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Cancer Stem Cells: to be or not to be, is that the problem?

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Introduction

Cancer Stem Cells features and Tumour Progression

The Cancer Stem Cell Concept

Human tissues are characterized by the ability to maintain, under physiological conditions, their mass and architecture over time, thanks to high regulated self-renewal and differentiation programs. This balance is regulated by a minor subset of long-lived cells, called stem cells (SCs), able to perpetuate themselves with self-renewal capacity, and at the same time generating mature cells, specific for each tissue, through differentiation processes. In the last years tumours have been considered as aberrant organs, containing a small fraction of tumour cells, called cancer stem cells (CSCs), with tumor initiation capacity, which with their indefinite proliferative potential can accumulate several mutations, thus starting and driving tumorigenesis. Like their normal counterpart, tumours are composed by heterogeneous cell populations with different degrees of accumulated mutations and differentiation. Actually this way to look at cancer is not new, since the idea of a subpopulation of tumour cells with stem cell properties (then called CSCs), responsible of tumour development and progression, was proposed more than a century ago [(1-2)]. This cell subset is considered to be responsible of intra-tumour heterogeneity, malignant behaviour and tumour recurrences, following chemo- or radio-therapy. Of course this idea was well accepted among the clinicians, frustrated by the partial and often lack of success of current anti-cancer therapies.
The concept of CSCs arose from the human acute myeloid leukaemia (AML) model, in which the majority of cells, with different differentiation patterns, originated from undifferentiated pluripotent stem cells [(3-5)]. This led to the idea that CSCs, such as normal stem cells, could reproduce ad infinitum thus generating the limited lifespan, multi-lineage differentiated majority of cells that compose the tumour bulk, called derived population (DC) [(6)].
This concept was strongly supported by *in* vivo assays based on xenotransplants using immunodeficient mice: in these experiments only a minor fraction of injected tumour cells would recapitulate human tumours, the CSCs. *In vitro* this cell subset grows in particular culture conditions (ultra-low adhesion flasks, serum-free media, growth factors as EGF and b-FGF), as spheres.
Even if the idea was simple and easy to validate, the difficulty in the identification and studying of these cells, which became the mark of new target therapies, made this field more complicated than expected [(5, 7-8)]. From a clinical point of view the definition and characterization of CSCs could allow to design new combinations of diagnoses and treatments, personalized for each patient.

CSC Features and Tumour Heterogeneity

The CSC origin concept, from normal SCs, is due to the idea that only the SCs would have the time to accumulate all the mutations necessary for cell transformation. However if we imagine a first oncogenic event conferring an increased lifespan to the cells, this would not be necessary.
The interest of the researchers about CSCs is mainly due to their resistance to conventional anti-cancer treatments, thus generating the idea that it is needed to couple to those treatments a specific anti-CSC therapy to avoid resistance and tumour recurrences. Many patients with solid tumours indeed respond poorly to existing treatment regimens, or they often relapse quickly after the initial remission. This capacity to survive to conventional therapies is due to several characteristics including high expression of drug transporters, cell cycle quiescence, high levels of DNA repair

machinery, resistance to apoptosis, and last but not least the over-activation of some signalling pathways, such as EGFR, VEGF, Wnt and Notch, and to the effect of the microenvironment in which they reside and growth (tumour niche) [9-10].

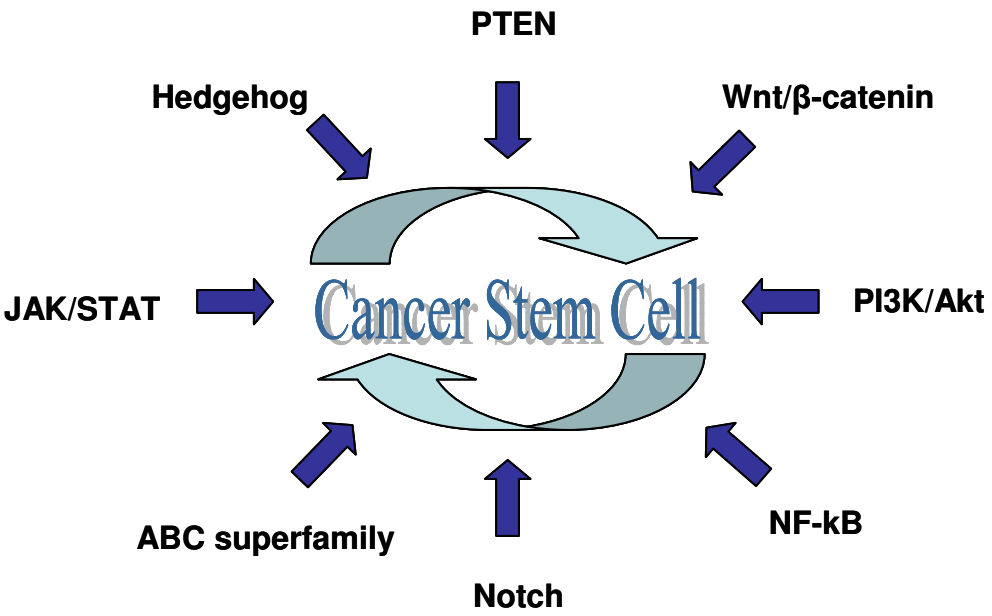

Fig. 1 CSCs characteristic signalling pathways

The dysregulation of signal pathways network is important in the maintenance of CSC stemness. Such as the normal stem cells, the pathways and the elements important in controlling CSC stemness and differentiation include PI3K/Akt, JAK/STAT, Wnt/β-catenin, Hedgehog, Notch, NF/kB and ABC super family. The ability to initiate tumour growth, and promote tumour recurrences is due to the acquisition of these aberrant pathways.

The nature of CSC drug resistance is multifactorial and it involves the alteration in drug targets, the inactivation of the drug, the decreased drug uptake, increased drug efflux, and the dysregulation of apoptotic pathways [11-12].

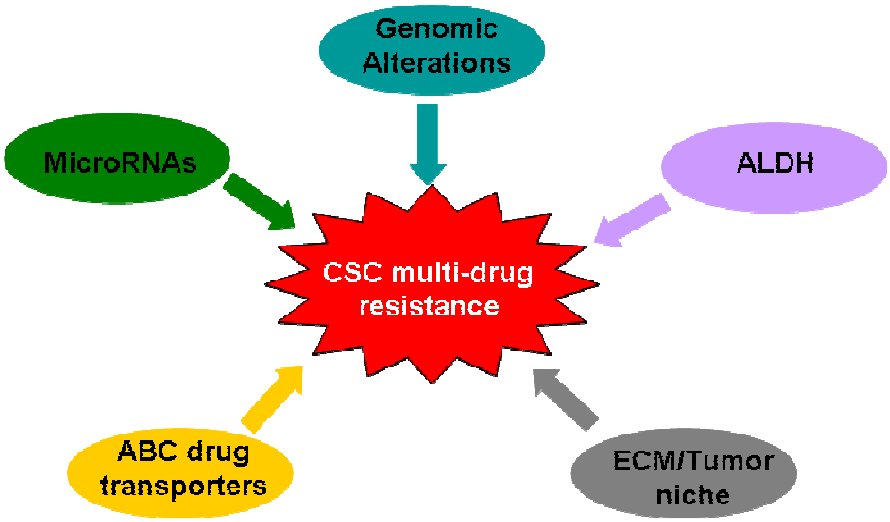

Fig. 2 CSC multi-drug resistance contributing factors

For this reason the molecular targeting of such highly tumorigenic cells becomes fundamental to improve the efficacy of current anti-cancer strategies, aiming to sensitize tumours to conventional therapies thus definitely abrogate tumorigenesis.
The main problem in CSC research is the identification and characterization of this cell subset, to study their biology and design new specific target therapy against them. In this regard the use of biomarkers, for CSC purification (most of the time by cell sorting, using membrane markers), has given a great contribute in the field. The panel of biomarkers used is different in different types of cancer, as shown in Table 1.

Table 1. CSC markers

Tumour type	CSC markers
Leukemia	CD34+ CD38- HLA-DR-CD71- CD90- CD117- CD123+
Multiple myeloma	CD138-
Breast cancer	ESA+ CD44+ CD24-/low ALDHhigh
Liver cancer	CD133+ CD49f+ CD90+
Brain cancer	CD133+ BCRP1+ A2B5+ SSEA-1+
Lung cancer	CD133+ ABCG2high
Colon cancer	CD133+ CD44+ CD166+ CD24+ EpCAM+
Prostate cancer	CD44+ CD133+ α2β1high
Pancreatic cancer	CD133+ CD44+ CD24+ EpCAM+
Head and neck cancer	CD44+
Melanoma	CD20+

The specificity of each marker is often questioned by different research groups for several reasons, i.e. the sequential cell sorting purifying CSCs having all the CSC markers has never been reported in literature (it is not rare to find a CSC population expressing one marker and not another one) [(13)] and sometimes these markers are also good markers of normal tissue adjacent to the tumour [(14)].
CSC state is increasingly being seen as a flexible, rather than fixed, quality of tumour cells that can be lost and gained over time [(10)]. These events could lead to a spatial/temporal intra-tumour heterogeneity (ITH) that may contribute to some of the difficulties in validating biomarkers for clinical use, despite the continued discovery of potential novel biomarkers [(15)]. CSCs are indeed used nowadays as a marker of tumour aggressiveness and drug resistance, the CSC number and phenotype is also used by clinicians to decide the best treatment to apply.
Despite the considerable progress made in cancer research, the majority of patients still do not show advantages by the use of a particular anti-cancer therapy, this phenomenon could be explained by ITH [(16)].
The ITH was initially proposed in 70's and justified by the continuous selection of tumour cells due to the applied regimen and by the CSCs differentiation.
It was recently demonstrated that about two thirds of the mutations found in single biopsies of renal cell carcinoma were not expressed through all the sample regions of the patient's tumour [(17)]. An important role in the generation and maintenance of ITH is certainly played by the microenvironment (the tumour, such as the normal tissue, possess a variable architecture, in terms of vascularisation, infiltration degree and connective tissue components) and the chemo- or radio-treatment. Recent studies using sequential sequencing through different lines of therapy highlighted the effect of DNA damaging agents, leading to the expansion of resistant clones, or generating

new cell populations as a result of new genomic alterations gained during the therapy [18-19].

According to the same model of spatial and temporal ITH, it was recent demonstrated that even if the metastatic lesions are related to primary tumours, they sometimes carry additional mutations in functionally important loci completely absent in the primary tumour [20-22], this discovery resulted fundamental since most of the therapeutic decisions are based on the primary tumor analysis.

In Vitro / *In Vivo* Models

Despite the 68 drugs that have been developed and approved for oncology over the last several decades [23] the success rate is still low. The reason could be found not only in the phenomenon of ITH, but also in the study and predictive models used in cancer research. In the late 70's, the colony forming soft agar assay was suggested to study the nature and differentiation potential of hematopoietic stem and progenitor cells, both in normal haematopoiesis and hematopoietic malignancies [24-26].

For the study of solid tumours the researchers used and studied the traditional cell lines originally derived from patient tumours but adapted to proliferate i*n vitro*, for more than a half century. These lines have been so far used thanks to their ability to be easily propagated and studied under defined conditions.

Unfortunately their continuous passage leads to a selection for cells adapted to that particular *in vitro* condition, thus eliminating the all the variables present in the tumour, such as the supporting non-tumour stroma, the hematopoietic cells and other tumour microenvironment factors. Even if these cell lines led to the development and execution of highly reproducible studies about drug sensitivity, basic tumor cell biology, and signalling pathways [27-28], the tumoral cells are selected over passages: recent studies showed that also brief periods of *in vitro* culture irreversibly change gene expression, suggesting that even low-passage cell lines could be compromised [29-30].

Although initially promising, showing high effects *in vitro*, chemo- and radio-treatments often fail to reproduce equal results *in vivo.* This is due to the higher complexity of the *in vivo* model, including the difficulty of the compound to reach the right action site, the different binding/action dynamic and last but not least the possible side effects. For all these reasons the researchers started to use *in vivo* models for the study of cancer biology, in particular the genetically engineered mouse models (GEMMs) and the patient derived xenografts (PDXs).

GEMMs are really interesting models to study cancer development and tumor progression, unfortunately they also have their intrinsic shortcomings.

The main issue in GEMMs generation comes from the evidence that for mice and men tumorigenesis it is needed at least one driver mutation [31], even if efficient tumorigenesis is usually driven by the introduction of two oncogenes and/or one mutated oncosuppressor. The generation of GEMMs bringing more than one driver mutation is quite difficult and time-consuming, and it is often difficult to exactly control the expression levels of the protein of interest (i.e. mutated KRAS), thus generating a model that doesn't reproduce the patients situation [32].

Another weakness of this model is the sporadic growth of the tumours thus rendering the study of significant number of animals quite hard. On the contrary the best advantage in using GEMMs is the presence of all the non-tumoral cells, such stromal and hematopoietic components in the tumour context, not possible in human tumour

xenograft setting, making this model useful for the study of all the small molecules, immunomodulatory and other agents that can cross-react with mouse antigens [33].
The introduction of the immunocompromised mice strains has permitted the engraftment, the passage and the study of human tumour cells *in vivo* [34-38].
This *in vivo* study was firstly used by the researchers to study the angiogenesis and tumor cell invasion, using traditional tumour cell lines (i.e. for the development of bevacizumab and sorafenib) [39-40]. Then the researchers started to use the PDX models to preserve the genomic integrity and tumour heterogeneity for their study, thus saving the stress and the selection for the cell, over passaging *in vivo* [30].
The most common sites where the cell are transplanted for primary xenografts are underneath the skin and mammary fat pad, even if many groups also do primary xenograft under the kidney capsule or orthotopically (the latter has the benefit to reproduce exactly the microenvironment that appears to be clearly the best suited for the growing of cancer cells of the same organ). These settings ensure physiological levels of oxygen, nutrients, hormones, natural physical substrates for cell adhesion and growth, and the preservation of the chromosomal architecture.
Many different PDXs have been validated over the last years, many of which have been also tested for chemoresponsiveness to anti-tumour agents, exhibiting promising results [41].
Of course also this model has its shortcomings, in particular about the efficiency of engraftment and growth, highly variable by tumour type and subtype, tumour grading, affected by the dependence on hematopoietic cells or microenvironment, the time that passes between tumour resection and its transplantation, and the presence/absence of appropriate support matrix and/or growth factors.

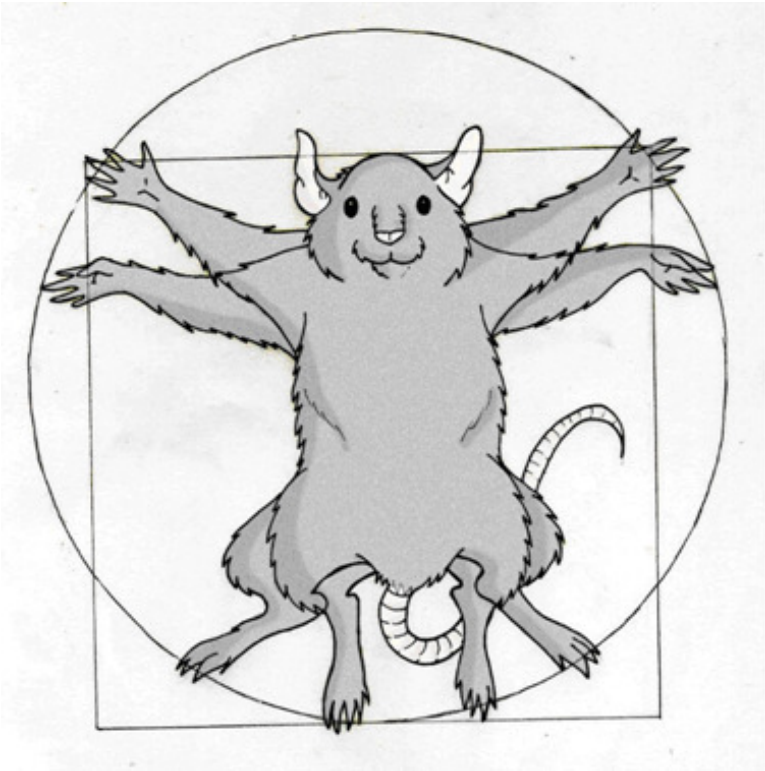

Fig. 3 *Pro et contra* of two *in vivo* models for the study of cancer cell biology: GEMMs Vs PDXs

The *in vivo* model for the study cancer biology is nowadays represented mostly by mice. GEMMs are characterized by the presence of all the non-tumoral cells, such stromal and hematopoietic components in the tumour context; they are uuseful for the study of all the small molecules, immuno-modulatory and other agents that can cross-react with mouse antigens. Their generation is quite difficult and time-consuming; they are characterized by the difficulty in controlling the expression levels of the genes/proteins, sporadic tumor growth, and difficulty to monitor.
PDXs are useful for the preservation of the genomic integrity and tumor heterogeneity. They are characterized by physiological levels of oxygen, nutrients, hormones, natural physical substrates for cell adhesion and growth, and by promising results about chemoresponsiveness to anti-tumor agents

In this thesis I will show all the results (mostly published) of my research, mainly focused on colorectal CSC characterization. A better understanding of this cell subset could lead in the next future in designing a new target therapy to be coupled to conventional chemo- or radio-therapy, aiming to completely eradicate tumor cells and recurrences.

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Aim of the Research

Cancer stem cell research has been developed to bypass and solve the current resistance phenomenon often found in cancer patients following standard regimens, as chemo- and radio-treatments. CSCs seem to be the main players of tumour initiation, progression, aggressiveness, resistance to standard treatments and recurrences. For this reasons their characterization could help researchers to better understand…

Chapter| 1

Colon Cancer Stem Cells: Bench-to-Bedside—New Therapeutical Approaches in Clinical Oncology for Disease Breakdown

Simone Di Franco, Pietro Mancuso, Antonina Benfante, Marisa Spina, Flora Iovino, Francesco Dieli, Giorgio Stassi, Matilde Todaro

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Abstract

It is widely accepted by the scientific community that cancer, including colon cancer, is a "stem cell disease". Until a few years ago, common opinion was that all neoplastic cells within a tumor contained tumorigenic growth capacity, but recent evidences hint to the possibility that such a feature is confined to a small subset of cancer-initiating cells, also called cancer stem cells (CSCs). Thus, malignant tumors are organized in a hierarchical fashion in which CSCs give rise to more differentiated tumor cells. CSCs possess high levels of ATP-binding cassette (ABC) transporters and anti-apoptotic molecules, active DNA-repair, slow replication capacities and they produce growth factors that confer refractoriness to antineoplastic treatments. The inefficacy of conventional therapies towards the stem cell population might explain cancer chemoresistance and the high frequency of relapse shown by the majority of tumors. Nowadays, in fact all the therapies available are not sufficient to cure patients with advanced forms of colon cancer since they target differentiated cancer cells which constitute most of the tumor mass and spare CSCs. Since CSCs are the entities responsible for the development of the tumor and represent the only cell population able to sustain tumor growth and progression, these cells represent the elective target for innovative therapies.

Keywords: cancer stem cell; colorectal cancer (CRC); CD133; differentiation

Introduction

Colorectal cancer (CRC) is characterized by progressive mutations in oncogenes, or tumor suppressor genes: scientific evidences show that at least 4–5 mutations are necessary for a malignant tumor formation [1]. Some of these mutations seem to elapse often within the same sequences, and they are then shared by most individuals with this tumor, while others are different and therefore determine the final phenotype of cancer [2].

Most of the information on the mutations that cause CRC derived from studies on hereditary forms of cancer, making up 5% to 10% of all colon cancer cases. Familial adenomatous polyposis (FAP) is an autosomal dominant CRC syndrome caused by a mutation in the APC (adenomatous polyposis coli) gene which characterizes multiple CRC [3].

APC is at the base of the signaling pathway called wingless/wnt. The main function of APC is to modulate the cytoplasmic β-catenin levels, a protein that can migrate into the nucleus and activate transcription of protein complexes called TNA (transcription of cMyc and cyclin D1), responsible for the regulation of proliferation, differentiation, migration and apoptosis [4]. For subsequent progression, cancers need more mutations, including KRAS and TP53 and deletion on chromosome 18q [5].

According to the old paradigm of carcinogenesis, tumor cell population is heterogeneous and all neoplastic cells within a tumor have an equal capacity to proliferate and thereby to sustain tumor growth. Different from this notion, current evidences suggest that cancer growth is dictated by a small population called cancer stem cells. These cells have a self-renewal property and generate a progeny of non-tumorigenic cells. The latter gives rise to the non-tumorigenic differentiated population which represents the majority within tumor mass. Cancer stem cells can derive from either normal stem cells or progenitor cells as a consequence of genetic and/or epigenetic alterations [6]. Cancer stem cells commonly survive conventional treatment; even if therapy results in an apparent complete regression of primary tumor, remaining CSCs are able to induce the minimal residual disease (MRD). Therefore understanding the mechanism that maintains the immature state becomes crucial in order to develop new anti-tumor approaches.

Colonic Crypt Organization

The colon wall is composed of several layers: mucosa, submucosa, muscularis and serosa.

The mucosa consists mainly of two cell types: epithelial cells, with cylindrical shape, whose function is to reabsorb water and salts, and goblet mucipare cells, whose function is to secrete a slimy substance in the lumen, in order to lubricate the same and facilitate stool passage. The epithelial cells show on their outer surface, toward the lumen, a series of invaginations, called crypts of Lieberkuhn, which are designed to increase the absorbent surface. The submucosa lies immediately under the mucosa and is very rich in vascular structures, lymph and nerve fibers, that regulate peristalsis (intestinal propulsive movements that promote the progression of the stool toward the rectum). The muscolaris consists of two layers of muscle: an inner, cross-trending, and an outer longitudinal trending. They give the bowel a characteristic saccular appearance. The serosa, also called the peritoneum, constitutes an outer coating, covering the entire colon and also all other abdominal organs and viscera [6].

The epithelial layer presents about 14,000 crypts/square centimeter in the adult human colon, each of these crypts contains 2,000 to 3,000 cells, and the colonic stem cells are located at the base, surrounded by mesenchymal cells to form the stem niche [5-11]. Each crypt in the intestine is mainly composed of three different cell types: the colonocytes or columnar cells, the mucin-secreting goblet cells and the endocrine cells. All these cells are generated starting from a colonic stem cell that, via asymmetric division, can generate a cell identical to itself (self-renewal capacity), and a transit cell that can proliferate and differentiate by migrating up to the top of the crypt. This "unitarian theory" (one single cell can generate all the cell types) was first formulated in 1974 [12] and later experimentally demonstrated [13,14]. These stem cells are responsible for the perpetual turn-over of the colonic epithelial cells during the whole lifetime of an individual. There is a continuous supply of these cells, every 2–7 days under normal conditions, and an increased turnover of them in tissue damage conditions.

The complexity of the crypt structure was an obstacle in understanding the key mechanisms that lead to the formation of the crypt from a single stem cell. The first studies to identify the colonic stem cell population were based on Chang *et al.* studies by using 3H-thymidine injection [15], and recently confirmed by bromodeoxyuridine DNA-labeling dye [16] for slow-cycling stem cells localization.

There are two models regarding the positioning of the stem cells: the "stem cell zone" model, and the "+4 position" model. The "stem cell zone" model describes the colon stem cells, the crypt base columnar cells (CBC), at the very bottom of the crypts. On the contrary, the "+4 position" model, related to the intestinal crypt, claims that the intestinal stem cells are located at the +4 position above the Paneth cells at the base of the crypt [17]. Actually the absence of specific colonic stem cell markers makes their identification and positioning rather difficult.

Adult stem cells are defined by two fundamental properties: self-renewal and differentiation capacity to generate all the cyto-types of that tissue. An important aspect in studying stem cells is the mechanism of cell division: stem cells seem to divide more slowly than the progenitor cells and differentiated cells [18]. Stem cells may undergo asymmetric division, thus generating two different cells, one stem cell identical to the mother cell, and a specialized one; but they can also make symmetrical division, generating two identical stem cells. The asymmetric division is slower and ensures the persistence of a pool of adult stem cells, and through cell differentiation, the continuous regeneration of organs and tissues [19]. According to the cell type division, it is possible to obtain a "lineage expansion" if stem cells are generated, or "lineage extinction" if differentiated cells are propagated [20]. The idea is widely accepted that the stem cells are responsible for giving rise to cancer, just because their slow cycles of division and longevity of life allow them to accumulate different mutations over time that could lead to so-called cancer stem cells [18].

Intestinal Niche

The intestinal niche is defined as the environment responsible for stem cells maintenance that is controlled by fine signals that ensure stem cells proliferation. The most determining effect seems to be due to the population of intestinal sub-epithelial myofibroblasts (ISEMFs), whose role is to regulate the organogenesis and tissue repair, and whose growth appears to be regulated by several growth factors [21,22]. Recent findings show that maintenance of stem niche is controlled by Wnt, Bone Morphogenetic Protein (BMP), Notch and Sonic hedgehog (Shh) pathways (Figure 1).

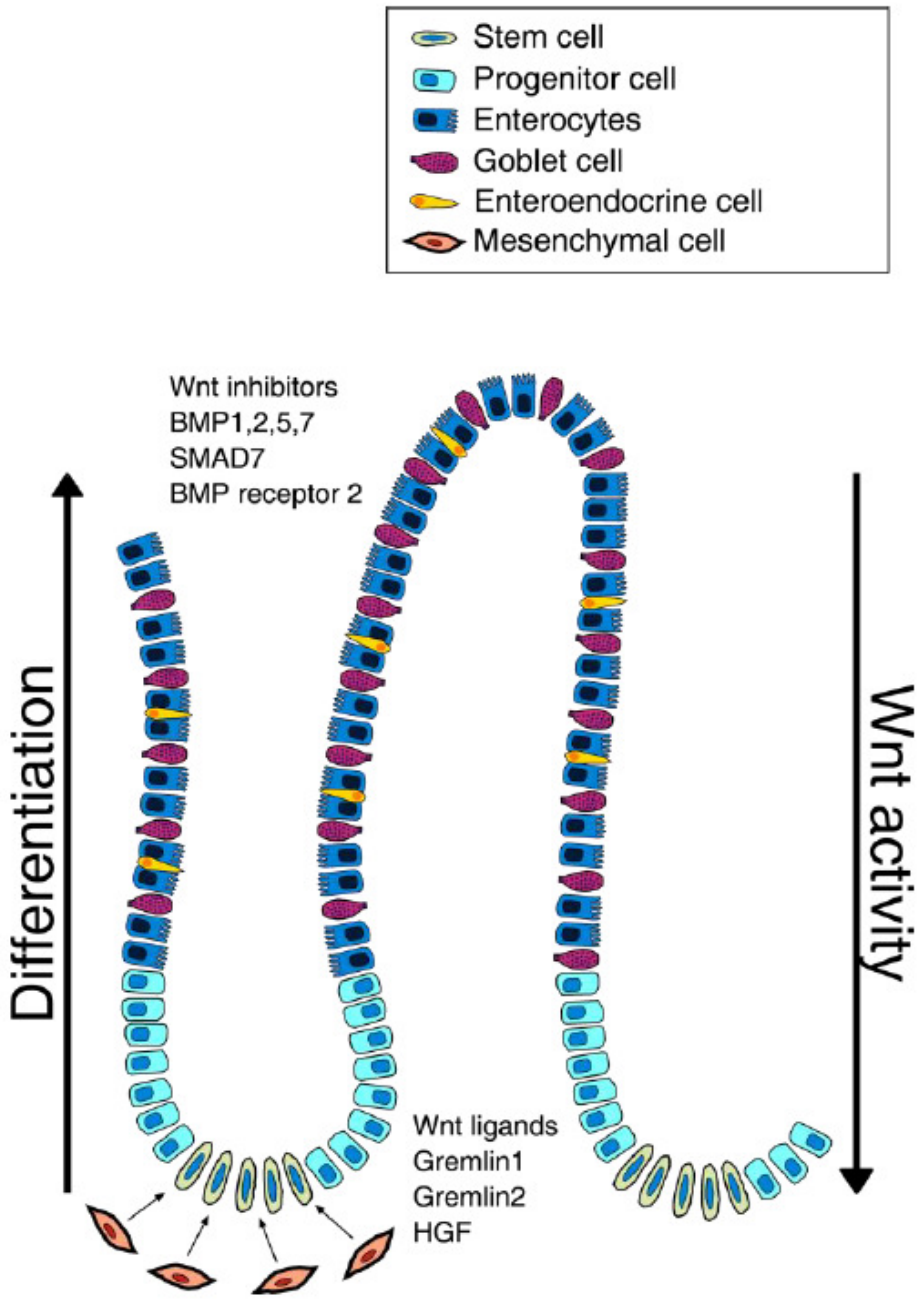

Figure 1. Graphic representation of a colon crypt.

This image shows the distribution of different cell types along the colon crypt unit. At the base of the crypt the mesenchymal cells (ISEMFs) are represented and the factors responsible for the stem cell niche maintenance. The progressive cellular differentiation toward the villus apex is also shown, where many factors that inhibit Wnt activity are over-expressed.

In this signal network, the Wnt pathway definitely has a key role: the central role is played by β-catenin, that, in the absence of Wnt ligands, binds the APC protein, the glycogen synthase kinase 3β (GSK3β) and axin, to be then phosphorylated, ubiquitinated and finally degraded by the proteasome machinery [23]. Instead, Wnt activation requires the binding of Wnt family proteins to their receptors of the Frizzled family (Fz) that subsequently promotes β-catenin accumulation into the nucleus, which binds TCF4, activating the transcription of several genes involved in

cell cycle regulation and proliferation [24]. β-catenin also induces the expression of Ephrin receptors EphB1 and EphB2, which regulate stemness maintenance, cell migration and differentiation [25]: these receptors, following interaction with ephrin ligands, extend the cell proliferation domain in areas higher up the crypts [26].
Interestingly, Wnt pathway members are differently distributed along the axis of the crypt *i.e.* the mRNA for secreted Fz-related protein (sFRP)-5, Wnt-3, Wnt-6, Wnt-9b and Fz-5 were found at the base of the crypts, with decreasing concentration towards the apex of the crypts where more differentiated cells reside. Moreover the cells at the top of the crypt seem to express Wnt inhibitor factors [27].
Recently, Vermeulen *et al.* [29] have demonstrated the important effect of myofibroblasts and the factors secreted by them, such as the hepatocyte growth factor (HGF) in maintaining the stem cell niche of the intestinal crypts. Differentiated cells are able to revert to their phenotype, reverting to stem cells (tumorigenic) in response to the addition of myofibroblasts or HGF.
In addition to Wnt, BMP, Notch and Shh pathways play crucial roles in niche homeostasis. BMP proteins are a subset of the TGF-β super-family members that, after linking their receptors, trigger different biological processes [28]. This pathway leads to the phosphorilation of Smad1, Smad5, Smad8/R-Smad [30], that together with Smad4 (co-Smad), move to the nucleus, and in cooperation with other transcription factors, can regulate the target genes expression [31]. It was recently demonstrated that BMP promotes terminal differentiation and apoptosis, increasing the conventional therapeutic activity in tumors that do not show concomitant mutation of SMAD4 and constitutive activation of PI3K [32].
Moreover, Kosinski *et al.* [33] demonstrated that there is a precise distribution of the different factors along the crypt: at the apex of the crypt the cells express high levels of BMP1, BMP2, BMP5, SMAD7, BMP7, and BMP receptor 2, while cells at the base of the crypt, probably due to the presence of myofibroblasts, produce high levels of BMP antagonists as GREM1, GREM2 and chordin-like-1, which contribute to the maintenance of stemness.
Notch pathway is one of the most studied cell signaling systems that includes four different type I trans-membrane receptor: Notch1, Notch2, Notch3 and Notch4. Its activation involves the binding of five different ligands including Jagged-1 (JAG1), -2 (JAG2), Delta-like (DLL) 1, 2 (DLL2) and 4 (DLL4): the extracellular binding of these ligands triggers the release of the intracellular domain (NICD) through proteolytic cleavage mediated by some metallo-proteases, such as ADAM10 or ADAM17. The NICD moves to the nucleus where it forms a complex with some DNA-binding proteins, converting them from inhibitors to activators of all the target genes transcription [34].
Finally, Sonic hedgehog (Shh) plays an important role during gut organogenesis. The activation requires Shh binding to its receptor, Patched (PTCH), which allows the release of the G-coupled protein Smoothened (SMO) that, together with the GLI transcription factors, migrates into the nucleus inducing target genes activation [35].

Cancer Stem Cell Theory

The idea that cancer is composed of a morphologically heterogeneous population of cells, differing in markers expression, proliferation capacity and tumorigenicity, has been described more than a century ago [2,36-38]. It is widely recognized that this heterogeneity is caused by genetic/epigenetic hits and micro-environmental differences that determine several degrees of cell differentiation [39]. In recent years,

novel insights in cancer research have suggested that the capacity to initiate and sustain tumor growing is a unique characteristic of a small subset of cancer cells with stemness properties within the tumor mass, called “cancer stem cells” (CSCs) or “tumor-initiating cells” [40].
This discovery has profoundly changed the way to look at cancer, which has previously only been seen as a genetic disease [41]. There are two different models of cancer that could explain the development of tumor: the first one, the “classic model” of tumorigenesis, postulated by Vogelstain and Nowell [2,36], describes the tumor development through sequential mutations in oncogenes and tumor suppressor genes. According to this theory, tumors consist of a heterogeneous cell population that, acquiring new mutations, undergoes uncontrolled proliferation and invasivity. This stochastic model considers all cancer cells able to reform a tumor, after implantation in immuno-compromised mice [42]. Contrarily the second theory, the “cancer stem cells” model, is based on evidence that only a small subset of cells, the CSCs, within the tumor population, can initiate and sustain tumor growth [43].
Emerging evidences suggest that CSCs, isolated from a variety of tumor types, retain tumorigenic capacity and are responsible for the propagation, relapse and metastatic dissemination. CSCs are defined by sharing stem cell-like features with the normal stem cells, such as self-renewal and pluripotent differentiation capacity. CSCs could derive from self-renewing of normal cells after genetic/epigenetic changes, or from progenitor cells that acquire self-renewal capacity. The link between cancer and normal stem cells has also been demonstrated on the basis of common signaling pathways that regulate self-renewal, including Wnt, Notch and Sonic Hedgehog (Shh): the deregulation of these pathways plays a key role in the tumorigenesis process [44]. Many studies have shown the importance of self renewal pathway activation for CSCs maintenance [45]. Jamieson and colleagues [46] first identified the aberrant Wnt/βcatenin self renewal pathway activation in leukemic stem cell propagation; Wnt pathway has been later considered important also in breast cancer stem cells (BCSCs). Korkaya *et al.* [47] showed that the increased activity of Wnt/β-catenin was mediated by activation of Akt signaling activation. Defects in Notch pathway, normally implicated in stem cell growth and differentiation, have been seen in the colon CSC (CCSC) subset. It was observed that using antibody anti DLL4, an important component of Notch pathway, the growth of human colon cancer xenograft was inhibited, directly inhibiting Notch signaling. Notch pathway is also activated in breast [48] and glioblastoma CSCs model. Finally, alterations in Hedgehog signaling pathway, have been reported in many tumors: leukemia [49,50], pancreatic, gastric, prostate, breast [51,52], glioblastoma [53] and colon cancer [54].
The discovery of CSCs has changed the view of carcinogenesis and therapeutic approaches over recent years. Tumors are considered to be able to evade death signals induced by therapeutic drugs through multiple mechanisms, even if the molecular bases concerning the failure of chemotherapy have not yet been defined. The CSCs are characterized by high resistance to drugs and general toxins, which target rapidly proliferating cells and spare the slow dividing cells, due to an up-regulation of several ATP-binding cassette transporters, active DNA-repair capacity, over-expression of anti-apoptotic molecules that cause changes in the signaling pathways controlling proliferation, differentiation and apoptosis [55].
The first CSCs were isolated from acute myeloid leukemia (AML) and then characterized by the presence of immature cells, the blasts, detected in blood and bone marrow by John Dick and colleagues [56,57]. They have indeed isolated a sub-population of CD34+ CD38- leukemic stem cell from patients with AML and they

observed that just a small number of leukemic cells were able to form colonies growing *in vitro*. They have also found that there was a sort of hierarchy in leukemic cells and that only CD34+ CD38- cells, if transplanted into immunodeficient mice, were able to reproduce the parental tumor phenotype [55]. Using similar approaches, many types of tumor stem cells have been identified from a variety of solid tumors. In particular Al Hajj *et al.* [58] showed that CD44+/CD24- cell population was enriched in breast cancer stem cells (BCSCs). After the publications about leukemia and breast cancer, many reports showed how to isolate the CSCs in several malignancies including: brain [59], colon [60-62], head and neck [63], pancreas [64,65], melanoma [66], mesenchymal [67], hepatic [68], lung [69], prostate [70], and ovarian [71] tumors.
Despite several scientific evidences about CSCs existence, there is still an alternative theory sustaining that this cell population would not behave as an entity, but as a phenotypic state, which was observed in stem cells of melanoma [72] as well as during epithelial-mesenchymal transition [73], where stem cells acquire stemness properties.

Colon Stem Cell Markers

Stem cells characterization is yet unclear even if several molecules have been identified as putative stemness markers because none are considered exclusive. There are indeed important debates about the value of each marker: scientific evidences have shown that it is possible to obtain a cell population enriched in colon stem cells through cell sorting, using different combinations of markers (Table 1) [62].

Putative marker	Alternative name	Roles	
CD34		Stemness maintenance	Normal colon
DCAMKL1		Kinase, resistance to apoptosis	
EphB receptors		Stemness maintenance, cell migration	
Msi-1		RNA-binding protein, asymmetric division	
Bmi-1		Polycomb group repressor, self-renewal, senescence inhibitor	Colorectal cancer
CD24	HSA	Cell adhesion molecule	
CD29	β1 Integrin	Proliferation, matrix-cell interaction	
CD44		Cell-cell interaction, hyaluronic acid receptor, cell migration	
CD133	Prominin1	Self-renewal, tumorigenesis	
CD166	ALCAM	Cell adhesion molecule	
ESA	EpCAM	Cell adhesion molecule	
Lgr-5	Gpr49	G protein-coupled receptor, unclear function	
ALDH1		Detoxifying enzyme	
nuclear β-catenin		Cell cycle regulation, proliferation	

List of putative stem cell markers and their specific roles.

