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Cell Stem Cell
Review

Colorectal Cancer Stem Cells: From the Crypt to the Clinic

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http://dx.doi.org/10.1016/j.stem.2014.11.012

Since their first discovery, investigations of colorectal cancer stem cells (CSCs) have revealed some unexpected properties, including a high degree of heterogeneity and plasticity. By exploiting a combination of genetic, epigenetic, and microenvironmental factors, colorectal CSCs metastasize, resist chemotherapy, and continually adapt to a changing microenvironment, representing a formidable challenge to cancer eradication. Here, we review the current understanding of colorectal CSCs, including their origin, relationship to stem cells of the intestine, phenotypic characterization, and underlying regulatory mechanisms. We also discuss limitations to current preclinical models of colorectal cancer and how understanding CSC plasticity can improve the development of clinical strategies.

Introduction

Colorectal cancer (CRC) is the third leading cause of cancer death in the industrialized world. Although the occurrence of CRC has begun to decline in the wealthiest countries, the rate of incidence still maintains a steep increase in the developing world (WHO, 2014). Twenty-four years have passed since the seminal discovery of genetic alterations associated with adenoma-carcinoma progression (Fearon and Vogelstein, 1990). Since then, genomic and epigenomic approaches as well as transgenic mouse models have led to impressive insights into the nature of CRC, revealing unpredicted layers of complexity. New determinants of heterogeneity have been recognized to exist across individual CRC patients (intertumoral heterogeneity), which calls for a shift in cancer treatment toward more personalized therapies (De Sousa E Melo et al., 2013a). Surprisingly, intertumoral heterogeneity has been shown to rely on patterns of gene expression and methylation rather than on genetic factors, leading to novel CRC classifications that may profoundly affect future clinical practice (De Sousa E Melo et al., 2013a; Sadanandam et al., 2013). In parallel, our knowledge regarding the complexity within the same tumor (intratumoral heterogeneity) is continuing to increase. Intratumoral heterogeneity was first recognized in CRC more than 2 decades ago with the discovery that multiple clones bearing different genetic mutations exist within the same tumor (Wersto et al., 1991). Recently, even genetically identical CRC cells have been shown to display intraclonal heterogeneity in terms of proliferation and therapeutic tolerance (Kreso et al., 2013), indicating that epigenetic factors crucially contribute to defining the functional properties of tumor propagation and therapy resistance. The discovery of colorectal cancer stem cells (CSCs) exposed a further layer of intratumoral heterogeneity by revealing the existence of tumor cells characterized by markers of immature cells and by an increased ability to self-renew, resist chemotherapy, and seed secondary tumors (O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Todaro et al., 2007, 2014). CSCs were initially considered a population with well-defined phenotypic and molecular features. However, accumulating evidence suggests instead that CSCs are a dynamic population continuously shaped by a convergence of genetic, epigenetic, and microenvironmental factors (Kreso and Dick, 2014). In this scenario, our view of colorectal CSCs is facing a profound transformation, in parallel with a rapidly evolving concept of stemness itself, both in cancer and in normal stem cells. Stemness is increasingly viewed not only as a cell-intrinsic characteristic but rather as a property of cell populations that is highly dependent on contextual conditions (MacArthur, 2014; MacArthur and Lemischka, 2013; Sánchez Alvarado and Yamanaka, 2014). In accordance with this view, traditionally opposite effects such as stochastic and deterministic, genetic and epigenetic, and cell-intrinsic and population factors can all be regarded as cooperating forces that contribute to stemness determination and ultimately to the functional diversity of single tumor cells (Figure 1). Based on these advancements, this Review will focus on the evolving concept of colorectal CSCs, which in very recent years has been characterized by unexpected discoveries and unresolved questions. Particular emphasis will be placed on recent studies that have revolutionized previous theories on CSCs derivation, phenotype, and function and on the clinically relevant implications of such discoveries. In fact, the finding that CSC profiles are highly prognostic for CRC patients (de Sousa E Melo et al., 2011; Merlos-Suárez et al., 2011; Todaro et al., 2014) has reinforced the hypothesis that colorectal tumorigenesis is strongly linked to the presence of an altered stem cell pool. Therefore, unraveling the mechanisms through which CSCs drive tumor progression may allow clinicians to interfere with these processes and ultimately improve CRC treatment.

The Adult Intestine: A Complex Environment Sustained by Stem Cells

Understanding the mechanisms that regulate intestinal stem cells (ISCs) is instrumental to gain insights into the biology of their malignant counterparts. Even if colorectal CSCs may not necessarily derive from normal ISCs, normal and malignant stem cells can still share several basic signaling pathways (Beck and

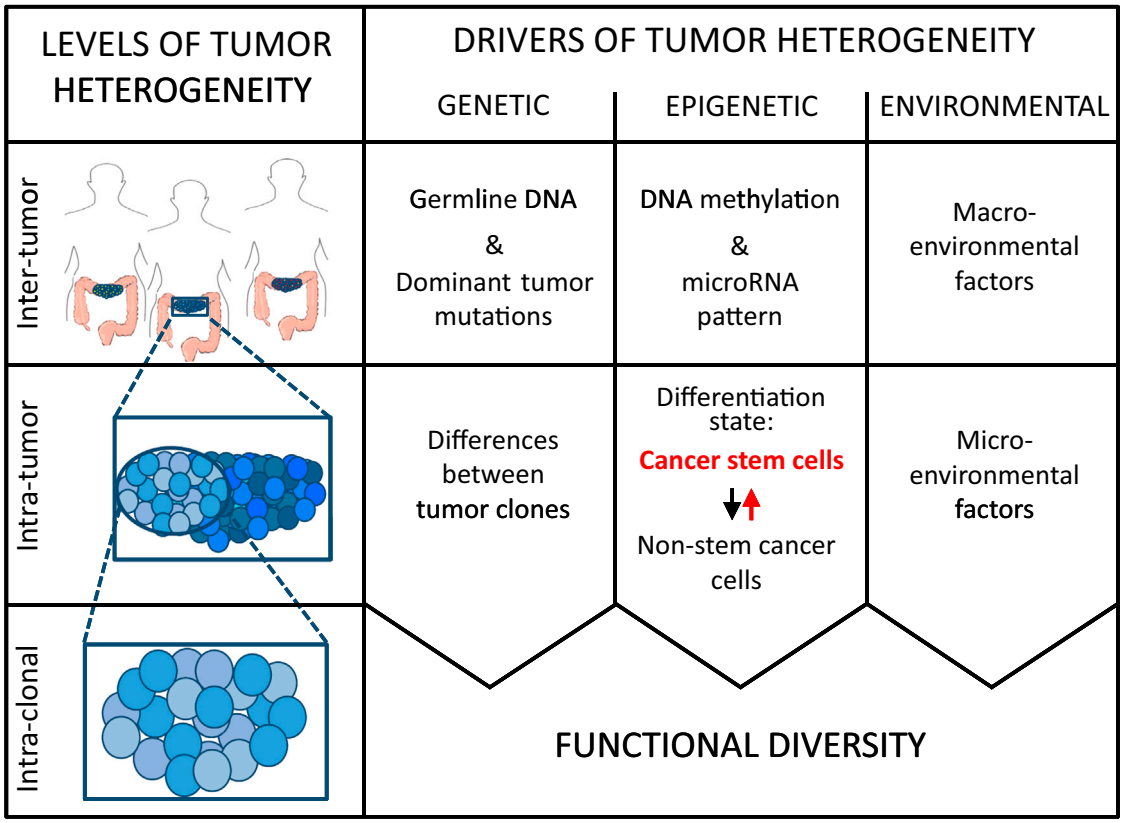

Figure 1. Colorectal CSCs in the Context of Tumor Heterogeneity
Tumor heterogeneity is driven by a combination of genetic, epigenetic, and microenvironmental factors, which all together result in functional diversity at the individual, clonal, and intraclonal level.

