal and hematopoietic homeostasis is a predictable bystander effect of nonspecific Wnt inhibition [87]. It is anticipated that drug design will require agents providing great specificity and a certain therapeutic window between normal stem cells and CSCs. For example, drugs that have been studied in other clinical settings also have substantial therapeutic impact partially dependent on their Wnt inhibitory properties. The use of nonsteroidal antiinflammatory drugs (NSAID), like sulindac and aspirin, has been suggested in a number of epidemiologic studies to have a chemoprotective role in CRC [88]. Preclinical studies have shown a correlation between efficacy of chemoprevention and the Wnt modulatory effects of these compounds [89]. NSAIDs have complex modes of action, and only part of them converges to an inhibition of the cyclooxygenase (COX) enzymes [90]. COX-2 expression is seen increasingly in early stages of CRC [91]. This enhanced expression drives the production of the prostaglandin E2 (PGE2), which mediates tumor progression, angiogenesis, and metastasis [92]. Mechanistically, COX-2–induced PGE2 can prevent β-catenin degradation by inhibiting both GSK-3β and Axin2 and, as a result, activate Wnt signaling (Fig. 4B; refs. [93, 94]). The inhibition of COX-2 can only partially account for the beneficial effect of NSAIDs and COX-2 specific inhibitors (coxibs), such as celecoxib and rofecoxib, on CRC. NSAIDs and celecoxib can also induce CRC cell death independently of COX-2 expression [95]. Furthermore, NSAIDs deprived of COX-2 inhibitory capacities also have an effect on CRC [96]. For example, growth inhibition via up-regulation of the cell cycle inhibitor p21Waf1 is one of COX-2’s independent modes of celecoxib action [97]. Another interesting mechanism involves the tyrosine kinase receptor c-MET [98]. C-MET, also known as hepatocyte growth factor (HGF) receptor, is known to influence Wnt signaling. Binding of HGF to its receptor induces dissociation of membrane-bound β-catenin from the E-cadherin complexes [99]. Additionally, c-MET activation can activate PI3 kinase signaling and subsequent phosphorylation and inactivation of GSK-3β [12, 100]. As a result, β-catenin that is part of the destruction complex is no longer degraded but stabilized. Moreover, β-catenin phosphorylation on ser552 by pAKT/PKB is a nuclear translocation mark (Fig. 4A; ref. [101]) also triggered by PI3 kinase activation. These concomitant events initiated by HGF ultimately boost β-catenin levels in the cytosol and nucleus and, therefore, regulate Wnt activity. On the other hand, celecoxib can block c-MET–dependent
phosphorylation of various substrates that are accompanied by an increase in GSK-3β activity, thus resulting in β-catenin degradation and in Wnt signaling inhibition [98]. Despite other modes of coxib action that require further clarification, celecoxib is approved by the U.S. Food and Drug Administration (FDA) for the treatment of FAP [102]. It is, however, important to note

Figure 4. Targeting Wnt signaling (A), HGF is mainly produced by stromal myofibroblasts. Binding to its receptor c-MET triggers activation of PI3 kinase signaling and, in turn, AKT/PKB phosphorylation. Activated AKT/PKB phosphorylates GSK3-β at a specific serine residue, which renders it inactive and unable to prime β-catenin for degradation. Additionally, AKT/PKB phosphorylates β-catenin at a specific serine residue, which enhances its nuclear translocation. Together, this contributes to an increase in nuclear TCF–β-catenin complexes. (B), elevated levels of COX-2 are observed in cancer cells. This finding results in increased prostaglandin PGE2 production. Via its receptor, PGE2 can efficiently prevent β-catenin degradation by interfering with both GSK3-β and Axin2 function. A panel of direct or indirect Wnt inhibitors (orange) and their molecular targets are also depicted. For instance, IWR stabilizes Axin2. Celecoxib inhibits COX-2 downstream signaling but also targets the receptor c-MET. TNKS is a poly-ADP-ribosylating enzyme that promotes Axin2 degradation and is targeted by XAV-939. Monoclonal antibodies (R13 and R28) can block HGF/c-MET interaction.
that the potential benefit of coxibs in CRC prevention in the general population is hampered by cardiovascular side effects [102, 103].

In the future, these types of drugs that are rationally identified based on their ability to target CSCs, or pathways that are critical for these cells, will be an important addition to the arsenal of anticancer drugs, and will provide an opportunity to improve patient outcome. However, these drugs must pass clinical testing, which is another aspect of cancer research that is greatly affected by the notion that not all cancer cells are created equal.