Msi-1 is an RNA-binding protein, it was one of the first molecules studied as a colon stem cell marker and its role was mostly studied in *Drosophila Melanogaster*, where it seems to be essential in the mechanisms of asymmetric cell division that regulate neural development [74]. It is also considered fundamental in the development of the nervous system of mammals [75]. Its location in murine and human small intestine, at the base of the crypts, makes it very important in the characterization of colon stem cells.

Among all cell surface putative stemness markers, β1 integrin (CD29), reported as a marker of high-proliferation, was found at high expression levels at the base of the crypts, detecting both stem cells and progenitor cells [76]. According to these data, the EphB receptors expression consists in a gradient with the highest levels at the base of the crypts, and lower ones at the crypt-villus junction [77,78].
Bmi-1, a repressor of the Polycomb group, was found essential for self-renewal of hematopoietic stem cells and adult neural stem cells, through repression of genes involved in senescence, suggesting that stem cells developed specific mechanisms to extend their proliferative capacity. It is indeed expressed in the small intestine near to the crypt's bottom, in line with the idea that this zone is the residence of colon stem cells [79]. Bmi-1 is over-expressed in patients and results in very poor survival [80].
DCAMKL-1 is proposed as a putative colon stem marker: it is a microtubule-associated kinase that can undergo auto-phosphorylation. DCAMKL-1+ cells are resistant to apoptosis after ionizing radiation injury.
More recently Lgr5 protein (Gpr49), a G protein-coupled receptor, whose gene is a Wnt regulation target, has been recently studied as an elective colorectal stem marker, even if its function remains unclear. It was demonstrated, in agreement with the multi-lineage capacity, that a single Lgr5+ cell is able to generate a whole crypt-like structure *in vitro*, generating any cell type present in the colonic epithelium [81]. Recently some reports showed Lgr5 over-expression in advanced CRCs and its correlation with cancer progression [82].
The first direct evidence supporting the CSC hypothesis came from the recent finding of self-renewal and tumor-initiating cells with a common and distinct surface-expressed polypeptide, the CD133 pentaspan trans-membrane glycoprotein, also known as Prominin-1. This protein was first released as a marker for hematopoietic stem cells and progenitor cells and it was subsequently used to identify many tumors [83]: brain [59], prostate, hepatocellular and colon tumors [60,61,84,85].
The stemness value of CD133 has been much debated, in particular the tumorigenic potential of colon CD133+ cells and the ability of these cells to give rise to a tumor in NOD-SCID mice. Many research groups showed that only the CD133+ cells within a colon carcinoma are able to initiate and sustain tumor growth [77,78,89,90].
CD133+ cells are maintained in culture for a long time without losing their ability to reproduce the parental human phenotype: CCSCs, after enzymatic digestion, can be expanded as tumor spheroids *in vitro* with a serum-free medium complemented with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), using low adhesion conditions (to induce differentiated cells death due to the *anoikis*) [86]. In differentiation conditions CD133+ cells are able to generate particular structures similar to crypt; moreover, during *in vitro* or *in vivo* differentiation these cells gradually acquire typical colon epithelial markers, such as CK20, and at the same time decrease CD133 expression. According to these findings much clinical data identify CD133 as an independent negative prognostic marker [87]; its expression in combination with nuclear β-catenin is very important to determine poor patient survival [88,89]. Since the use of one single marker it is considered insufficient for the identification and isolation of CCSCs, many researchers usually perform a sorting using several different putative markers. Dalerba *et al.* [62] showed that CD133+ cells express the stem-like epithelial specific antigen (EpCAM), CD44 and CD166. In their study the injection of CD44+ and EpCAMhi cells, into NOD-SCID mice, reproduced a tumor xenograft phenotypically similar to parental one.
Supporting this thesis, Du's group [90] has shown that CD44 could be considered as an important marker in CCSCs that give rise to spheres *in vitro* and to a xenograft

similar to the original tumor *in vivo*. More recently, aldehyde dehydrogenase-1 (ALDH1), a detoxifying enzyme, has been proposed as a marker to identify, isolate and track, human CSCs during CRC development [91].
The possibility to isolate and to study CSCs represents a revolutionary approach in cancer research to better understand the pathogenesis of cancer, so these cells are an elective target for new therapies.

Alternative and Synergistic Therapies

Today most of the existing conventional therapies are insufficient to permanently eradicate the tumor or to treat patients with advanced forms of CRC. Almost all colon cancers begin as benign polyps that can slowly develop into malignant tumors.
To timely remove precancerous polyps, before malignant transformation and subsequent metastasization (the liver is the most common site), would be appropriate especially for patients with familiarity. Preventive colonoscopy could lead to the the surgical removal of the cancer as soon as possible.
Nowadays, for patients with metastatic CRC to the liver, there are two useful treatments available, FOLFOX (Folinic acid/Fluorouracil and Oxaliplatin) and FOLFIRI (FOLFOX plus vitamin B and irinotecan). Sometimes Cetuximab, a monoclonal antibody, is added to FOLFIRI [92]. FOLFOX and FOLFIRI have demonstrated good efficacy in Phase III trials and are actually employed more frequently in younger than in older patients with metastatic CRC [93]. Neoadjuvant chemotherapy has been combined with anti-angiogenic drugs in metastatic colon cancer patients, treated with Bevacizumab, a humanized monoclonal antibody that targets the vascular endothelial growth factor (VEGF) [94], which is an important angiogenic factor in primary and metastatic human CRC [95]. VEGF expression is observed early in the progression from premalignant adenoma to invasive and metastatic disease. Additionally, VEGF expression has been correlated with increased micro-vessel count in colon tumors, and both VEGF and micro-vessels count have been associated with poor outcomes, as measured by tumor size, metastasis and patient survival. Another neo-adjuvant drug for colorectal cancer is Cetuximab, also known as Erbitux, a monoclonal antibody that inhibits the epidermal growth factor receptor (EGFR), involved in cell differentiation and proliferation [92]. Cetuximab is indicated for the treatment of EGFR expressing patients, KRAS wild-type metastatic colorectal cancer, alone or in combination with FOLFIRI. Two large clinical studies of cetuximab, OPUS and CRYSTAL, have recently been published, and have provided further evidence that cetuximab significantly improves response rates and disease-free survival in metastatic CRC patients with KRAS wild-type tumors [92].
New targeted therapies under investigation are directed not only against downstream factors of the EGFR pathway, but also toward correlated pathways, to overcome growth factor–mediated resistance. An alternative therapy could selectively target CSCs pathways such as IL-4, that is a cytokine produced in an autocrine way by CCSCs; it is known for its involvement in activated B-cell stimulation, T-cell proliferation and the differentiation of CD4+ T-cells into Th2 cells [96].
In CCSCs, the inhibition of IL-4 signaling transduction pathway with anti–IL-4 neutralizing antibody or IL-4 receptor α antagonist, leads to the sensibilization of these cells to chemotherapeutic agents through down-regulation of anti-apoptotic proteins, such as cFLIP, Bcl-xL, and PED. IL-4 antibodies treatment, in combination with standard chemotherapeutic agents (5-fluorouracil or oxaliplatin) reduces tumor growth: this phenomenon is confirmed also *in vivo* where this treatment significantly

reduces xenograft tumors growing [85]. Recent studies have demonstrated that the up-regulation of IL-4 cytokine in CD133+ CCSCs stem cells is an important mechanism that protects these tumorigenic cells from apoptosis [97].
BMP4 is another important molecule because of its ability to activate a differentiation program and stimulate apoptosis in CCSCs, reducing β-catenin activation through inhibition of PI3K/AKT pathway and up-modulation of Wnt-negative regulators. Also in this case chemotherapeutic agents, such as oxaliplatin and 5-flourouracil, increase the anti-tumor activity of BMP4 since their concomitant administration induces complete long-term regression of colon CSC-derived xenograft tumors [32].
Cancer immunotherapy could be considered an important approach taking advantage of the forcefulness and specificity of the immune system. Although cancer cells are less immunogenic than their normal counterpart, the immune system is clearly able to recognize and eliminate them. Thus, the challenge for immunotherapy is to use advances in cellular and molecular immunology to develop strategies that effectively and safely increase antitumor responses [98].
Most cancers are resistant to current therapies due to the slow-cycling CSCs and because of the location of these cells within hypoxic niches [99,100]. Clinical studies have demonstrated that, in terms of survival, the synergic use of chemotherapy and immunotherapy greatly benefited the health of the patient compared to chemotherapy alone [101]. Chemotherapeutic agents can also stimulate tumors to immune cell-mediated killing, increasing sensitivity of tumor cells to cytotoxicity through T cells across the up-regulation of death receptors Fas and TRAIL-R2 (DR5) ligands to FasL (CD95L) and TRAIL, respectively [102].
Most current immunotherapeutic approaches aim at inducing antitumor response sensitizing the adaptive immune system, depending on MHC-restricted αβ T cells. Anyway, in cancer cells, loss of MHC molecules is recurrently observed, making tumor cells resistant to αβ T cell-mediated cytotoxicity. γδ T cells show potent MHC-unrestricted lytic activity *versus* different tumor cells *in vitro*, suggesting their potential employment in anticancer therapy. Moreover, γδ T cells have been isolated and identified from tumor infiltrating lymphocytes in different cancer types, including prostate carcinoma [103]. Antigen recognition of γδ T-cell receptors is strictly selective and the responses frequently exhibit native characteristics. Furthermore peripheral γδ T cells exert several regulatory functions, rapidly producing cytokines, such as interferon (IFN)-γ and IL-17, and they also promote inflammation. Nevertheless, γδ T cells improve tumor clearance, directly through target cell lysis. The fruitful interaction of γδ T-cell and other immune cells may be critical for immune regulation and host defense [104]. Moreover, the incubation of the CCSCs with bisphosphonate zoledronate leads to a relevant γδ T-cell response against different tumor cells *in vitro*, even if this experiment represented the first report in employing γδ T cell to target CSCs [103]. All the therapies mentioned above should be validated in order to avoid survival of CSCs responsible for tumor recurrences.

Concluding Remarks

CSCs might derive from normal stem cells or SC-like progenitor cells that acquire genetic/epigenetic hits necessary for tumorigenesis; they also retain important biological features in common with normal stem cells, such as self-renewal and pluripotent capacities. Many self-renewal pathways undergo deregulation during neoplastic development. Moreover, CSCs' plurypotency properties support the idea

that a tumor is an aberrantly developed organ, constituted by a heterogeneous cell population.
The role of CSCs in CRC is gaining interest, since this hypothesis could explain carcinogenesis, helping to define innovative therapeutic strategies focused on the tumorigenic sub-population. The highly negative prognosis of CRC is due to the inefficacy of current treatments in definitively eradicating the tumor. Accordingly, tumor growth/progression arrest requires the targetted elimination of CSCs considered responsible for minimal residual disease in order to prevent recurrences and metastasization.

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Chapter| 2

CD133 as a target for colon cancer

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Introduction

Recent evidence based on cancer stem cell (CSC) models, is boosting the progress of translational research and providing relevant clinical implications in many tumour types, including colorectal cancer. The current failure of standard therapies is attributed to a small fraction of the primary cell population with stem-like characteristics, such as self-renewal and differentiation.
Identification of CSCs is based on two different criteria of selection: stemness-selective conditions and direct isolation based on putative stem cell markers expression. CD133, a transmembrane glycoprotein, was associated with tumor-initiating cells derived from several histological variants of tumors, including colon.
Areas covered: In this review the current understandings about CD133 as putative marker of tumour-initiating cells in colorectal cancer (CRC) is described. The focus of the discussion is on the need for additional markers to better identify the cell population able to recapitulate the parental tumor in immunocompromised mice.
Expert opinion: Identification and characterization of CSCs represents a relevant issue to define innovative therapeutic approaches, overcoming the emergence of cancer cell clones capable of evading standard therapy.

Keywords: CD133, colon carcinogenesis, colorectal CSCs, stemness markers

Introduction

According to the stochastic model, tumours are clonally derived from somatic cells following genetic mutations of cancer-critical genes together with a disregulation of microenvironmental factors. A defect in DNA stability is the early event occurring in tumor transformation followed by tumour suppressor gene loss or oncogene activation. These setbacks reflect a major probability of accumulating mutations in long-lived somatic stem cells rather than short-lived differentiated cells [1]. Thus, self-renewal ability, quiescence and asymmetric division render stem cells natural candidates for oncogenic process.

Unlike the tumorigenesis paradigm, in the new concept of cancer an abnormal small population within the tumor called 'cancer stem cells' has been suggested to drive and sustain tumor growth. This cell subset represents the forefather of the heterogeneous differentiated cell population.

CSCs share similar hallmarks with normal stem cells, including slow cycling, altered DNA repair machinery, high expression levels of anti-apoptotic genes and ATP-binding cassette (ABC) transporters, which could elucidate the failure of current anti-cancer drugs [2]. Identification and isolation of CSCs have been useful to create a reliable preclinical model for accurately defining an effective anti-cancer therapy.

The scientific community established a model to validate the tumorigenic potential of this primary tumor cell fraction through their ability to generate a tumor xenograft with a similar phenotype of the original one.

Dick's group, as first, demonstrated that a small cell population expressing CD34+ and CD38-, within leukemic cells, retains tumorigenic capacity [3].

Later on Bonnet et al. showed that the leukemic tumorigenic cells also reside within the CD34+CD38+ fraction. The anti-CD38 antibody used in the original studies essentially depleted the CD38+ population [4].

Successively, tumor-initiating cells were identified in many human cancers, including breast [5], brain [6], prostate [7], colon [8,9], pancreas [10] and thyroid [11]. Although several putative stem-like markers have been proposed, CD133 remain the most promising candidate.

Colonic crypt organization and carcinogenesis

Adult colonic epithelial layer consists of a continuous sheet of columnar epithelial cells, folded into finger-like invaginations and embedded in the connective tissue forming the colon functional unit, the crypt.

Colon epithelial turnover occurs within every five days fuelled by adult multipotent stem cell population. They reside at the very base of the crypt where it subsists a crosstalk with pericryptal myofibroblasts which are components of the niche. Myofibroblasts secrete wingless-related mouse mamaary tumor virus integration site protein (Wnt), which binds Frizzled receptor on epithelial stem cells, promoting b-catenin-induced proliferation. Furthermore, Wnt induces expression of ephrin type-B (EphB) receptors, which interact with their ligands distributed along the crypt, enhancing the proliferation rate [12].

Colon stem cells maintenance is also regulated by other factors including bone morphogenetic protein (BMPs) which counteract with the proliferative effect of Wnt driving differentiation. Noggin, secreted by myofibroblasts and smooth muscle cells, antagonizes BMPs [13].

Despite the paucity of reliable markers, the colonic crypts have long been known to represent a functional stem cell ompartment [14]. Multipotent stem cells generate progenitor cells which rapidly expand the epithelial renewal and give rise to the colonic differentiated cells [12].
There are two models regarding the positioning of the stem cells: the 'stem cell zone' model, and the '+4 position' model. According to the 'stem cell zone' model the colon stem cells, called crypt base columnare (CBC), resides at the very base of the crypts. On the contrary, the '+4 position' model, related to the intestinal crypt, claims that the intestinal stem cells are located at the +4 position just above the Paneth (Figure 1) [15].

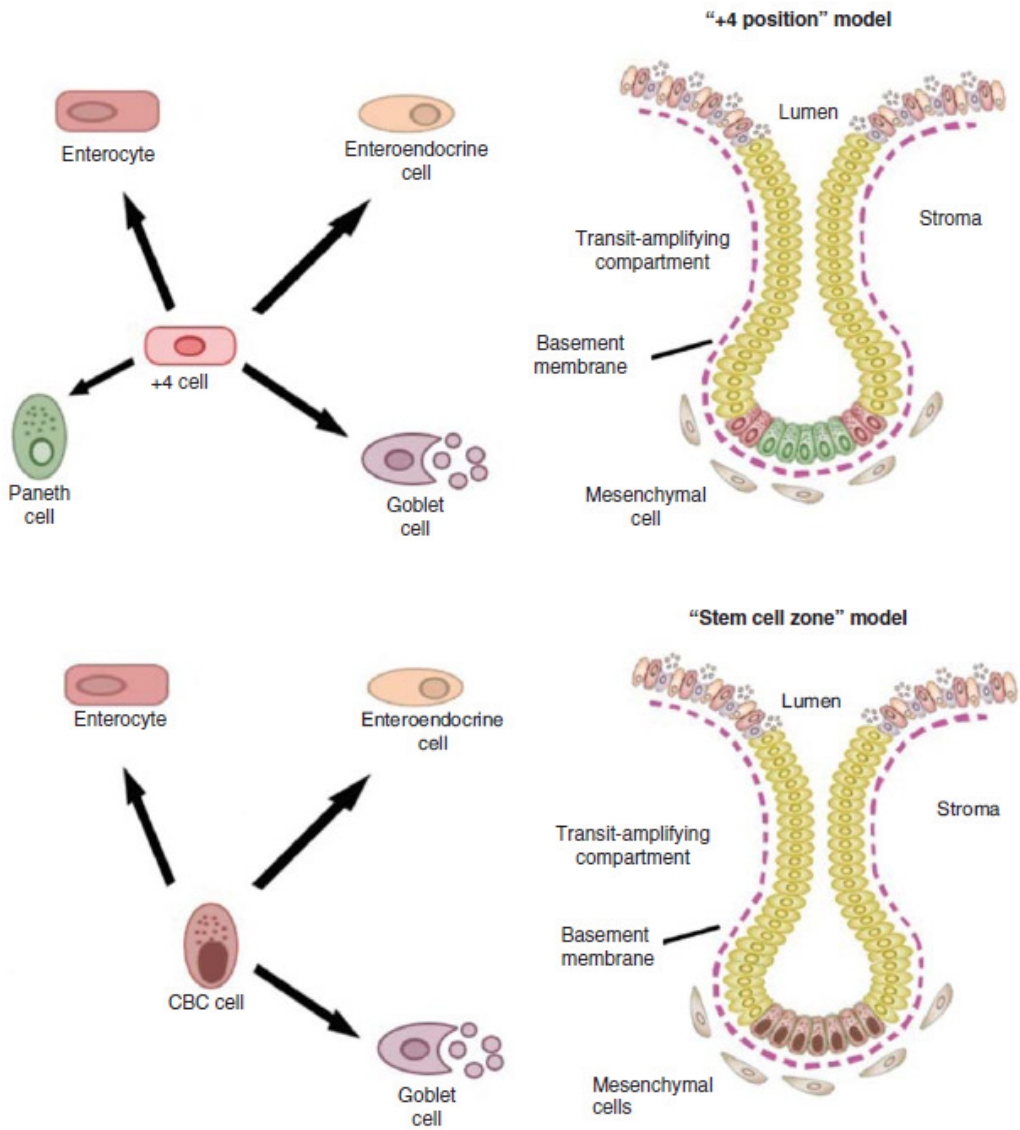

Figure 1

Models for localization and identification of colorectal stem cells. The "+4 position" model places stem cells at the +4 position into the crypt bottom, just above the Paneth cells. The "Stem cell zone" model suggests that stem cells, called crypt base columnar (CBC) cells, are located at the very base of the crypt.

The absence of specific colonic stem cell markers makes their identification and positioning difficult.

The first studies, aimed to identify the colonic stem cell population, were based on the use of 3H-thymidine injection [16], and then confirmed by bromodeoxyuridine DNA-labeling dye [17].
Several stem-like markers have therefore been proposed, including RNA-binding protein Musashi-1 (Msi-1), B lymphoma Moloney-murine leukemia virus insertion region 1 polycomb ring finger oncogene (BMI-1), doublecortin and calcium/calmodulin-dependent protein kinase-like 1 (DCAMKL1), CD133 and activated leukocyte adhesion molecule (ALCAM/CD166) [18]. However their role still remains to be validated.
Among all the proposed putative stem cell markers leucinerich repeat-containing G protein-coupled receptor 5 (Lgr5) a Wnt target gene, is considered the most promising [19].
Whatever would be the marker used, stem cells with their self-renewal capacity and quiescence are the ideal candidates for oncogenic processes. According to the Fearon and Vogelstein model, healthy crypt transformation starts with the mutation in adenomatous polyposis coli (APC), a tumour suppressor gene that control the Wnt/b-catenin axis pathway [20,21]. Most of its mutations lead to the production of an abnormally short and nonfunctional APC protein. This short protein cannot suppress the cell growth leading to the formation of polyps, which can become cancerous. This first hit is associated with several changes in crypt appearance and behaviour, the colon stem cells indeed acquire a more immature phenotype and a higher proliferative rate. In this context of growing genomic instability normal myofibroblasts, activated by factors produced by pre-malignant and infiltrating cells, increase their hepatocyte growth factor (HGF) secretion. This phenomenon can stimulate the Wnt pathway, contributing to maintaining and also reinstalling stem-cell fate in the more differentiated tumour cells (dedifferentiation) (Figure 2).

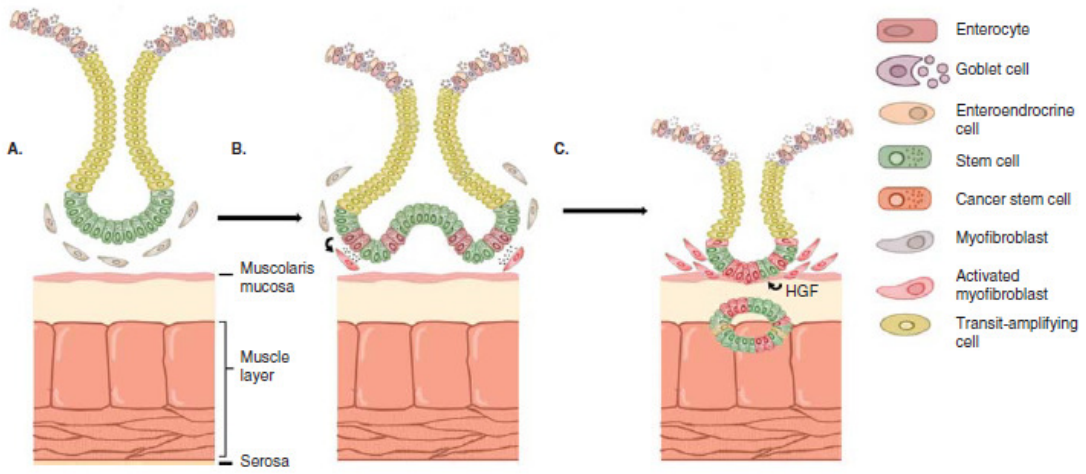

Figure 2

Adenoma-carcinoma sequence. A. Normal colorectal crypt; B. Transformation of healthy crypt towards an adenoma; C. Tumour progression.
HGF: Hepatocyte growth factor.

Then the accumulation of other genetic lesions, including activation of k-RAS and inactivation of P53, smalland mothers against decapentaplegic 4 (SMAD4) and

phosphatase and tensin homologue (pTEN), drives clonal expansion and progression towards an invasive growing CRC [22-24].
Recent findings indicate that the loss of microenvironment-derived signals mediated by BMP, that normally control the epithelial proliferation through inactivation of SMAD4 or BMPreceptor2 (BMPR2), is associated with CRC progression [13].

Colorectal CSCs identification through CD133 expression

Recent studies have revealed a possible key role of CD133 in the identification of cancer stem cells and colon cancer progression. It was first identified through its surface antigen, AC133, in the hematopoietic system. The AC133+ population held longterm self-renewal capacities effectively engrafting in a fetal sheep transplantation model. [25].
AC133 antibody recognizes a glycoprotein that consists of a single polypeptide chain with a reduced molecular weight of about 120 kDa with 20-kDa glycosidic-linked polysaccharides later named CD133 and also known as Prominin-1. It is a pentaspan membrane protein which contains five transmembrane domains, two large N-glycosylated extracellular loops, two small intracellular domains and a cytoplasmic C-terminal domain (Figure 3) [26].

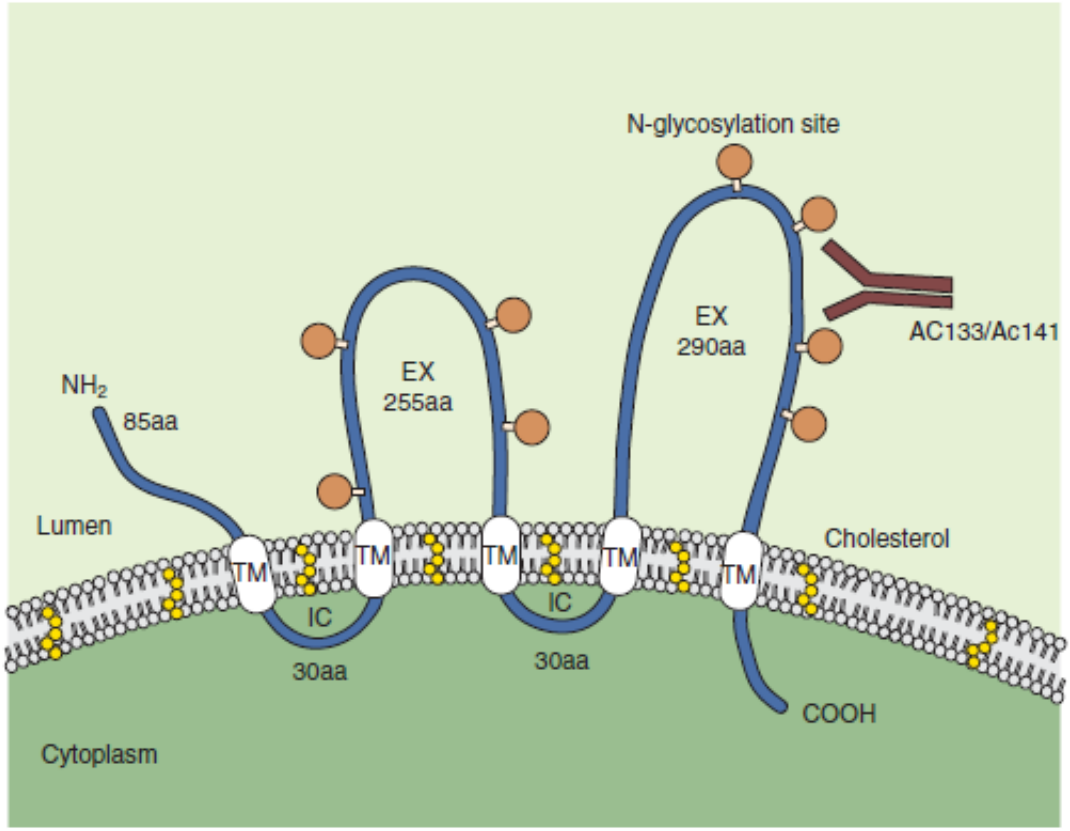

Figure 3

The predicted structure of CD133 consists of an 85 amino acids (aa) N-terminal extracellular domain, five transmembrane domains (TM) with two extracellular loops (EX) of 255 aa and 290 aa, two 30 aa intracellular domains, and a 50 aa cytoplasmic tail. Eight N-linked glycosylation sites are the binding sites of AC133 and AC141 antibodies.

CD133 is confined into particular membrane structures that protrude from the planar areas of plasmalemma [27], where it interacts with plasma membrane cholesterol-based lipid 'raft' [28].
Although its function remains unclear, CD133 could play a role in cell polarity and integration via cell--cell and cell--matrix interactions [29]. CD133 was reported to be located into the membrane microdomains forming active transduction complexes [30]. The latter seem to be involved in maintaining stem cell properties, such as symmetric/asymmetric balance division [31].
CD133 involvement in colon cancer was demonstrated through the analysis of its expression levels in normal and cancer colon tissues by using flow cytometric counter plots (0.4 -- 2.1% healthy tissue versus 8.9 -- 15.9 % in cancer). To test the tumourigenic potential CD133- and CD133+ fractions were injected into the renal capsule of NOD/SCID mice. The results showed that about 92% of the mice the received injections of CD133+ cells developed tumours that resemble the morphological features of the parental ones, including CD133 expression levels. These findings were not established in the CD133- cell population which was unable to give rise tumours.
It was indeed determined that the frequency of tumourigenic colorectal cancer stem cells (CR-CSCs) within the CD133+ fraction was one in 262 cells. A possible explanation for these findings is that there may be a different subclasses of CR-CSCs to be established using additional cell surface markers, in combination with CD133 [8]. This evidence provides strong support to the hierarchical organization of human colon cancer. With a similar approach and independent research group isolated CD133+ cells, accounting for 2.5% of total cells, from colon specimens, and compared their ability to reproduce the original tumour in immunodeficient mice respect to the CD133- cells. They demonstrated that only the CD133+ fraction was able to generate xenografts, and also that during the in vivo passages, CD133+ cells progressively increased their aggressiveness, as showed by the faster growth and the increased number of CD133+ cells in the following tumours.
Interestingly, the tumour cells isolated from these xenografts grew in vitro as undifferentiated floating colonies or 'tumour spheres' in serum-free medium in the presence of EGF and basic fibroblast growth factor (bFGF), maintaining the ability to generate tumours after serial transplantation. In presence of fetal calf serum (FCS) or extracellular matrix, the above mentioned spherical aggregates differentiate into large and adherent cells, gaining cytokeratin-20 (CK20) and caudal type homeobox transcription factor 2 (CDX2) expression.
Importantly, under differentiation conditions, CD133+ cells lost their ability to form tumours, in line with the CSCs hypothesis [9]. Additional supporting data have been obtained with freshly purified CD133+ cells which, once seeded in a threedimensional culture system, generated colonies organized in a structure similar to a colonic crypt and acquired the expression of colonic epithelial markers with the gradually lost of CD133. The CD133+ subset was relatively increased in tumour xenografts treated with conventional drugs, thus suggesting that the chemoresistance is confined to the cancer stem fraction [32].
Following studies suggest use of the hyaluronic acid receptor CD44 and the epithelial cell adhesion molecule (EpCAM) as a alternative markers for the isolation of CR-CSCs since the tumourigenicity was restricted to the EpCAMhigh/CD44+ subpopulation.

A further enrichment of CR-CSCs within the EpCAMhigh/ CD44+ population was obtained by using the mesenchymal stem cell marker CD166, whose high expression levels were associated with poor clinical outcome in CRC patients [33].
Recently, it was found that the CD133+ CD44+ population is highly enriched in tumour-initiating cells in HCT116 cells and that precisely this cell fraction maintain the metastatic property, confirming that CD133 together with CD44 could be more accurate in defining CRCs [34].
Since the stemness seems to correlate with a combination of putative markers, the sole use of extracellular epitope AC133 becomes more and more questionable.
Shmelkov et al. suggested that CD133 is not a specific marker of organ-specific stem and progenitor cells, since the gene is expressed both in undifferentiated and differentiated colonic epithelial cells. They also showed that both CD133+ and CD133- subpopulations, isolated from metastatic colon cancers, are capable of tumour initiation.
Further analysis demonstrated that metastatic CD133- fraction expresses the typical phenotypic markers of cancer initiating cells, including CD44, while CD133+ cells are CD44low/CD24+ cells [35].
This discrepancy with previously published data could be due to the different glycosylation [36] and/or splice variants of CD133 [37]. Moreover, the CD133 expression profile can be influenced by the detection procedures used, in particular those involving cell fixing and/or permeabilization, which could modify the CD133 tertiary structure [12].
Although the use of CD133 as a CSC marker is questionable, clinical data support a functional role as an independent negative prognostic marker, with an increased overall survival in patients with lower levels of CD133 [38].
A promising new marker for CR-CSCs identification is aldehyde dehydrogenase1 (ALDH1), a detoxifying enzyme that oxidizes intracellular aldehydes conferring resistance to alkylating agents. Accordingly, a recent study reported that ALDH+/CD133+ cells showed an increased ability to generate new tumours in immunocompromised mice respect to ALDH+/CD133- or ALDH+ alone [39]. An independent research group confirmed that ALDH and CD133 are CSCs markers, underlining also the importance of the signal transducer and activator of transcription 3 (STAT3) signalling pathway in tumour survival and sphere forming capacity [40].
Table 1 summarizes the bibliography data supporting the role of CD133 as a putative colorectal CSCs marker and the opposed points of view, according to which CD133+ cells do not represent stem compartment.

Authors	Title	Ref.	Opinion
O'Brien CA, Pollett A, Gallinger S, Dick JE	A human colon cancer cell capable of initiating tumour growth in immunodeficient mice	[8]	+
Ricci-Vitiani L, Lombardi DG, Pilozzi E *et al.*	Identification and expansion of human colon-cancer-initiating cells	[9]	+
Todaro M, Alea MP, Di Stefano AB *et al.*	Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4	[32]	+
Kemper K, Sprick MR, de Bree M *et al.*	The AC133 epitope, but not the CD133 protein, is lost upon cancer stem cell differentiation	[36]	+
Artells R, Moreno I, Díaz T *et al.*	Tumour CD133 mRNA expression and clinical outcome in surgically resected colorectal cancer patients	[38]	+
Pohl A, El-Khoueiry A, Yang D *et al.*	Pharmacogenetic profiling of CD133 is associated with response rate (RR) and progression-free survival (PFS) in patients with metastatic colorectal cancer (mCRC), treated with bevacizumab-based chemotherapy	[41]	+
Pilati P, Mocellin S, Bertazza L *et al.*	Prognostic value of putative circulating cancer stem cells in patients undergoing hepatic resection for colorectal liver metastasis	[42]	+
Kemper K, Rodermond H, Colak S, Grandela C, Medema JP	Targeting colorectal cancer stem cells with inducible caspase-9	[43]	+
Chen KL, Pan F, Jiang H *et al.*	Highly enriched $CD133^{+}CD44^{+}$ stem-like cells with $CD133^{+}CD44^{high}$ metastatic subset in HCT116 colon cancer cells	[34]	*
Huang EH, Hynes MJ, Zhang T *et al.*	Aldehyde dehydrogenase 1 is a marker for normal and malignant human colonic stem cells (SC) and tracks SC overpopulation during colon tumorigenesis	[39]	*
Lin L, Fuchs J, Li C, Olson V, Bekaii-Saab T, Lin J	STAT3 signaling pathway is necessary for cell survival and tumorsphere forming capacity in $ALDH^{+}/CD133^{+}$ stem cell-like human colon cancer cells	[40]	*
Dalerba P, Dylla SJ, Park IK *et al.*	Phenotypic characterization of human colorectal cancer stem cells	[33]	–
Shmelkov SV, Butler JM, Hooper AT *et al.*	CD133 expression is not restricted to stem cells, and both $CD133^{+}$ and $CD133^{-}$ metastatic colon cancer cells initiate tumors	[35]	–

Table 1

This panel represents the opinion of several authors with respect to the CD133 value in defining the CRCSC population: those marked + represented the papers supporting this hypothesis, those marked * propose the use of CD133 in combination with other markers, Thhose marked -- are the opponents to its use in CRCSC identification.

CD133: clinical outcome and target therapy

Although CD133's function in colon progression remains unclear, an excellent food for thought about its role is provided by recent publications.
In particular, Pohl et al. demonstrated that there is a strong relationship between CD133, VEGF,and VEGFR (VEGFR-1, -2 and -3) expression. They studied a panel of 54 patients with metastatic colorectal cancer (mCRC) treated with bevacizumab, showing a correlation between tumour response and CD133 expression levels, with a significantly better response rate (RR) in the patients with high CD133 expression levels (RR 86%) than those with low expression (RR 38%) [41]. In the same study they analyzed the expression of three different polymorphisms in the CD133 gene proving that two of them (rs2286455 and rs3130) are associated with favourable benefits in progressionfree survival (PFS). All these results make CD133 a good candidate for a predictive role in anti-VEGF treatment.
An alternative approach was used to understand the importance of CD133 in patients with colorectal liver metastasis undergoing hepatic resection. The idea arose from the consideration that the liver resection results in 5-years overall survival rates of just 20

--40% of patients. This could be caused by circulating tumour cells (CTCs), believed to be responsible for minimal residual disease (MRD), which can be easily obtained from the peripheral blood. They analyzed the expression of seven genes, that identify epithelial cells (cytokeratin 19 (CK19) and CK20) or malignant cells (carcinoembryonic antigen (CEA) and survivin), or that play a key role in cancer progression (CD133, VEGF, EGFR and survivin), founding out that only the expression levels of CD133 and survivin correlate with patient survival. Only the transcriptional amount of CD133 was significantly associated with disease-specific survival (DSS) indicating its predictive power for this patient group. The authors also showed that the expression of CD133 was higher in the liver metastasis specimens compared with both primary colon carcinoma and normal human cells. All these findings support the idea that the detection of CD133 could be associated with the amount of metastatic CRC cells in the peripheral blood [42].
Further research could be useful to consider CD133 as a responsiveness marker to adjuvant therapy, for instance, as a marker for early detection of disease recurrence during follow-up.
As cancer stem cells are considered to be responsible for tumour progression, new studies are aimed at defining new ways to selectively target this sub-population, characterized in particular by over-regulation of anti-apoptotic factors.
A recent study performed by Kemper et al. highlighted the role of caspases as a way to induce CSCs death. In particular they showed that the dimerization of an inducible caspase 9 (iCasp9) is able to induce apoptosis in cells that are resistant to conventional therapy, both in vitro and in vivo. This apoptosis induction is independent of the mitochondrial pathway, because it acts downstream of it, affecting the cell subpopulation unresponsive to 5-fluorouracil (5-FU) treatment in vitro. The in vivo experiments resulted in a strong decrease in tumour size, an increase in cell apoptosis and a loss of CD133+ cells, suggesting that the dimerization of iCasp9 circumvents the apoptosis block. This approach could be useful to design new way to selectively target the cancer stem cell compartment within the tumour, thus reducing the risk of tumour progression [43].