Blanpain, 2013). However, the vast majority of studies on ISC biology have focused on stem cells of the small intestine while, paradoxically, stem cells in the colon are much less characterized. The small intestine crypt has been extensively studied and traditionally considered as the prototype stem cell compartment, leading to the identification of functionally different populations of ISCs (Barker, 2014; Clevers, 2013). Two ISC populations, named +4 cells (characterized by prevalent expression of BMI1, HOPX, TERT, and LRIG1) and crypt base columnar cells (expressing high levels of LGR5) have both been recognized for their capacity to self-renew and give rise to all the differentiated cells of the intestinal epithelium, thus meeting the criteria of "true" stem cells (Barker, 2014; Barker et al., 2012). The two ISC populations were initially believed to represent distinct stem cell pools with different functional activities, though capable of bidirectional interconversion (Takeda et al., 2011). Proliferating LGR5$^+$ cells were considered to be responsible for intestinal homeostasis, while quiescent BMI1$^+$ cells were viewed as a reserve stem cell pool able to regenerate the LGR5$^+$ population (Yan et al., 2012). However, the subsequent finding that LGR5$^+$ cells can also express +4 markers including BMI1 (Muñoz et al., 2012) challenged the distinction between the two ISC populations. Subsequent studies largely based on lineage tracing techniques provided new insights into the nature of the ISC pool. Actively cycling LGR5$^+$ cells were found to generate a transient population of quiescent Paneth cell progenitors expressing both LGR5 and +4 markers, which, in case of injury, acquire the properties of a functional stem cell population (Buczacki et al., 2013). Similar observations were made for a population of LGR5$^-$ secretory precursors characterized by expression of the Notch ligand DLL1, which can regenerate the ISC compartment upon damage (van Es et al., 2012). Although the use of tamoxifen typically administered in lineage tracing experiments has been proposed to induce apoptosis of +4 cells and alter the balance of ISC populations (Zhu et al., 2013), lineage tracing studies appear to remain a considerable resource for ISC research. In fact, such an approach has been recently used to define the numbers of functional stem cells in crypts and adenomas (Kozar et al., 2013) and to identify a new subpopulation of DCLK1$^+$ quiescent cells in the small intestine and colon (Westphalen et al., 2014). Additional technologies have exploited live imaging of genetically marked ISCs or double LGR5-GFP;Ki67^{RFP} transgenic mice to investigate stem cell dynamics in the intestine and highlight the heterogeneous nature of ISCs (Basak et al., 2014; Ritsma et al., 2014).

As compared to the small intestine, the colon presents several differences in crypt structure and cell composition. The colonic crypt does not protrude to form villi at the mucosal surface, and it does not contain Paneth cells, +4 cells, or BMI1$^+$ cells. The colonic stem cell has been described as LGR5$^+$ or EphB2^{high} (Barker et al., 2007; Jung et al., 2011) and has been shown to be capable of generating an entire self-renewing

crypt that engrafts the mouse colon (Yui et al., 2012). Slow-cycling stem cells have also been detected in the colonic crypt and have been identified by elevated Notch signaling (Hirata et al., 2013) or LRIG1 expression (Powell et al., 2012) or as a subpopulation of DCLK1$^+$ tuft cells (Westphalen et al., 2014). Further studies are needed to fully characterize the mechanisms leading to colonic stem cell transformation, particularly in light of the high bacteria concentration present in the crypts and the potential tumorigenic role of microbiota alteration (Song et al., 2014).

ISCs and the Origin of CRC

The CRC cell of origin likely contributes to intertumoral heterogeneity and plays an important role in defining tumor features, as suggested by the distinct phenotype and clinical features of tumors that develop through the WNT/serrated pathway (De Sousa E Melo et al., 2013b). Specific activation of the β-catenin pathway in ISCs expressing LGR5, BMI1, or CD133 results in adenoma generation, pointing to ISCs as the prevalent cells of origin of CRC (Barker et al., 2009; Sangiorgi and Capecchi, 2008; Zhu et al., 2009). However, several differences exist between tumor development in mouse models and that in CRC patients, suggesting that further factors will have to be taken into account when describing the origin of CRC. First, the majority of CRC genetic mouse models lead to the formation of adenomas that rarely progress to full carcinomas (Su et al., 1992; Taketo and Edelmann, 2009). Second, adenoma formation in CRC genetic mouse models occurs in the small intestine, whereas human malignancies appear almost exclusively in the colon. Third, the development of human CRC is strongly influenced by environmental factors such as chronic inflammatory conditions (Itzkowitz and Yio, 2004) that are not usually present in genetically modified mice. Although the complex conditions underlying CRC development are difficult to recapitulate in mouse models, significant advancements have been made in understanding how common genetic mutations in CRC (*APC*, *P53*, and *KRAS*) influence stem cell dynamics in tumor initiation. While stem cells in normal crypts continuously replace each other in a random fashion (Lopez-Garcia et al., 2010), oncogenic mutations confer an advantage to the clone in which they originate, which is less subject to replacement by wild-type stem cells (Snippert et al., 2014; Vermeulen et al., 2013). It is of note that mutated stem cells do not become deterministically fixed but are still subject to some stochastic replacement by wild-type cells, thus rendering the accumulation of mutations a complex process (Vermeulen et al., 2013). This model is supported by the observation that adenomas appear to be mitotically old populations in which occasional events may trigger the rapid growth of aggressive subclones, leading to cancer development (Humphries et al., 2013). Interestingly, the competition between normal and mutated stem cells in the crypt has been shown to be influenced by inflammation: *P53* mutated clones do not have a benefit over wild-type stem cells in normal conditions but tend to prevail in an inflamed intestine (Vermeulen et al., 2013). These observations clearly show that both genetic and environmental factors play a role in CRC initiation by influencing the degree of stem cell clonal advantage, which follows a trend of constant increase during tumor progression generating increasingly competitive CSCs. While, during the early phases of tumor growth, competition occurs between normal and neoplastic stem cells, in more advanced tumors CSC clones compete with each other, with more aggressive clones emerging as the combined result of genetic mutations and environmental pressures (including cancer therapy). However, in the highly competitive microenvironment of an advanced tumor, cancer cells may raise their survival chances not only through an enhanced proliferative ability but also by increasing genetic drift through a mechanism of segregation (Sottoriva et al., 2011). In this process, metastatic CSCs may emerge at the apex of tumor evolution (Figure 2), not necessarily by virtue of their superior fitness (strictly defined as the ability to overcome competitor clones) but rather by their pioneering capacity. It is important to note that the last step of tumor evolution is always followed by a reduction in CSC expansion either when the patient undergoes remission or ultimately succumbs to the tumor. We refer to the phenomenon by which uncontrolled CSC expansion results in their ultimate destruction as "CSC overshoot," which is analogous to what occurs in ecology when a population exceeds the carrying capacity of its environment. Recently, the concept of ISCs as the colon cancer cell of origin has been challenged by studies showing that CRC may arise from more differentiated cells as the consequence of constitutive NF-kB activation (Schwitalla et al., 2013). CRC was also shown to arise from a subpopulation of differentiated quiescent tuft cells positive for the marker DCLK1 upon combined *APC* deletion and chemically induced inflammation (Westphalen et al., 2014). Taken together, these studies indicate that tumors can originate from both stem cells and non-stem cells, thus providing an unexpectedly variegated picture of the cell of origin in CRC.