**Clinical trial design**

**RECIST criteria.** Clinical testing of novel anticancer drugs develops through several phases. In phase 1 the safety of the drug is assessed, and phase 2 studies aim to provide a proof of principle in patients with late-stage disease who have typically been exposed to extensive pretreatment schedules. In phase 3, the superiority of the novel drug against the prevailing standard is tested in randomized controlled trials. In oncology, most drugs (around 70%) do not make it from phase 2 to 3, in many cases because of a lack of favorable effect on the malignancy. At that point, an immense amount of money has been spent by pharmaceutical companies, and there is usually extensive preclinical data showing efficacy in animal models. Still, lack of effect in a phase 2 trial mostly leads to abandoning the drug. In most cases, evaluation of drug efficacy in phase 2 studies is standardized by the Response Evaluation Criteria In Solid Tumors (RECIST) [104]. These criteria depend on radiological assessment of target lesions, for example tumor locations in lungs or liver (Fig. 5A). Response to the drug is reported in patients in whom the cumulative longest diameters of target lesions decreased by more than 30% (a complete response occurs when there is no radiological evidence for remaining tumor locations) [104]. RECIST criteria have been useful in many instances; however, judging drug responses by RECIST criteria only, particularly with the insight provided by the CSC model, has important shortcomings. Short-term decrease in tumor volume seems to be unrelated to the effect of the drug on the CSC population. It is possible that drugs causing radiological complete remission do not affect CSCs, which then cause rapid recurrence or new metastasis (Fig. 5B). Conversely, therapeutic interventions that show no evidence of inducing radiological response, or that show short-term progression of the disease, might be very successful in eliminating the clonogenic core of the malignancy and preventing metastasis (Fig. 5C). Therefore, drugs that make it to phase 3 clinical testing might have only induced a cosmetic response of the tumor in phase 2, and potentially potent agents are discarded when they fail to induce rapid tumor shrinkage even if they have successfully eliminated the CSC population. Similar considerations have been reported with other therapeutic approaches; for example, effective cancer immunotherapy might result in increased target lesion size because of an influx of immune cells [105]. These problems could be prevented by extensive preclinical assessment of the drug, with special emphasis on its relative efficacy against differentiated cells compared with CSCs within the malignancy. This might provide strong clues as to whether the drug is effective against CSCs, and this knowledge should be incorporated in the subsequent clinical evaluation of the drugs.
Readouts of drug efficacy. How can the efficacy of drugs in controlling the CSC population be tested? Preferentially, the efficacy of a drug should be tested in a large phase 3 trial; however, this is usually not feasible because of ethical and financial reasons. There is a need for phase-2-like protocols to assess the potential of novel drugs in patients before studies in non-heavily pretreated and early stage patients. In several cases, survival analysis can be used as a primary...
endpoint in phase 2 trials, although this is only possible in malignancies that have an overall poor prognosis (e.g., pancreatic cancer). In all other cases, surrogate endpoints need to be formulated and reliably evaluated. The CSC model provides potential clues to address this issue. Long-term clonogenicity mediated by the self-renewal capacity of stem-like cells is the crucial hallmark to be targeted by effective therapy, since it is associated with the most important clinical features, such as expansion and progression of the malignancy and formation of distant metastasis. Therapeutic failure and recurrence also ultimately depend on expansion of cells with self-renewal capacity. Therefore, direct assessment of clonogenicity provides a promising readout. Indeed, studies show the feasibility of using clonogenicity as readout in therapeutic intervention studies. In patients with multiple myeloma, enhanced decrease in tumor-cell clonogenicity was reported with a chemotherapy regime containing cyclophosphamide [106]. The applicability and clinical relevance of clonogenic analysis in solid tumors has also been shown; in a study of patients with glioblastoma, high in-vitro clonogenicity was related to poor clinical prognosis [107]. Determining post-treatment clonogenicity could only be successfully pursued in settings where neoadjuvant chemotherapy is given. In all other cases, surrogate markers of clonogenicity need to be assessed (Fig. 5D). CSC markers might serve such a purpose, since expression of these markers directly correlates to tumorigenic capacity. Biopsies from the primary tumor or metastasis could be taken to evaluate the fraction of tumor cells in treated (and pretreated) populations. A limitation with this method might be that it is an invasive procedure with a risk of serious morbidity (e.g., bleeding); moreover, the small amount of tissue that can be obtained only allows for a thorough estimation of CSC percentage when this population is sufficiently common. A less invasive method would be to use fine-needle biopsies, in which expression of CSC markers or target genes of CSC-associated pathways should be assessed in the whole tissue fragment. An indirect assessment of the efficacy of treatment in targeting the clonogenic CSC fraction could be measurement of the initiation of novel metastatic lesions. Practically, this would imply modification of the RECIST criteria to also take into account the emergence of small, new metastatic lesions.

Another challenging approach to assess treatment efficacy is enumeration of circulating tumor cells (CTCs) with CSC features. CTCs can be identified in many malignancies, and their numbers correlate with prognosis [108]. In patients with breast cancer, CTCs show a CSC phenotype (CD44+/CD24−, ALDH+) in 18–35% of cells analysed [109]; suggesting an important CSC enrichment in CTCs compared with the primary tumor, in line with the proposed role of CSCs in metastasis formation. A recent report revealed that detection of CD133 mRNA in combination with cytokeratin and carcinoembryonic antigen in peripheral blood predicted poor prognosis in patients with Dukes’ stage B and C colorectal cancer [110]. We envision that similar techniques could be applied to monitor treatment responses—i.e., the number of CTCs and fraction of CSC-marker-positive CTCs before, during, and after treatment. An important advantage of this approach is that it is much less invasive than tumor sampling by biopsies, since the analysis can be done with peripheral blood. Potentially, several other biomarkers that serve as surrogate markers for CSC numbers could be evaluated during treatment, including cytokines produced by CSCs, or degradation products of CSC-specific cytoskeleton components (e.g., Nestin) [111].
any case, basic research aimed at improving the assessment of drug efficacy in clinical trials is crucial for taking the next steps to optimise anticancer treatment.

Conclusion

This review describes how the CSC concept has developed from a rigid hierarchic model, with a fixed population of stem cells, towards a more nuanced view in which the CSC population is flexible and regulated by the environment. The latter dynamic view explains why drugs specifically aimed at the CSC population are most likely insufficient as anticancer drugs. Still, the CSC theory provides a framework for development of anticancer drugs and ways to assess their efficacy in clinical trials. It is important that we do not ignore the immense complexity of the disease and that we continue to relate the CSC concept to other developments in fundamental and clinical cancer research. We believe that novel acquired insights will shape the next generation of cancer therapies and hopefully fulfill the long-awaited promise to transform cancer into a curable or chronic disease.

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