Conclusion

The colon CSC phenotype has been associated to the expression of several putative markers, including CD133, CD44, CD166 and CD24, even if their function in cancer biology is not yet clear. These markers are involved in cell--cell and cell—matrix interaction, which could be fundamental for the capacity to recapitulate the parental tumour in immunocompromised mice. It becomes therefore necessary to further investigate the contribution of the above mentioned markers in determining the stem cell state such as ALDH1 or the Wnt pathway. This latter could be considered specific targets for the development of drugs against the CSC compartment. Further studies on tumoral niche and microenvironment, still now a little neglected, will lead to better understanding of the CSC biology from which one could speculate alternative therapies.

Expert opinion

CSC model proposes that tumour is organized in a hierarchical scale with a differentiation grading that could explain the resistance to conventional treatment. Cancer response to standard therapies may be only apparent due to the presence of

quiescent and drug-resistant CSCs. It is attracting the attention of the scientific community in this field, since it reflects the need to characterize the CSCs and understand the molecular mechanisms involved in cancer resistance to outline a target therapy. One of the most promising stem cell markers is CD133, although its role in tumour progression has been subject of several debates in recent years. Although the role of CD133 is not clearly established, nowadays its connection between normal and cancer stem cells is strongly defined. High expression levels of CD133 are associated with chemoresistance, relapses, and decreased survival in many tumours, including colorectal cancer. More importantly it seems that CD133 mRNA levels of circulating tumour cells could have prognostic value in advanced colorectal cancer. Since CD133+ cells, such as CSCs, are extremely plastic, the next studies will be focused on integrated strategies that will target molecules which regulate the survival of both differentiated and CS cells and control tumour niche pathways.

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Declaration of interest

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Chapter| 3

Proliferation State and Polo-Like Kinase1 Dependence of Tumorigenic Colon Cancer Cells

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Abstract

Tumor-initiating cells are responsible for tumor maintenance and relapse in solid and hematologic cancers. Although tumor-initiating cells were initially believed to be mainly quiescent, rapidly proliferating tumorigenic cells were found in breast cancer. In colon cancer, the proliferative activity of the tumorigenic population has not been defined, although it represents an essential parameter for the development of more effective therapeutic strategies. Here, we show that tumorigenic colon cancer cells can be found in a rapidly proliferating state in vitro and in vivo, both in human tumors and mouse xenografts. Inhibitors of polo-like kinase1 (Plk1), a mitotic kinase essential for cell proliferation, demonstrated maximal efficiency over other targeted compounds and chemotherapeutic agents in inducing death of colon cancer-initiating cells in vitro. In vivo, Plk1 inhibitors killed $CD133^+$ colon cancer cells leading to complete growth arrest of colon cancer stem cell-derived xenografts, whereas chemotherapeutic agents only slowed tumor progression. While chemotherapy treatment increased $CD133^+$ cell proliferation, treatment with Plk1 inhibitors eliminated all proliferating tumor-initiating cells. Quiescent $CD133^+$ cells that survived the treatment with Plk1 inhibitors could be killed by subsequent Plk1 inhibition when they exited from quiescence. Altogether, these results provide a new insight into the proliferative status of colon tumor-initiating cells both in basal conditions and in response to therapy and indicate Plk1 inhibitors as potentially useful in the treatment of colorectal cancer.

Introduction

Every year more than 1 million people worldwide develop colorectal cancer, with a mortality of approximately 33% in developed countries [1]. In the past 5 years, the use of targeted agents has brought an improvement of survival outcomes for selected categories of colorectal cancer patients. However, a large portion of patients will not benefit by treatment with monoclonal antibodies and relies on 5-fluoropyrimidine-based chemotherapeutic regimens. The discovery of cancer stem cells (CSC) in solid tumors drew a different perspective on antineoplastic therapies, following the demonstration that CSC are extremely resistant to cytotoxic stimuli in vitro and in vivo as a result of hyperactive DNA repair pathways, drug efflux mechanisms, or decreased production of reactive oxygen species [2–4]. Colon CSC were characterized on the basis of CD133, Lgr5, or aldehyde dehydrogenase 1 (ALDH 1) expression [5] and were subsequently shown to be particularly resistant to chemotherapeutic agents [6], possibly explaining the inability of chemotherapy to eradicate colorectal tumors. Therefore, it is of primary importance to identify pathways active in CSC that could represent the target for new antitumor therapies.

CSC proliferation status is highly relevant for cancer therapy. However, while the proliferative activity of stem cells in normal adult tissues and hematologic cancers has been thoroughly investigated, the proliferation of CSC from solid tumors has only recently started to be explored. Mammary CSC have been shown to possess an increased replicative potential as compared to their normal counterparts and to undergo more frequent self-renewing divisions [7]. In breast cancer, these properties are likely responsible for the continuous expansion of the CSC pool, while the ability of CSC to divide asymmetrically accounts for the production of more differentiated tumor cells. Normal colon stem cells have been shown to be actively cycling [8] and to stop dividing when they differentiate, but the proliferative status of their tumor counterpart has never been investigated in detail.

Polo-like kinase1 (Plk1) is a serine-threonine kinase that was originally identified in *Drosophila* as an essential mitotic regulator [9]. The expression of Plk1 is low in most adult tissues except for those with a high proliferative index. Conversely, a broad spectrum of human tumors overexpress Plk1, supporting the theory that an elevated expression of PLK1 is a general feature of human cancer [10]. Plk1 inhibition is considered a promising therapeutic approach in anticancer treatment, and a first wave of phase I studies aimed at identifying the maximum-tolerated dose has been recently completed. The Plk1 inhibitor volasertib (BI 6727) has moved to phase II in order to determine its antitumor activity and safety profile. Preliminary results from a clinical trial with volasertib as single-agent showed encouraging antitumor activity, with nonhematologic adverse events that were mild and uncommon [11]. We show that colon cancer-initiating cells (CCIC) are actively proliferating both in vitro and in vivo, overexpress Plk1, and are sensitive to Plk1 inhibitors. Importantly, Plk1 inhibitors showed enhanced antitumor activity on CCICderived xenografts as compared to chemotherapy, pointing to Plk1 as a potential therapeutic target in colorectal cancer.

Material and Methods

Cells

Colon cancer specimens were obtained from patients undergoing surgical resection upon informed consent and approval by the Institutional Ethical Committee, and subsequent studies were conducted according to the Helsinki Declaration guidelines. CCIC were selected as previously described (6) and cultured as multicellular spheroids composed of $CD133^+$ cells in serum-free medium containing epidermal growth factor 20 ng/ml and basic fibroblast growth factor 10 ng/ml (PeproTech, London, U.K., http://www.peprotech.com). CCIC differentiation was obtained by culture in keratinocyte serum-free medium (KSFM) medium or in 10% serum-containing Dulbecco's modified Eagle's medium (both from Gibco-Invitrogen, Carlsbad, CA, http://www.invitrogen.com) for 10 days and confirmed by a decrease in CD133 and Lgr5 expression. Freshly isolated colon cancer cells were isolated from human tumor specimens or from CCIC-derived xenografts by magnetic cell separation with CD133/1 antibody-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec. com). To obtain the human CD133-fraction, xenograftderived CD133- cells were additionally sorted with an anti-human epithelial cell adhesion molecule (EpCAM) antibody. Antibodies and Reagents BI 2536 was purchased from Axon Medchem (Groningen, The Netherlands, http://www.axonmedchem.com). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was from Roche Molecular Biochemicals (Indianapolis, IN, http://www.roche.com). CD133/1-phycoerythrin (PE) (used for flow cytometry/ sorting) and CD133/2 (used for immunofluorescence) were from Miltenyi Biotec. Alexa Fluor-conjugated secondary antibodies were from Invitrogen-Molecular Probes (Eugene, OR, http:// www.invitrogen. com). Anti-PLK1, Ki67, p-ATM, and anti-cyclin B1 antibodies were from Santa Cruz (Santa Cruz, CA, http:// www.scbt.com). Caspase 3 and 9 antibodies were from Millipore- Upstate Biotechnology (Billerica, MA, http://www.millipore.- com). Monoclonal anti-a-tubulin was from Sigma Aldrich (St Louis, MO, http://www.sigmaaldrich.com). Aurora aurora/IPL1- related kinase (A/AIK) and anti-phospho-histone H3 (Ser10) were from Cell Signaling Technology (Beverly, MA, http://www.cellsignal. com). Secondary anti-mouse and anti-rabbit antibodies coupled to horseradish peroxidase (HRP) were from GE Healthcare (Uppsala, Sweden, http://www.gehealthcare.com). M30 antibody was from Peviva AB (Bromma, Sweden, http://www.peviva. com), and Lgr5 antibody was from Abgent (San Diego, CA, http://www.abgent.com).> The ALDEFLUOR assay was from Aldagen, (Durham, NC, http://www.aldagen.com). The APC bromodeoxyuridine (BrDU) Flow Kit was from BD Biosciences (San Diego, CA, http://www.bdbiosciences.com).

PKH Staining

CCIC spheroids were dissociated with TrypLE Express (Invitrogen) and stained for 2 minutes at 37_C with 1:500 PKH-26 dye (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com), then washed extensively with PBS, and cultured for 10 days. Cells were then sorted with a fluorescence-activated cell sorting (FACS) Aria (Becton Dickinson, Franklin Lakes, NJ, http://www.bd.com) into PKH-low/negative, -intermediate, and -high fractions. Control cells did not undergo PKH-

26 staining but were mock-sorted to induce similar stress conditions as in the other sorted samples.

Plk1 RNA Interference

A total of 1.25 X 10^5 CCSC were plated on six-well plates in antibiotic- free culture medium and incubated for 4 hours at 37°C in the presence of 320 nM ON-TARGETplus SMARTpool shortinterfering RNA (siRNA) (J-003290-00-0005, Human PLK1, NM_005030) or control siRNA (nontargeting siRNA D-001810- 01) (Thermo Scientific, Lafayette CO, http://www.thermoscientific.com) and 5 ll Lipofectamine 2000 (Invitrogen). After 4 hours, the transfection mixture was substituted with normal growth medium, and cells were analyzed for Plk1 expression, viability, and morphology at the indicated times.

Library Screening

CCIC spheroids were dissociated with TrypLE Express and 3,000 cells per well were seeded in 96-well microtiter plated at day 0. On day 2, cells were treated with a library of kinase inhibitors (Enzo Life Sciences, New York, NY, http://www.enzolifesciences.com) used at a 100 nM concentration. In addition, the following compounds were used at a concentration of 100 nM: BI 2536, Met kinase inhibitor (PHA-665752), and Aurora kinase inhibitor (PHA- 680632) (both from Selleck Chemicals, Houston, TX, http:// www.selleckchem.com). 5-Fluorouracil (5-FU) and Oxaliplatin (OXA) (Sigma-Aldrich) were added at a final concentration of 10 lM, which approximates peak plasma values in treated patients. Cells were processed after 48 hours with the CellTiter-Glo viability assay (Promega, Madison, WI, http://www.promega.com) according to manufacturer's instructions, and the luminescent signal was read on a DTX880 microplate reader (Beckman Coulter, Brea, CA, http://www.beckmancoulter.com). Viability was calculated as follows: Vs/Mc x 100, where Vs represents the integrated intensity of luminescence counts from the treated sample and Mc represents the average of the integrated intensities of luminescence counts from the dimethyl sulfoxide (DMSO) control.

Cell cycle, Apoptosis, and Clonogenicity Assays

The cell cycle status of CCSC was assessed by staining dissociated spheroids with 50 µg/ml propidium iodide dissolved in buffer 0.1% trisodium citrate, 9.65 mM NaCl, 0.1% Nonidet P40, and 200 µg/ml RNase for 1 hour at room temperature. Samples were analyzed with a FACSCanto flow cytometer (Becton Dickinson) equipped with a DIVA software. The CellTiter-Glo assay (Promega) was used to determine cell viability. A total of 2 x 10^3 dissociated cells were plated at day 0 in 96-well flat bottom plates. 100 nM BI 2536, 200 mg/ml His-Flag TRAIL, or 40 µM zVAD (Bachem, Bubendorf, Switzerland) were added at day 1, and cells were processed at day 3 according to the manufacturer's instructions. Luminescent signal was read using DTX880. Clonogenicity assay of CCSC was performed by plating 500 single cells per well in triplicate suspended in 0.3% agarose with or without BI 2536 over a layer of 0.4% agarose. Plates were incubated in a 5% CO2 humidified incubator at 37°C, and colony counts were performed 20–25 days after plating. The colonies were stained with crystal violet (0.1% in 10% MetOH) and counted under a light microscope. Data shown represent the percentage of colony numbers relative to plated cells.

Mice Treatment

Animal experiments were conducted according to the national Animal Experimentation guidelines (D.L.116/92) upon approval of the experimental protocol by the Institutional Animal Experimentation Committee. 6-8 week-old female non-obese diabetic (NOD). Cg- $Prkdc^{scid}$ $Il2rg^{tm1Wjl}$/SzJ (NSG) mice (The Jackson Laboratory, Bar Harbor, ME, http://www.jax.org) were subcutaneously injected with the following number of cells resuspended in 100 µl Matrigel/CCIC medium 1:1. For BI 6727 or chemotherapy treatment, we used 2.5 x 10^5 cells; for the PKH experiment of Figure 1, we used 2.5 x 10^3 cells; for PKH limiting dilution experiments, we used cell numbers indicated in Supporting Information Table 1. Pharmacological treatments with BI 6727 or chemotherapeutic agents were started when tumor xenografts reached an approximate volume of 100 mm^3. The BI 6727 group (n = 6) was treated by injecting endovenously the compound for 2 consecutive days each week (60 mg/kg weekly) dissolved in 1 N HCl 0.9% NaCl. The chemotherapy group (n = 6) was treated by injecting intraperitoneally the compounds (OXA 10 mg/kg, 5-FU 25 mg/kg) resuspended in phosphate buffer saline (PBS) once a week. The vehicle group (n = 6) was treated by injecting endovenously 100 µl 1 N HCl 0.9% NaCl twice a week. Tumor volume was evaluated by using an external digital caliper, and mice weight was evaluated at the indicated times. Mice were sacrificed 3 weeks after the beginning of the treatment, and tumors were removed, weighted, and either embedded in optimal cutting temperature compound (OCT) and frozen at -80°C or processed for subsequent flow cytometry/sorting. For short-term treatments, the BI 6727 group (n = 6) was injected endovenously with the compound (40 mg/kg) dissolved as described above, while the vehicle group (n = 6) was injected with 1 N HCl 0.9% NaCl. After 24 or 48 hours, mice were sacrificed and tumors were removed, embedded in OCT, and frozen at -80°C.

Immunofluorescence

CCIC spheroids were cytospun at low speed on polylysine-coated glass slides, whereas differentiated colon cancer cells were grown on Matrigel-coated coverslips. Typically, cells were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X-100 (Bio-Rad Laboratories, Richmond, CA, http://www.bio-rad.com) then incubated overnight at 4°C with primary antibodies dissolved in PBS containing 3% bovine serum albumin (BSA), 0.1% Triton X-100. After two washes in PBS, cells were incubated with Alexa Fluor-conjugated secondary antibodies for 30 minutes at room temperature in the dark, stained for 15 minutes with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen), diluited in PBS 3% BSA, and mounted with Prolong- Gold antifade (Invitrogen). For actin staining, cells were incubated for 30 minutes at room temperature with Alexa Fluor 488 phalloidin 1 µM (Invitrogen). For mitochondrial staining, live cells were incubated for 30 minutes at 37°C with 100 nM Mitotracker CMXRos (Invitrogen) then processed for staining with cytochrome c antibody as described above. Slides were analyzed on a FV1000 confocal microscope (Olympus, Tokyo, Japan, http://www.olympus-global.com) equipped with x60 and x40 oil immersion objectives.

Flow Cytometry and Sorting

Mitochondrial membrane depolarization was measured by flow cytometry following incubation of dissociated cells with 10 µg/ml tetramethyl rhodamine methyl ester (Invitrogen Molecular Probes)

for 30 minutes at 37°C. For isolation of cells from xenograft tissue, cells were labeled with EpCAM-fluorescein isothiocyanate (FITC) (Dako, Glostrup, Denmark, http://www.dako.com) and CD133/1 PE (Miltenyi Biotec) for 30 minutes at room temperature and sorted with a FACS Aria (Becton Dickinson). 10 µg/ml 7-aminoactinomycin D (Sigma-Aldrich) was always added for dead cell exclusion.

Western Blotting

Cell lysates were obtained from approximately 2.5 x 105 colon cancer initiating cells (CCIC) by incubation of cell pellets in 1% Nonidet P40 lysis buffer (20 mM Tris-HCl pH 7.2, 200 mM NaCl, 1% Nonidet P40) supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktails I and II (all from Sigma-Aldrich). Lysate concentrations were determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA), and equal amounts of proteins were loaded on a 4%–12% precast gel (Invitrogen) and transferred to nitrocellulose membranes. Blots were blocked with tris-buffered saline Tween-20 (TBST) 5% nonfat dry milk and incubated overnight at 4°C with primary antibodies, then incubated for 45 minutes with secondary HRP-conjugated antibodies dissolved in TBST 1% BSA. Chemiluminescent signals were detected with Super SignalWest Pico (Pierce, Rockford, IL, http://www.piercenet.com).

Immunohistochemistry

Tumor sections (5–8 µm) were obtained using a cryomicrotome (Kriostat 1720 MGW Leitz, Melville, NY, http://www.leitz.com). For CK18 staining, frozen sections were fixed in ice-cold methanol for 4 minutes, endogenous peroxidase activities were blocked using 0.03% hydrogen peroxide for 15 minutes in the dark, and then the sections were incubated at room temperature for 1 hour with M30 antibody. Sections were then processed using avidin-biotin-peroxidase complex (ULTRATEK HRP SCY, Tek Laboratories, West Logan, UT, http://teklabs.com), counterstained with hematoxylin, and mounted with DPX (Sigma-Aldrich). For immunofluorescence analysis, frozen sections were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, and blocked with 5% serum in PBS containing 1% BSA and 0.1% Triton X-100. Sections were stained overnight at 4°C with primary antibodies, incubated with fluorochrome-conjugated secondary antibodies with 5 mg/ml RNase A, and counterstained with DAPI. TUNEL staining was performed with the In Situ Cell Death Detection Kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. Azan-Mallory staining was performed on xenograft cryosections with the Mallory Tricromica kit (Bio-Optica, Milan, Italy, http://www.bio-optica.it) according to the manufacturer's instructions.

Statistical Analysis

Unless otherwise specified, statistical analysis was conducted by using two-way ANOVA, and differences between groups/pairs were evaluated with Bonferroni post tests. Analysis of Figure 6E was performed with two-tailed unpaired t test. Statistical analysis was performed with GraphPad Prism v.4.0 for Windows (Graph- Pad Software, San Diego, CA, www.graphpad.com), and statistical significance was accepted up to .05. p values are displayed using a single asterisk for significances ranging from .05 to .01, two asterisks for values between .001 and .01, and three whenstatistical differences produced significance below .001.

Results

Tumorigenic Colon Cancer Cells Are Present in All Proliferative Subsets of CD133$^+$ Cells

CD133$^+$ colon cancer cells have been previously shown to be enriched for cells with tumorigenic ability [12, 13]. To investigate the proliferative state of CCIC, we separated different subsets of CD133$^+$ CCIC according to their proliferative activity and then tested their ability to generate tumors in immunocompromised mice. To do so, CD133$^+$ cells derived from surgical specimens were cultured as multicellular spheroids and stained with the fluorescent dye PKH-26, which binds cell membranes and segregates in daughter cells after each cell division, thus providing a signal that correlates with the cell's proliferation history. After 10 days of PKH staining, CCIC were FACS-separated into three subsets, $PKH_{LOW/NEG}$ (very rapidly proliferating), PKHMED (rapidly proliferating), and PKHHIGH (slowly proliferating). Sorted cells were immediately inoculated in NSG mice (2.5 x 10^3 cells each), and subcutaneous tumor formation was observed after approximately 2 months. Cells belonging to the PKHMED and PKHHIGH fractions gave rise to tumors in 100% of mice, as did bulk control cells from unstained cultures (Fig. 1).

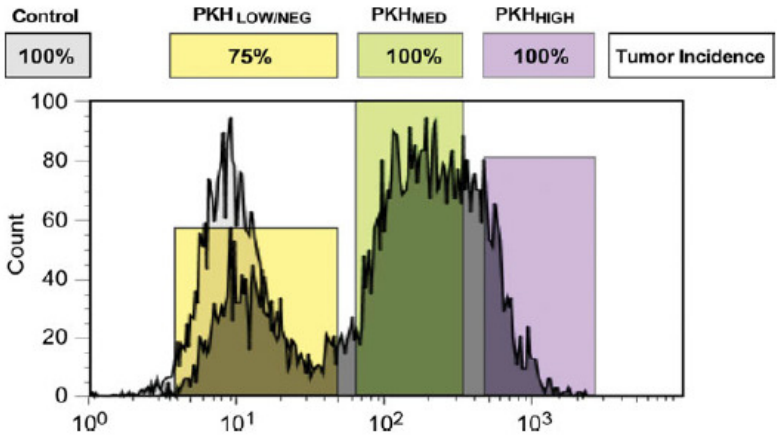


Figure 1

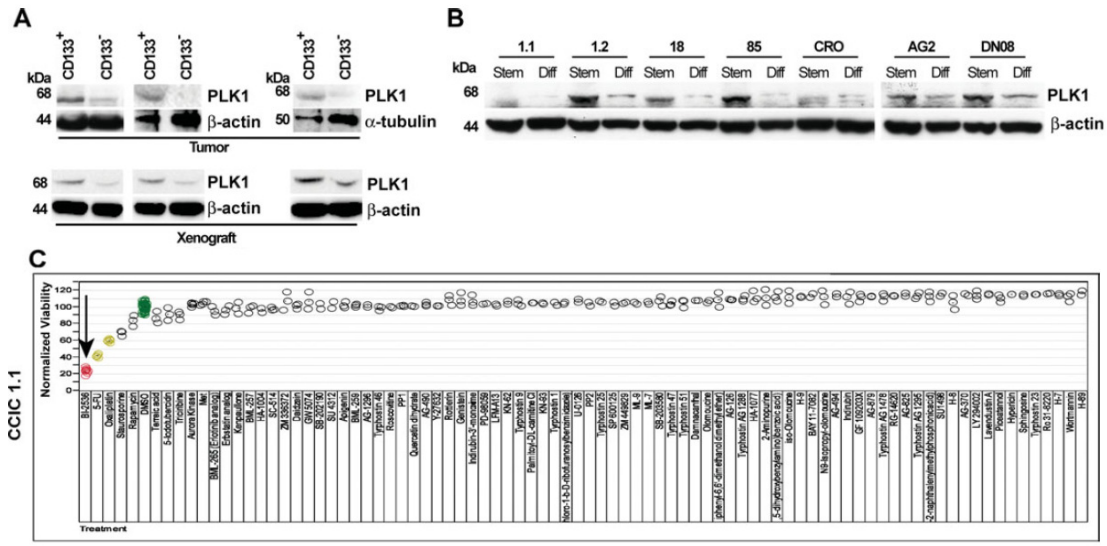
All the proliferative subsets of CD133$^+$ cells generate tumors in mice. CD133$^+$ cells derived from a colon tumor specimen were cultured as multicellular spheroids (DN08 colon cancer-initiating cell [CCIC] line), stained with the PKH-26 dye and, after 10 days, separated into three fractions according to PKH-26 retention. Sorted cells were inoculated subcutaneously

into NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ mice (eight per group). The percentage of tumors that arose from the different fractions (tumor incidence) is shown above.

Surprisingly, however, also cells of the $PKH_{LOW/NEG}$ subset gave rise to tumors, although with lower efficiency as compared with the two other subsets (Fig. 1). These results were substantiated by limiting dilution experiments showing, in three different CCIC samples, the presence of tumorigenic cells in variable amounts in the PKHLOW/NEG fraction (Supporting Information Table 1). Results from in vitro colony forming assays confirmed these observations, showing that all three PKH cell subsets were endowed with colony-forming efficiency (data not shown). PKH distribution was also investigated in cultured CCIC in relation to ALDH activity, which was previously indicated as a colon stem cell marker [14]. The starting cell population, composed of 100% $CD133^+$ cells, was separated in $PKH_{LOW/NEG}$, PKH_{MED}, and PKH_{HIGH} 10 days after PKH staining, and ALDH activity was measured in the three fractions with the ALDEFLUOR kit. $ALDH^+$ cells were comparably distributed among the different PKH fractions, again suggesting an equivalent content of tumorigenic cells (Supporting Information Fig. S1). These observations indicate that colon tumor-initiating cells are found both in slow and fast proliferative states and they can undergo rapid cell division without losing their tumorigenic potential.

CCIC Express High Levels of Plk1 and Are Highly Sensitive to Plk1 Inhibition

Proliferating cells express high levels of the mitotic kinase Plk1. To determine whether high Plk1 levels were associated with the CCIC compartment, we investigated Plk1 expression in colon cancer cells freshly isolated from surgical specimens or from tumor xenografts. $CD133^+$ tumor cells isolated either from human tumors or from CCIC-derived mouse xenografts expressed higher levels of Plk1 as compared with $CD133^-$ cells (Fig. 2A), thus suggesting that a highly proliferating state may be typical of the colon CSC population in vivo.



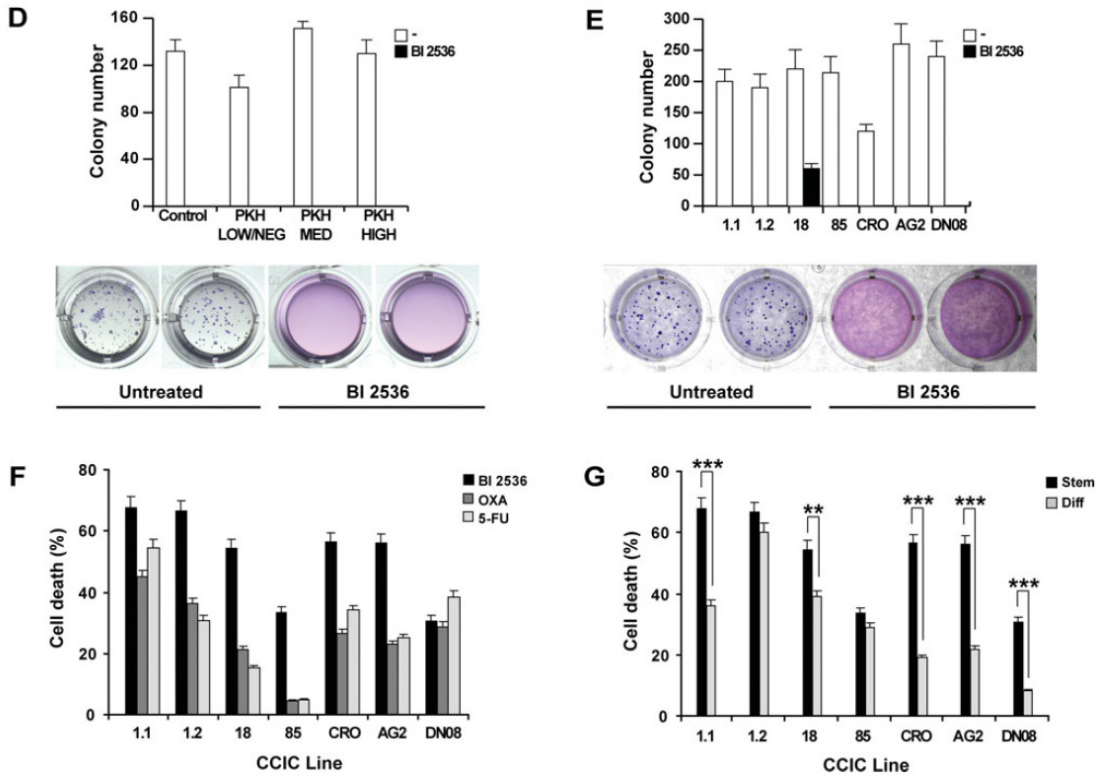


Figure 2

CCIC overexpress Plk1 and are specifically sensitive to Plk1 inhibitors. (A): Plk1 expression in $CD133^+$ and $CD133^-$ cells freshly isolated from three different colon adenocarcinoma samples (tumor) or from mouse xenografts derived from three different CCIC lines (xenograft). (B): Plk1 expression in CCIC (Stem) and in their differentiated counterparts (Diff). (C): Screening of kinase inhibitor library and chemotherapeutic agents on one CCIC line (CCIC 1.1). Cells were treated for 48 hours with the indicated compounds (shown in greater detail in Supporting Information Table 3), and cell viability was measured as described in Materials and Methods. The arrow indicates the BI 2536-treated sample. (D): Top: Colonies grown in soft-agar from $PKH_{LOW/NEG}$, PKH_{MED}, and PKH_{HIGH} fractions separated from one CCIC line (CCIC DN08), stained as in Figure 1 and plated in the presence (BI 2536) or in the absence (-) of 100 nM BI 2536. Bottom: Representative picture of the plates. (E): Top: Colonies generated in soft-agar by seven CCIC lines in the presence (BI 2536) or in the absence (-) of 100 nM BI 2536. Bottom: Representative picture of the plates. (F): Cell death induced by 100 nM BI 2536 (BI 2536) or chemotherapeutic agents OXA or 5-FU (10 lM each) in CCIC lines after 48 hours of treatment. (G): Cell death induced by 48 hours of treatment with BI 2536 in seven lines of CCIC (Stem) as compared with their differentiated progeny (Diff). ANOVA comparison of stem versus differentiated cells resulted in $p < .001$. Data shown in (D–G) are the mean 6 SD of four independent experiments. **, $p < .01$; ***, $p < .001$. Abbreviations: CCIC, colon cancer-initiating cells; OXA, oxaliplatin; Plk1, Polo-like kinase1; 5-FU, 5-fluorouracil.

The correlation between ‘‘stemness’’ and Plk1 expression was confirmed by comparing seven patient-derived spheroid cultures (described in Supporting Information Table 2) with their differentiated progeny, composed of adherent cells that have lost CD133 and Lgr5 expression (Supporting Information Fig. S2). As expected, Plk1 was more expressed in the stem cell fraction (Fig. 2B) and was mainly localized on centrosomes of dividing cells (Supporting Information Fig. S3A). The higher Plk1 expression in $CD133^+$ CCIC observed in vitro and in vivo parallels the elevated proliferative activity of $CD133^+$ cells. In fact, we observed that in vitro CCIC proliferate more than their differentiated counterparts (Supporting Information Fig. S4A, S4B), and that in CCIC-derived tumor xenografts, CD133 expression was mainly associated with Ki67 staining (Supporting Information Fig. S4C). Due to the high expression of Plk1 in the CCIC compartment, we ought to investigate the effect of the Plk1 inhibitor BI 2536 on CCIC in comparison to a panel of 80 targeted agents and to chemotherapeutic agents used for colon cancer treatment (5-FU and OXA). Only the Plk1 inhibitor BI 2536 resulted in a >50% reduction of CCIC viability after 48 hours in two CCIC lines tested (Fig. 2C; Supporting Information Fig. S3B; compounds’ names are shown in greater detail in Supporting Information Table 3). A doseresponse assessment of BI 2536 efficacy revealed that CCIC death was induced already at a 25 nM concentration (Supporting Information Fig. S3C). However, we chose a working concentration of 100 nM, as this dose was previously shown to maximally and specifically inhibit Plk1 [15]. To investigate the in vitro sensitivity to Plk1 inhibitors of differently proliferating CCIC subsets separated according to PKH staining, we evaluated the colony-forming capacity of $PKH_{LOW/NEG}$, PKH_{MED}, and PKH_{HIGH} fractions in the presence of BI 2536. None of the three CCIC subsets was able to generate colonies in semisolid culture in the presence of BI 2536 (Fig. 2D), prompting us to use bulk CCIC cultures for subsequent experiments. The effect of BI 2536 was then tested on seven patient-derived CCIC lines, where it was able to inhibit colony formation in 6/7 cases (Fig. 2E). Importantly, BI 2536 was more effective than chemotherapeutic agents in reducing CCIC viability in six of seven CCIC lines tested (Fig. 2F). Finally, we compared the effect of BI 2536 on stem and differentiated colon cancer cells. As expected, CCIC resulted more sensitive than their differentiated progeny to Plk1 inhibition (Fig. 2G), according to their higher proliferative activity.

Plk1 Depletion Recapitulates the Effects of BI 2536 on Colon CSCs

Although highly specific for Plk1, BI 2536 can affect the activity of other Plks kinases such as Plk2 and Plk3 [15]. To confirm that the effects observed on CCIC were due to Plk1 inhibition, we specifically knocked down Plk1 expression by treating two CCIC lines with targeted short hairpin RNA (shRNA) sequences. Plk1 protein expression was effectively decreased in RNA interference (RNAi)-treated cells, as shown by immunoblot analysis of the two CCIC lines (Fig. 3A). After 48 hours of RNAi, CCIC with decreased Plk1 expression were characterized by spheroid disaggregation and by the appearance of cells with aberrant mitotic spindles (Fig. 3B). The viability of CCIC treated with Plk1 RNAi, but not with control oligonucleotides, massively decreased 72 hours after transfection (Fig. 3C), indicating that Plk1 is essential for CCIC survival and that its downregulation initiates cell death.

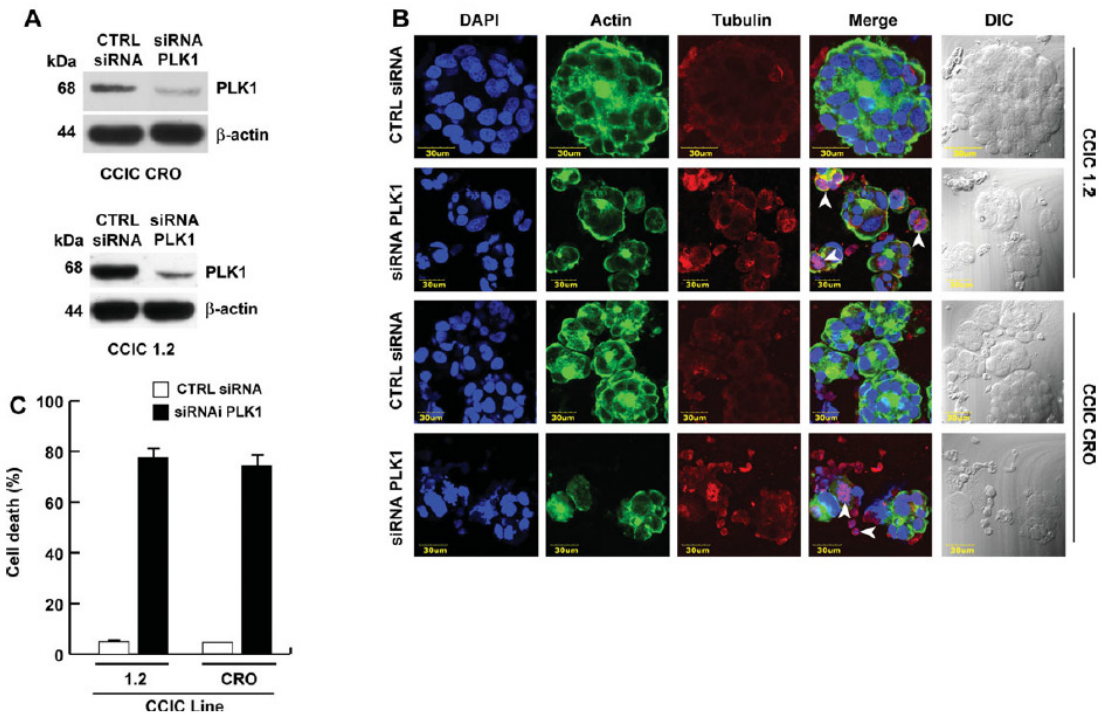


Figure 3

RNA interference against Plk1 results in CCIC death. (A): Plk1 expression detected 48 hours after transfection in two CCIC lines (CCIC 1.2 and CRO) transfected with control (CTRL siRNA) or anti-Plk1 (siRNA Plk1) RNA sequences. (B): Actin/tubulin staining performed 48 hours after transfection of two CCIC lines treated as in (A); white arrows indicate aberrant mitotic spindles. (C): Cell death detected 72 hours after transfection in two CCIC lines treated as in (A). Data shown are the mean 6 SD of three independent experiments. Abbreviations: CCIC, colon cancerinitiating cells; DAPI, 4',6-diamidino-2-phenylindole; DIC, differential interference contrast; Plk1, Polo-like kinase1; siRNA, short-interfering RNA.

Plk1 Inhibition Subverts Cell Cycle Kinetics in CCIC

To investigate the consequences of Plk1 inhibition in CCIC, we treated spheroids with BI 2536 for 24 hours and observed the appearance of mitotic nuclei in 20%–80% of cells (Fig. 4A, left) which, upon actin/tubulin staining, showed a disorganized cytoskeleton and mitotic spindles typical of the ''Polo'' phenotype (Fig. 4A, right) [16]. Propidium iodide staining of BI 2536-treated CCIC showed an accumulation of cells in the G2/M phase of the cell cycle (Fig. 4B). An evaluation of mitosis-associated proteins showed that BI 2536 treatment resulted in dephosphorylation/activation of Cdc2 and in cyclin B1 accumulation typical of prophase entry, as confirmed by massive histone H3 phosphorylation (Fig. 4C). At the same time, the Plk1 substrate Cdc25 was progressively dephosphorylated (Fig. 4C). In line with previous studies [15], we found cyclin B1 accumulation in the nucleus of BI 2536- treated cells, which was maximal at 8 hours (Fig. 4D). These observations confirm that Plk1 is dispensable for Cdc2 activation and cyclin B1 nuclear translocation at mitotic entry [17], whereas it is essential for Cdc25 phosphorylation in mitotic cells. Aurora A expression was also investigated upon treatment with BI 2536, as Aurora A plays an important role in CCIC and is tightly linked to Plk1 in the control of mitotic progression [18–20]. In untreated CCIC, intense Aurora A

expression was detectable only in dividing cells, whereas the majority of BI 2536-treated cells expressed high Aurora A levels (Fig. 4E).