Portrait of a Colorectal CSC: Phenotypic and Functional Traits

Human colorectal CSCs were first isolated on the basis of CD133 expression and demonstrated to induce tumors in mice that resembled the original malignancy (O'Brien et al., 2007; Ricci-Vitiani et al., 2007). The search for other surface markers of colorectal CSCs proceeded in hopes of finding a CSC-specific biomarker, which would greatly facilitate the development of prognostic and therapeutic tools. Several CSC phenotypes have been described. However, CSC surface markers identified so far are expressed also by normal ISCs, preventing their potential use as therapeutic targets. Markers that have been described to characterize colorectal CSCs include EphB2^{high} (Jung et al., 2011), EpCAM^{high}/CD44$^+$/CD166$^+$ (Dalerba et al., 2007), ALDH$^+$ (Huang et al., 2009), LGR5$^+$ (Kemper et al., 2012a), and CD44v6$^+$ (Todaro et al., 2014). Recently, expression of the DCLK1 kinase has been proposed to specifically mark CSCs, but not normal ISCs (Nakanishi et al., 2013), but its intracellular localization limits its potential therapeutic utility. Many questions remain open in the field of colorectal CSC identification. First, the consistency of CSC-associated markers deserves further investigation, because the CSC phenotype itself has been shown to be unstable. In fact, it has been demonstrated that CSC populations that are positive and negative for LGR5 can interconvert upon chemotherapy (Kobayashi et al., 2012). Moreover, cytokines produced by tumor-associated cells can induce increased CSC self-renewal (Kryczek et al., 2014; Lotti et al., 2013; Vermeulen et al., 2010) and reprogram transit-amplifying

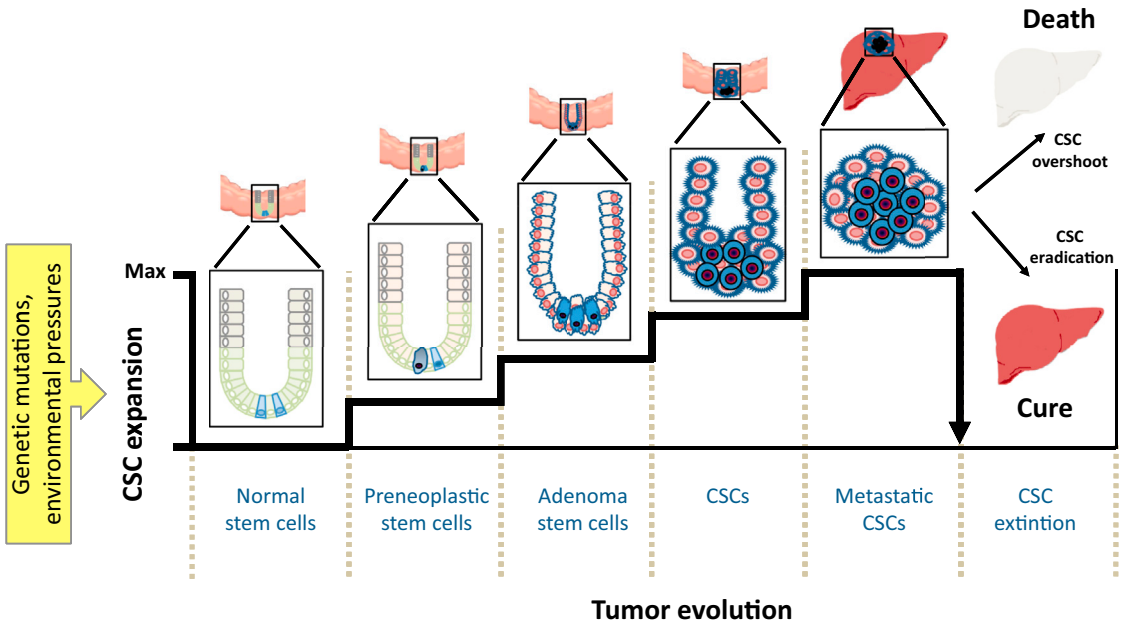

Figure 2. CSC Expansion versus Tumor Evolution in Colorectal Cancer
The expansion of colorectal CSCs increases during tumor development as the result of genetic mutations and environmental pressures. At an advanced stage, CSCs expand not only by selecting clones with increased competitiveness but also by colonizing distant tissues (Metastatic CSCs), but this state is necessarily followed by an extinction of the whole CSC population in case of either therapy success or failure.

progenitors to CSCs (Todaro et al., 2014). It is thus possible that the proportions of cells expressing CSC markers may vary depending on tumor stage (because hierarchical organization has been proposed to be strict in the early stage and relaxed in advanced tumors; Kreso and Dick, 2014), on the type and timing of therapy, and finally on a series of microenvironmental and individual factors that are predictably difficult to define. Therefore, in a dynamic scenario where CSCs may vary in quantity and phenotype during tumor progression, the expression of CSC markers should be seen as a relative and contextual parameter rather than a general property of the tumor. Besides phenotypic markers, another way to identify CSCs is through molecular and/or functional features. From a molecular standpoint, the hallmark of colorectal CSCs has been shown to be a hyperactivated β-catenin pathway, which translates into the ability to generate serial tumors in vivo (Vermeulen et al., 2010). Another stem cell functional trait is self-renewal, which in colorectal CSCs has been shown to depend on the transcriptional regulators ID1 and ID3 (O'Brien et al., 2012). Recently, the transcriptional regulator BMI1 has also been shown to be a key player of self-renewal in colorectal CSCs, as its inhibition results in stem cell loss and impairment of tumor growth (Kreso et al., 2014). Finally, important insights into the function of colorectal CSCs were obtained by molecular tracking studies, which have the ability to monitor CSC behavior in an in vivo setting, thus allowing a functional definition of their properties. Such studies demonstrated the existence of multiple types of colorectal CSCs with different roles in tumor maintenance and metastasis formation (Dieter et al., 2011), allowing the field to appreciate a further level of cellular heterogeneity among the CSC compartment and to redefine cellular hierarchies in CRC.

Colon CSCs: Elusive or Plastic?

Compelling evidence indicates that stemness is a dynamic state (Huang, 2009; MacArthur and Lemischka, 2013; Vermeulen and Snippert, 2014). Such dynamism derives from a pattern of epigenetic states associated with different propensities for proliferation, differentiation, and apoptosis, thus producing functional variability within stem cell populations that results in high adaptability to environmental conditions (Easwaran et al., 2014; MacArthur, 2014). A fundamental aspect of stem cell dynamics is plasticity, intended here as the capability of cells to shift between different functional states including quiescence/proliferation, drug sensitivity/resistance, symmetric/asymmetric division, epithelial-mesenchymal transition/mesenchymal-epithelial transition, and stem/nonstem state (Meacham and Morrison, 2013). CSCs are no exception to this rule: they have been shown to be plastic with regard to drug resistance, asymmetric division, and differentiation state. In solid tumors, the interconversion between CSCs and non-stem cells has been shown to occur in melanoma, breast cancer, and CRC (Kreso and Dick, 2014). In particular, colorectal CSCs have been shown to originate from the dedifferentiation of progenitor cells as a consequence of enhanced WNT activation driven either by elevated NF-kB signaling (Schwitalla et al., 2013) or through stimulation by cytokines produced by tumor-associated cells (Kryczek et al., 2014; Todaro et al., 2014; Vermeulen et al., 2010). It remains to be elucidated whether the plasticity of colorectal CSC depends only on extrinsic factors or also on stochastic factors, which have been shown to play a key role in the interconversion of CSCs and non-stem cells in breast cancer cell lines (Gupta et al., 2011). In fact, stochastic variations of gene expression have been shown to increase cellular fitness in a changing