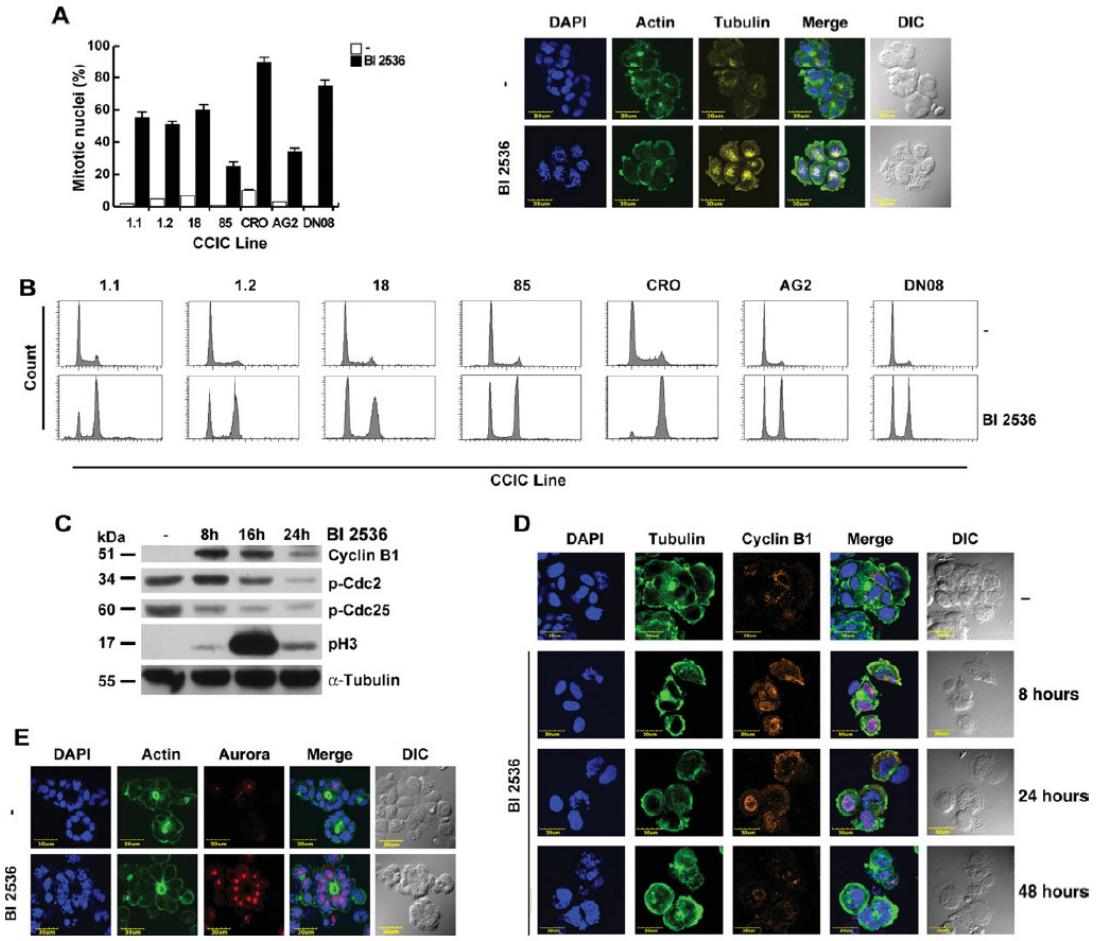


Figure 4

Plk1 inhibition by BI 2536 alters cell cycle kinetics in CCIC. (A): Percentage of mitotic nuclei in CCIC lines untreated (-) or treated for 24 hours with 100 nM BI 2536 (left) and actin/tubulin immunofluorescence staining of CCIC (AG2) treated for 24 hours with 100 nM BI 2536 (right). (B): Cell cycle analysis of CCIC treated for 24 hours with 100 nM BI 2536. Data shown are the mean 6 SD of three independent experiments. (C): Western blot analysis of cyclin B1, p-Cdc2, p-Cdc25, and p-histone H3 (pH3) in the CCIC line AG2 untreated or treated for the indicated times with 100 nM BI 2536. (D): Immunofluorescence analysis of cyclin B1 localization in the CCIC line AG2, untreated or treated for the indicated times with 100 nM BI 2536. (E): Immunofluorescence analysis of Aurora A expression in the CCIC line AG2 untreated (-) or treated for 24 hours with 100 nM BI 2536 (BI 2536). Abbreviations: CCIC, colon cancer-initiating cells; Cdc, cell division cycle; DAPI, 4',6-diamidino-2-phenylindole; DIC, differential interference contrast; Plk1, Polo-like kinase1.

CCIC Respond to Plk1 Inhibitors with DNA damage, Mitochondrial Depolarization, and Mitotic Death

Acute Plk1 inhibition has been previously shown to induce apoptosis in cancer cell lines [15, 21–24]. Therefore, we analyzed the effect of BI 2536 in CCIC by assessing signs and mechanisms of cell death. First, we observed ATM phosphorylation/activation in CCIC after 4 hours of BI 2536 treatment and a

progressive H2AX phosphorylation peaking at 48 hours, indicating the occurrence of a DNA damage response (Fig. 5A, 5B; Supporting Information Fig. S5A). Then, we evaluated typical hallmarks of apoptosis in treated cells. We found consistent mitochondrial depolarization both in intact spheroids (Fig. 5C) and in dissociated CCIC (Fig. 5D; Supporting Information Fig. S5B), cytochrome c release from mitochondria (Fig. 5E) and activation of caspases 3 and 9 (Fig. 5F). However, BI 2536-induced death of CCIC appeared to be mainly caspase-independent, as the pan-caspase inhibitor carbobenzoxy-valyl-alanyl- aspartyl-[O-methyl]-fluoromethylketone (zVAD-FMK) was unable to inhibit CCIC death, while it effectively blocked TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in Jurkat cells (Supporting Information Fig. S5C). In BI 2536-treated CCIC, the majority of cells acquired a giant size (Supporting Information Fig. S5D) and up to 10% of the cells became polyploid (Fig. 5G), indicating that CCIC undergo mitotic cell death in the presence of Plk1 inhibitors.

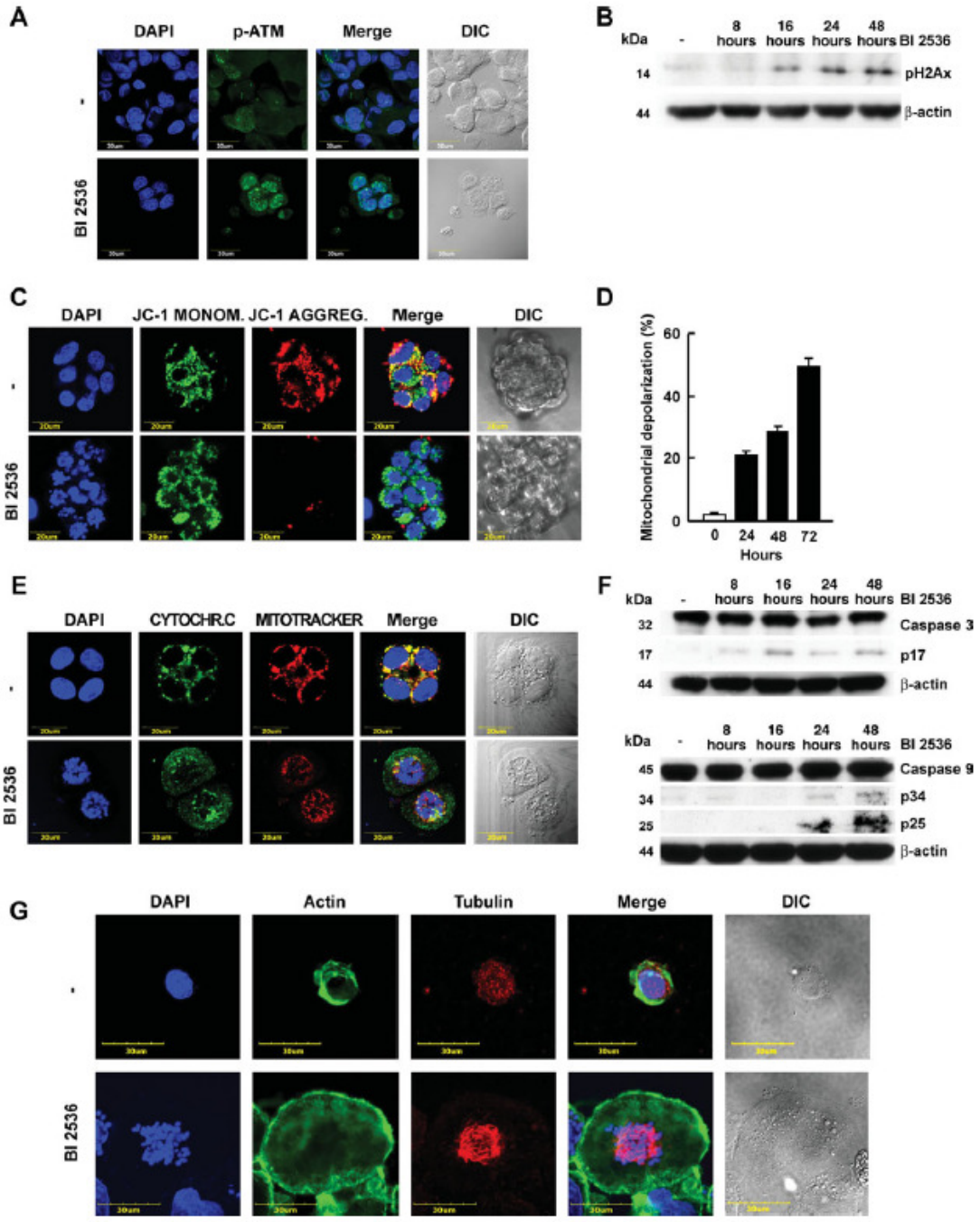


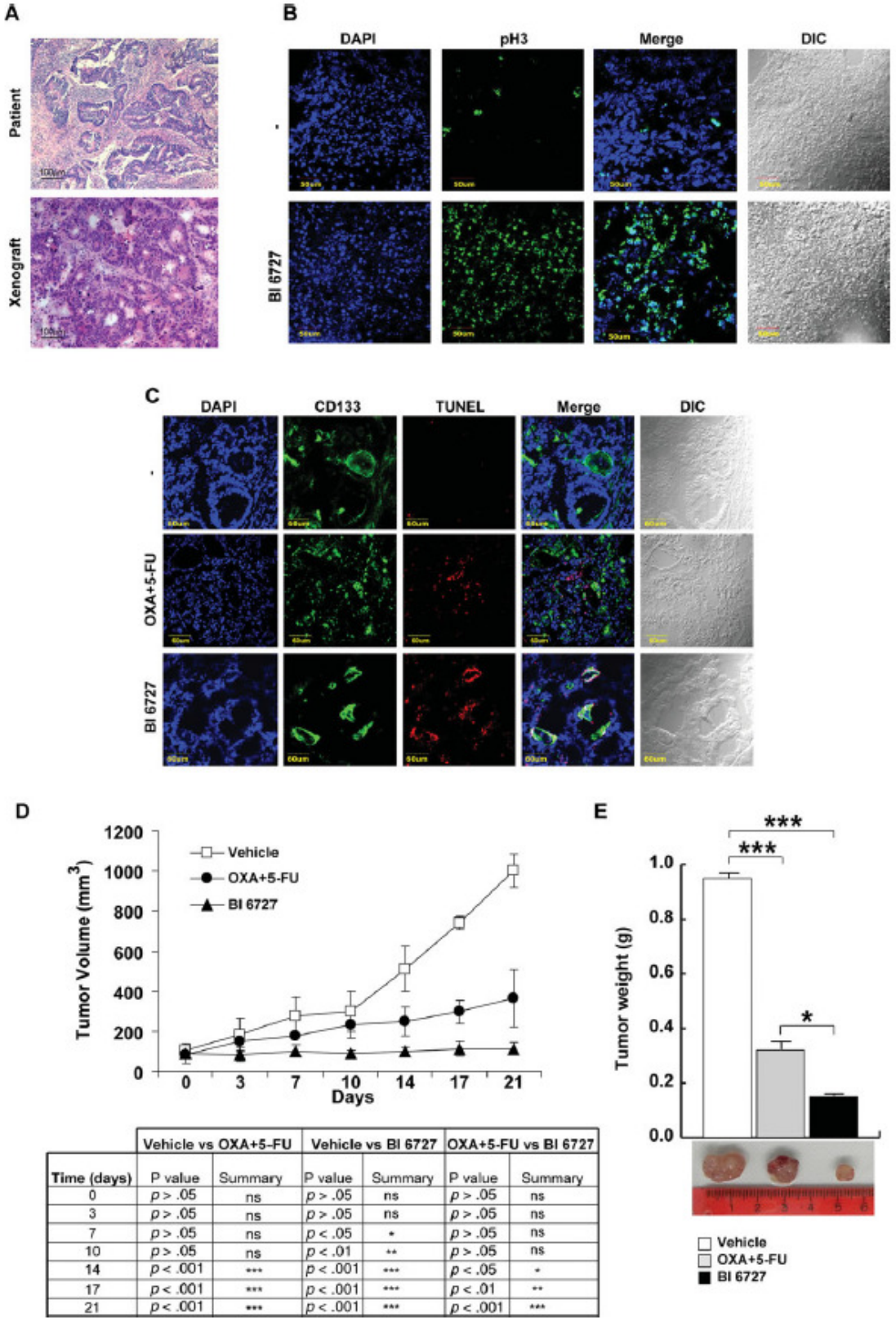
Figure 5

BI 2536 induces DNA damage and mitotic death of CCIC (line AG2). (A): ATM phosphorylation in CCIC, untreated (-) or treated for 4 hours with100 nM BI 2536. (B): Histone H2AX phosphorylation in CCIC untreated (-) or treated for 24 hours with 100 nM BI 2536. (C): Mitochondrial depolarization in live intact spheroids (JC-1 staining) treated for the indicated times with 100 nM BI 2536. x60 magnification, x2 zoom. (D): Mitochondrial depolarization in dissociated CCIC (TMRM staining) treated for the indicated times with 100 nM BI 2536. Data shown are the mean 6 SD of three datasets obtained in independent experiments. (E): Cytochrome c release from mitochondria in CCIC untreated (-) or treated for 24 hours with 100 nM BI 2536 (BI 2536); x60 magnification, x2 zoom. (F): Activation of caspases 3 and 9 in CCIC treated for the indicated times with 100 nM BI 2536. (G): Actin/tubulin staining in untreated CCIC (-) compared with a giant polyploid CCIC obtained

by treatment for 72 hours with 100 nM BI 2536 (x60 magnification, x3 zoom). Abbreviations: ATM, ataxia-telangiectasia mutated; CCIC, colon cancer-initiating cells; DAPI, 4',6-diamidino-2-phenylindole; DIC, differential interference contrast; Plk1, Polo-like kinase1; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide; TMRM, tetramethylrhodamine methyl ester.

Plk1 Inhibition In Vivo Results in CCIC Death and Tumor Growth Arrest

CSC are characterized by the ability to reproduce the original human tumor in immunocompromised mice, thus representing a powerful tool to evaluate the effect of anticancer agents in vivo. We investigated the effect of Plk1 inhibition on CCICderived tumor xenografts obtained through subcutaneous injection of CCIC, which reproduced the histological structure of the parental colorectal tumor (Fig. 6A).



	Vehicle vs OXA+5-FU		Vehicle vs BI 6727		OXA+5-FU vs BI 6727	
Time (days)	P value	Summary	P value	Summary	P value	Summary
0	$p > .05$	ns	$p > .05$	ns	$p > .05$	ns
3	$p > .05$	ns	$p > .05$	ns	$p > .05$	ns
7	$p > .05$	ns	$p < .05$	*	$p > .05$	ns
10	$p > .05$	ns	$p < .01$	**	$p > .05$	ns
14	$p < .001$	***	$p < .001$	***	$p < .05$	*
17	$p < .001$	***	$p < .001$	***	$p < .01$	**
21	$p < .001$	***	$p < .001$	***	$p < .001$	***

Figure 6

Plk1 inhibitors target CCIC in vivo and block the growth of CCIC-derived xenografts. (A): Hematoxylin/Eosin staining of a CCICderived xenograft section (lower panel, cryosection) and of the parental patient tumor (upper panel, paraffin-embedded), _10 magnification. (B): Phospho-histone H3 (pH3) staining of xenograft sections obtained from mice treated for 24

hours with vehicle (- or with BI 6727 (BI 6727), x60 magnification. (C): Immunofluorescence staining (x40 magnification) for CD133 and TUNEL of xenograft sections derived from mice treated for 48 hours with vehicle (-) or with BI 6727 (BI 6727). (D): Tumor volume of xenografts treated with vehicle only (vehicle), with chemotherapeutic agents (OXA+5-FU) and with BI 6727 (BI 6727) as described in Materials and Methods section. Statistical analysis is shown in the table beneath, where ns stands for ''nonsignificant''. Two-way ANOVA resulted in $p < .001$. The results shown are representative of four independent experiments. (E): Tumor weight at the end of treatment and representative picture. Data shown are the mean 6 SD of six samples per group relative to the experiment shown in (D). *, $p < .05$; ***, $p < .001$. All the experiments in this figure were performed with the CCIC line AG2 and replicated on the CCIC line DN08 with similar results. Abbreviations: CCIC, colon cancer-initiating cells; DAPI, 4',6-diamidino-2-phenylindole; DIC, differential interference contrast; OXA, oxaliplatin; Plk1, Polo-like kinase1; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; 5-FU, 5-fluorouracil.

For in vivo experiments we used BI 6727, a dihydropteridinone Plk1 inhibitor similar to BI 2536 with comparable activity toward CCIC in vitro (data not shown) and an improved pharmacokinetic profile in vivo [25]. Analysis of cell cycle and apoptosis markers performed on CCIC-derived xenografts upon a short-time treatment with BI 6727 revealed a widespread induction of histone H3 phosphorylation at 24 hours (Fig. 6B) indicating the occurrence of mitotic arrest in the majority of cells. Caspase 3 was massively activated in BI 6727-treated xenografts at 48 hours of treatment as shown by extensive staining for cleaved cytokeratin (Supporting Information Fig. S6A). Importantly, a double staining for CD133 and TUNEL on xenograft sections revealed that areas of cell death widely overlapped with stem cell areas identified by CD133 positivity in BI 6727-treated samples but not in chemotherapy-treated samples (Fig. 6C), indicating that BI 6727 specifically induces CCIC apoptosis in vivo. Similar results were obtained by double staining of xenografts sections with Lgr5 and TUNEL (Supporting Information Fig. S6B). A 21-day treatment with BI 6727 resulted in complete block of tumor growth, whereas standard chemotherapeutic agents (OXA plus 5-FU) were only able to slow xenograft expansion (Fig. 6D). Assessment of tumor size and weight at the end of treatment confirmed that the growth of CCIC xenografts was strongly inhibited by BI 6727 and at a lesser extent by chemotherapeutic agents (Fig. 6E).

Quiescent CD133 Cells Escape BI 6727 Treatment but Retain Sensitivity to Subsequent Plk1 Inhibition

To investigate the long-term effects of BI 6727 on xenograft stem cell populations, we examined xenografts treated for 21 days with either chemotherapeutic agents or BI 6727. Azan- Mallory staining of xenograft sections revealed that BI 6727-treated tumors contained large fibrotic areas, whereas chemotherapy-treated tumors showed a dense cellular structure (Fig. 7A).

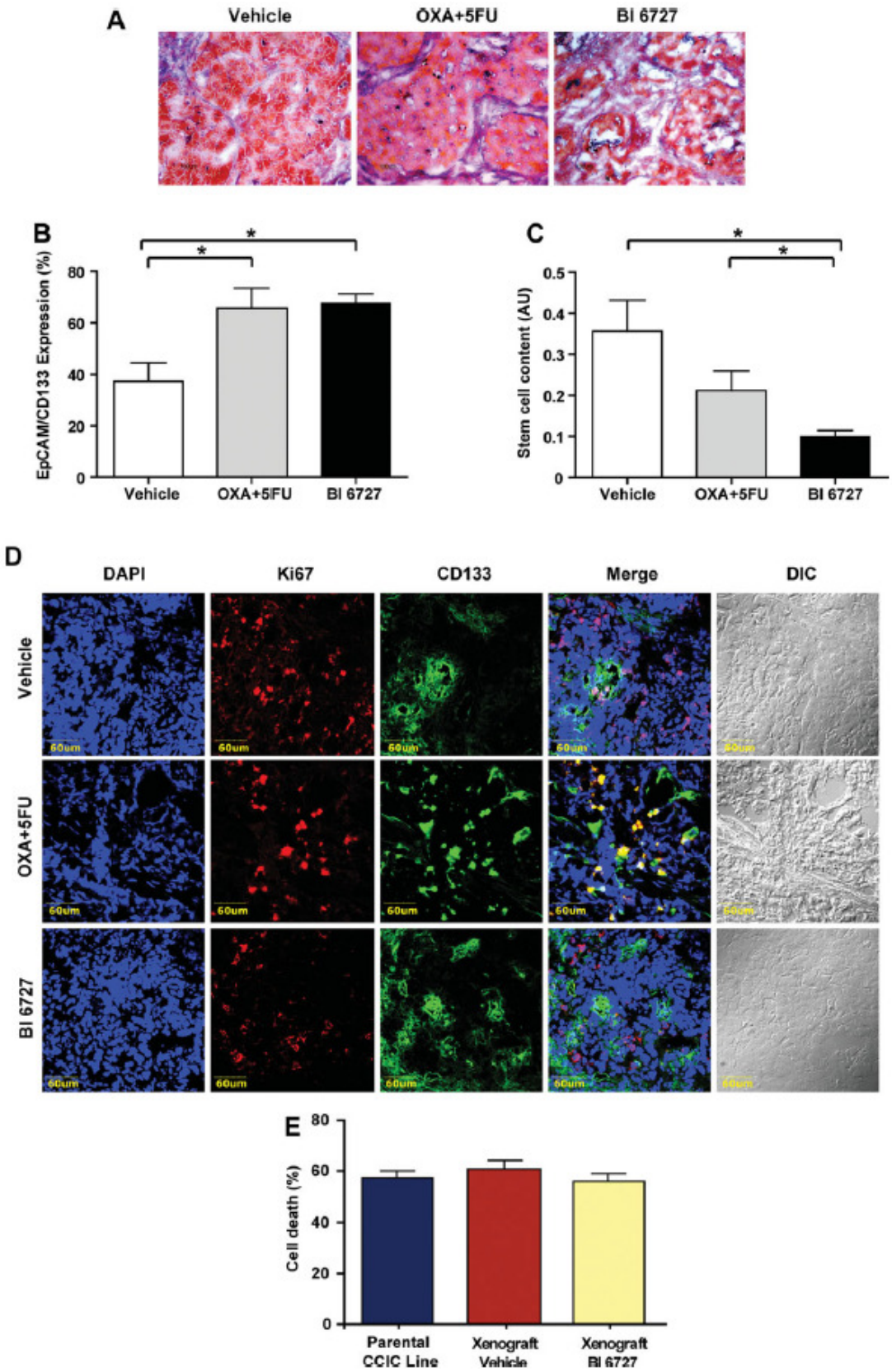


Figure 7

BI 6727-treated xenografts contain quiescent CD133^{+} cells that retain sensitivity to Plk1 inhibitors. (A): Azan-Mallory staining of xenograft sections derived from vehicle-treated

(vehicle), chemotherapy-treated (OXA+5-FU), and BI 6727-treated mice after 3 weeks of treatment. x10 magnification. (B): Percentage of $CD133^+/EpCAM^+$ cells present in xenografts after 3 weeks of treatment with vehicle only (vehicle), chemotherapy (OXA+5-FU), or BI 6727. Data shown are the mean 6 SD of five samples per group. (C): Total stem cell content in tumor xenografts relative to the experiment shown in Figure 6B. Values were calculated as tumor weight _ percentage of $Epcam^+CD133^+$ cells/100. (D): Immunofluorescence staining (x40 magnification) of Ki67 and CD133 in xenograft sections obtained after 3 weeks of treatment with vehicle only (vehicle), chemotherapy (OXA+5-FU), or BI 6727. (E): Cell death induced by treatment in vitro with 100 nM BI 6727 for 48 hours of the parental CCIC line and of $EpCAM^+/CD133^+$ cells extracted from vehicle-treated (xenograft vehicle) or BI 6727-treated (xenograft BI 6727) xenografts after 3 weeks of in vivo treatment. Data shown are the mean 6 SD of three samples per group. All the experiments in this figure were performed with the CCIC line AG2. Abbreviations: A.U.: arbitrary units; CCIC, colon cancer-initiating cells; DAPI, 4' ,6-diamidino-2-phenylindole; DIC, differential interference contrast; EpCAM, epithelial cell adhesion molecule; OXA, oxaliplatin; Plk1, Polo-like kinase1; 5-FU, 5-fluorouracil.

A quantitative evaluation of $CD133^+$ cells present in tumor xenografts revealed that the percentage of $CD133^+$ cells was increased both in chemotherapy-treated samples and in BI 6727-treated samples as compared with vehicle-treated controls (Fig. 7B). However, taking into account xenograft volume at the end of the treatment, the absolute CCIC content at the end of the treatment proved to be significantly lower in BI 6727-treated samples as compared to both controls and chemotherapy-treated samples (Fig. 7C). Moreover, a double staining for CD133 and Ki67 on xenograft sections revealed that, in chemotherapy-treated tumors, $CD133^+$ cells were actively proliferating. Conversely, in BI 6727-treated tumors, residual $CD133^+$ cells were completely quiescent (Fig. 7D). These observations indicate that treatment with Plk1 inhibitors, although does not completely eradicate the $CD133^+$ population, eliminates all the proliferating tumorigenic cells. To understand whether CCIC that survived BI 6727 treatment developed resistance to Plk1 inhibition, we isolated $EpCAM^+/CD133^+$ cells from vehicle-treated and BI 6727-treated xenografts, kept them in culture for 4 weeks, and then treated them with BI 6727 in comparison with the parental CCIC line. Sensitivity to BI 6727 was virtually identical in the three populations (Fig. 7E), indicating that $CD133^+$ cells that survive treatment with Plk1 inhibitors in vivo do not acquire resistance to Plk1 inhibitors. Altogether these observations indicate that, in vivo, CCIC respond to chemotherapeutic agents with increased proliferation, whereas in the presence of Plk1 inhibitors CCIC are found in a quiescent state resulting in inhibition of tumor growth.

Discussion

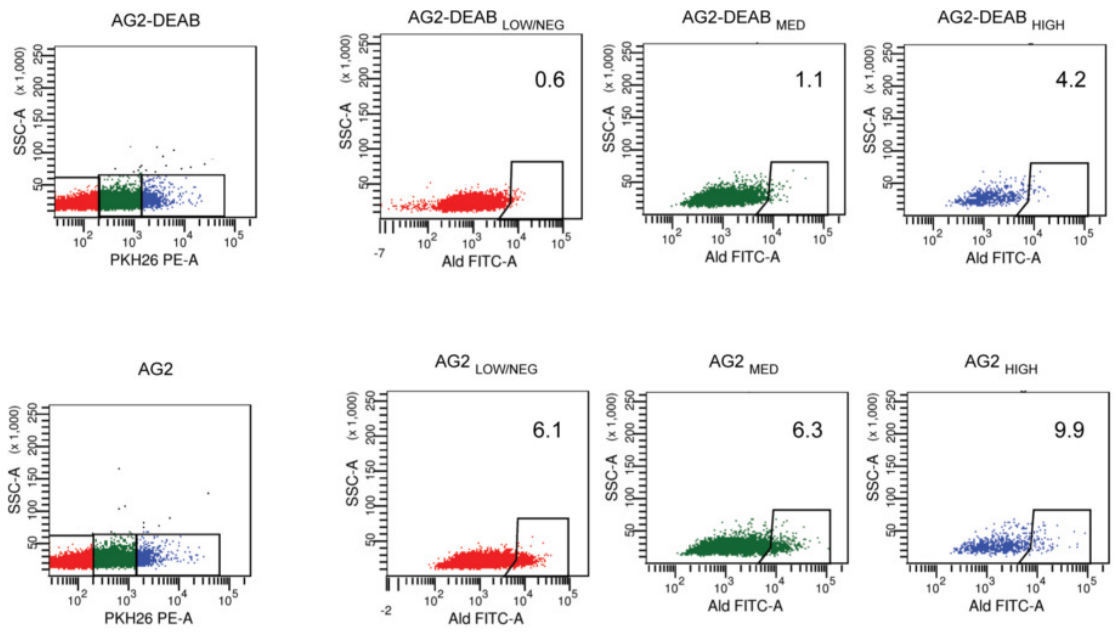
Stem cells of normal adult tissues are predominantly found in a quiescent state, from which they egress to replenish the pool of more differentiated cells. A similar hierarchy of proliferating cells is found in hematologic malignancies, where a population of quiescent stem cells retains the ability to transfer the disease in experimental animals, likely being responsible for drug resistance and relapse in leukemia patients. Differently from other stem cell types, normal colon stem cells have been found to be actively proliferating, both in mice and in humans [26, 27]. Proliferation of CSCs from solid tumors has been mainly investigated in breast cancer, where tumorigenic cells have been demonstrated to possess a replicative activity higher than their normal counterparts [7]. This feature, together with their

predilection toward symmetric division, may be responsible for continuous expansion of the breast CSC population. Characterizing the proliferation state of CSCs has strong therapeutic implications, as identifying altered cell division mechanisms present in tumorigenic cells may indicate effective routes to eradicate this population. We found that CCIC comprise both rapidly and slowly proliferating cells. Consistently with a sustained proliferative activity in vivo, CCIC freshly isolated from colon cancer specimens express high levels of Plk1, a kinase essential for multiple steps of cell division. Although not specifically considered as part of the classic stem cell equipment, increased Plk1 expression was previously identified as part of a gene signature concomitantly altered in both normal stem cells and metastatic prostate cancer cells [28], suggesting the existence of a link between stemness and deregulated expression of Plk1. More generally, high Plk1 activity is considered as a tumor-promoting force, as it has been demonstrated to stimulate mitosis, override DNA damage checkpoints, support cell invasion, and facilitate the insurgence of aneuploidy [10]. Plk1 inhibitors have been recently demonstrated to be particularly cytotoxic for neuroblastoma tumor-initiating cells and for breast cancer-initiating cells, suggesting that they may represent an interesting therapeutic option in multiple tumor types [29, 30]. Our results demonstrate that, in colon tumors, the use of Plk1 inhibitors resulted in CCIC death both in vitro and in vivo, showing an increased efficacy over chemotherapeutic agents. Due to their enhanced antitumor effect as compared with conventional chemotherapy, Plk1 inhibitors may prove particularly useful in the preoperative setting, when a rapid reduction of tumor volume is required in order to perform radical surgery and/or preserving organ function. For instance, Plk1 inhibitors may be indicated for the preoperative treatment of colorectal cancer patients with unresectable liver metastatic disease. In this case, Plk1 inhibitors (which are not associated to significant hepatobiliary toxicity) would have a clear advantage over chemotherapeutic agents, which cause considerable liver damage further increasing liver injury due to hepatic metastases and surgical resection. To a similar extent, BI 6727 could be exploited in the neoadjuvant setting for treating rectal cancer patients; in this setting, there is an urgent need for identifying therapeutic options safer than standard chemoradiation protocols as well as to increase the rate of sphincter-preserving surgery when tumors arise from the distal rectum. Specific analysis of CCIC proliferation state in xenografts treated for 3 weeks with chemotherapy or Plk1 inhibitors showed that chemotherapy-treated tumors, although smaller than control tumors, contained actively proliferating $CD133^+$ cells. Conversely, tumors treated with Plk1 inhibitors had a smaller size and contained only quiescent $CD133^+$ cells. These observations indicate that Plk1 inhibitors block tumor growth by eliminating all the proliferating tumorigenic cells. This situation reminds the effect of imatinib in chronic myeloid leukemia (CML), where the breakpoint cluster region-Abelson (BCR-ABL) inhibitor selectively eliminates proliferating leukemia cells [31, 32]. The inability of imatinib to eliminate quiescent stem cells, together with the propensity of neoplastic cells to acquire BCR-ABL kinase domain mutations results, in most cases, in persistence of the malignant CML clone. Differently, quiescent CCIC that survive treatment with Plk1 inhibitors in vivo do not acquire resistance to these drugs suggests that, in line of principle, they may be targeted by sequential rounds of BI 6727 treatment or by alternate rounds of chemotherapy/Plk1 inhibitors.

Conclusions

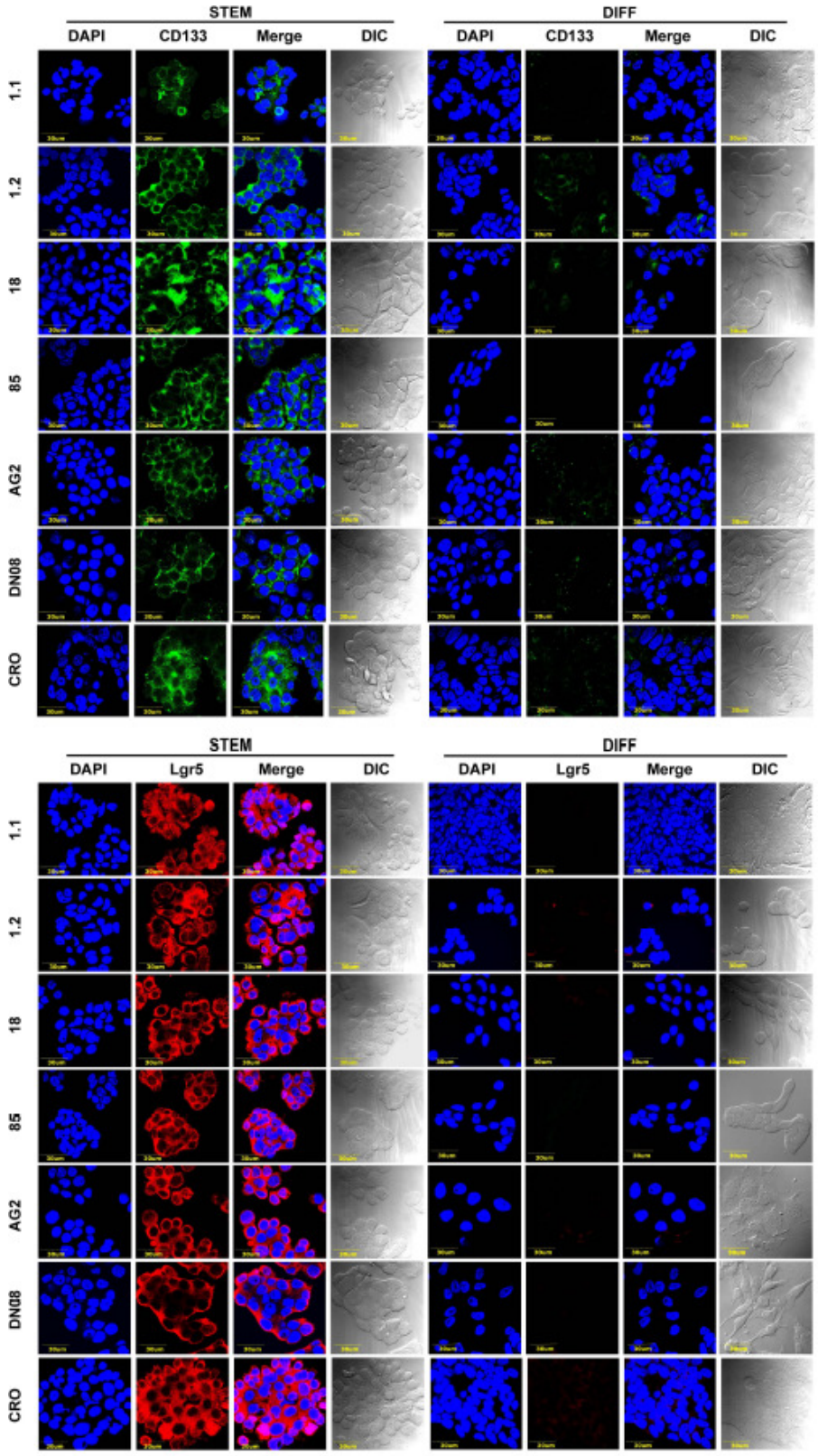
Our results indicate for the first time that colon cancer tumorigenic cells actively proliferate in vivo and are characterized by an elevated expression of Plk1, which constitutes a relevant therapeutic target. Future studies will be crucial to elucidate the mechanisms that control CSC quiescence and proliferation, as influencing this balance may render tumorigenic cells susceptible to targeted therapeutic strategies.

Supplementary Figures



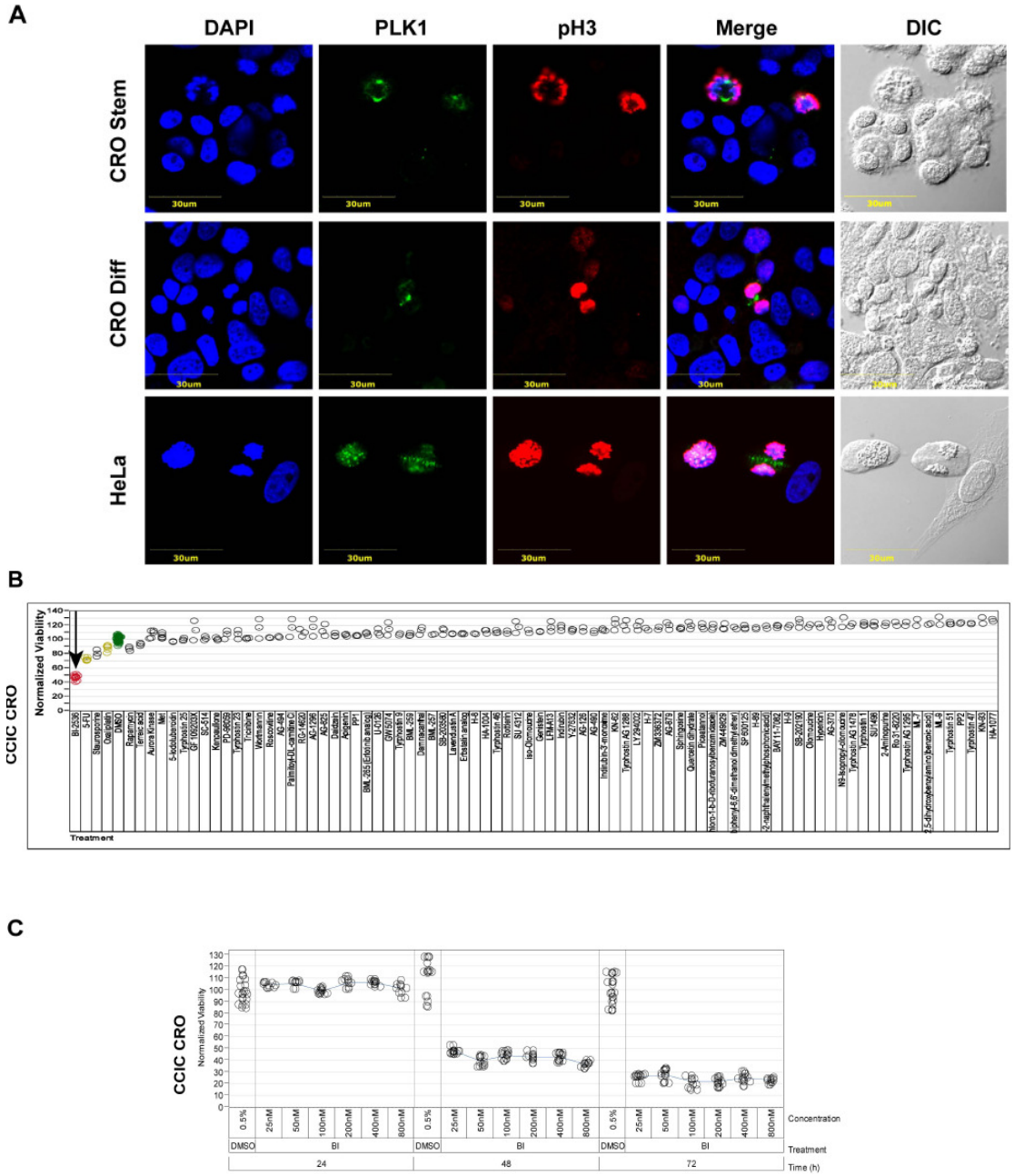
Supplementary Figure 1

Distribution of ALDH activity in one CCIC line (AG2) stained with PKH26 and, after 10 days, separated in three PKH-retaining fractions and assayed with the ALDEFLUOR kit as described in Materials and Methods. Percentages of ALDH-positive cells, deducted of the respective DEAB (control) samples, are 5.5 (PKHLOW/NEG), 5.2 (PKHMED) and 5.7 (PKHHIGH).



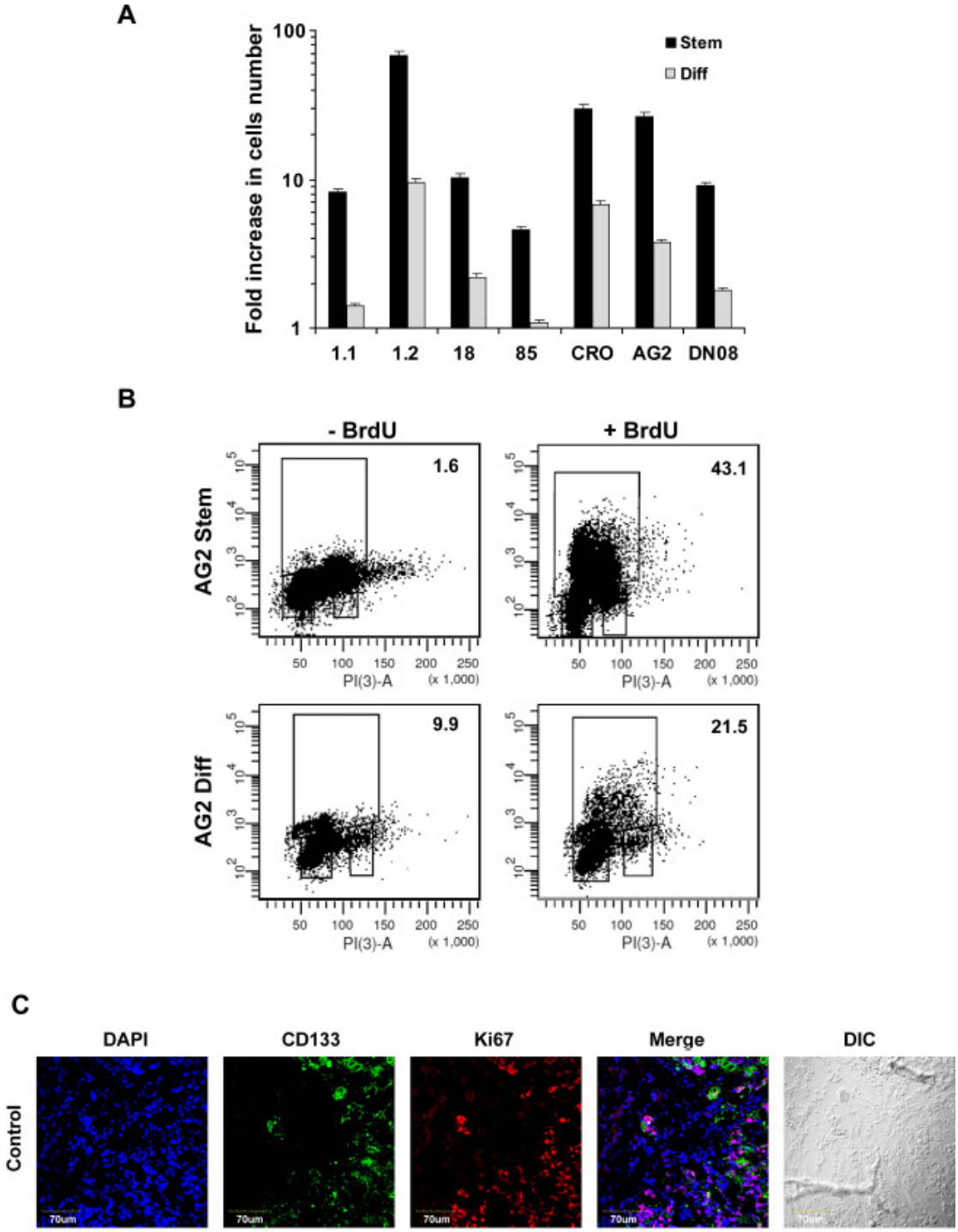
Supplementary Figure 2

Immunofluorescence staining of CD133 (top panels) and Lgr5 (bottom panels) expression in seven lines of CCIC (STEM) and their differentiated progeny (DIFF). 60x magnification, 2x zoom. Abbreviations: Lgr5, leucine-rich repeat containing G protein-coupled receptor 5.



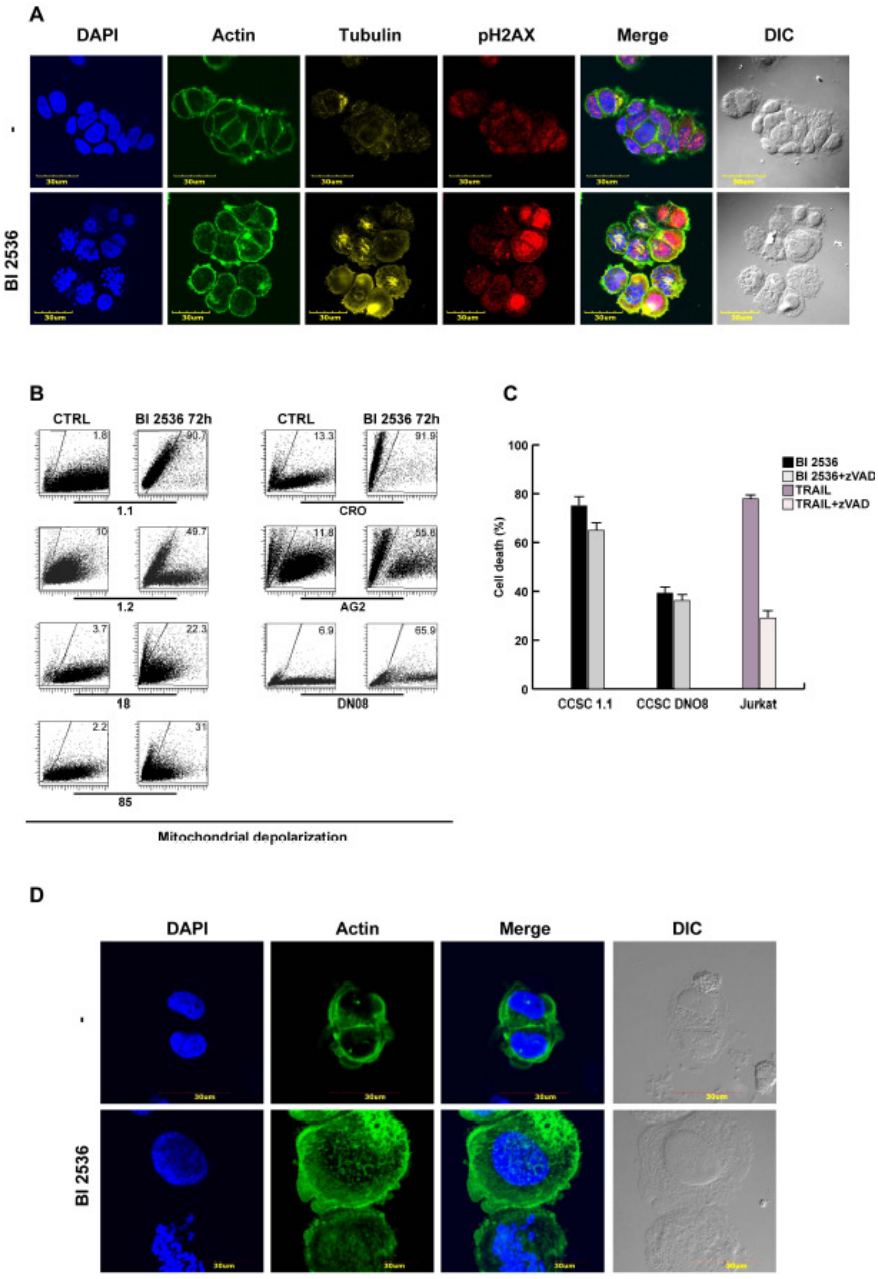
Supplementary Figure 3

(A) Immunofluorescence analysis of Plk1 localization and phosphohistone H3 (pH3) expression in one CCIC line (CRO stem), its differentiated progeny (CRO Diff) and HeLa cells. 60x magnification, 3x zoom. (B): Screening of a kinase inhibitor library and chemotherapeutic agents on one CCIC line (CCIC CRO). CCIC were treated for 48 hours with the indicated kinase inhibitors and chemotherapeutic agents and cell viability was measured with the Cell Titer Glo assay as described in the Materials and Methods section. The black arrow indicates the BI 2536-treated sample. (C): Dose-response treatment of a CCIC line (CCIC CRO) with BI 2536 (BI) at the indicated times. Cell viability was measured as described in the Materials and Methods section. Abbreviations: CCIC, colon cancer-initiating cells; Plk1, Polo-like kinase1; pH3, phospho Histone H3.



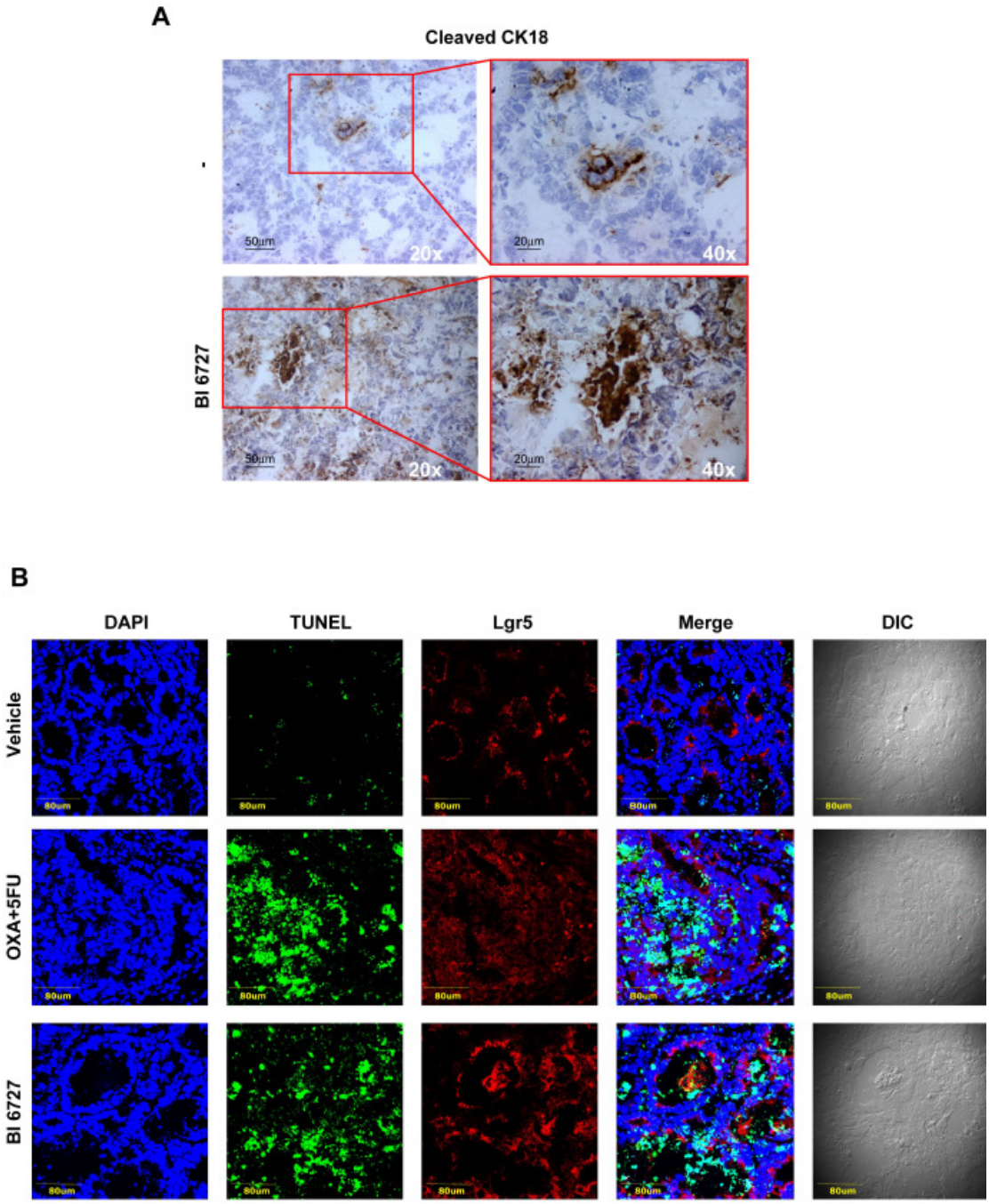
Supplementary Figure 4

(A): Proliferative activity of colon cancer stem (Stem) versus differentiated (Diff, CCIC-derived) cells. 2,5 x 10^4 cells were plated in 12-well plates and proliferation was monitored by manual counting. Bars represent the fold increase in cell number assessed 10 days after plating. (B): BrDU staining of CCIC (AG2 stem) and of the same cells differentiated for 10 days in serum-containing medium (AG2 Diff) as described in Materials and Methods. (C): Double Ki67/CD133 staining of an untreated CCIC (AG2)-derived xenograft.



Supplementary Figure 5

(A): Histone H2AX phosphorylation (pH2AX) in CCIC AG2 untreated (-) or treated for 24 hours with 100 nM BI 2536 (BI 2536) detected by immunofluorescence (60x magnification, 2x zoom). (B): TMRM staining and flow cytometry analysis of CCIC untreated (CTRL) and treated for 72 hours with 100 nM BI 2536. Numbers indicate the percentage of cells with depolarized (TMRM-negative) mitochondria contained in the left inset. (C): Cell death in two CCIC lines (1.1 and DN08) untreated or treated 48 hours with 100 nM BI 2536 in the presence or in the absence of zVAD-FMK. Death induced in Jurkat cells by TRAIL in the presence or in the absence of zVAD-FMK is shown as a control of zVAD-FMK efficacy. Data shown are the mean ± SD of three independent experiments. (D): Photomicrograph showing the size of CCIC AG2 untreated (-) or treated for 48 hours with BI 2536 (BI 2536) and stained with Dapi and Alexa488- conjugated phalloidin (60x magnification, 3x zoom). Abbreviations: CCIC, colon cancer-initiating cells; Plk1, Polo-like kinase1; pH2AX, phospho Histone H2AX; zVAD, benzyloxycarbonyl-Val- Ala-Asp (OMe) fluoromethylketone; TRAIL, TNF-related apoptosis-inducing ligand.



Supplementary Figure 6

(A): Immunohistochemistry staining for cleaved cytokeratin 18 (CK18) on CCIC (AG2)-derived xenograft sections after 48 hrs of mice treatment with vehicle (-) or with BI 6727 (BI 6727), 20X magnification. (B): Immunofluorescence staining (40x magnification) for Lgr5 and TUNEL on CCIC (AG2)-derived xenograft sections derived from mice treated for 48 hrs with vehicle (Vehicle) or with BI 6727 (BI 6727). Abbreviations: CK18, cytokeratin 18; Lgr5, leucine-rich repeat containing G protein-coupled receptor 5

DN08	100 cells	500 cells	1000 cells
Control	100%	100%	100%
PKH $_{LOW/NEG}$	50%	75%	75%
PKH $_{MED}$	100%	75%	100%
PKH $_{HIGH}$	100%	100%	100%

AG2	100 cells	500 cells	1000 cells
Control	100%	100%	100%
PKH $_{LOW/NEG}$	100%	100%	100%
PKH $_{MED}$	100%	100%	50%
PKH $_{HIGH}$	100%	100%	100%

18	100 cells	500 cells	1000 cells
Control	100%	100%	100%
PKH $_{LOW/NEG}$	100%	100%	100%
PKH $_{MED}$	75%	75%	50%
PKH $_{HIGH}$	50%	75%	100%

Supplementary Table 1

Percentages of tumor incidence in NSG mice inoculated with the indicated number of cells of three different CCIC lines (DN08, AG2, 18). Cells were stained with PKH26, sorted according to PKH26 retention 7-10 days after staining and immediately inoculated in NSG mice (2 for control groups and 4 for PKH groups). Tumor incidence was evaluated approximately two months after injection.

CCSC Line	Origin	TYPE	DUKES	P53	K-RAS	PI3CA
1.1	Sigma	Adenocarcinoma	G3/C	wt	wt	wt/E542K
1.2	Sigma	Adenocarcinoma	G2/C	wt	wt	wt/E542K
18	Colon right	Adenocarcinoma	G2/B	wt/fs	G12V/wt	wt
85	Sigma	Adenocarcinoma	G3/B	mut	wt	wt
CRO	Sigma	Adenocarcinoma	G2/D	mut	wt	wt
AG2	Caecum	Adenocarcinoma	G3/D	mut	G13D	wt
DN08	Colon left	Adenocarcinoma	G2/C	mut	G13D	wt

Supplementary Table 2

Tumor type, Dukes' classification and staging of colorectal tumors that were used to derive the indicated CCIC lines. The mutational status of p53, KRAS (G12V and G13D) and PI3 kinase (PI3CA) of the respective CCIC lines is shown (wt, wild type; mut, mutated; fs, frameshift).

1	BI 2536
2	5-Fluorouracil
3	Oxaliplatin
4	Staurosporine (cytotoxicity control)
5	Rapamycin
6	DMSO (dimethylsulfoxide) (negative control)
7	Terreic acid
8	5-Iodotubercidin
9	Triciribine
10	Aurora Kinase inhibitor (PHA-680632)
11	Met inhibitor (PHA-665752)
12	BML-265 (Erlotinib analog)
13	Erbstatin analog
14	Kenpaullone
15	BML-257
16	HA-1004
17	SC-514
18	ZM 336372
19	Daidzein
20	GW 5074
21	SB-202190
22	SU 4312
23	Apigenin
24	BML-259
25	AG-1296
26	Tyrphostin 46
27	Roscovitine
28	PP1
29	Quercetin dihydrate
30	AG-490
31	Y-27632
32	Rottlerin
33	Genistein
34	Indirubin-3'-monoxime
35	PD-98059
36	LFM-A13
37	KN-62
38	Tyrphostin 9
39	Palmitoyl-DL-carnitine Cl
40	KN-93
41	Tyrphostin 1
42	DRB (5,6-Dichloro-1-b-D-ribofuranosylbenzimidazole)
43	U-0126
44	PP2
45	Tyrphostin 25
46	SP 600125
47	ZM 449829
48	ML-9
49	ML-7
50	SB-203580
51	Tyrphostin 47

52	Tyrphostin 51
53	Damnacanthal
54	Olomoucine
55	HHBPDDE (2,2',3,3',4,4'-hexahydroxy-1,1'-biphenyl-6,6'-dimethanol dimethyl ether)
56	AG-126
57	Tyrphostin AG 1288
58	HA-1077
59	2-Aminopurine
60	HDBA (2-Hydroxy-5-(2,5-dihydroxybenzylamino)benzoic acid)
61	Iso-Olomucine
62	H-9
63	BAY 11-7082
64	N9-Isopropyl-olomoucine
65	AG-494
66	Indirubin
67	GF 109203X
68	AG-879
79	Tyrphostin AG 1478
70	RG-14620
71	AG-825
72	Tyrphostin AG 1295
73	HNMPA (Hydroxy-2-naphthalenylmethylphosphonic acid)
74	SU1498
75	H-8
76	AG-370
77	LY 294002
78	Lavendustin A
79	Piceatannol
80	Hypericin
81	Sphingosine
82	Tyrphostin 23
83	Ro 31-8220
84	H-7
85	Wortmannin
86	H-89

Supplementary Table 3

Compounds included in the screening of potentially cytotoxic agents on CCIC, as shown in Figs. 2C and Supplementary 3B.

Acknoledgements

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Chapter| 4

Human NK cells selective targeting of colon cancer-initiating cells: a role for natural cytotoxicity receptors and MHC class I molecules

Tallerico R, Todaro M, **Di Franco S**, Maccalli C, Garofalo C, Sottile R, Palmieri C, Tirinato L, Pangigadde PN, La Rocca R, Mandelboim O, Stassi G, Di Fabrizio E, Parmiani G, Moretta A, Dieli F, Kärre K, Carbone E.

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Abstract

Tumor cell populations have been recently proposed to be composed of two compartments: tumor-initiating cells characterized by a slow and asymmetrical growth, and the "differentiated" cancer cells with a fast and symmetrical growth. Cancer stem cells or cancer-initiating cells (CICs) play a crucial role in tumor recurrence. The resistance of CICs to drugs and irradiation often allows them to survive traditional therapy. NK cells are potent cytotoxic lymphocytes that can recognize tumor cells. In this study, we have analyzed the NK cell recognition of tumor target cells derived from the two cancer cell compartments of colon adenocarcinoma lesions. Our data demonstrate that freshly purified allogeneic NK cells can recognize and kill colorectal carcinoma–derived CICs whereas the non-CIC counterpart of the tumors (differentiated tumor cells), either autologous or allogeneic, is less susceptible to NK cells. This difference in the NK cell susceptibility correlates with higher expression on CICs of ligands for NKp30 and NKp44 in the natural cytotoxicity receptor (NCR) group of activating NK receptors. In contrast, CICs express lower levels of MHC class I, known to inhibit NK recognition, on their surface than do the "differentiated" tumor cells. These data have been validated by confocal microscopy where NCR ligands and MHC class I molecule membrane distribution have been analyzed. Moreover, NK cell receptor blockade in cytotoxicity assays demonstrates that NCRs play a major role in the recognition of CIC targets. This study strengthens the idea that biology-based therapy harnessing NK cells could be an attractive opportunity in solid tumors.

Introduction

Cancer-initiating cells (CICs) have been proposed to play a major role in the metastatic process and in the recurrence of tumors (1, 2). Metastasis formation is a complex, multistep process that involves a sequence of events; namely, cancer cells must leave the original tumor anatomical site, migrate through the blood or lymph, move from the circulation into the local tissue, form micrometastases, develop a blood supply, and grow to form macroscopic metastases. It has been estimated that <2% of solitary cells that successfully migrate to a new site are able to initiate growth once there. Moreover, <1% of cells that initiate growth at a secondary site are able to maintain this growth sufficiently to become macroscopic metastases (3). These observations suggest that a small, and most likely specialized, subset of cancer cells drives the spread of disease to distant organs. Recently, CICs have been proposed as responsible for this phenomenon. According to this hypothesis, the metastatic efficiency may reflect the relative amount of CICs present within the tumor population and their interaction with the tumor microenvironments (2). It has been demonstrated that CICs and metastatic cancer cells share several properties that are essential to the metastatic process, including the requirement of a specific microenvironment (or "niche") to support growth and provide protection (1). Metastatic sites for a given cancer type could therefore represent those tissues that provide or promote the development of a compatible CIC niche, from which CICs could expand through cellular signaling. Initiating cells tend to be quiescent unless activated to divide (3–6). CICs express multidrug resistance genes that make them resistant to the common antineoplastic treatments: chemotherapy and radiotherapy (7, 8). As such, this subpopulation could form the kernel of cells responsible for metastasis and cancer recurrence following treatment and remission. Colorectal carcinoma (CRC) is the second most common cause of death from cancer (9); CICs have been recently isolated from CRC tumor biopsies and have been biologically and functionally characterized (10). The CRC-derived CICs have been demonstrated previously to be the key tumor compartment in establishing this neoplastic disease in animal models (11, 12). Although different immunotherapies have been considered in relation to CRC tumor, there is little information available concerning immunologically important properties of the CRC-derived initiating cells. NK cells are large granular lymphocytes that are potent effectors of the innate immune system, with a critical role in early host defense against invading pathogens (13, 14). Historically, the NK cells have been defined for their ability to recognize and kill virus infected and cancer cells, making them appealing effector cells for immune therapy strategies in the treatment of human cancer (15). Human NK cells comprise ~10% of PBLs and are characterized phenotypically by the presence of the cell surface marker CD56 and the lack of CD3. Most (~90%) human NK cells are $CD56^{dim}$ and express high levels of FcgRIII (CD16), whereas a minority (~10%) are $CD56^{bright}$ and $CD16^{dim/neg}$. Additionally, $CD57^{+}CD56^{dim}$ NK cells were recently identified as the major NK cell cytotoxic subpopulation (16).

NK cells participate in innate immune responses by recognizing, without prior specific sensitization, virus-infected, transformed, and allogeneic cells while sparing autologous healthy cells (17). This capability depends on the integrated balance of input to activating and inhibitory NK cell receptors that scrutinize the surface of potential target cells. Some ligands for activating receptors are cellular stress inducible molecules such as NKG2D ligands; these include, among others, MICA, MICB, and a group of ULBPs (18). Other triggering receptors include the group of

natural cytotoxicity receptors (NCRs): NKp30, NKp44, and NKp46 (19, 20), as well as the DNAX-activating molecule-I (DNAM-I) (21). The main inhibitory receptors are killer Ig-like receptors, CD94/NKG2A heterodimers, and Ig-like transcript (LIR, CD85) (20–22), most of which recognize classical MHC class I molecules. Increased NK susceptibility can thus be caused by increased expression of activating ligands, decreased expression of MHC class I molecules or other inhibitory ligands, or a combination of these two events (23).
Studies on NK cells function in vivo, mainly in murine models, have shown that they can contribute to control and prevent tumor growth and dissemination (24, 25). The capacity of human NK cells to exert antitumor effects ex vivo has been documented in several reports (26–30). Given the proposed use of NK cells in immunotherapy approaches against cancer (31) and the emerging concept of CICs as discussed above, it appears relevant to ask how NK cells interact with, for example, CRC-initiating cells, particularly because these may be relatively resistant to cytostatic drugs and radiotherapy (32). We therefore set out to test whether NK cells can kill CRCinitiating cells, and to further investigate which molecules may be involved in regulating a possible difference between NK susceptibility of CRC-initiating cells and the complete tumor population.

Materials and Methods

Cell culture: CRC-initiating cells and CRC cell lines

The human colon carcinoma cell lines HCT116 and RKO (allogeneic CRC lines) were originally obtained from the American Type Culture Collection and were cultured in complete DMEM (EuroClone) supplemented with 10% FBS (BioWhittaker/Lonza, Treviglio, Italy) and 1% penicillin/streptomycin (Invitrogen).
All the CRC-initiating cells (CRC-derived CICs) (DV29, AV9, AP24, DN08, AG2, CC09, 1247, and 1076) (Supplemental Table I) were obtained by digesting human colon carcinoma specimens from patients undergoing colorectal resection admitted at the University of Palermo (Palermo, Italy) or at the San Raffaele Hospital (Milan, Italy) in accordance with the ethical standards of the institutional committees.
Tumor diagnosis was based on the morphologic microscopic features of tumor cells. Tumor tissues were mechanically and enzymatically digested using collagenase (1.5 mg/ml; Life Technologies) and hyaluronidase (20 mg/ml; Sigma-Aldrich).
The tumor digest was divided into three different culture conditions: primary tumor cells, hereafter denominated autologous CRC tumor cells (1247 CRC tumor and 1076 CRC tumor), were obtained using Advanced RPMI 1640 (Lonza) supplemented with 5% heat-inactivated FBS, antibiotic/antimycotic (EuroClone), penicillin/streptomycin (EuroClone), and L-glutamine (EuroClone); the CICs were selected plating tumor cells on ultralow adhesion flasks (Corning, Lowell, MA) in DMEM F12 serumfree medium (Life Technologies) with the addition of epidermal growth factor (20 ng/ml) and basic fibroblast growth factor (10 ng/ml; both from Sigma-Aldrich, St. Louis, MO) to promote the growth of the spheres (33, 34). The sphere cultures were validated for the ability to form a xenograft in immunocompromised mice, resembling the parental tumor (10), and for a colorectal CIC phenotype by assessing the expression of CD166, CD44, CD24, CD133, Lgr5, EpCAM, CEA, Nanog, Sox2, and Aldefluor/ALDH1. Briefly, CD44, CD24, EP-CAM, and ALDH-1 were homogeneously (60–80%) expressed by CIC and overexpressed (2- to 6-fold) as compared with the non-CIC counterparts (A. Volonte´, T. Di Tomaso M. Spinelli, F.

Sanvito, L. Albarello,M. Bissolati, L. Ghirardelli, E. Orsenigo, S. Ferrone, C. Doglioni, P. Dellabona, C. Staudacher, G. Parmiani, and C. Maccalli, submitted for publication). To achieve the in vitro differentiation of CRCderived CICs, dissociated sphere cells were cultured in DMEM supplemented with 10% FBS in adherent conditions, obtaining three differentiated CRC-derived CICs (AG2D, DN08D, CC09D).
All cell lines were cultured at 37°C in a 5% CO2 humidified incubator. The cells were passed by trypsinization (trypsin 0.05%/EDTA 0.02% in PBS without calcium, magnesium, and phenol red; EuroClone) every 4 d to avoid reaching confluence, for cells in adhesion, or to avoid the formation of big spheroids, which would lead to the death of the inner cells. For CRC-derived CICs, this treatment requires cell sedimentation, removal of supernatant, and subsequent suspension in an appropriate volume of trypsin solution. The cells were then placed at 37°C for cycles of 2 min and then subjected to mechanical disruption. The cells were eventually resuspended in their growth medium (inhibition by dilution) and centrifuged at 192 x g; the cell pellet was resuspended in the appropriate growth medium and finally plated.

CICs stem-like features

When colon cancer samples from patients were dissociated into single cells and cultured in a serum-free medium containing epidermal growth factor and fibroblast growth factor 2, a sphere-like culture was obtained. The sphere-like aggregates could be expanded for several months in this medium. We tested common stemness markers such as CD133, b-catenin, or Nanog on human colorectal tumor tissues through confocal microscopy analysis. The immunofluorescence was performed on 5-mm-thick paraffinembedded tumor sections using the following Abs: CD133/1 (AC133, mouse IgG1; Miltenyi Biotec, Bergisch Gladbach, Germany), b-catenin (H-102, polyclonal rabbit IgG; Santa Cruz Biotechnology), and nanog (N-17, polyclonal rabbit IgG; Santa Cruz Biotechnology).
In single-cell cloning experiments, 1, 2, 4, or 6 cells were seeds in wells from a 96-well plate and their growth was followed. The clonogenic potential was evaluated by extreme limiting dilution analysis (35).

Tumorigenesis capacity of CICs

For the in vivo experiments, 5-wk-old NOD/SCID mice from Charles River Laboratories were maintained in accordance with the institutional guidelines of the University of Palermo and San Raffaele Foundation Center Animal Care Committee. Freshly dissociated cells (0.5 x 10^6/injection) from CIC lines were resuspended in a 1:3 mixture of growth factor–depleted Matrigel (BD Biosciences, Palo Alto, CA) and medium, for a final volume of 100 µl, and s.c. injected. These CICs display the tumor-initiating ability as reported elsewhere (10).
Serial dilution of CIC and non-CIC counterparts to be inoculated in NOD/SCID mice was performed, resulting in a high rate of efficiency in tumor formation, even by the inoculation in NOD/SCID mice of 10 x 10^3 and 10 x 10^2 cells only by CIC (A. Volonte` et al., submitted for publication).

mAbs and immunofluorescence procedures

The colon cancer–initiating cell lines were analyzed by indirect immunofluorescence and flow cytometry analysis using the following Abs: W6/32 (anti-HLA class I, IgG2a; BioLegend, San Diego, CA); clone BAM 195 2382 NK CELLS RECOGNITION OF COLON CANCER–INITIATING CELLS Downloaded from http://www.jimmunol.org/ at Universiteit van Amsterdam on August 30, 2013 (anti-MICA, IgG1) (36) and mAb 6D4 (anti-MICA/B, IgG1) were provided by Veronika Groh (Fred Hutchinson Cancer Research Center, Seattle, WA); M295 (anti-ULBP1, IgG1), M310 (anti-ULBP2, IgG1), M550 (anti-ULBP3, IgG3), and M478 (anti-ULBP4, IgG1) were gifted by D. Cosman (Amgen, Seattle, WA); mAb L95 (anti-PVR, IgG1) and mAb L14 (anti–Nectin-2, IgG2a) were developed and characterized as described in Bottino et al. (21).
After the cell incubation with appropriate primary mAbs, cells were incubated by FITC-conjugated goat anti-mouse secondary Abs (Bio-Legend). In all experiments, as a first step cells were incubated with human serum for 15 min and isotype-matched controls were used to set up the negative values. Samples were analyzed by a FACSVantage (Becton Dickinson, Mountain View, CA). The NK cells were analyzed using the following Abs: anti-CD56, clone B159; anti-CD57, clone HNK1; anti-CD3, clone UCHT1; anti-CD16, clone 3G8; and anti-CCR7, clone 3D12 (Becton Dickinson).
A double flow cytometry staining on CICs was perforemed to understand the correlation between CD133 and HLA-I in CICs and primary tumor cells. The colon cancer–initiating cell lines were analyzed by indirect immunofluorescence and flow cytometry analysis using the following Abs: CD133/2 (293C3, mouse IgG2b; Miltenyi Biotec) and HLA-I as previously described. CICs and their tumor cell counterparts were characterized by immunofluorescence and cytofluorimetric analysis for the expression of CD133, CD24 clone ML5, CD44 clone G44-26, Ep-CAM clone EBA-1, SOX2 clone 245610, and CEA clone B1.1/CD66 (Becton Dickinson). CICs expressed homogeneously (70–90% of positive cells) these markers (Ref. 10 and A. Volonte` et al., submitted for publication).

Fluorescence staining of cells by NCR-Fc molecules

To measure NCR ligand expression, immunofluorescence and flow cytometry analysis were performed using NCR-Fc fusion protein: NKp30-Fc, NKp44-Fc, and NKp46-Fc.
Cells (2×10^5) were sequentially incubated with 200 µl heatinactivated human serum for 15 min at room temperature and then with 2.5 µg/µl NCR-Fc fusion protein for 2 h on ice. Binding of NCR-Fc was revealed by secondary incubation with R-PE–conjugated $F(ab')_2$ fragments of goat anti-human Fc secondary Ab (Jackson ImmunoResearch, Baltimore, MD). As control staining for recombinant soluble NCR proteins, secondary Ab alone was used. Cells were washed and analyzed by FACSVantage (Becton Dickinson) and the results were analyzed using FlowJo software version 9.3.1.

NK cell generation assay

NK cells preparation was done as described elsewhere (37). Briefly, PBMCs were isolated by Biocoll separating solution (Biochrom AG, Berlin, Germany) density

gradient centrifugation. Enriched NK cells were isolated from the separated PBMCs utilizing the NK cell isolation kit and VarioMACS (Miltenyi Biotec) according to the manufacturer's instructions. The purity of the isolated $CD3^{-}CD56^{+}$ NK cell populations was >95%. This protocol was also used to isolate NK cells from frozen PBMCs of cancer patients. Freshly enriched NK cells were suspended in RPMI 1640 culture medium (Life Technology, Milan, Italy) supplemented with penicillin (100 IU/ml) and streptomycin (100 µg/ml) and 10% FBS.

Cytotoxicity assay

Cytotoxicity assays were performed using the fluorescent 5,6-carboxyfluorescein diacetate (CFDA) NK assay. In CFDA NK assays, cytotoxicity was analyzed by flow cytometry using the protocol described elsewhere (38). Briefly, the target cells were labeled with CFDA-mixed isomers (Invitrogen, Milan, Italy). Target cells were mixed with effector cells at different E:T ratios. The incubation was performed in 96-well U-bottom plates at 37°C in a humidified 5% CO2 incubator for 3 h. The specific lysis of target cells was calculated as follows: % specific lysis = (CT 2 TE/CT) x 100, where CT indicates mean number of fluorescent target cells in control tubes and TE indicates mean number of fluorescent cells in target plus effector tubes.
In the receptor blocking experiments, freshly purified NK cells were incubated for 30 min at room temperature with various mAbs before the addition of target cells. To block NKG2D and NCRs, the mAb clone BAT221 (IgG1) and a combination of anti-NCR mAbs were used: anti- NKp46 clone KL247, anti-NKp44 clone KS38, and anti-NKp30 clone F252 provided by S. Parolini (University of Brescia, Brescia, Italy) (39). To block DNAM-I, mAb F5 (IgM) was used. As isotype control mAb (TIB200) was used, which recognizes a lymphocyte membrane–associated CD57 glycoprotein. All mAbs were used at a final concentration of 10 µg/ml.