microenvironment (Feinberg and Irizarry, 2010). Therefore, it is likely that colorectal CSC plasticity may be driven by a combination of stochastic and microenvironmental variations, thus resulting in an efficient adaptation strategy at the population level (MacArthur, 2014; Vermeulen and Snippert, 2014). An exacerbated plasticity, as compared to that of normal stem cells, may in fact represent a strategy exploited by colorectal CSCs to enhance their adaptation potential. While normal stem cells *respond* to challenges by attempting to restore homeostasis through tissue regeneration, CSCs may *react* to challenges by increasing in number and adopting a more aggressive phenotype. This behavior has previously been hypothesized and mathematically inferred (Harless, 2011; Sottoriva et al., 2011). Indeed, CSC numbers have been shown to increase after chemotherapy or irradiation, both in CRC and in other tumors (Dylla et al., 2008; Hu et al., 2012; Lee et al., 2011). Quiescent CSCs are spared by cytotoxic therapies, which usually result in a relative increase in tumor stem cell content, due to the selective survival of the CSC fraction. Moreover, after initially targeting proliferating CSCs, multiple cycles of chemotherapy may promote CSC proliferation (Francescangeli et al., 2012) and self-renewal (Hu et al., 2012; Lee et al., 2011; Lotti et al., 2013). Besides chemotherapy, targeted therapies that partially or totally ablate the CSC pool may have to face the problem of CSC regeneration due to the ability of non-stem cells to recreate CSCs. In an even worse scenario, targeted therapies may elicit a reactive response resulting in the resurgence of more aggressive tumors. During or after therapy, CSCs with new functional properties may be generated under the pressure of microenvironmental signals or as a consequence of genetic mutations responsible for drug resistance (Sottoriva et al., 2011). Taking into account the interconversion of CSCs and transit-amplifying progenitors, the stochastic selection of more aggressive clones may occur at both levels. Thus, the CSC model and the stochastic model could be integrated by taking into consideration the cancer progenitor pool, which may be regarded as a reservoir of the CSC compartment. Although CRC progenitors do not self-renew or migrate to metastatic sites (Todaro et al., 2014), a permissive microenvironment may foster their conversion into CSCs that are able to contribute to minimal residual disease and metastasis formation. A possible solution for avoiding posttherapy regeneration of CSCs would be to combine CSC-targeted therapies with drugs that inhibit either microenvironmental or epigenetic mechanisms responsible for the reprogramming of transit-amplifying progenitors into CSCs. Interfering with tumor cell plasticity may therefore offer new tools to support the activity of both conventional and targeted anticancer drugs.

Signaling Pathways Implicated in the Regulation of Colorectal CSCs

The comprehension of signaling pathways active in normal ISCs has advanced tremendously in recent years. By contrast, the knowledge of how such pathways are deregulated in colorectal CSCs is still in its infancy. Here, we will focus on three pathways that are emerging as key players of colorectal CSC regulation: the WNT pathway, the BMP pathway, and the Notch pathway.

WNT growth factors play a prominent role in the regulation of normal and malignant stem cell maintenance (Clevers, 2006). In CRC, 90% of tumors bear a mutation in *APC* or another key regulatory factor of the WNT/β-catenin pathway, resulting in the enhanced transcription of WNT target genes (Kinzler and Vogelstein, 1996). However, the dysregulation of β-catenin levels is not sufficient for CRCs to develop. Other events such as additional mutations, epigenetic silencing, microenvironmental signals, or pathway crosstalk are necessary to generate the nuclear levels of β-catenin that confer tumorigenic activity (Fearon, 2011). In fact, colorectal tumors harboring activating mutations in the WNT/β-catenin pathway show variable levels of WNT pathway activation. Only cells with the highest levels of WNT pathway activation actually display nuclear localization of β-catenin and possess CSC properties (Vermeulen et al., 2010). Additional genetic mutations can contribute to hyperactivate the WNT/β-catenin pathway in cells that already have basal dysregulation of β-catenin activity due to *APC* mutation. A *KRAS* mutation has been recently shown to hyperactivate the WNT/β-catenin pathway in the genetic background of *APC* loss, generating cells with a CSC phenotype and an increased metastatic potential (Moon et al., 2014). Additionally, WNT/β-catenin pathway activation can be positively or negatively influenced by other morphogenetic pathways such as the Notch, Hedgehog, PI3K, and BMP pathways (He et al., 2004; Kwon et al., 2011; Todaro et al., 2014; van den Brink et al., 2004), likely resulting in a fine-tuning of the CSC compartment. A meaningful example of how multiple pathways cooperate with *APC* loss to drive stem cell expansion in CRC is provided by recent studies on the role of the reactive oxygen species (ROS), which have been shown to connect the small GTPase Rac1 with the NF-kB pathway, allowing the initiation of colon tumorigenesis (Myant et al., 2013). Increased ROS levels that are found at the crypt base likely play an important, yet largely unknown, role in the regulation of both ISCs and their transformed counterparts.

Recently, new potential clues regarding CSC regulation have been unraveled by showing the intersection of the WNT pathway with the Hippo pathway, the latter being a key regulator of organ size control and mechanotransduction. The Hippo transducers YAP/TAZ have been shown to be integral components of the β-catenin cytoplasmic destruction complex and their translocation into the nucleus was shown to be essential for WNT/β-catenin signaling in cells where the WNT pathway is activated (Azzolin et al., 2014). YAP/TAZ regulation of the ISC compartment appears to be complex: YAP has been demonstrated to act either as an oncogene or a tumor suppressor in the colon (Barry et al., 2013; Camargo et al., 2007). In colorectal CSCs, the specific role of YAP/TAZ is unknown, but TAZ has recently been demonstrated to confer CSC traits and chemoresistance in breast cancer (Bartucci et al., 2014; Cordenonsi et al., 2011). This finding, together with the observation that YAP/TAZ is not essential in homeostatic conditions but plays an essential role during intense intestinal proliferation (Azzolin et al., 2014; Cai et al., 2010), depicts a scenario wherein YAP/TAZ may be also involved in the expansion of colorectal CSCs.

While WNT signaling prevails at the crypt base where it supports ISC proliferation, TGF-β/BMP signals predominate at the open end of the crypt, where they promote cell differentiation and apoptosis at least in part by counteracting WNT/β-catenin effects (He et al., 2004; Kosinski et al., 2007). In spite of their highly compartmentalized and orderly activity in the normal colon, the WNT/β-catenin and the TGF-β pathways are

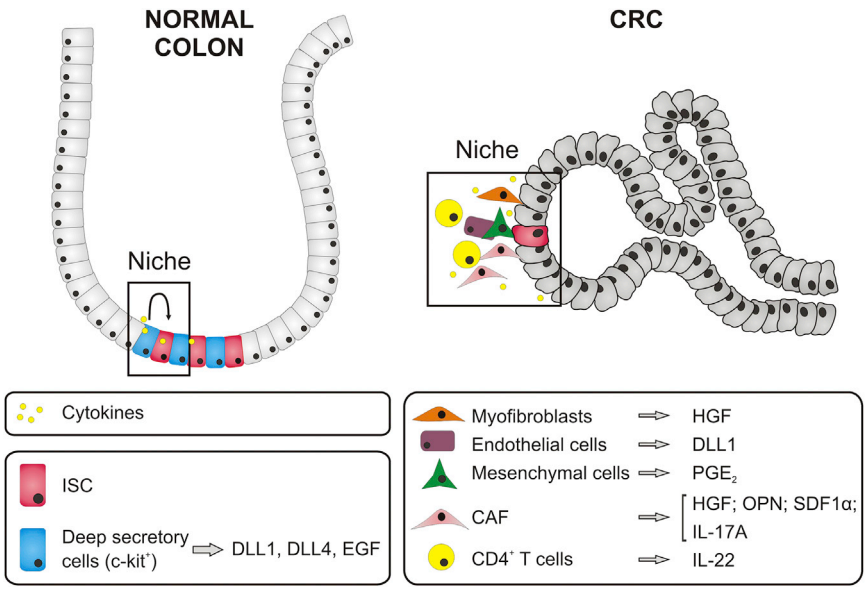

Figure 3. Schematic Model of the Stem Cell Niche in Normal Colon and CRC
ISCs, intestinal stem cells; CAF, cancer-associated fibroblast; DLL, Delta-like ligand; EGF, epidermal growth factor receptor; HGF, hepatocyte growth factor; PGE_2, prostaglandin E_2; OPN, osteopontin; SDF1α, stromal-cell-derived factor 1-alpha; IL, interleukin.