Mixed lymphocyte tumor cell cultures

PBMCs from the peripheral blood of 1247 CRC patients were cultured in vitro at a 5:1 ratio with autologous irradiated (300 Gy) CRC-derived CICs or CRC tumor cells with 100 IU/ml recombinant human IL-2 (Chiron, Emeryville, CA) and 10 ng/ml recombinant human IL-7 (PeproTech, Rocky Hill, NJ) in X-VIVO 15 (Cambrex/Lonza, Basel, Switzerland) plus 10% human serum. Cell cultures were weekly restimulated with irradiated autologous CRC-derived CICs or CRC tumor cells. Following two rounds of in vitro stimulation the specificity of PBMCs was assessed by IFN-g secretion ELISPOT assay as previously described (40) (data not shown) or by the determination of the cytotoxic activity. The phenotype analysis of PBMCs was performed by immunofluorescence and cytofluorimetric analyses (LSRFortessa II; BD Biosciences) using the following Abs: CD3, CD4, CD8, CD16, CD45RA, CD45RO, CD56, CD57, CD27, CD28, CCR7, CD25, CD127, CD134, CD137 (BD Pharmingen), and NKG2D (eBioscience, San Diego, CA).

Confocal microscopy analysis

Tumor cells and related initiating cells ($2x10^5$) were treated with inactivated human serum (200 µl), washed by addition of 1x PBS, pelleted, and stained with MHC class I W6/32 (anti-HLA class I, IgG2a, 3 µg/ml; eBioscience) revealed by anti-mouse FITC secondary Ab, and with NCR-Fc fusion proteins (NKp30 and NKp44) stained

with R-PE–conjugated F(ab9)2 fragments of goat anti-human Fc secondary Ab (Jackson ImmunoResearch). Stained cells were fixed with fixation solution (Cytofix/Cytoperm kit; BD Biosciences) and washed twice with permeabilization solution (53 PBS, 5% BSA, 5% Triton X-100), with the first wash containing DAPI (1 mg/ml stock, final dilution 1:1000; Molecular Probes)
Stained cells were recovered in mounting medium (ProLong antifade; Molecular Probes) and mounted on a glass coverslip. The images were collected on a Leica TCS SP2 confocal microscope (Leica Mycrosystems, Wetzlar, Germany) with a 363 Apo PLA oil immersion objective (numerical aperture, 1.4) and 60-µm aperture. Cells were scanned from the bottom to the top (usually 7–10 horizontal scans) to identify the central plane for the evaluation of MHC class I, NKp30, and NKp44 distribution; Z-stacks of images were collected using a step increment of 0.2 µm between planes; at least 30 independent fields were scanned for each experimental point. The distribution patterns were analyzed by scanning the fluorescence intensity with ImageJ software 1.45s around the perimeter of the cells.
Human paraffin-embedded tumor tissues and CICs were stained for CD133, NKp30, NKp44, and NKp46.Both of the human tissue sections and cytospins of CICs were immobilized onto polylysine slides to prevent loosening of the samples during the following step of the staining. The slides of human tissues were placed in a rack, deparaffinized, and rehydrated according to the following washes: xylene, 10 min; xylene 1:1 with 100% ethanol, 3 min; 100% ethanol, 5 min; 95% ethanol, 5 min; 70% ethanol, 5 min; 50% ethanol, 5 min; and finally running cold tap water to rinse. All slides were kept in PBS buffer solution until ready to perform Ag retrieval.
Cytospins were prepared with freshly dissociated cells, which were washed twice with PBS buffer solution. After spinning, the samples were fixed with 2% paraformaldehyde for 30 min at 37°C. The slides were then washed twice with PBS and maintained in this same buffer until staining.
After incubation with the appropriate primary Ab (8 h at 4°C), all samples were incubated with Rhodamine Red–conjugated secondary Ab (goat anti-mouse IgG [H+L]) for CD133 detection, or with Alexa Fluor 488–conjugated secondary Ab (goat anti-human IgG [H+L]) for NCR ligand detection. Both of the secondary Abs were diluted in the appropriate buffer and used for 1 h at 37°C. The nuclei were then counterstained with TOTO-3 iodide (Molecular Probes/Invitrogen). All slides were finally mounted with fluorescent mounting medium (Dako) and covered with slim coverslip. The fluorescent mounting medium increases the display of samples when subjected to observation with a fluorescence microscope. The slides mounted with fluorescent mounting medium were stored in the dark at 2–8°C, allowing them to retain the fluorescent signal for at least 1 mo.

Micro-Raman spectroscopy measurements and spectra analysis

Micro-Raman analysis is an unbiased method that allows determination of numerous chemical changes among different biological samples. Our previous study successfully demonstrated that this method could discriminate the difference in MHC class I molecule expression on several kinds of cells (41). Micro-Raman spectra are acquired by means of an inVia Raman microscope from Renishaw, equipped with an 832-nm laser source. All Raman measurements are recorded with a total laser power of ~10 mW at the sample level and an accumulation time of 20 s, in the range from 800 to 1800 cm^{-1}. All cells were washed three times with PBS to eliminate all medium contaminants and then placed on calcium fluoride substrates, The Journal of

 used for their negligible Raman intensity. Principal component analysis (PCA) is carried out on the preprocessed spectra to highlight the spectral differences between CRC-derived CICs (DV29, AV9, AP24, DN08, and AG2) and one allogeneic CRC line (HCT116). PCA is a multivariate statistical tool to handle problems described by a large number of variables. For spectroscopic studies, the total number of variables is given by all of the recorded frequencies (channels), as each one of these could provide useful information regarding the probed sample. PCA works on the data covariance matrix to extract few parameters, the PCs, which account for most information in terms of variance of the recorded spectra. Each PC is a linear combination of the former variables and it retains a certain amount of useful information, usually referred to as latent PCs. Typically only few PCs are needed to describe nearly the entire information hidden in the numerous old variables.

Statistical analysis

Results of experimental points obtained from multiple experiments were reported as mean ± SD. Significance levels were determined by a pairwise, two-tailed Student t test. A p value <0.05 was considered significant. ANOVA was performed to compare the CIC lines group and the relative CRC tumor cell lines using GraphPad Prism 5.0 software.

Results

CICs and tumorigenesis

Because CD133 is considered to be a selective marker for CICs in colon cancer (42), we tested its association with common stemness markers such as b-catenin, or Nanog, on human colorectal tumor tissues by confocal microscopy analysis (Fig. 1A).
Their close association demonstrates that CD133 indeed is a suitable stemness marker in our colorectal cancer cell system. Therefore, we measured the percentage of $CD133^+$ cells in our CIC lines. The flow cytometry analysis showed that ~75% of the spheroid cells were expressing this protein on their cell surface. Moreover, the CICs also express the Lgr5, CD166, and CD44 markers (Fig. 1C).
To clarify whether a single colon CIC retains the capability of multilineage differentiation, a single-cell cloning experiment was performed. We have calculated a mean percentage of 28% by extreme limiting dilution analysis. We therefore conclude that CICs represent a subpopulation of cells that contain stem-like features and have the capacity to differentiate in vitro (Fig. 1D).
The derived xenograft confirmed that CICs retain the capacity to initiate and sustain tumor growth in NOD/SCID mice. Histological examination of xenografts derived from CIC cultures showed that they present the same histopathological features as do their related human tumors (Fig. 1E). These data therefore confirm that the CICs within colon carcinoma represent cancer stem-like cells that contain tumorigenic capacity.

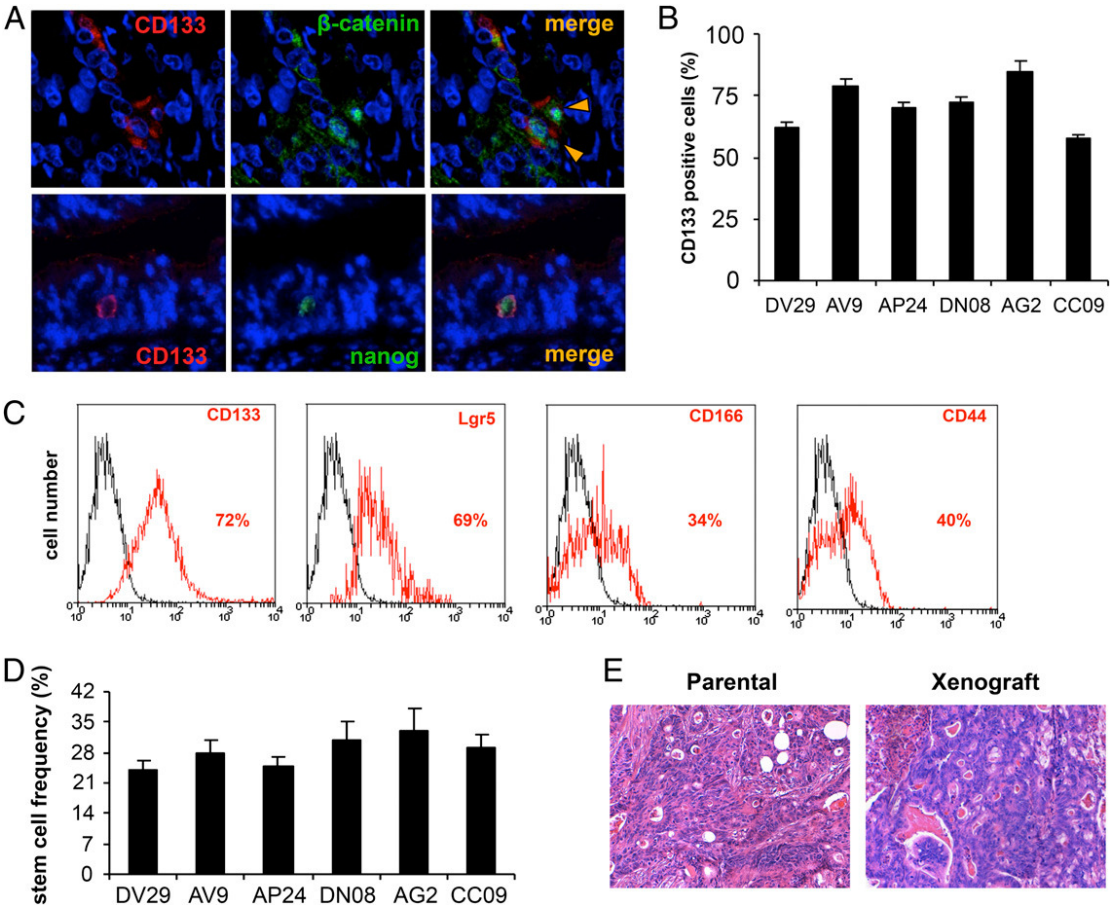


Figure 1

Characterization of CICs. (A) Confocal microscopy analysis of CD133 (red), b-catenin (green, upper panel), and Nanog (green, lower panel) on human colorectal tumor tissue. Nuclei were counterstained by TOTO-3 iodide (blue staining). Original magnification x40. (B) Flow cytometry analysis of CD133 on dissociated colon CICs. (C) Flow cytometry analysis of CD133, Lgr5, CD166, and CD44 in one representative of six independent tumors (black histogram represents isotype control). (D) Clonogenic assay performed on colon CICs. One, two, four, or six cells were manually cultured in wells from a 96-well plate and their growth was followed. The clonogenic potential was evaluated by extreme limiting dilution analysis as reported in Materials and Methods. (E) In vivo growth of colon CICs. Histology panels represent H&E staining of parental and xenografted tumors confirming a CRC phenotype. Original magnification x20.

Recognition of CRC-derived CICs by allogeneic and autologous NK cells

To address whether colon CICs can be recognized by NK cells, we initially performed cytotoxicity assays with allogeneic NK cells purified from the blood of different healthy donors and challenged them in vitro with four different colon CIC lines. These had been derived from colon or rectum adenocarcinoma biopsies. As shown in representative experiments in Fig. 2A and 2B, all four of these CIC lines were more susceptible to NK cell lysis than were the adenocarcinoma cell lines included as controls. Fig 2C and 2D summarize the results from five consecutive experiments comparing the NK susceptibility of CIC lines (DV29, AV9, AP24, DN08, AG2, CC09) with conventional adenocarcinoma cell lines (HCT116, RKO). The CIC lines

showed significantly increased NK susceptibility compared with adenocarcinoma lines.

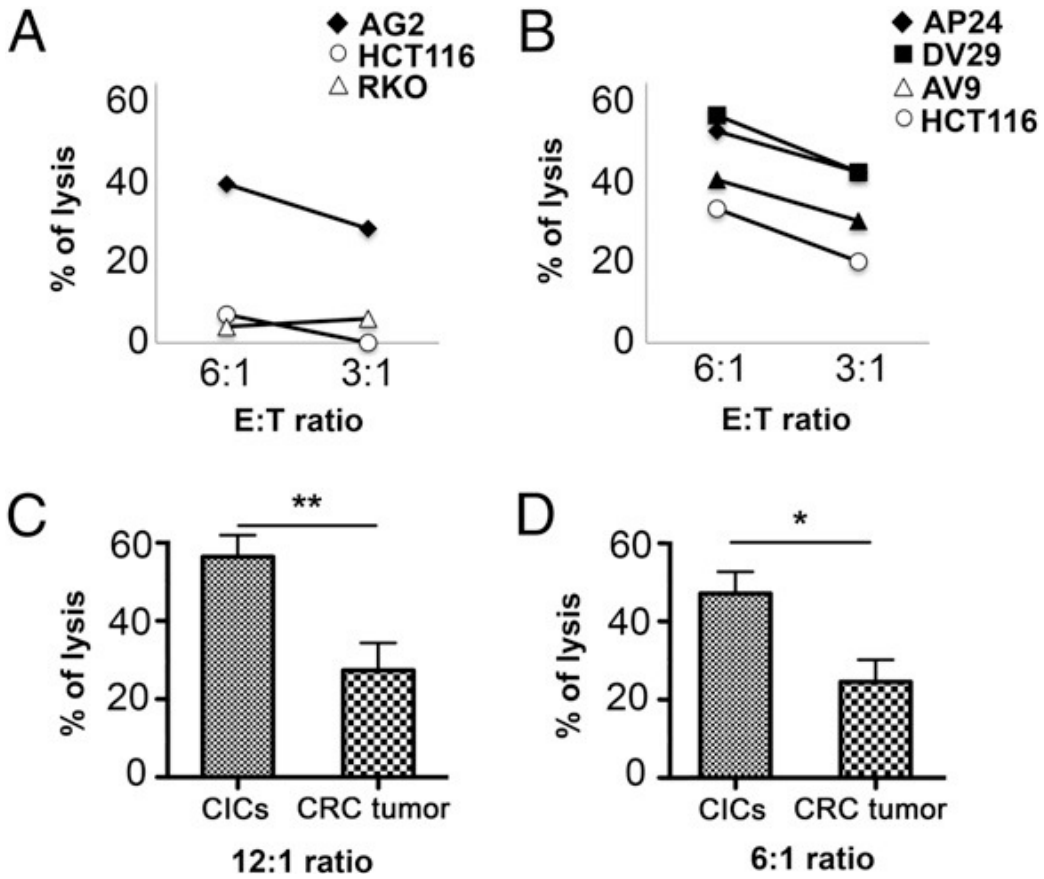


Figure 2

NK susceptibility of CRC-derived CICs and CRC tumor cell lines. (A) Representative experiment where one CRC-derived CIC line, AG2 (♦), and two colorectal carcinoma cell lines, HCT116 (O) and RKO (Δ), were tested for their susceptibility to highly purified peripheral blood NK cells. (B) Representative experiment where three different CRC-derived CIC lines, AP24 (♦), AV9 (Δ), and DV29 (■), and one CRC cell line, HCT116 (O), were tested for their susceptibility to highly purified peripheral blood NK cells. (C and D) Combined data from five experiments using CRC-derived CICs (DV29, AV9, AP24, DN08, AG2, CC09) and CRC cell lines (RKO and HCT116) as targets for NK cells. There was a statistically significant difference between the two types of target cells at the 12:1 E:T ratio ($p = 0.007$) as well as at the 6:1 E:T ratio ($p = 0.02$). *$p < 0.05$, **$p < 0.01$.

To validate these observations and to introduce a more stringently controlled comparison, we next applied the same experimental approach using pairs of cell lines derived from the same tumors. One of the cell lines in each pair was established and maintained in stem cell–specific medium, leading to growth of CICs in the form of spheroids. In parallel, a cell line representing the whole colorectal cancer population from the same biopsy was established in conventional culture medium. This made it possible to directly compare the NK susceptibility of different tumor cells derived from the same neoplastic lesion. The representative experiment in Fig 3A illustrates that there was some variability between patients/tumors, but within each patient/tumor, the CIC line showed a higher susceptibility than did the "complete" CRC tumor. In a series of six comparative experiments, including CICs from two

different patients and their autologous complete tumor lines as controls, each of the CICs showed a significant elevation of NK susceptibility (Fig. 3B–E).

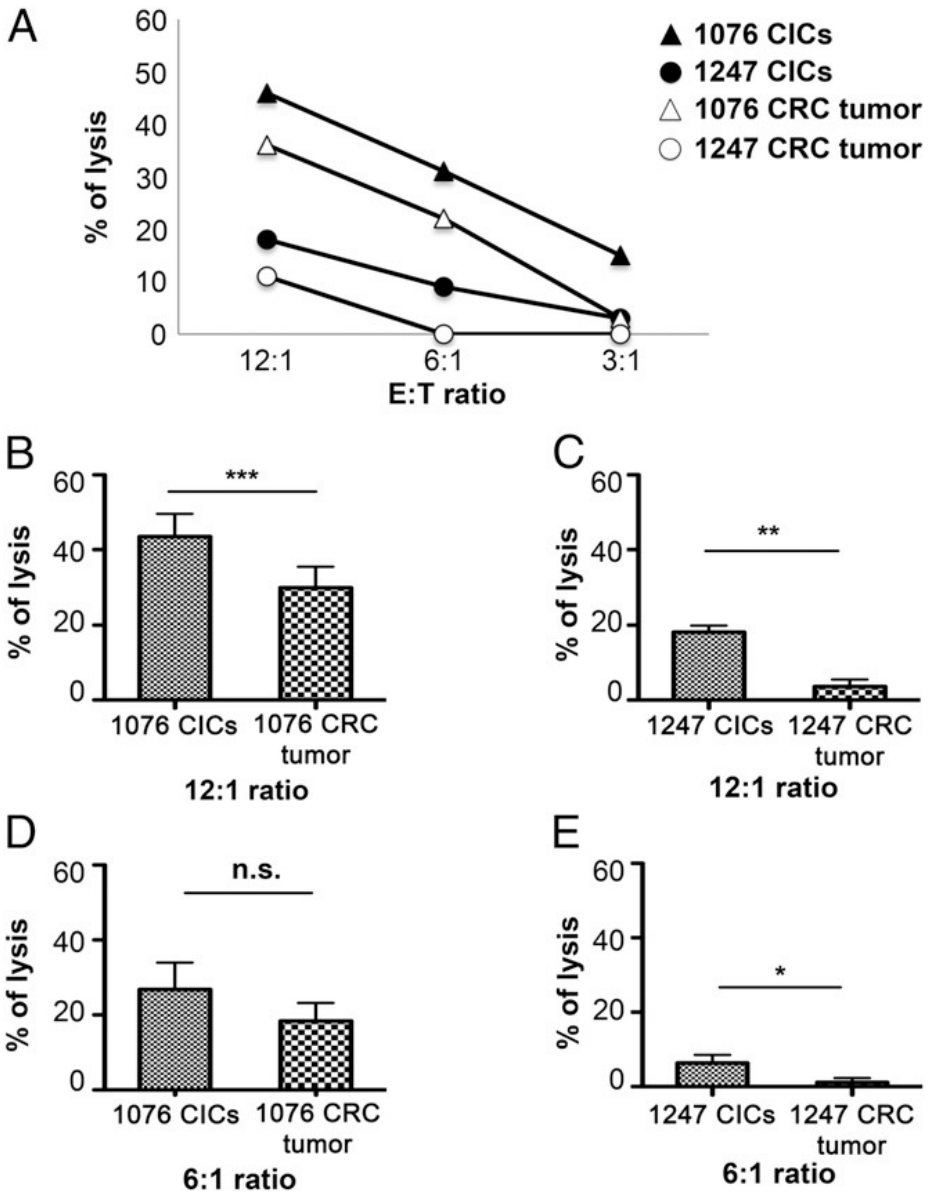


Figure 3

Pairwise comparison of NK susceptibility of CRC-derived CICs and complete CRC cell lines derived from the same tumor. (**A**) Representative experiment where the colon CICs from patient 1076 (▲) and 1247 (●), maintained in stem cell medium as spheroids, and the complete autologous CRC tumor cells derived from the same patients 1076 (Δ) and 1247 (O) were tested for susceptibility to highly purified peripheral blood NK cells. (**B–E**) Combined data from six experiments where the NK susceptibility of CRC-derived CICs were compared with complete CRC cell lines from the same tumor [patient 1076 in (B) and (D), patient 1247 in (C) and (E)] at E:T ratios of 12:1 (B, C) and 6:1 (D, E). Using a pairwise Student t test two-tailed analysis on both cell systems there was statistically significant difference between the two types of target cells at an E:T ratio of 12:1 [(B) 1076 $p = 0.0005$; (C) 1247 $p = 0.005$] and at an E:T ratio of 6:1 [(D) 1076 p = NS; (E) 1247 $p = 0.04$]. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

In one patient it was possible to analyze the NK cells autologous response to either CICs (Fig. 4B) or related tumor cell stimulation (Fig. 4A). By culturing PBMCs in the presence of autologous CICs, a clear expansion of NK cells was observed (Fig. 4B). The subset of $CD16^{+}CD56^{dim}$ NK cells expands with CIC stimulation (Fig. 4C), and half of these cells have a fully matured phenotype, as shown by CD57 staining (Fig. 4D), whereas there was no staining using an anti-CCR7 Ab (Fig. 4E). The NK cells purified from autologous PBMC/CIC cocultures were used in autologous cytotoxicity assays (Fig. 4F) where they showed a preferential recognition of autologous CICs, confirming the data obtained in the allogeneic experimental setting.

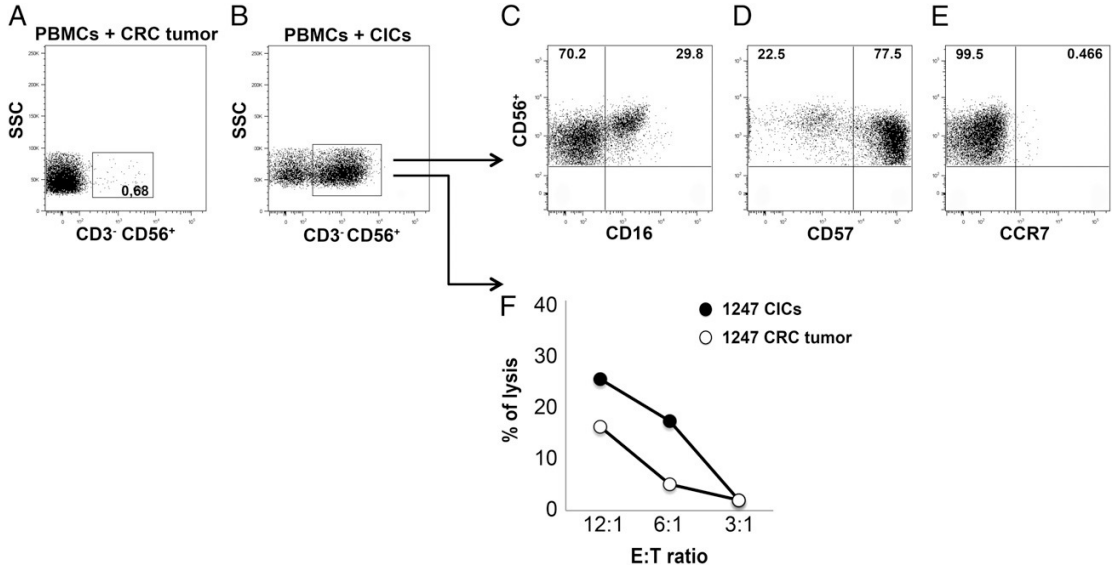


Figure 4

Expansion and cytolytic activity of autologous NK cells stimulated either with CICs or related tumor. PBMCs from patient 1247 were cultured in the presence of autologous tumor cells (A) and autologous CICs (B). Analysis of NK cells performed gating on $CD3^{-}CD56^{+}$ population. The $CD3^{-}CD56^{+}$ population was analyzed for the expression of CD16 (C), CD57 (D), and CCR7 (E). Autologous NK cells mediated cytotoxicity against CICs and related tumor target cells (F).

The molecular dissection of NK cell–mediated recognition of CRC-derived CICs

To decipher the molecular mechanisms behind NK cell recognition of CICs, we first focused on MHC class I molecules. These are the most potent inhibitory ligands for NK cell recognition, and it was thus conceivable that these molecules might play a role in regulating the recognition of CICs. As shown in Fig. 5, the MHC class I expression was barely detectable on CICs, whereas significantly higher levels were measured on autologous or allogeneic cancer lines representing the whole population (Fig. 5A, 5B). Our data are in agreement with a previous report (40). We also carried out a double flow cytometric analysis using HLA-I and CD133 Abs to evaluate a possible correlation in the expression of these two proteins, both in CIC lines and in their differentiated counterpart. The results show that CD133 is overexpressed in CICs compared with their differentiated counterparts, whereas HLA-I expression is downregulated in CICs (Fig. 5C).

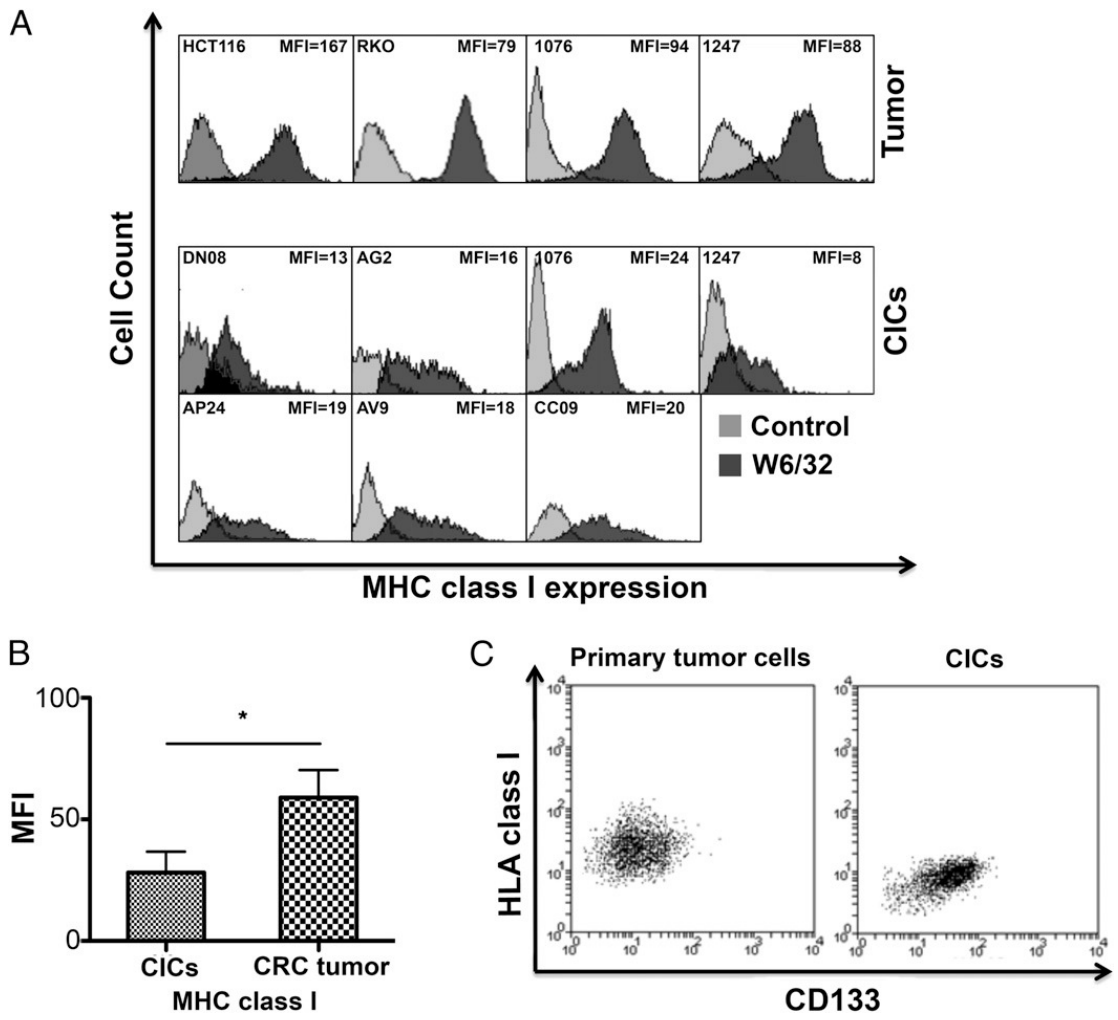


Figure 5

Expression of MHC class I membrane molecules on CRC-derived CICs and CRC tumor lines. (A) CICs and CRC tumor cells were analyzed by flow cytometry for the expression of MHC class I molecules stained with W6/32 mAbs (dark gray) or with isotopic control mAbs (light gray). (B) Mean fluorescence intensity (MFI) of MHC class I was detected by flow cytometry in 11 experiments using five CICs and two CRC tumor lines and was compared by pairwise, two-tailed Student t test. $p = 0.012$. (C) Flow cytometry analysis for HLA-I and CD133 performed on primary tumor cells (upper panels) and colon CICs (lower panels). Data are representative of six cell lines analyzed. $*p < 0.05$.

We validated this observation by single-cell analysis of MHC class I expression by Raman microspectroscopy, as previously described (41). This biophysical analysis addresses a large variety of membrane properties without bias (e.g., introduced by Ab). Supplemental Fig. 1A shows that the PCA discriminated well between the colon CICs and the control line HCT116. The PC2 component alone was enough to account for the total spectral differences between these two classes of cells. The spectral composition (i.e., the loadings curve) of the PC2 component (Supplemental Fig. 1B) showed a pronounced peak located in the region 1650–1680 cm^{-1}, thus indicating the mean peak corresponding to detection of MHC class I molecules. Collectively, the Raman analysis indicated that there are some differences between the CICs and the whole tumor populations, and that one of these concerns MHC class I expression. When looking for molecules that might account for the differential NK susceptibility

of CICs, we tested cell lines in our target cell systems for expression of ligands for key activating receptors on NK cells. No major differences correlating with NK susceptibility were observed for the ligands of LFA-1, DNAM-I, and NKG2D (ICAM-1, CD155 and CD112, and MICA/B and ULPBP1–4, respectively; Supplemental Fig. 2. ICAM-1 showed a tendency to be more expressed on CICs, but none of these molecules was found to be expressed at statistically significant different levels on CICs and tumor cell lines. We next probed the target cells with Fc fusion proteins with each of the three members of the NCR group of activating receptors: NKp30, NKp44, and NKp46. Fig. 6 shows the FACS plot from one representative experiment (Fig. 6A–C) and a compilation of the total (n = 15) experiments (Fig. 6D–F) measuring binding of each of these fusion proteins to target cells.

Ligands for NKp30, NKp44, and NKp46 were readily detectable on the surface of CIC lines, but not on cell lines representing the complete tumor population of either HCT116, RKO, or the autologous tumor. Interestingly, the expression of NKp30 and NKp44 ligands decreased dramatically when the CICs were cultured in differentiation-inducing media (Supplemental Fig. 3). The binding of NKp46-Fc fusion protein to CICs was less impressive, and no significant changes when comparing differentiated cells or complete tumor cell populations were found (Fig. 6C, 6F, Supplemental Fig. 3).

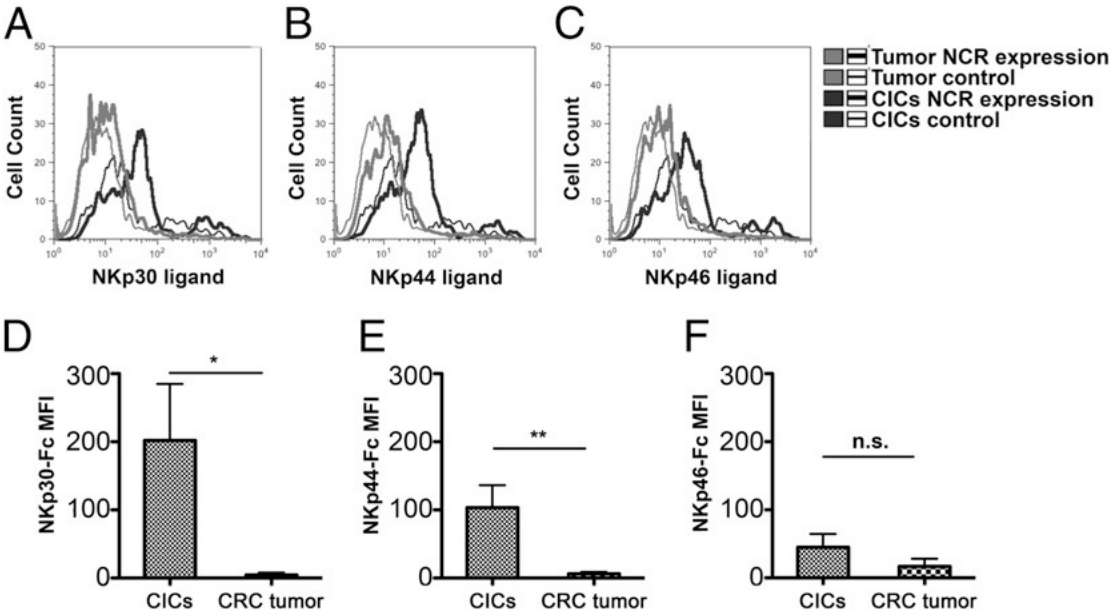


Figure 6

Expression of NCR ligands on CRC derived CICs and CRC tumor cell lines. Representative overlay analyses of expression of NCR ligands determined by flow cytometry after staining with fusion proteins (A, NKp30; B, NKp44; C, NKp46) on the CIC line (black line) and CRC tumor line (gray line) from patient 1247. The thin black and gray lines refer to the isotypic control. (D–F) Combined data from 15 experiments where CRC-derived CICs and CRC complete tumor cell lines (from the same lesion as well as established allogeneic cell lines) were compared by statistical analysis with a pairwise, two-tailed Student t test (NKp30, CICs versus tumor lines, $p = 0.03$; NKp44, CICs versus tumor lines, $p = 0.009$). $*p < 0.05$, $**p < 0.01$.

Note that the same expression pattern of the NCR ligands and MHC class I molecules, as well as the same NK susceptibility, was found on xenografted explanted CICs (Supplemental Fig. 4). To better understand whether NCRs ligands could correlate and/ or colocalize with CD133 expression, a confocal microscopy analysis with immunofluorescence was performed on primary tumor tissues and CICs/primary tumor cells. The results show that in colon tumor tissues the expression of CD133 and NCRs ligands is relatively low and is found preferentially at the very base of the crypts of the colon, often with a strong colocalization (Fig. 7A). As positive control we used the decidual tissue (43). In the tumor cell cultures the results show that primary tumor cells maintain a very low expression of both CD133 and NCR ligands, whereas CICs expressed both proteins at higher levels (Fig. 7B).

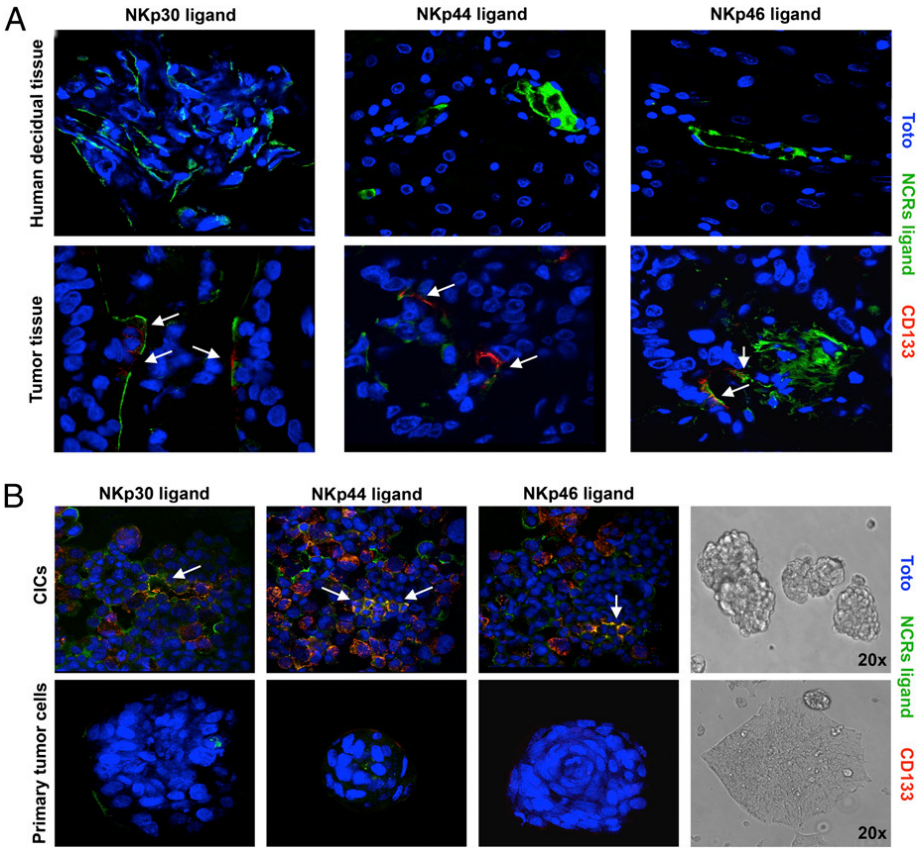


Figure 7

Confocal microscopy analysis of distribution of CD133 and NCR ligands on CRC-derived CICs and complete tumor cell line derived from the same neoplastic lesion. (A) Confocal microscopy analysis of NKp30, NKp44, and NKp46 (green) on human decidual tissue (positive control, upper panels) and tumor tissue (lower panels). Nuclei were counterstained by TOTO-3 iodide (blue staining). Arrows indicate cells in which a correlation of expression of CD133 (red staining) and NKp30, NKp44, and NKp46 is observed. Original magnification x40. (B) Confocal microscopy analysis of NKp30, NKp44, and NKp46 (green) on colorectal CICs (upper panels) and primary adherent CRC cells (lower panels). Nuclei were counterstained by TOTO-3 iodide (blue staining). Original magnification x40. The phase-contrast microscopy images permit observation of cell morphology. Original magnification x40. Arrows indicate cells in which a correlation of expression of CD133 (red staining) and NKp30, NKp44, and NKp46 is observed. One representative experiment of six different lines is shown.