subverted in CRC and can act synergistically to promote colorectal tumorigenesis (Takaku et al., 1998). The TGF-β superfamily members, BMPs, have been shown to play a key role in the regulation of normal and neoplastic ISCs. *BMPR1* intestinal conditional knockout mice develop multiple intestinal polyps due to enhanced WNT signaling and stem cell proliferation (He et al., 2004). Among the BMP family, BMP2 and BMP4 have been specifically involved in colorectal CSC regulation by promoting CSC differentiation and antagonizing the WNT/β-catenin signaling (Lombardo et al., 2011). Recently, the transcription factor GATA6 has been identified as a key regulator of the WNT and BMP pathways in the neoplastic colon. In adenomas, which are hierarchically similar to CRC (Merlos-Suárez et al., 2011; Vermeulen et al., 2010), GATA6 has been found to be a direct regulator of LGR5 expression and its knockdown in an *APC* null background was able to suppress colon tumorigenesis. Mechanistically, GATA6 promoted CSC expansion and self-renewal through the repression of *BMP* gene expression by competing with β-catenin/TCF4 to bind to a regulatory region of the *BMP4* locus and thus lowering BMP signaling (Whissell et al., 2014). These findings provide novel and important insights on how the BMP pathway suppresses CSC self-renewal and unveil a new link between the WNT and BMP pathways, contributing to deciphering the interplay of events responsible for colorectal CSC regulation.

Notch is another relevant actor in the control of colorectal CSCs. Notch signaling is required for the homeostasis of normal mouse ISCs (Pellegrinet et al., 2011; van Es et al., 2005). In cancer, inhibition of the Notch pathway, and specifically of the Delta-like 4 ligand (DLL4), has been shown to reduce CSC frequency and enhance chemosensitivity (Hoey et al., 2009). Levels of Notch signaling have been shown to be particularly elevated in colorectal CSCs, where they prevent apoptosis and contribute to maintenance of an undifferentiated state (Sikandar et al., 2010). Recently, the Notch pathway has emerged as a regulator of cell fate in colorectal CSCs, acting through microRNA-mediated circuits that control the rates of symmetric versus asymmetric cell division. In fact, it was demonstrated that different levels of miR-34a define the cell division modality of colorectal CSCs by targeting Notch1 (Bu et al., 2013). Interestingly, early-stage colorectal CSCs express high levels of miR-34 that sequester Notch1 mRNA, thus balancing asymmetric and symmetric divisions, whereas late-stage CSCs lose this regulation and undergo prevalent self-renewing divisions. Subsequent studies pointed in the same direction, showing the existence of a signaling axis involving Snail, miR-146a, and the Notch inhibitor Numb that act in concert to tune WNT signaling, which in turn regulates self-renewing divisions and stem cell expansion (Hwang et al., 2014). Altogether, these studies depict an increasingly complex picture of signaling pathways active in colorectal CSCs.

A Dynamic Niche for Colorectal CSCs

The microenvironment has enormous power in determining the fate and function of cancer cells. In an extreme situation, the microenvironment can even reprogram cancer cells to normalcy, as was first shown by the observation that embryonic carcinoma cells produce teratocarcinomas when injected subcutaneously in mice whereas they generate normal chimeric mice when injected into a blastocyst (Mintz and Illmensee, 1975). In CRC, the microenvironmental control of cancer cells is strongly supported by the fact that chronic inflammation favors tumor development (Itzkowitz and Yio, 2004) and is strongly related to the regulation of the CSC pool (Medema and Vermeulen, 2011). The tumor microenvironment is composed of nonmalignant cells such as endothelial cells, fibroblasts, and immune cells and a noncellular matrix composed of proteoglycans, hyaluronic acid, and fibrous components. The different tumor-associated cells together with the extracellular matrix form the supportive framework of the tumor, or tumor stroma. Predictably, the stroma is not a static scaffold but undergoes dramatic changes during tumor progression and has been shown to play an active role in influencing tumor growth and chemoresistance (Egeblad et al., 2010; Junttila and de Sauvage, 2013). The specialized microenvironment responsible for stem cell maintenance represents the stem cell niche, which for normal ISCs is reportedly represented by Paneth cells in the small intestine and by a subpopulation of $cKIT^+$ goblet cells in the colon (Rothenberg et al., 2012; Sato et al., 2011b). Since in CRC crypt organization is subverted, colorectal CSCs cannot count on a traditional supportive niche and lie within a much more anarchic environment composed of multiple cell types that provide them with noncanonical signals (Figure 3). Crucially, signals provided by the colorectal CSC niche have been demonstrated to play a key role in defining

the CSC state and are responsible for the induction of CSC phenotype and function. The initial work of Vermeulen et al. (2010) demonstrated that HGF released by cancer-associated fibroblasts (CAFs) activated β-catenin-dependent transcription and self-renewal in colorectal CSCs. Moreover, HGF was able to induce the CSC phenotype in more differentiated tumor cells, providing the first compelling evidence of CSC plasticity (Vermeulen et al., 2010). Subsequent discoveries further expanded the concept of niche as a key determinant of CSC properties, identifying a plethora of functional factors released by different subsets of tumor-associated cells. Endothelial cells were shown to promote the CRC stem cell phenotype through production of the Notch ligand DLL1 and activation of Notch signaling (Lu et al., 2013). In this case, colorectal CSCs were shown to reside in the perivascular regions of human colon tumors, similarly to what occurs in glioblastoma. Mesenchymal cells belonging to the tumor stroma have been proposed to contribute to the CSC phenotype by secreting prostaglandin E_2 and cytokines that induce β-catenin activation and CSC formation (Li et al., 2012). CAFs have recently been shown to promote reprogramming of CRC progenitors into CSCs through the release of HGF, OPN, and SDF1 (Todaro et al., 2014). Even immune cells, and specifically $CD4^+$ T cells, have been shown to influence the self-renewal of colorectal CSCs through secretion of IL-22 and activation of the DOT1L methyltranferase responsible for the transcription of stem-cell-associated genes (Kryczek et al., 2014). In agreement with a dynamic view of the CSC niche, CAFs have been shown to secrete specific cytokines and chemokines upon chemotherapy treatment, including IL-17A, which increased colorectal CSC self-renewal and invasion (Lotti et al., 2013). Importantly, the latter observation indicates that chemotherapy induces a remodeling of the tumor microenvironment promoting an aggressive evolution of the CSC population. It can be predicted that many other studies will follow on how the microenvironment influences CSC dynamics and functions, possibly exposing weak points in CSC survival strategy that could be exploited therapeutically.

Stemness and Metastasis: Two Faces of the Same Coin?

The clues that link CSCs and metastasis are numerous and compelling, beginning with the evidence that only cancer cells with the attributes of tumor-initiating cells can succeed in forming a new tumor at a distant site. An initial report suggested that a subpopulation of CSCs expressing CD26 was responsible for the development of CRC metastasis (Pang et al., 2010). Later, the hypothesis of an overlap between metastatic cells and CSCs was supported by two key studies based on the clonal analysis of lentivirally marked tumor cell populations, showing that metastases arise from a subpopulation of cells present in the primary tumor. These cells were quiescent, resistant to chemotherapy, and endowed with long-term self-renewal capacity, thus possessing typical CSC features (Dieter et al., 2011; Kreso et al., 2013). A relevant question concerns the potential ability of colorectal CSCs to colonize different organs, which could be part of a stochastic dissemination or a molecularly driven process. A recent report showed that only CSCs expressing the thrombopoietin receptor (CD110) are able to colonize the liver, while the expression of the CUB-domain-containing protein 1 (CDCP1) is associated with the propensity to make lung metastasis (Gao et al., 2013). Thrombopoietin production by hepatocytes increases the self-renewal of $CD110^+$ CSCs and allows their extravasation into the liver parenchyma, while $CDCP1^+$ CSCs seem to home preferentially to the lung microvascular endothelium (Gao et al., 2013). Notably, both $CD110^+$ and $CDCP1^+$ CRC cells were included in the $CD133^+$ population and possessed the functional properties of CSCs. Although both CD110 and CDCP1 are negatively correlated with patient outcome and may be used as prognostic biomarkers, genetic and epigenetic events driving their differential expression and the consequent metastatic proclivity are still unclear.

While it appears intuitive that the tumor cells able to initiate metastases should be CSCs, until recently it was unclear whether all colorectal CSCs were able to form metastases. For a long time, it seemed reasonable that the acquisition of specific genetic mutations would account for the emergence of metastatic potential in CSCs. However, a comparison of primary colorectal tumors and matched metastases found no mutations that were specifically and consistently associated with metastasis (Jones et al., 2008). On the other hand, increasing evidence suggests that mutations in epigenetic regulators or in genes that control them through metabolic pathways favor the emergence of altered states that provide cancer cells with an increased adaptability to environmental pressures (Oskarsson et al., 2014). Therefore, the genetics of the primary lesion, together with epigenetic and microenvironment components, jointly dictate the metastatic features of single tumors. In this context, TGF-β has been shown to play a key role in the interactions between metastatic tumor cells and the microenvironment in CRC. Although TGF-β acts as tumor suppressor during the initial transformation, it plays a predominant oncogenic role during tumor progression. This switch seems to occur after the acquisition of additional mutations, such as *P53* (Adorno et al., 2009) or *SMAD4* (Zhang et al., 2010). In advanced tumors, increased TGF-β levels are associated with poor disease outcome due to the induction of a prometastatic program activated by crosstalk between tumor cells and stromal cells, which results in increased survival of metastatic cells and organ colonization (Calon et al., 2012). Furthermore, it seems likely that CRC cells capable of initiating metastasis possess the capacity to raise TGF-β levels in the environment by either secreting TGF-β or recruiting TGF-β-producing cells such as macrophages, CAFs, or platelets (Calon et al., 2012). The metastasis-initiating cells in CRC have been phenotypically identified as cells expressing CD44v6, a coreceptor for MET (Todaro et al., 2014). The formation of metastatic colorectal CSCs is counteracted by BMPs, which induce CSC differentiation and loss of both CD44v6 and metastatic potential. In contrast, cytokines that activate PI3K and increase the activation of β-catenin, such as HGF, SDF1, and OPN, turn non-metastatic progenitors into metastatic CSCs (Todaro et al., 2014), in line with clinical data showing the requirement of concomitant PI3K and β-catenin activation for metastasis formation in CRC (Ormanns et al., 2014). Both constitutive and reprogrammed colorectal CSCs express CD44v6 and display epithelial-mesenchymal transition genes that collectively contribute to enhance cell motility, invasiveness, and metastatic potential of CSCs (Todaro et al., 2014). Such antagonism between BMPs and the PI3K/β-catenin pathway has been proposed to

be altered in tumors with *SMAD4* mutations, which in NCI60 cell lines may turn the tumor suppressor activity of BMPs into metastasis-driving signals through the activation of the ROCK pathway (Voorneveld et al., 2014). However, we found that BMP4 promotes the differentiation of primary colorectal CSCs regardless of *SMAD4* status (Lombardo et al., 2011), suggesting that additional investigations are required to understand BMP signaling and the role of *SMAD4* mutations in these cells. Finally, the homeobox transcription factor PROX1 has been recently proposed as an essential player of metastatic CSC expansion (Wiener et al., 2014). PROX1 contributes to the metabolic adaptation of colorectal CSCs to an unfavorable microenvironment by sustaining autophagy, which has been previously shown to be essential for the survival of CRC cells (Ragusa et al., 2014; Sato et al., 2007).

Potential Relevance of Colorectal CSCs for Tumor Prognosis and Therapy

Recent studies on the molecular signatures of colorectal CSCs point to a strong link between levels of CSC-associated genes and patient outcome. In accordance to what was shown for breast and other tumors, a "stemness" signature was shown to predict disease relapse in CRC patients (Giampieri et al., 2013; Merlos-Suárez et al., 2011). In contrast to the clear significance of CSC-associated molecular profiles, the expression of CSC surface markers as predictors of patient outcome is controversial. A meta-analysis evaluating 15 studies found that high CD133 expression was actually an independent prognostic marker for both overall survival and disease-free survival (Chen et al., 2013) whereas, conversely, LGR5 expression may be unrelated to patient prognosis (Ziskin et al., 2013). Additionally, germline polymorphisms in the CSC-associated genes *LGR5*, *CD44*, and *ALDH1A1* seem to define a subset of stage II and stage III patients with a significantly shorter recurrence time (Gerger et al., 2011). CSCs represent an attractive target for more effective therapies against CRC. A number of reports have shown that colorectal CSCs display an intrinsic tendency toward chemoresistance and may be responsible for tumor regeneration and relapse after conventional therapy (Colak et al., 2014; Dylla et al., 2008; Lombardo et al., 2011; Lotti et al., 2013). Direct CSC targeting can be achieved by inhibiting self-renewal pathways, by interfering with vital antiapoptotic or metabolic pathways, by activating differentiation pathways, or by acting on the protective microenvironment. Several potential anti-CSC targeted drugs have emerged in past and recent studies, some of which are making their way to the clinic (Table S1 available online). In past studies, blocking IL-4 autocrine production led to CSC sensitization to drug-induced death by lowering the levels of antiapoptotic proteins (Todaro et al., 2007), while inhibition of the Notch pathway was able to reduce CSC frequency (Hoey et al., 2009). Subsequently, a screening of kinase inhibitors identified Polo-like kinase 1 as a therapeutic target required for the survival of proliferating colorectal CSCs (Francescangeli et al., 2012). More recently, mitochondrial targeting agents have been shown to increase the efficacy of chemotherapy (Colak et al., 2014) and target quiescent colorectal CSCs (Zhang et al., 2014), which are particularly difficult to eradicate (Francescangeli et al., 2012). Finally, promising early clinical data with WNT-targeting agents are emerging (Le et al., 2014). These agents may have an anti-CSC activity similar to that of BMPs, which appear as strong endogenous and exogenous CSC inhibitors in CRC (Lombardo et al., 2011; Todaro et al., 2014) and may potentiate the antitumor activity of chemotherapy, provided that the protumor cytokines are not largely diffused in the niche. In summary, it appears increasingly clear that the size of the CSC compartment in CRC can be regulated by the relative proportions of pro-CSC and anti-CSC factors (Figure 4).