We also studied the expression and cell surface distribution of NK receptor ligands on the target cells with confocal microscopy– assisted immunofluorescence. We focused on the NKp30 ligand (NKp30L), NKp44 ligand (NKp44L), and MHC class I, because the expression of these molecules showed interesting differences between target cells in the experiments described so far. CICs and a CRC cell preparation from the same neoplastic lesion were probed using anti-MHC class I mAbs stained with anti-mouse FITC secondary Ab (green) and NCR-Fc fusion proteins (NKp30 and NKp44), revealed by secondary incubation with R-PE–conjugated anti-human Fc secondary Ab (red). As expected from the FACS analysis, the NCR ligand fluorescence intensity of both NKp30 (Fig. 8A, 8C) and NKp44 (Fig. 8B, 8D) was more abundant in CICs compared with the complete tumor cell line derived from the same neoplastic lesion. Moreover, the overlay of the two stainings demonstrated that the two molecules tended to colocalize in the same membrane region (Fig. 8A, 8B, yellow staining, and in surface scanning curves of Fig. 8C, 8D).

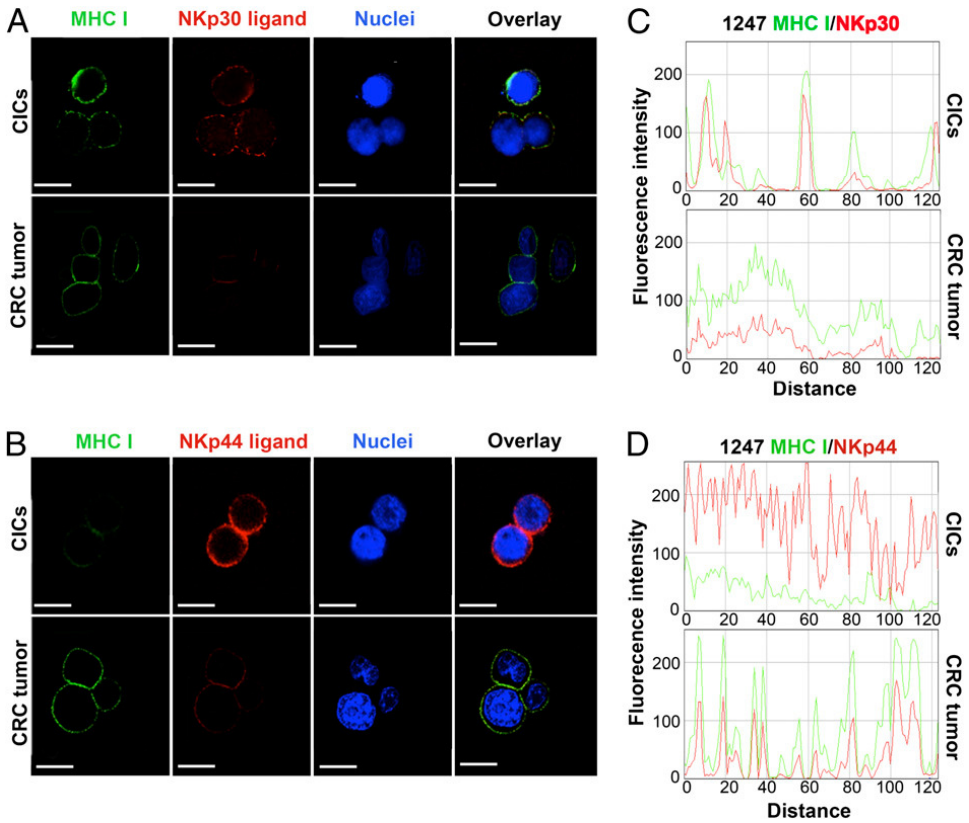


Figure 8

Confocal microscopy analysisof distribution of MHC class I, NKp30, and NKp44 ligands on CRC-derived CICs and complete tumor cell line derived from the same neoplastic lesion. (A and B) The distribution of MHC class I molecule stained with W6/32 mAb (green) and NCR-Fc fusion proteins (red) in CICs and CRC tumor cells from patient 1247 was detected by confocal microscopy. The cell nuclei were stained with DAPI (blue). For each panel, a single plane confocal image shows the central section of the cell. Scale bars, 5 µm. (C and D) Relative fluorescence intensity profiles for MHC class I and NCR-Fc fusion protein channels, along a line scan through a representative cell, are shown. One representative comparison is shown from a series of three experiments.

The colocalization data suggest that MHC class I molecules may efficiently inhibit the formation of an activating synapsis between NK cells and CRC by being prelocalized close to areas harboring NKp30 and NKp44 ligands (44), whereas their low expression levels on the CRCderived CIC cells surface could lead to a dominant activating effect of NKp44 and NKp30 ligand recognition by NCRs.

NK susceptibility of CICs after Ab blockade of activating receptors

To test the role of activating receptors more directly, we finally performed NK receptor blockade experiments. Fig. 9A summarizes the data obtained from three independent experi-ments where CICs from patient DN08 was used as a target for NK cells. Different activating NK receptors were blocked using specific mAbs. The most prominent effect was obtained when an anti-NCR receptor mAbs mixture was added. Similar results were obtained using as target the 1247 CRC-derived CICs (Fig. 9B).

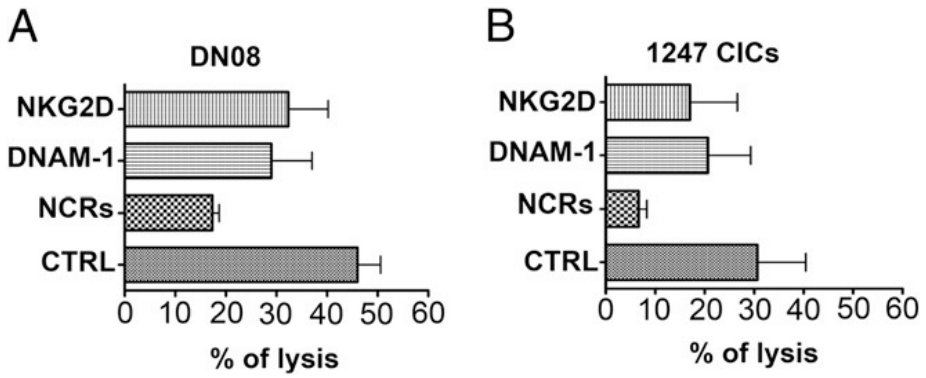


Figure 9

Effect of Ab-mediated receptor blockade on NK cell recognition of CRC-derived CICs. NK cell–mediated killing in the absence and presence of Abs to different activating receptors (NKG2D, DNAM-1, a mixture of Abs to NKp30, NKp44, and NKp46 and isotype control). (A and B) Results from a representative CFDA cytotoxicity assay with CRC-derived CICs from patients DN08 (A) and 1247 (B) at E:T ratio 10:1.

These experiments demonstrate an important dominant role for NCRs in driving NK cell killing of CRC-derived CICs. Note that the blockade by the NCR mixture was not complete. The residual lysis may reflect the involvement of additional activating receptors.

Discussion

We have explored the possibility of targeting colorectal-derived CICs with allogeneic NK cells. Moreover, we have compared the NK cell recognition patterns of the two colon adenocarcinoma cell compartments; that is, the initiating cancer cells (CICs) and the related “differentiated” tumor. Our results reveal that resting allogeneic NK cells show a robust cytotoxicity effect on CRCderived CICs, whereas killing of related differentiated cancer cells was lower.
Autologous NK cell recognition of CICs derived from patient 1247 has been performed using PBMCs stimulated with autologous CICs. In this coculture a preferential expansion of NK cells was observed, and when they were purified and

tested in autologous cytotoxicity assays against CICs and complete tumor, a preferential recognition of the former was observed. The observed expansion of the $CD56^{dim}CD57^{+}$ NK cell subset is reminiscent of that observed during virus infections (45, 46). To our knowledge, we report in this study for the first time that CICs express NKp30L and NKp44L and that their levels are higher than those in the related differentiated and CRC cell lines. These data define a new biological context where NCR ligand expression occurs, that is, the early stage of tumor formation.

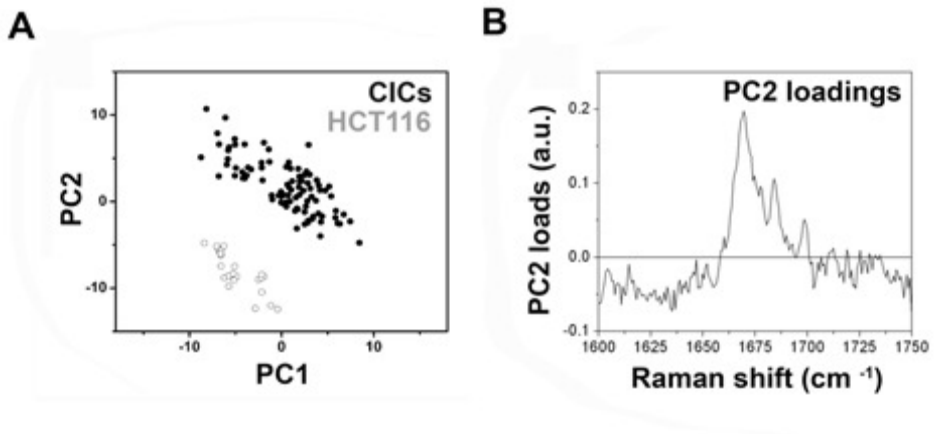
Moreover, MHC class I molecules, known to inhibit NK recognition, showed the reverse expression pattern: low on CICs, high on cells in the complete tumor population. Our confocal microscopi studies of the topographical membrane distribution of NKp30L /NKp44L and MHC class I are in line with the flow cytometry data and, additionally, they suggest that these molecole may colocalize in the membrane prior to binding of NK cells. It is thus conceivable that this high expression of inhibitory ligands and low expression of activating ligands may contribute to the relative resistance of the more differentiated tumor lines. The notion that ligands for the activating receptors of NKp30 and NKp44 are partly responsible for the high NK susceptibility of CICs was verified by NK receptor blockade experiments. Abs directed against NCRs reduced the killing considerably, but not completely, thus leaving the possibility that additional activating receptors may be involved.

Three earlier studies analyzed the NK cell interactions with glioblastoma, melanoma, oral carcinoma, and mesenchymal-initiating cells (47–51). A common pattern emerges from the reports. Melanoma and glioblastoma cancers initiating cells were highly resistant to resting allogeneic NK cells; they became susceptible to NK cytotoxicity only after effector cells had been activated by IL-2. Their phenotypes are characterized by low MHC class I expression and the presence of activating ligands for NK receptors. These cells, although resistant to freshly isolated NK cells, were highly susceptible to lysis mediated by both allogeneic and autologous IL-2 (or IL-15)–activated NK cells. The analyzed glioblastoma-initiating cell culture did not express protective amounts of HLA class I molecules, whereas they did express DNAM-I and NKp46 ligands (39). Probably the killing activity of resting or activated NK cells is modulated by the different derivation compartment of the specific tumor: epithelial in CRC, whereas ectoderm for the others (melanoma and glioblastoma). A direct correlation between NK cell infiltrates of the colon cancer lesion and a better prognosis has been proposed (52). It is tempting to speculate that the main contribution of NK cell infiltrates in the colon adenocarcinoma lesion may be to eliminate the CICs, therefore limiting the disease burden.

Because CICs have been reported to be both drug resistant and radioresistant, encouraging results reported in literature (45–49) indicate that NK cells could target CICs. Todaro et al. (53) have recently shown that bisphosphonate zoledronate treatment of colon CICs induces a high susceptibility to γδT cell killing. This cytotoxic interaction was regulated via TCR and to a lesser extent by NKG2D receptors. Our data add to this observation, indicating that CICs can be targeted also by another effector mechanism, NK cells. It has recently been reported that bisphosphonate zoledronate triggers NK cell activation via dendritic cell maturation in a γδT cell–dependent manner (54). Considering the data from Todaro et al. (53) together with data in the present study, it is interesting to speculate that CICs could be optimal targets for immunotherapy intervention based on activation and/or adoptive transfer of NK and γδ T cells.

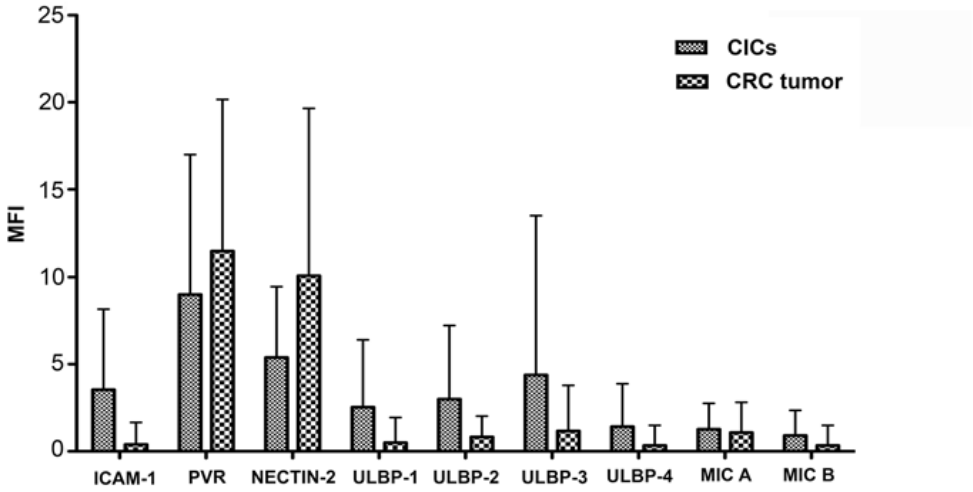
Numerous innate and adaptive immune effector cells and molecole participate in the recognition and destruction of cancer cells, a process often referred to as cancer immunosurveillance. Cancer may sometimes avoid such immunosurveillance through the outgrowth of nonimmunogenic tumor cell variants (immunoselection) and through subversion of the immune system (immunosubversion) (55). It will be important to study both of these processes in relationship to the CIC compartment.
Definition of the CIC compartment plays a crucial role in the natural history of the disease and metastatic progression, as well as in its chemo- and radioresistance. This may provide a piece in the puzzle of the frustrating history of anticancer therapy and the limited success of immunotherapy. This calls for new approaches to target the tumor-initiating cells. In this study, we provide evidence that NK cells can eliminate CICs with high efficiency. This study provides a base for further exploration of NK cells as a possible key player in immunotherapy of solid tumors.

Supplementary Figures



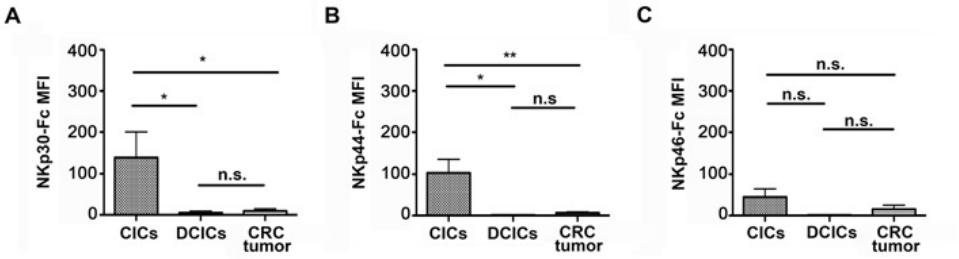
Supplementary Fig. 1
Raman micro-spectroscopy analysis of MHC class I expression at the single cell level.

(A, B) The deconvolution analysis PC2/PC3 is reported as main difference dots, between CRC derived CICs indicated with (close circle) and CRC indicated with (open circle) (panel A) and as main spectra difference peak reported was that defined for the MHC class I α-helix of alpha 1-2 domain are mapping (panel B).



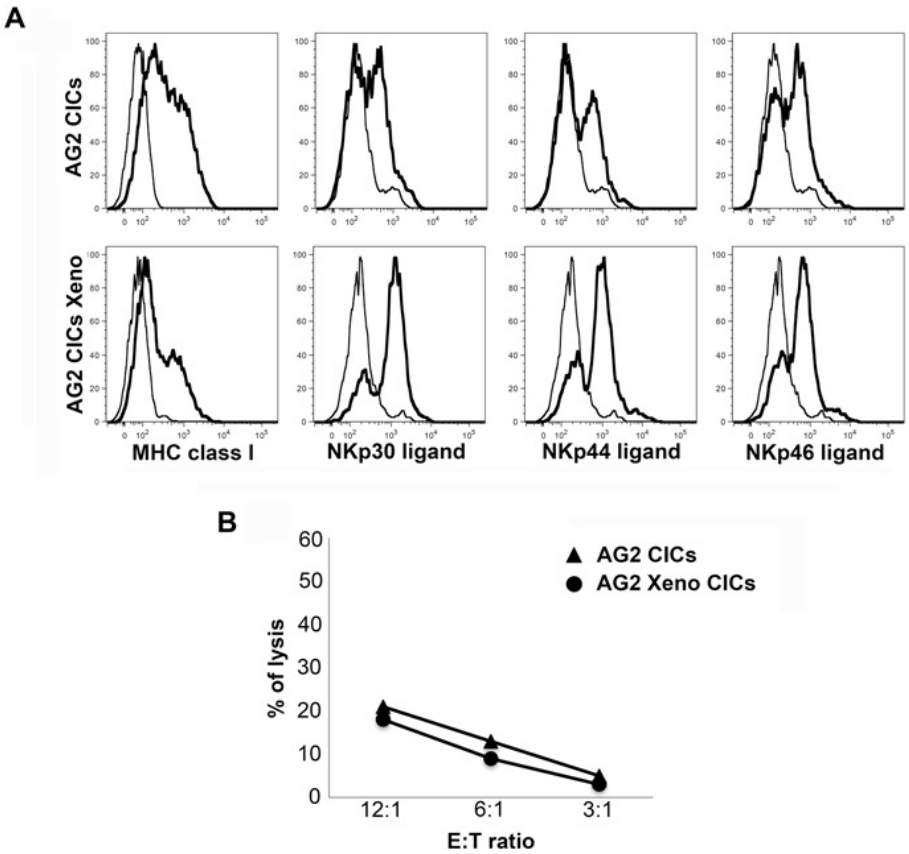
Supplementary Fig. 2
Expression of ligands for activating NK receptors on CRC-derived CIC and CRC tumor cell lines.

Mean fluorescence intensity of different NKG2D and DNAM-1 ligands as determined by flow cytometry after staining with the corresponding antibodies. The diagram shows one representative experiment from nine, performed where a total of five CRC-derived CICs (closed bars) and two CRC tumor cell lines (open bars) were used.



Supplementary Fig. 3
NCRs ligands expression on CRC-derived CICs their redifferentiated variant and CRC tumor cell lines.

This figure show combined data from fifteen experiments were CRC derived CICs, their redifferentiated variant (DCIC) and CRC complete tumor cell lines (CRC tumor) were compared by statistical analysis with Student´s t-test two tails, (NKp30: CICs vs tumor lines p value = 0.03, CICs vs redifferentiated CICs p value = 0.04; NKp44: CICs vs tumor lines p value = 0.009, CICs vs redifferentiated CICs p value = 0.007), N.S. not significative.



Supplementary Fig. 4
CIC cell lines immunophenotype recapitulated that foumd ex vivo in xenografted explanted CICs

Xenografted freshly explanted CIC and related in vitro cultured cell lines were comparatively analysed for MHC class I and NCR ligands expression (thick line) (A), isotopic mAb control staining (thin line). NK cells susceptibility comparative analysis of Xenografted freshly explanted CIC and related in vitro cultured cell lines (B).

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Chapter| 5

Colorectal cancer defeating? Challenge accepted!

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Abstract

Colorectal tumours are actually considered as aberrant organs, within it is possible to notice a different stage of cell growth and differentiation. Their origin is reported to arise from a subpopulation of tumour cells endowed with, just like the healthy stem cells, self- renewal and aberrant multi-lineage differentiation capacity likely to be called colorectal cancer stem cells (CCSCs). Cancer stem cells (CSCs) fate, since their origin, reflects the influences from their microenvironment (or niche) both in the maintenance of stemness, in promoting their differentiation, and in inducing epithelial–mesenchymal transition, responsible of CSCs dissemination and subsequent formation of metastatic lesions. The tumour cells heterogeneity and their immuno-response resistance nowadays probably responsible of the failure of the conventional therapies, make this research field an open issue. Even more importantly, our increasing understanding of the cellular and molecular mechanisms that regulate CSC quiescence and cell cycle regulation, self-renewal, chemotaxis and resistance to cytotoxic agents, is expected to eventually result in tailor-made therapies with a significant impact on the morbidity and overall survival of colorectal cancer patients.

Introduction

Colorectal cancer (CRC) is one of the major causes of death worldwide (Jemal et al., 2011). Despite the prompt surgical removal followed by adjuvant therapy, often suitable in the early stages of the disease, the majority of patients undergo to recurrences and metastases. This phenomenon frequently correlates with an acquired resistance to conventional therapies such as chemo- and radio-therapy (Janne and Mayer, 2000).

Increasing evidences recently claimed that tumours are structured by heterogeneous populations of cells hierarchically organized, with CSCs at the top of this pyramid model. The concept that this subset of cells may arise from normal stem cells (or progenitor cells), as a result of genetic and/or epigenetic mutations (Barker et al., 2009) is appealing for several reasons. Healthy stem cells and CSCs share many properties, including the self-renewal and aberrant multi-lineage differentiation capacity, altered DNA repair machinery, high expression levels of anti-apoptotic genes and ATP-binding cassette (ABC) transporters, which could explain the failure of current anti-cancer treatments (Todaro et al., 2007). Moreover, the CSCs are highly clonogenic and can generate a serially transplantable phenocopy of the primary malignancy in immuno-compromised mice (Clarke et al., 2006), highlighting their tumourigenic capacity.

The amazing cellular turnover of the colon epithelium makes this tissue the ideal subject for the study on the healthy stem cells biology, and then on cancer stems cells during tumour progression. Under physiological conditions, colon homeostasis is highly regulated, and it is the result of a perfect balance between stem cells, differentiated cells and the microenvironment. Sometimes, however, this balance is missing, so throwing the foundations for the emergence and progression of the tumour.

It has been noticed that the colon stem cells (SCs) reside at the base of the intestinal crypts, where the microenvironment seems to orchestrate the stemness status, the proliferation and the resistance to apoptosis of stem cells, regulating different signaling pathways. This network of signals link up different stromal cells, such as mesenchymal cells with immune cells, blood vessels, soluble factors and extracellular matrix components (Kosinski et al., 2007) building the complex tumour architecture. Likewise the CSCs are strictly dependent from their residing environment, the tumour niche, which not only play a role in determining the cell type, but also provides protection by sheltering CSCs from diverse genotoxic insults, contributing to their enhanced therapy resistance (Sun and Nelson, 2012).

Most conventional therapies affect differentiated cells, which constitute the bulk of tumour mass, thus saving CSCs. This phenomenon seems to be the cause of the initial tumour shrinkage followed by relapses, often more aggressive of the primary tumour of origin (Al-Hajj et al., 2004).

Nowadays, thanks to new quick and low-cost technologies, it is possible to achieve genomic and proteomic analysis to better characterize the tumour patient ‘‘phenotype’’ considering that the ‘‘one-size-fits-all’’ approach for cancer treatment is not longer sufficient. These ‘‘omics’’ analyses have pushed a new personalized approach in the cancer field, trying to optimize the treatment options, to avoid the resistance phenomena, bypassing unnecessary side effects (Chang et al., 2009; Wilson et al., 2007).

The origin and tumour progression are tightly regulated by aberrant oncogenic pathways activation, in concert with an inactivation of tumour suppressor signals.

This phenomenon, however, seems to follow a branched trend, rather than linear, thus generating a large clonal diversity, and contributing to the intra-tumoral genetic heterogeneity (Marusyk et al., 2012). The huge inter-tumour variability depends from several aspects, first of all the variables related to the host (age, hormonal status), while the intra-tumour cellular organization is differentially influenced by several stimuli coming from the contiguous microenvironment (differences in vascularity, infiltration degree, connective tissue components). Last but not least it is also important to consider the cellular state diversity, as the cell cycle, the exposure to antigens, and the membrane composition (Heppner, 1984).
This great heterogeneity poses a considerable number of questions on how to address the issue of tumour, both from the point of view of diagnosis and of the possible treatment suggested (Gerlinger et al., 2012), as we will discuss later in this review.
We recent demonstrated the role of different components of the immune system against the tumours (Tallerico et al., 2013; Todaro et al., 2009). Although there is strong evidence of how the cells of the immune system can limit tumour growth, other data indicated that prolonged and unresolved immune responses, such as chronic inflammation, can act promoting cancer growth and progression (Grivennikov et al., 2010; Shiao et al., 2011).
In spite of, it is evident that the tumour cells negatively regulate the immune system through the release of immunosuppressive factors (Sidler et al., 2011), and that the tumour infiltration by immune cells can be considered as a positive prognostic factor for the overall patient's survival (Pages et al., 2010).
In light of the recent evidence, it will be interesting to deepening elucidate all the mechanisms regulating the immune surveillance within the tumour microenvironment to develop important new therapeutic strategies to be coupled to conventional therapies for better response to treatment.

Colorectal cancer

CRC progression is characterized by the progressive acquisition of at least 4–5 oncogenes mutations, or of tumour suppressor genes, determining a malignant tumour formation (Vogelstein et al., 1988), some of them occur often in the same genes and are commonly shared by most people affected by this malignancy, otherwise some mutations are acquired differently and they determine the final cancer phenotype (Fearon and Vogelstein, 1990).
Most of knowledge about colon cancer progression derived from the study of the its inherited form the familial adenomatous polyposis (FAP), an autosomal dominant CRC syndrome caused by the Adenomatous Polyposis Coli (APC) gene mutation which include about 10% of cases (Galiatsatos and Foulkes, 2006). The APC protein promotes the Wnt signaling pathway activation, and its main role is in modulating the cytoplasmic B-catenin levels, a protein that migrating to the nucleus can activate the transcription of several genes involved in cell proliferation, differentiation, migration and apoptosis (Fearnhead et al., 2001).
Tumour progression in these cases required also other related mutations such as KRAS, SMAD2/4, TP53 and deletion of chromosome 18q (Kinzler and Vogelstein, 1996).Recent mounting evidences suggest that cancer growth is fuelled by a cell subpopulation called cancer stem cells, not only important in establish the primary tumour but mostly in the metastatic processes and tumour recurrences (Croker and Allan, 2008; Li et al., 2007b).

Tumour metastasis formation is a complex process that involves a sequence of pathological events, starting with local invasion by tumour cells which must leave the primary tumour site, migrate through the blood and lymphoid vessels, to evade from the circulation and reach the distant tissue, in order to form a micrometastasis. A study on melanoma cells has shown that only 2% of these migrating tumour cells actually manage to form metastases to distant tissue (Luzzi et al., 1998). According to this hypothesis, this number actually reflects the amount of CSCs within the tumour population.
It was recently noticed that CSCs as the metastatic tumour cells, need to be closely in contact with a specific microenvironment to support their growth and to provide protection and contrary to differentiated tumour cells the CSCs are resistant to conventional therapies, (Lobo et al., 2007; Moitra et al., 2011), making them an ideal target to develop new therapy strategies.

Colon crypt and stem cells

Colon tissue is composed by the serosa, the muscolaris, the sub-mucosa and mucosa, as outer layer. It mainly consists of epithelial and goblet mucipare cells and it is surrounded by an absorptive and secretory epithelium, folded in a set of invaginations (about 14,000/square centimeter in the adult human colon), called crypts of Lieberkuhn.
Each crypt contains about 2000/3000 cells, belonging to different populations: the columnar cells, the muco-secreting goblet cells and a small fraction (about 1%) of entero-endocrine cells (Booth and Potten, 2000; Brittan and Wright, 2002). According to the ‘‘unitarian theory’’ all these cell types are generated by a colonic SC (4–6 SCs/crypt), located at the base of the crypt that, through an asymmetric division generate one stem cell (identical with self-renewal capacity) and one progenitor cell that can proliferate, differentiate and migrate to the top of the crypt (Cheng and Leblond, 1974; Kirkland, 1988; Paulus et al., 1992).
Several markers have been linked to the colon SCs, among these: Musashi-1 (Msi-1), B lymphoma Mo-MLV insertion region 1 homolog (Bmi1), Aldehyde Dehydrogenase 1 (ALDH1), EphrinB (EphB) receptors, and Leuchin-rich repeat-containing G protein-coupled receptor 5 (Lgr5).
Msi-1 is a RNA binding protein responsible for Drosophila melanogaster asymmetric division (Nakamura et al., 1994), it was the first molecule identified as a putative human colon SC marker. Its expression was reported in mouse small intestine and in human colon crypt SCs (Nishimura et al., 2003; Potten et al., 2003). Msi-1 appears also to regulate p21 and Notch-1 signaling, as demonstrated by its silencing that causes mitotic catastrophe and xenograft tumour growth arrest by Notch inhibition and p21 up-regulation (Sureban et al., 2008).
Another important putative marker for colon SC is Bmi-1 that has an crucial role in self-renewal of several tissues, as in hematopoietic system, breast and neural one, and it is predominantly expressed in the small intestine at the base of the crypts at the ‘‘+4’’ position, directly adjacent to the Paneth cells (Sangiorgi and Capecchi, 2008). Its expression marks the quiescent stem cells that proliferate in response to injury (Yan et al., 2012).
A more promising marker might be ALDH1, since its expression seems to correlate with the cells that exhibit stem cell properties: a small subset of colonic cells are ALDH1+ (less than 5%), they localize at the bottom of normal colon crypts, and

ALDH1+ sorted cells are able to generate a xenograft once engrafted in mice, contrary to the ALDH1_ population (Huang et al., 2009).
It was recently demonstrated that colon SCs are marked by high expression of EphB2 receptor, which is gradually silenced during cell differentiation (Merlos-Suarez et al., 2011). These important colon SC markers act regulating the migration and proliferation in colon epithelium, and their expression follows a gradient along the crypt, with the highest levels at bottom of the crypt, the so called ‘‘stem cell niche’’ (Holmberg et al., 2006).
In the same compartment at the base of crypt, some Lgr5 (Gpr49) positive cells were founded. Lgr5 is a trans-membrane protein coupled with G-protein. In murine system, it is expressed only in active cycling columnar cells that can differentiate into functional colonic epithelium lineages (Barker et al., 2007). Moreover it was recently demonstrated that a single Lgr5+ cell is able to regenerate a complete crypt-like structure in vitro (Sato et al., 2009). Moreover Lgr5 is a target gene of the well defined Wnt signaling pathway and its expression identify mitotically active stem cells important in the homeostatic regeneration of the tissue (Yan et al., 2012).

Stem cell niche

The organization of the intestinal niche includes fibroblasts, endothelia and inflammatory cells, forming the appropriate environment that guarantees the pluripotency of SCs.
The intestinal sub-epithelial myofibroblasts (ISEMFs) are considered the most important players in the maintenance of the niche, which surround the colon SCs, at the base of the crypt, regulate organogenesis and tissue repair. Their growth is finely controlled by many factors that together with different cytokines maintain the fine balance between self-renewal and differentiation (Adegboyega et al., 2002; Powell et al., 1999).
We have recently demonstrated that the myofibroblasts and their secreted factors, as well as hepatocyte growth factor (HGF) have a key role in establishing the colon niche in colon crypts. We have shown that HGF can have an effect on differentiated cells restoring their stem cell phenotype (Vermeulen et al., 2010).
In addition, current data show that Wnt, Phosphoinositide 3-kinase (PI3K), Bone Morphogenetic Protein (BMP), Notch and Sonic Hedgehog (Shh) pathways are a prominent force controlling cell proliferation, differentiation and apoptosis along the crypt-villus axis, thus maintaining stem cell fate and niche homeostasis.

Wnt pathway

In the last 20 years much it was published about the role of Wnt induced-signal in the regulation of colon tissue development. The binding of Wnt ligands to their Frizzled receptors (Fz) prevents b-catenin degradation (by a complex containing APC and Axin1/2), thus permitting its translocation to the nucleus where it acts as a transcriptional factor, interacting to the TCF/LEF complex, and inducing several genes transcription involved in cell cycle regulation and proliferation (He et al., 1998). An aberrant activation of this pathway, due to mutation in APC or b-catenin genes is clearly linked to colon cancer development (Clevers, 2006).
The identification of all the b-catenin target genes has led to the awareness about the role of this pathway in the maintenance of stemness, even if recent studies suggest that different cell types respond differentially to Wnt signal, according to the cell

localization along the crypt. In line with this model, it has also been demonstrated that there is a different expression of Wnt members along the crypt: mRNA for secreted Fz-related proteins were found at the base of the crypts where they act maintaining the cell stemness, otherwise their expression decrease toward the top of the crypt, where reside the differentiated cells, and where are also expressed Wnt inhibitors (Gregorieff et al., 2005).
Moreover, the best characterized stemness target gene of Wnt/b-catenin pathway is Lgr5 (Barker et al., 2007), that such as c-myc and cyclin D1, is involved in cell proliferation of the transit-amplifying compartment (He et al., 1998; Shtutman et al., 1999; Tetsu and McCormick, 1999).
Furthermore the identification of EphB as a Wnt target has improved our knowledge about the role of Wnt in the intestinal morphogenesis regulation (Batlle et al., 2002), such as in the induction of epithelial differentiation and epithelialmesenchymal transition (regulating the expression of MMP7, laminin c2, Slug), important also for the tumour invasion (Brabletz et al., 1999; Crawford et al., 1999; Hlubek et al., 2001; van Es et al., 2005).
Of course the effect of Wnt pathway is the result of a combined effect with all other actors of the colon stem niche.

Pten–PI3K–Akt pathway

A second important pathway with a central role in stem niche regulation is the PTEN–PI3K–Akt pathway. PI3K is composed by the regulatory subunit p85 that can bind the receptor tyrosin-kinase (RTK), thus activating the catalytic subunit p110, that can phosphorylate its substrates. This event leads to the phosphorylation of the Akt kinase (p-Akt) by PDK1. PI3K pathway has just one negative regulator, the phosphatase PTEN, which convert PIP3 in PIP2, inhibiting Akt function (Cully et al., 2006).
This pathway is important in stem niche regulation since is linked to the Wnt one: p-Akt can phosphorylate b-catenin (He et al., 2007), the main player of Wnt pathway, inducing its nuclear accumulation and enhancing its transcriptional activity (Persad et al., 2001).
It seems indeed to be activated according to a gradient which shows the greater expression in the epithelial cells of the lumen, making guess that it could have a role in confining the effect of the Wnt pathway just in the colon crypts (Kim et al., 2002).
PI3K deregulation was also noticed in many different human tumours, and it may be attributed to its role in cell survival and proliferation, in particular almost 40% of human colorectal cancer bring a constitutively PI3K activated pathway, mainly due to PTEN inactivation (Parsons et al., 2005).

BMP pathway

BMP pathway is a key regulator of colon cell differentiation (Auclair et al., 2007). BMPs belong to the Transforming Growth Factor beta (TGF-b) super-family members, and after linking their receptor they can trigger different biological processes (Chen et al., 1998). The activation of this pathway leads to the phosphorylation of Smad1, Smad5, and Smad8/R-Smad (Miyazono et al., 2010) that in collaboration with the co-Smad, Smad4, translocate into the nucleus and regulate target gene expression (Derynck and Zhang, 2003).
It was recently demonstrated (Kosinski et al., 2007; Rider and Mulloy, 2010) that there is a fine distribution of the different factors along the crypt: BMP1, BMP2,

BMP5, SMAD7, BMP7 and BMP receptors are highly expressed in the apex of the crypt, otherwise BMP antagonist, such as GREM1, GREM2 and chordin-like-1, are expressed at the base of the crypt (Hsu et al., 1998), probably because of the presence of myofibroblasts, which contribute to the stemness maintenance. Interestingly it was shown that BMP pathway can control the number of colon SCs and their self-renewal (He et al., 2004).
There is a strong interaction between BMP with Wnt and PI3K pathway. In particular BMP inhibition by Gremlin leads to the activation of Wnt (Kosinski et al., 2007), as well as BMPs stabilizes PTEN, leading to the Akt activity reduction and thereby reducing b-catenin intranuclear accumulation (Persad et al., 2001; Waite and Eng, 2003).