Currently, most of the interventional clinical trials are carried out on metastatic patients with the aim to temporarily control tumor progression. However, with the increasing knowledge of colorectal CSC biology, several therapeutic options are becoming available. The effectiveness of CSC-targeted therapies may be maximal on CSCs disseminated outside the tumor context, which may be vulnerable to combined therapies due to the absence of a protective niche. In fact, a partial targeting of disseminated CSCs may be empirically carried out in the clinic during adjuvant treatment. Although there is no formal proof that CSCs can be effectively targeted in CRC patients, it is extremely likely that adjuvant chemotherapy can kill disseminated CSCs that escape surgery. This would explain why there is a statistically significant curative advantage in the administration of adjuvant therapy in stage II and III patients. An example of the need to target CSCs in the right clinical setting may be represented by the PI3K/AKT pathway. Although previous clinical studies indicated a low efficacy of PI3K/mTOR inhibitors as single agents in advanced patients (Janku et al., 2014), the same treatment in a more appropriate setting may have a considerably higher success. Based on experiments with cell lines overexpressing β-catenin and FOXO3a, it has been proposed that, in the subset of tumors with upregulation of both genes, PI3K inhibition can promote CRC cell migration rather than antitumor activity (Tenbaum et al., 2012). However, we subsequently found that colorectal CSCs have a constitutive activation of β-catenin and PI3K/AKT pathways and die in vitro upon PI3K inhibition. Moreover, we observed that in mice carrying primary orthotopic tumors, treatment with PI3K inhibitors considerably reduces metastasis formation (Todaro et al., 2014). Our data are in line with recent clinical observations showing that in CRC the metastatic activity of β-catenin depends on PI3K activation (Ormanns et al., 2014). Thus, it is likely that the combination of anti-CSC agents and chemotherapy could be significantly more effective than the standard treatment, particularly when disseminated CSCs are targeted in the adjuvant setting (Figure 5).

A crucial issue in translating CSC discoveries into the clinics is the development of suitable tools used to assess the efficacy of CSC-directed therapies in CRC patients. In fact, both in humans and in mice, reduction of tumor volume is not informative of therapy efficacy on the CSC pool and, by contrast, may prelude the development of a more aggressive CSC-driven tumor. Moreover, the traditional structure of phase I/II clinical trials, which are conducted on advanced metastatic and heavily treated patients, may not be suitable to investigate the activity of new CSC-targeted drugs. In fact, in the context of advanced tumors the hierarchical structure dominated by CSCs may be lost (Kreso and Dick, 2014) and the protective effects of the tumor microenvironment may heavily interfere with targeted drugs. Besides these potential issues, there is also a need for methods suitable to evaluate therapy efficacy on the CSC population. These

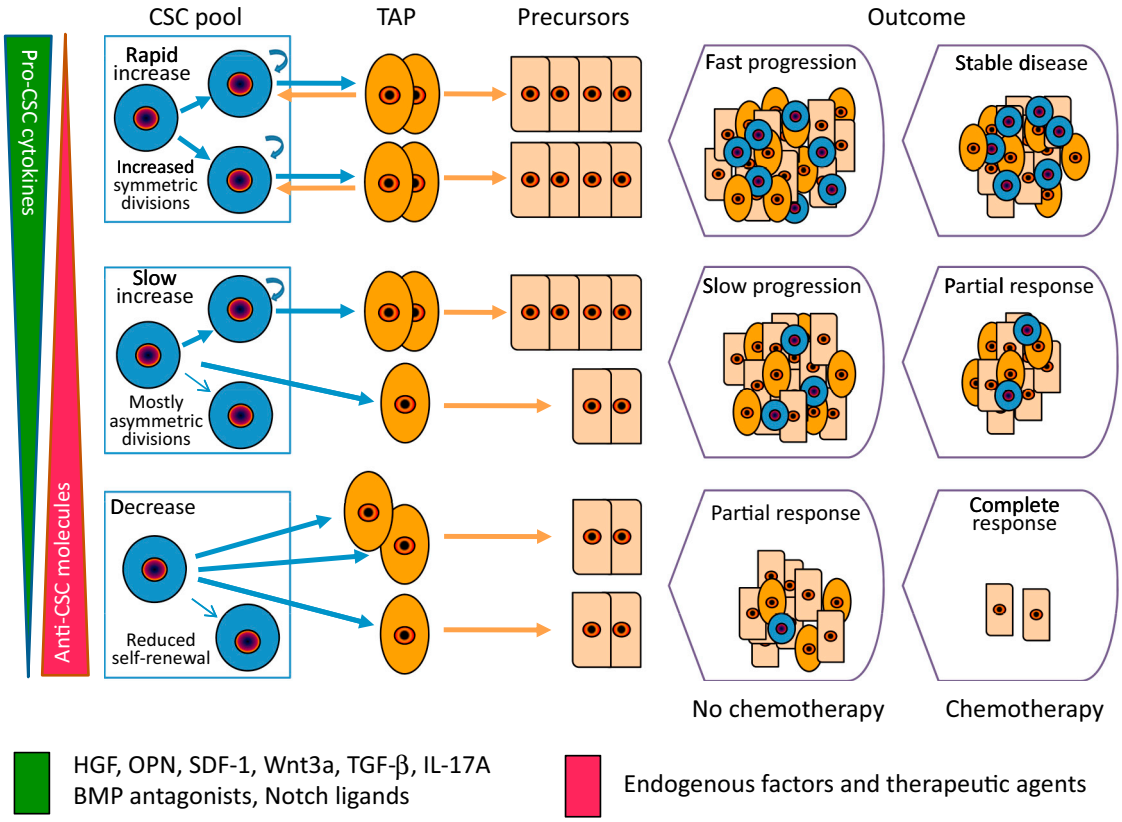

Figure 4. The Balance of pro-CSC and anti-CSC Molecules Influences Disease Outcome
Pro-CSC cytokines produced by the tumor microenvironment increase the CSC pool, while endogenous and exogenous anti-CSC molecules decrease CSC number, forcing the sequential differentiation into transient-amplifying progenitors (TAP) and precursors. Chemotherapy is scarcely effective in the presence of a protumor microenviroment and requires the contribution of therapeutic molecules able to target CSC self-renewal or survival. HGF, hepatocyte growth factor; PGE_2, prostaglandin E_2; OPN, osteopontin; SDF1, stromal-cell-derived factor 1; BMP, bone morphogenetic protein; IL, interleukin; TGF-β, transforming growth factor beta.

approaches may include detection techniques based on the evaluation of CSC phenotypic traits, which would be easily manageable and suitable also to evaluate circulating CSCs. However, it should be kept in mind that CSC markers appear to vary both with time and among different patients, possibly being context dependent and related to the type and mutational profile of the tumor cell of origin. Therefore, it would be preferable to establish clinical endpoints related to CSC function in order to evaluate therapy efficacy on the CSC population in clinical trials. One potential CSC-related endpoint would be evaluating the formation of new metastases in stage IV patients, particularly in those who underwent hepatic metastasis resection and may benefit significantly from subsequent treatment with CSC-targeted drugs. Therefore, administration of therapeutic CSC-targeting molecules in the appropriate clinical setting appears to be a path worth pursuing. However, for this to be possible, an increased understanding of CSC biology should proceed in conjunction with a CSC-oriented planning of clinical trials.

Limitations of Preclinical Models: Current Evidences and Pitfalls

Methods used to isolate and expand colorectal CSCs strongly influence our knowledge of this cell population. For this reason, it is important to clarify as much as possible the limitations and possible biases associated with different culture methods. This is particularly important for colorectal CSCs, which can be expanded relatively easily in vitro and in vivo. Commonly used methods to expand colorectal CSCs are represented by multicellular spheroid cultures (MSCs) and organoid cultures (OCs) in vitro and by xenografts in vivo. The latter can be obtained either by inoculating mice with dissociated CSCs to obtain subcutaneous or orthotopic (colonic) tumors or by subcutaneously transplanting primary tumor fragments to produce patient-derived xenografts (PDXs, also called "xenopatients").

Spheroid cultures of cells derived from primary or metastatic tumors have been widely used to isolate and expand colorectal CSCs (Dieter et al., 2011; Ricci-Vitiani et al., 2007; Todaro et al., 2007; Vermeulen et al., 2010). MSCs represent a convenient method to obtain large numbers of colorectal CSCs suitable for experimental purposes, for banking, and as a reservoir for in vivo experiments. However, many scientists rely on the use of conventional cell lines instead of primary samples as starting material for MSCs. Such cells underwent a number of passages in vitro and may be extremely different from those growing in patient tumors. There is a vast amount of scientific data continuously produced using these models, which may easily contain artifacts and lead to unreliable conclusions. Moreover, it is possible that the growth of MSCs is influenced by the presence

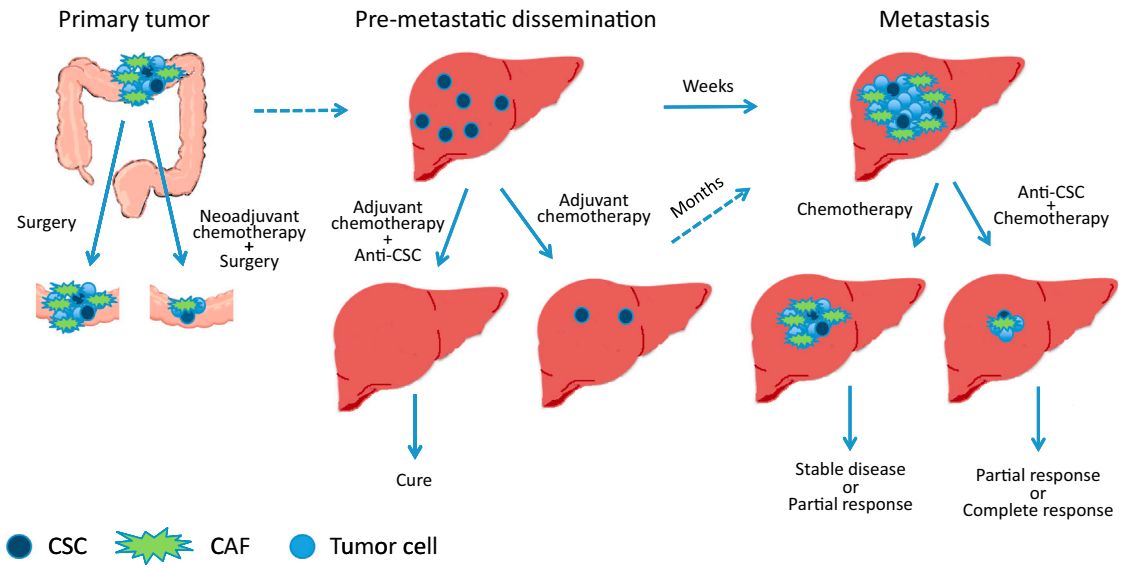

Figure 5. Impact of Colorectal CSCs on Clinical Process

After CRC diagnosis, the primary tumor is usually removed by curative or palliative surgery, which may follow a neoadjuvant chemotherapy. In case of CSC dissemination, chemotherapy may kill a significant proportion of CSCs, which are not protected by the tumor microenvironment. However, some residual CSCs that survive chemotherapy may produce liver metastases that, in turn, need to be treated with effective therapies. While chemotherapy is present in all the current therapeutic regimens for CRC, additional therapeutics targeting major CSC pathways (Anti-CSCs) are required to achieve a potential cure in advanced disease, particularly in the absence of specific therapies targeting driver oncogenes. CAF, cancer-associated fibroblast.

of specific driver mutations, possibly depending on culture conditions used by different investigators. In fact, the efficiency of successfully isolating colorectal CSC spheroids from surgical specimens is relatively low, being around 30% (Ricci-Vitiani et al., 2007), and factors that favor the selection of MSC-generating cells remain largely unknown.

The generation in vitro of 3D crypt-like structures called organoids has been exploited for growing normal and neoplastic ISCs (Sato et al., 2009, 2011a). Organoids have been recently shown to be amenable to the introduction of oncogenic mutations, making them a candidate for the model of choice for the study of genetic alterations associated with CRC development (Li et al., 2014). However, some limitations may still exist. *KRAS* and *P53* mutations have been recently correlated with the efficiency of OC development (Li et al., 2014). Moreover, OCs, similarly to MSCs, contain only a minority of stem cells. Selective expansion of the stem cell fraction in normal intestinal cell cultures has been recently achieved through the use of two small molecules, valproic acid and a GSK-3 inhibitor. In such conditions, $LGR5^+$ cells have been reported to grow as spherical colonies maintaining a high purity and self-renewal capacity (Yin et al., 2014).

Subcutaneous CSC-derived tumor xenografts have been broadly used for preclinical tests of new therapeutic agents. However, the technology for orthotopic implantation of CSCs is now available (Todaro et al., 2014) and offers the significant advantage of generating spontaneous lung and liver metastases. The generation of PDX platforms has also emerged as a reliable method for preclinical drug testing that preserves the individual diversity and the genetic heterogeneity typical of the tumors of origin (Bertotti et al., 2011). Moreover, PDXs also give rise to distant metastases (Puig et al., 2013). However, similarly to primary CSC cultures, PDX may also be influenced by the presence of specific mutations, as suggested by the fact that some investigators obtained a strong enrichment of PDX from *KRAS*-mutated tumors (Puig et al., 2013). The major limitation of all the in vivo methods for growing colorectal tumors concerns the xenogenic network of signals surrounding the tumor, because mouse and human microenvironments may impact differently on CSC behavior. Moreover, the absence of lymphocytes and related cytokines may reduce the predictive capacity of such preclinical models, which would appear more solid if complemented by experiments with transgenic models recapitulating the human disease.

A major area of debate has been the quantification of CSCs, both in terms of functional assays and markers for identification. The minimal frequency (1 in 57,000) of colorectal CSCs initially hypothesized based on transplantation efficiency (O'Brien et al., 2007) does not appear to be realistic in light of subsequent studies, which showed that CSC frequency in solid tumors is generally much higher and that current assays have a number of limitations (Quintana et al., 2008). An apparently solid marker such as CD133 has been criticized, possibly due to the absence of mRNA downregulation after colorectal CSC differentiation (Kemper et al., 2010). In general, the study of CSCs for clinical evaluation in the context of prognostic analysis and biomarkers for supporting therapy decisions suffers from the controversy generated by conflicting data, particularly on CSC markers. A possible explanation could be that genetic and epigenetic differences impact CSC marker expression (Kemper et al., 2012b), which may differ from sample to sample. The picture is further complicated by the ability of cancer progenitors and stem cells to interconvert from the pressure of the microenvironment. An increased availability of CSC-related information directly derived from CRC patients will be instrumental to fully understand the

limitations of current experimental models and to solve the controversies.

Concluding Thoughts

CRC is one of the most compelling examples of a hierarchically organized solid cancer dominated by a subpopulation of immature cells with peculiar molecular and functional features. However, initial assumptions on the nature of colorectal CSCs are progressively falling apart, making space for a new view where stemness arises from the continuous adaptation of cancer cell populations to microenvironmental signals. Increasing evidence suggests that, in both the normal and neoplastic intestine, stemness results from the incessant convergence of cell-intrinsic features (genetic mutations and epigenetic regulation), local signals (of a chemical, mechanical, and molecular nature), stochastic events, and population forces that continuously shape the stem cell pool. In this scenario, the future development of successful clinical strategies will be tightly linked to a deeper understanding of the dynamic, adaptable, and evolving nature of colorectal CSCs.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes one table and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2014.11.012.

ACKNOWLEDGMENTS

This work was supported by the Italian Association for Cancer Research (AIRC) with a "5 per mille" grant to R.D.M. and G.S. The authors would like to thank the director and staff of the ISS library for logistic support; Mauro Di Giovanni for figures; and Federica Francescangeli, Paola Contavalli, and Marcello Maugeri-Saccà for helpful discussions. We extend our most sincere thanks to all the colleagues that, with their efforts, have brought to light the knowledge collected in this review.

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