Notch pathway

The contribution of Notch signaling in colon SCs fate is extensively recognized by the scientific community. Notch pathway consists of four different trans-membrane receptors (Notch1, Notch2, Notch3 and Notch4), and its activation is based on the binding of five different ligands (Jagged-1, Jagged-2, Delta-like 1, Delta like 2 and Delta like 4). The ligands binding induces the cleavage of Notch intracellular domain (NICD) mediated by ADAM metallo-protease. The NICD thus moves to the nucleus where it activates the transcription of all its target genes after dimerization with RBP-jj/CSL (Dikic and Schmidt, 2010; van Es and Clevers, 2005).
The best characterized target gene of Notch pathway is the Hairy/enhancer-of-split (Hes-1) which is involved in the control of proliferation and differentiation (Bray, 2006).
Notch seems to push the proliferation of the transit-amplifying cells compartment, and its activity, in cooperation with specific factors, is fundamental for the differentiation into several epithelial lineages. Recent studies have shown how Notch and Math1 are essential for colon homeostasis and neoplastic transformation. Moreover, the transgenic expression of the NICD blocks cell differentiation, through the expansion of immature progenitors (Kim and Shivdasani, 2011; van ES et al., 2010).

Hedgehog pathway

Sonic hedgehog (Shh) pathway is known to play an important role during colon development. Its activation is mediated by the binding of Shh and Indian hedgehog (Ihh), which are secreted by epithelial cells, to its receptor Patched (PTCH), which is expressed in the sub-epithelial myofibroblasts. The activation leads to the release of the G-coupled protein Smoothened (SMO) that, in collaboration with the GLI transcription factors, migrates into the nucleus to induce target genes activation (Hegde et al., 2008).
The importance of this pathway is not directly the effect on epithelial cell fate, but in the correct development of crypts and villi structures in the mucosa (Madison et al., 2005).

Cancer stem cells

CSCs concept have been long discussed and investigated in the last years, furthermore also the term CSC was used many times, often changing its meaning. For these

reasons, during the Year 2011 Working Conference on CSCs, it was established the correct definition of CSCs opening new questions on this regard (Valent et al., 2012).
It was established that CCSCs arise from the colon SCs, following genetic and/or epigenetic changes, sharing with them several important properties such as the self-renewal and the aberrant multi-lineage differentiation capacity (Barker et al., 2009). CCSCs are often less sensitive to conventional treatments than the bulk of differentiated cells from them generated, and this could be the reason of the initial regression of the tumour, often followed by a more aggressive relapse. Another important aspect in this field is the continuing genomic and epigenomic changes that affect this population, thus contributing to the resistance to standard treatments and then to the amplification of such resistant clones (Baylin and Jones, 2011; Magee et al., 2012; Stratton, 2011). There are two major clinical issues about CSCs that we will try to deepen: the identification and characterization of the CSCs, and the design of new target therapies against them.
The CSCs were isolated and characterized for the first time from acute myeloid leukemia (Bonnet and Dick, 1997; Lapidot et al., 1994), and then this concept has been extended to many solid tumours, including brain (Singh et al., 2004), head and neck (Prince et al., 2007), pancreas (Li et al., 2007a), melanoma (Schatton et al., 2008), liver (Yang et al., 2008), lung (Eramo et al., 2008), prostate (Collins et al., 2005), ovarian (Curley et al., 2009), and colon cancer (O'Brien et al., 2007; Ricci-Vitiani et al., 2007).
A recent study showed that within the tumour population it is possible to identify not only the CSCs and the differentiated counterpart, but a more heterogeneous population with different biological properties (Dieter et al., 2011) (Fig. 1).

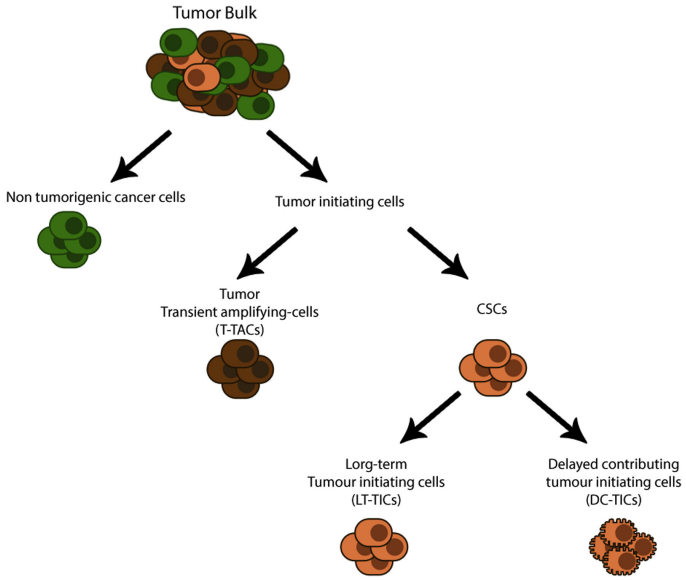

Fig. 1

Heterogeneity of colon tumor initiating cells. Human colon cancer was considered composed by non tumorigenic cells and a small subfraction of tumour-initiating cells (TICs) considered being a functionally homogeneous stem-cell-like population driving tumour growth and metastatic processes. This schema report the recent evidences about cell heterogeneity in TIC compartment, containing three different cell subsets with different biological properties and availability in primary tumour and metastasis. They include T-TACs and CSCs, these last can give rise to LT-TICs and DC-TICs.

Using a molecular tracking strategy and exploiting the tumorigenic ability by in vivo transplantation in immune-compromised mice, Dieter et al. (2011) identified three different subtypes of colorectal tumour initiating cells (TICs) with different roles on tumour growth and metastasis formation. The apex of the pyramidal model proposed is constituted by the self renewing long-term TICs (LT-TICs), a cell subset that was founded both in primary and in serial tumours, and delayed contributing TICs (DC-TICs), a cell population that was not detectable in primary tumours (probably due to their quiescent state) but present in subsequent transplants. These two cell populations are identified as CSCs. The third tumour population, the tumour transient-amplifying cells (T-TACs), was characterized by the ability to promote the primary tumour formation, but without being founded in subsequent serial transplants (Fig. 2).

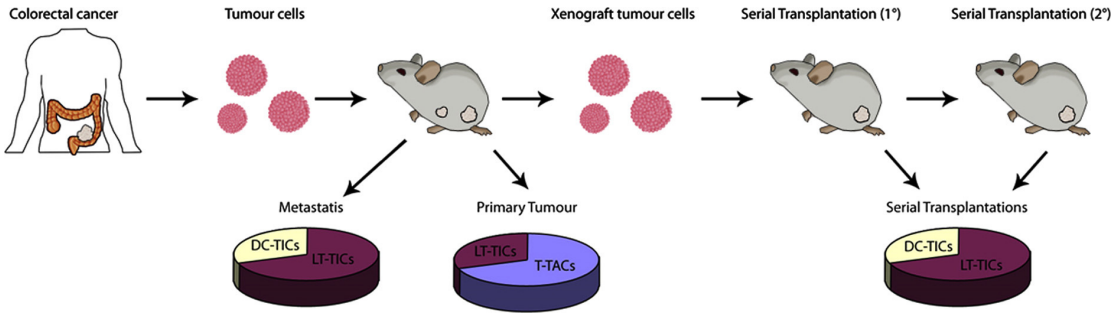

Fig. 2

Biological roles of different subset of TIC during tumour progression. Schematic distribution of colon TICs in xenograft mouse model. Colon tumours contain extensively self-renewing long term TICs (LT-TICs) that are able to maintain tumour formation in serial xenotransplants. Otherwise the tumour transient amplifying cells (T-TACs) possess limited or no self-renewal capacity contributing just to tumour formation in primary mice. Rare delayed contributing TICs (DC-TICs) were found only in secondary or tertiary mice. The metastasis formation seems to be driven by self-renewing LT-TICs demonstrating that tumour initiation, self-renewal, and metastasis formation are limited to different subsets of TICs in primary human colon cancer.

Nevertheless, the molecular mechanisms underlying the biological role of these different cell populations within the tumour are still poorly understood.

Colon CSC markers

Human CCSCs were firstly identified within the CD133+ population. CD133, also known as Prominin-1, is a glycoprotein formed by a single polypeptide chain with a molecular weight of about 120 kDa with 20-kDa glycosidic-linked polysaccharides. It is a pentaspan membrane protein, containing five trans-membrane domains, two N-glycosylated extracellular loops, two intracellular domains and a cytoplasmic C-terminal domain (Yin et al., 1997).
Although its function is not yet well known, CD133 may regulate the cell polarity and cell‑cell and cell‑matrix interactions (Giebel et al., 2004). Ricci-Vitiani et al. (2007) claimed that the CD133+ cell fraction is able to form tumour in vivo, maintaining a self-renewal capacity after serial transplantations, otherwise CD133_ fraction did not.

CD133+ subpopulation was also able to growth as spheres in undifferentiated state, preserving their capacity to differentiate when placed in serum supplemented media.
Although this protein is widely considered to be a marker for the identification of CSCs, its use it is still under debate as demonstrated by several controversial results from different research groups (Shmelkov et al., 2008). Based on this observation it was suggested that CD133 cannot be considered a specific marker for CCSCs since its gene is widely expressed both in undifferentiated and differentiated cells. It was also showed that both $CD133^+$ and $CD133^-$ subpopulations obtained from metastatic colon cancers are able to initiate tumour growth in in vivo transplantation experiments.
This discrepancy could be argued with the different glycosylation status (Kemper et al., 2010) and/or splice variants of CD133 (Fargeas et al., 2004), or with the experimental methodology applied for protein detection, in particular regard cell fixing and/or permeabilization, which could modify the CD133 tertiary structure.
CD44 was recently described as another important marker for CCSCs identification (Du et al., 2008). It is a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion and migration. Moreover, the existence of different splice variants (CD44v) of CD44 has gained a great interest since it was shown for the first time their prominent role to confer a metastatic behavior to tumour cells. Furthermore the role of this molecule in tumour progression was demonstrated in many cancers (Naor et al., 1997; Ponta et al., 2003). CD44 represents a family of glycoproteins encoded by a single gene that undergo to the alternatively splicing giving rise to variable exon products (Screaton et al., 1992). Contrary to the ubiquitous expression of standard isoforms (CD44s), CD44v aberrant expression was observed in many cancer types, conferring them a metastatic potential, resulting in poor prognosis (Harada et al., 2001; Reeder et al., 1998; Wielenga et al., 1993).
CD44 is described as the main receptor of hyaluronan (HA), the most abundant component of the extracellular matrix, highlighting its role as key regulator of cell adhesion (Aruffo et al., 1990). HA binding promotes cell motility and migration, two paramount processes involved on tumour cells dissemination, extravasation of CSCs (Lamontagne and Grandbois, 2008), and metallo-proteases production (Baronas-Lowell et al., 2004).
Finally several cytokines and chemokines such as Interferon gamma (IFNc) (Levesque and Haynes, 2001), osteopontin (Kazanecki et al., 2007), Hepatocyte Growth Factor (HGF) (Corso et al., 2005), basic Fibroblast Growth Factor (bFGF) (Bennett et al., 1995), Vascular Endothelial Growth Factor (VEGF) (Tremmel et al., 2009), heparin binding factor (Yu et al., 2002), can also bind CD44 with important implications in cell proliferations and survival.
Among the all splice variants of CD44, CD44v6 boast of a central role in metastatic progression and its expression was also associated with poor prognosis in CRC (Peng et al., 2008; Zlobec et al., 2009). CD44v6 is primary involved in the assembly of the matrix, supporting its function in tumour cell cross-talk with the stroma and in pre-metastatic niche formation. Many other CSC markers have been proposed including CD24 (Choi et al., 2009), ALDH1 (Huang et al., 2009), MSI1 (Potten et al., 2003) and CD29 (Vermeulen et al., 2008). Farther, several studies showed that the CCSCs could reside within different cell subsets, such as in the $EpCAM^{hi}/CD44^+$ fraction (Dalerba et al., 2007).
However it is common opinion that the cancer stemness is linked not to the exclusive expression of one of these markers but to a combined expression of them. For this reason our group decided to re-examine the expression of these markers: using the single cell sorting we found that the clonogenic potential resided in the $CD133^+$

population which co-expressed the other CCSC markers mentioned above (Vermeulen et al., 2008).

CSCs and pre-metastatic niche

It is widely accepted that the SCs need a stem cell niche for their maintenance, to control the correct balance between survival, self-renewal and differentiation programs (Morrison and Spradling, 2008).
This concept was also extended to the CSCs population, and convincingly demonstrated mostly during the metastasis formation processes (Bissell and Labarge, 2005).
According to the ‘‘seed and soil’’ theory the organ-preference patterns of tumour metastasis result in interaction between metastatic tumour cells and their specific microenvironment. More recently, it has been suggested that early changes in the microenvironment at the distant sites, could be induced by the primary tumour, reported as ‘‘pre-metastatic niche’’ formation, although the mechanisms and factors responsible for such pre-metastatic niches are not well defined (Kaplan et al., 2005).
Over recent years, there has been an appreciable increase in our understanding of the cross-talk that occurs between these two compartments on the systemic, cellular, and molecular bases. Continued investigations of the mechanisms that mediate site-specific metastasis and new insights to the differences between the normal and CSCs niches will likely lead to the identification of new targets for therapy.

Exosomes

As previously mentioned, the CSCs to form metastasis need to be supported by an educated environment necessary to lead extravasation, to implant and grow driven also by soluble factors, recently found likewise within the exosomes which assume on this context a decisive role.
Exosomes are small vesicles of 30–100 nm (Johnstone, 2006) generated upon fusion of multivescicular bodies (MVB) with the plasma membrane (Lakkaraju and Rodriguez-Boulan, 2008), their importance in cancer comes from their biological role and from the evidence that cancer cells release more exosomes than the normal cells (Johnstone, 2006).
The exosomes content is related to their cell of origin, such as the function of the protein there harbored, that is maintained over time (Schorey and Bhatnagar, 2008).
Exosomes contain a big set of both membrane and cytosolic molecules including integrins, ICAM, Major Histocompatibility Complex (MHC) molecules, vesicle transport associated proteins, Heat Shock Proteins (HSPs), signal transduction molecules, and tetraspanins (Gruenberg and Stenmark, 2004). Tetraspanins seem to have a key role in both the mechanisms that regulate the exosomes function, the selective proteins recruitment into exosomes, and the following interaction with the target cells.
Tetraspanins are a family of 34 proteins with four intramembrane domains (Boucheix and Rubinstein, 2001), which form a network with other transmembrane and intracellular signaling proteins (Levy and Shoham, 2005). Among their partners there are G protein coupled receptors (Little et al., 2004), peptidases (Le Naour et al., 2006), Ig superfamily members and, mostly important in CRC, CD44v6 and EpCAM (Kuhn et al., 2007). All these associated partners highlight the tetraspans as an important factor in the regulation of cell motility, adhesion, and invasion.

Furthermore, it has been assed that exosomes hold different mRNAs and miRNAs, which can be in turn transferred to the target cells, thus activating several pathways (Baj-Krzyworzeka et al., 2006). Based on this observation a higher concentration of exosomal mRNAs was indeed found in the serum of CRC patients (Fleischhacker and Schmidt, 2007), enriched in cell-cycle related mRNA leading to endothelial cell proliferation, suggesting their potential involvement in tumour growth particular refereed to angiogenesis. There are also paramount studies trying to define the miRNA profile in CRC, many of which are down-regulated, suggesting a tumour suppressor role. For this reason improvements on exosomal mRNA and miRNA profile characterization would be suitable to use as prognostic factor (Taylor and Gercel-Taylor, 2008; Tokarz and Blasiak, 2012).

EMT and MET in colon cancer progression

Metastases formation is the final product of a multistep process, which involves the dissemination of cancer cells to distant organs and the following adaptation and proliferation into the foreign tissue microenvironment. This process is determined by the genetic and/or epigenetic alteration of cancer cells, but also driven by non-neoplastic stromal cells (Valastyan and Weinberg, 2011).
The epithelial-mesenchymal transition (EMT) has been noticed as the first step of the metastatic cascade as a well defined biological event playing an important role not only in normal tissue development during the organogenesis, but also in the pathogenesis of diseases, with particular interest during the acquisition of the migratory phenotype of CRC cells (Thiery et al., 2009).
Cells that undergo to EMT are characterized by loss of some epithelial features, such as the apico-basal polarity and cell adhesion, the expression of E-cadherin, occludin and cytokeratins, and at the same time a marked up-regulation of N-cadherin, vimentin, fibronectin, Twist1, zinc-finger proteins (SNAIL, SLUG, ZEB2) and metalloproteinases, with consequent increase of cell mobility (Lee et al., 2006).
A paramount of different signals directly from the surrounding microenvironment may lead to the acquisition of an invasive phenotype in epithelial malignancies (Le et al., 2008), i.e. fibroblasts, myofibroblasts, granulocytes, macrophages and lymphocytes are defined as EMT-inducing factors: pathway as TGF-b (through the direct activation of Twist, SLUG and ZEB2), PI3K/Akt (increasing the mTOR kinase expression), Shh and Wnt are indeed potent inducers of EMT (Gulhati et al., 2011; Moustakas and Heldin, 2007).
Furthermore, the mesenchymal-epithelial transition (MET), the reverse process to EMT, is also considered as fundamental mechanism occurring in normal tissue development and in colon mucosa regeneration. This process could play a role both in the first steps of tumour formation, since a dedifferentiation process of the mesenchymal cells is suggested to be crucial in some cancers (Rubio et al., 2008), and in metastasis formation (Brabletz, 2012).
Despite considerable studies in cancer, to date there is not a reliable theory underling the mechanisms regulating the migrating cancer stem cells (mCSCs) (Brabletz et al., 2005) and the acquisition of the metastatic phenotype of tumour cells opening a new issue on tailored therapy (Fig. 3).

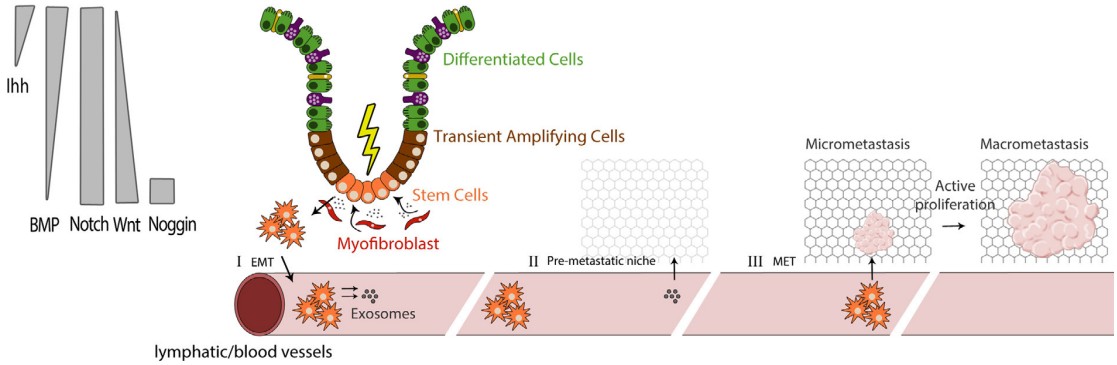

Fig. 3

Colon cancer progression. The formation of metastasis is considered a multistep cell-iological process that involves the dissemination of colon CSCs to anatomically distant organs and the following adaptation and growth in the foreign microenvironment. All these events are driven by the acquisition of genetic and/or epigenetic changes of tumour cells and the cooperation of non-neoplastic stromal cells. The first step consists of the local invasion of colon cancer cell through the EMT. The surviving cancer cells then produce exosomes and other soluble factors that can have an important role in determining the pre-metastatic niche. In the last step the cancer cells have to arrest at the target organ and to become proliferative active through the MET, to finally form clinically detectable macro-metastases.

Treatment option overview

A key role in the cure of colon cancer is represented by the prevention, followed by the surgery when the tumour is still in the early stages of development. This approach offers to the patients a good rate of success but unfortunately the symptoms of early disease, such as fecal occult blood (FOB), occur just in 5% of the cases.
Nowadays there are two strategies available for colon cancer screening: the fecal occult blood test (FOBT) and the colonoscopy, these analyses are suggested to be addressed to over-50 years old men and women every 1–2 years.
An important serological marker for early detection and diagnosis for colorectal cancer is the carcinoembryonic antigen (CEA), which can be used in the preoperative staging and postoperative follow-up, even if it possesses a poor predictive value in asymptomatic patients because of the low sensitivity and specificity (Labianca et al., 2010).
Table 1 shows the different colon cancer stages according to the American Joint Committee on Cancer (AJCC) and the TNM Classification of Malignant Tumours (TNM). Colon cancer staging it is useful for diagnostic and essential to determine the best treatment. As showed in Table 1, the staging depends on the local invasion extension, the degree of lymph node involvement and distant metastasis.

Table 1
Colon cancer staging.

AJCC stage	TNM stage			TNM stage criteria for colon cancer
Stage 0	Tis	N0	M0	Tis: tumor confined to mucosa; cancer-in situ
Stage I	T1	N0	M0	T1: invasion of submucosa
Stage I	T2	N0	M0	T1: invasion of muscularis propria
Stage II-A	T3	N0	M0	T3: invasion of subserosa or beyond (without other organs involvement)
Stage II-B	T4	N0	M0	T4: invasion of adjacent organs or perforation of the visceral peritoneum
Stage III-A	T1-2	N1	M0	N1: metastasis to 1 to 3 regional lymph nodes. T1 or T2
Stage III-B	T3-4	N1	M0	N2: metastasis to 1 to 3 regional lymph nodes. T3 or T4
Stage III-C	Any T	N2	M0	N2: metastasis to 4 regional lymph nodes. Any T
Stage IV	Any T	Any N	M1	M1: distant metastases. Any T, any N

For the detection of the metastasis it is needed some imaging techniques such as abdominal ultrasound, CT and PET scanning. Of course the definitive classification can only be determined after surgery, and pathology analysis.
The standard treatment for metastatic CRC (only about 39% of them are found at an early stage) is represented by a combination of 5-fluorouracil, leucovorin and oxaliplatin (FOLFOX), or a combination of 5-fluorouracil, leucovorin and irinotecan (FOLFIRI). These regimens are actually used more frequently in younger than older patients with metastatic CRC, maybe to improve the resection rate (Lenz, 2008).
In order to reduce the risk of relapses, the standard of care for patients with early-stage CRC remains the surgery, combined with adjuvant chemotherapy (most of them are given for about 6 months). Adjuvant therapy is generally used in cases of high risk, defined by the serosa infiltration of tumour (stages II B, III and IV). Patients with stage II A can be considered at high-risk if there is a poorly differentiated tumour, or more than 12 lymph nodes involved, or with a vascular/lymphatic invasion, or tumour perforation.
The infiltration of tumour cells in regional lymph nodes is today considered as the most accurate prognostic factor for colorectal cancer survival (Iddings and Bilchik, 2007).
CRC staging is useful not only for the prognosis but also to predict which patients will benefit from adjuvant therapy. The chemotherapy administered after surgery at stage III colon cancer patients improves their survival, enhancing both the timeto- recurrence up to 40% and the overall survival (OS) up to 30% (Krook et al., 1991; Wolmark et al., 1993).
CCSCs seem to resist death-inducing signals thanks to their slow cycling proliferation and to high levels of drug transporters (Dean et al., 2005). CCSC are also characterized by high levels of anti-apoptotic proteins and it makes them resistant to apoptotic stimuli (Todaro et al., 2008).
After chemo- or radio-treatments, these drug-resistant cells are responsible of the repopulation of treated-tumours, for this reason the identification and targeting of the characteristic pathways of this cell subset, such as of the microenvironment in which they reside could be important to completely eradicate the tumour.

Targeted therapy: how to selectively kill CCSCs?

CCSCs constitute a minority cell subset in the tumour bulk and they are considered to be the source of tumour cell renewal, thus affecting the tumour behavior in terms of cell proliferation and resistance to chemo- and radio-therapy.
Their ability to survive to conventional therapy is due to the over-activation of some signaling pathways (EGFR, VEGF, Wnt, Notch) and to the effect of the microenvironment in which they reside and growth.
For these reasons the molecular targeting of such highly tumorigenic cells must be considered the key to improve the efficacy of current anti-cancer strategies, aiming to sensitize tumours to conventional therapies thus definitely abrogate tumorigenesis.

CSCs targeting

Conventional therapies most of the times do not suffice in killing all tumour cells, since they affect the more differentiated cells (which constitute most of the tumour mass) thus saving the CCSCs, which are then able to repopulate the tumour bulk. This is mainly due to the quiescent state of CCSCs that protect them against conventional treatment, which mostly target active proliferating cells.
For this reason one of the most promising treatments that could be coupled to conventional ones is the differentiation therapy. The induction of differentiated state could force the CCSCs to acquire a mature phenotype, thus making them more vulnerable to standard therapies. In this regard Lombardo et al. (2011) have recently

demonstrated that BMP4 can promote terminal differentiation, apoptosis, and chemosensitization of CCSCs, suggesting that BMP4 might be considered as a therapeutic agent against CSCs in advanced colorectal tumours.
Since the CCSC state is a consequence of the EMT process, another therapeutic approach could involve the use of EMT inhibitors, by blocking TGF-b and Wnt signaling, which are the most characterized pathway known to positively affect this process (De sousa et al., 2011; Thiery et al., 2009).
A recent work revealed that CCSCs (identified as $CD133^+$ cells) produce and utilize IL-4 to protect themselves from cell death. Consistently to these data, the treatment with IL-4Ra antagonist or anti-IL-4 neutralizing antibody showed a sensitization of this cell subset to chemotherapeutic agents, paving the way for the development of new CCSCs target treatments (Todaro et al., 2007).
Finally, an important difference between normal and cancer cells is about their metabolism, the so-called Warburg's effect. Warburg et al. (1927) reported an increased uptake of glucose and production of lactate by tumours in vivo as compared with normal tissues.
This study suggests that cancer cells restrict use of fatty-acid oxidation in favor of glycolysis as an ATP energy source and even if this concept was recently revised (Koppenol et al., 2011), it could be exploited for therapy (Dang et al., 2011).

Targeting CCSCs pathway: VEGF

It is widely accepted that tumour growth is supported by a framework of new blood vessels, in a process called angiogenesis, which does not occur in the normal healthy tissues except for tissue repair, remodeling or inflammation (Kerbel, 2008).
Recent findings propose CSCs as strong promoters of angiogenesis, a multistep mechanism that leads to the formation of new or extended capillaries, involving many processes such as vasodilation, vessel permeability, and endothelial cell proliferation and migration.
The interplay between CSCs and vasculature was clearly demonstrated by Bao et al. (2006). In this study it is shown how the CD133+ cells give rise to strongly angiogenic tumours compared to $CD133^-$ cell subset. This effect can be explained by a 10–20-fold increase in VEGF release of these cells of $CD133^+$, giving them a significant angiogenic advantage.
The correlation between CSCs and tumour vascularization was also demonstrated by Folkins et al. (2009), who showed that CSC-high tumours exhibited increased microvessel density and blood perfusion compared with CSC-low ones, also inducing increased mobilization and tumour recruitment of bone marrow-derived endothelial progenitor cells (EPC).
Recent findings showed that also the endothelial cells have an effect on CCSCs. Lu et al. (2013) indeed described how the endothelial cells through paracrine signaling can induce colorectal cancer cells to acquire CSCs properties, in terms of $CD133^+/ALDH1^+$ compartment and sphere forming capacity, and that this conversion is mainly mediated by Notch pathway. Together these data suggest that CSCs can be the most important source of angiogenic factors in tumour microenvironment and that the targeting of pro-angiogenic factors could be critical for patient therapy.
In this regard a recent work by Blansfield et al. (2008) showed that the targeting of tumour microenvironment, in particular toward endothelial cells and inhibition of angiogenesis, could be considered a promising therapy to apply in clinic. In this work it was demonstrated how lenalidomide, sunitinib (both involved in inhibition of

angiogenesis, through different mechanisms) and cyclophosphamide (cytotoxic to endothelial cells) were able to inhibit the proliferation of endothelial cells in vitro (in an additive manner) and to completely inhibit the in vivo growth of primary tumour in mouse models. Tumour growth inhibition is due to the ability of these compounds to establish an inhospitable tumour microenvironment (Blansfield et al., 2008).
Among molecules that regulate tumour angiogenesis, the most characterized pathway involves VEGFs and receptors (VEGFRs), even if it can be also regulated by many other factors as platelet-derived growth factor (PDGF), FGF and transforming growth factor alpha (TGF-a) (Hicklin and Ellis, 2005).
The VEGF family encloses six members: the most important is VEGF-A, then the placenta growth factor-1 and -2 (PGF-1, PGF-2), VEGF-B, VEGF-C and VEGF-D. All these molecules are soluble factors secreted by tumour and stromal cells that binding the extracellular domain of their receptors leading to several intracellular signaling cascades endowed with survival, proliferation, migration, differentiation and permeability of endothelial cells.
It was assessed that the VEGF expression may play an important role in human colon cancer progression, in particular during the transition from premalignant adenoma to invasive and metastatic disease (Takahashi et al., 2003). Its overexpression it is correlated with tumour progression and a worse prognosis (Lee et al., 2000).
Judah Folkman in 1971 for the first time proposed a possible key role of angiogenesis in tumours, farther suggesting the anti-angiogenic agents as potential compound for cancer treatment (Folkman, 1971).
Many molecules have been tested and undergone to clinical trials, and among these the bevacizumab, a monoclonal antibody directed against VEGF-A was the first successfully accepted, in 2004, to use in combination with standard chemotherapy for metastatic CRC inhibiting angiogenesis and thus the tumour growth (Gordon et al., 2001). It is also functional in normalizing the tumour blood vessels structure, decreasing the intra-tumour hydrostatic pressure, enhancing the drug delivery to the tumour (Ellis, 2006).
The bevacizumab was initially use in combination to the IFL (irinotecan, 5-FU and leucovorin) (Hurwitz et al., 2004), and then to other regimens, in many of which it resulted fundamental for increased response rates (RR) and survival rate (Grothey et al., 2008).
Two studies also analyzed its possible negative effect on postoperative wound healing in patients were subjected to hepatic resection, both demonstrating no increase complications upon patients treatment (Gruenberger et al., 2008; Kesmodel et al., 2008).Unfortunately some important side effects were observed, such as gastrointestinal perforation (1.5%), arterial thrombosis, myocardial infections and strokes (Prat et al., 2007).
Even if the inhibition of angiogenesis has been emerging as an efficient strategy for treating CRC, recurrences often occur after the first period of suppression of tumour growth. Moreover some anti-angiogenesis drugs have been shown to positively influence metastasis of malignant progression in animal models (Ebos et al., 2009; Paez-Ribes et al., 2009).
In a recent study it was shown that CCSCs ($CD133^+$ cells) are mor e resistant to anti-angiogenesis treatments, and this could be the reason of the occurring of recurrences after this treatment. This phenomenon is mediated by the activation of anti-apoptotic signaling pathway involving Hsp27, and its inactivation can sensitize CCSCs to undergo cell death (Lin et al., 2013).

Targeting CCSCs pathway: EGF

As previously mentioned the CCSCs require the presence of growth factors produced by the microenvironment for their proliferation and maintenance of stem-like properties (Vermeulen et al., 2010). One of the best characterized factors that affect CCSCs behavior is the Epidermal Growth Factor (EGF), which is known to regulate intestinal epithelial cell and stem/progenitor cell growth and differentiation (Suzuki et al., 2010). In a recent study Feng et al. (2012) showed that EGF signaling activation is necessary to promote the formation of CCSCs and for their maintenance of self-renewal capacity. For these reasons EGF receptor (EGFR) became an important possible target in cancer therapy.

EGFR is a tyrosine kinase receptor belonging to the HER family that includes EGFR itself (Erb1/HER1), Erb2 (HER2/neu), Erb3 (HER3) and Erb4 (HER4). Upon activation by binding of its growth factor ligands EGF and TGF-a, EGFR switch from an inactive monomeric form to an active homo- or hetero-dimeric complex resulting in the stimulation of its intrinsic intracellular protein-tyrosine kinase activity, determining an auto-phosphorylation of several tyrosine residues in the C-terminal domain. Receptor activation, through the docking of cytoplasmic proteins, can initiate several cell signaling pathways, including the Ras–Raf–MAPK, PI3K/Akt, the protein kinase C, STAT and src kinase pathway, primarily involved in cell proliferation, inhibition of apoptosis, invasion and migration.

Nowadays, two different anti-EGFR therapies are currently use for colorectal cancer treatment, the Cetuximab, a monoclonal antibodies that recognize EGFR (a partially humanized monoclonal antibody) (Van Cutsem et al., 2009), or panitumumab (a fully human monoclonal antibody) (Andre et al., 2013), and small molecule inhibitors of EGFR tyrosine kinase activity (TKIs) (Marshall, 2006).

The monoclonal antibody cetuximab prevents receptor activation interfering with its dimerization through steric inhibition of the extracellular domain, leading also to receptor internalization and subsequent degradation. Moreover cetuximab can kill target cells by mediating antibody-dependent cell- mediated cytotoxicity (ADCC) and complement fixation (El Zouhairi et al., 2011).

On the contrary panitumumab blocks ligands binding determining receptor internalization, but without induced degradation, suggesting that it could be recycled to the cell surface.

Cetuximab was approved by the US Food and Drug Administration (FDA) in February 2004, in combination with irinotecan in irinotecan-refractory patients, or as a single agent in patients intolerant to irinotecan. It was tested both alone in patients that were refractory to irinotecan-containing regimens, inducing a response in 10.8% of them with a media time to progression (TPP) of 1.5 months, and in combination with irinotecan, obtaining 22.9% of response and a TPP of 4.1 months (Cunningham et al., 2004).

Panitumumab obtained the FDA approval based on similar studies, reporting an 8% of response in patients whose disease had progresses after FOLFOX and FOLFIRI regimens (Douillard et al., 2010; Gibson et al., 2006; Peeters et al., 2010; Van Cutsem et al., 2007).

The more investigated TKIs targeting EGFR for metastatic CRC are gefitinib, erlotinib, and EKB-569. Their activity in metastatic CRC was minimal, and in combination with FOLFOX and FOLFIRI the clinical response rate ranged from 24% to 74% in phase II studies. The worse aspects in the use of TIKs were the adverse effects, with a 3–4-fold increase grade of toxicity. Probably the most interesting

aspect of the anti-EGFR therapies is the role of mutant Kras in predicting response to these treatments. Kras is a small intra-membrane serine–threonine kinase, activated just downstream of EGFR, which acts propagating further signaling events.
Retrospective studies for both cetuximab and panitumumab, showed that there is a strong correlation between Kras status and the response to these treatments. Lievre et al. (2008) demonstrated for cetuximab treatment that in the mutated Kras tumours the response rate was of 0% versus the 40% in the tumours with wild-type Kras, with an increased overall survival of 4 months in the latter (Lievre et al., 2008).
A similar study was performed for panitumumab treatment, where the researchers showed that no mutated Kras tumours responded to panitumumab, versus the 17% of responding patients with wild-type Kras (Amado et al., 2008). As a consequence of these findings all the clinical trials for anti-EGFR treatments have to take in consideration the mutational status of Kras.

Immune system, tissue homeostasis and colon cancer development

The main role of the immune system is to maintain the tissue homeostasis, to protect against pathogens and to eliminate damaged cells.
In this context the tumour is a disease that arises from DNA mutations affecting crucial pathways that regulate cell proliferation, survival and cell death, impairing the tissue homeostasis. The aberrant tissue homeostasis was recently addressed as new field of study drawing complicate and multifunctional network between the microenvironment and tumour cells, in favor of the survival of the latter. Based on this observation the immune microenvironment could play a key role during the tumour progression.
The mammalian immune system is made up of different cell types (innate or adaptive immunity) and mediators, which interact with non-immune cells to create a dynamic network of signals thus providing protection against pathogens, while ensuring tolerance to the self-antigens.
The first-line of protection against perturbations in tissue homeostasis is represented by sentinel macrophages and mast cells which through the release of soluble mediators, such as cytokines, chemokines, matrix remodeling proteases and reactive oxygen species (ROS), attract additional leukocytes into the damaged area.
The dendritic cells (DCs) interconnect directly the innate and the adaptive immunity capturing the foreign antigens and presenting them to the adaptive immune cells into lymphoid organs. Also the natural killer cells (NKs) partecipate to the cross-talking between innate and adaptive immune cells interacting with the DCs, eliminating them or promoting their maturation (Hamerman et al., 2005; Raulet, 2004).
Once pathogens have been eliminated, the immune system cells are regulated in terms of proliferation and cell death to restore the normal levels of guard. Unbalance in the immune cells number in a particular district or tissue can dramatically affect the maintenance of tissue homeostasis, such as occurs during tumor formation (Finch and Crimmins, 2004). The existence of a link between immune cells and cancer has been known for many years (Balkwill and Mantovani, 2001), even if their properly role is not well defined.
Initially it was suggested that the infiltrate leukocyte could have a role in preventing the tumour growth, given the fact that excessive infiltration of NKs in gastric and colorectal cancer was associated with favorable prognosis (Coca et al., 1997; Ishigami et al., 2000). On the contrary the presence of other innate-immune cells within the tumour tissues such as macrophages in breast carcinoma, and mast cells in lung

adenocarcinoma and melanoma, were associated with poor clinical prognosis (Leek et al., 1996; Imada et al., 2000; Ribatti et al., 2003).
When colon tissue homeostasis is perturbed, several processes involved in the inflammation are induced, and if this phenomenon is prolonged, so becoming chronic, it can bring to an excessive tissue remodeling, loss of its architecture or completely destruction, as well as induce DNA and protein damages because of the oxidative stress, intensifying the risk of cancer.
The most prominent clinical evidence of a link between chronic inflammation and cancer arises from some epidemiological studies, which claimed that long-term usage of anti-inflammatory drugs, such as aspirin and cyclooxygenase-2 (COX-2) inhibitors, significantly reduce the risk to get a cancer (Dannenberg and Subbaramaiah, 2003), including colorectal one (Rahme et al., 2003).
The inhibition of COX2, involved in the production of prostaglandin, seems to reduce cancer risk according to recent epidemiological studies (Zha et al., 2004). In several human epithelial cancers the COX2 over-expression, mainly found in stromal cells, correlates with poor prognosis (Dannenberg and Subbaramaiah, 2003). The exact mechanism of action of the COX2 inhibitors is not yet entirely clear, but it seems to function by normalizing different pathways of stromal and innate immune cells.
A possible explanation of why the inflammatory process protects tumour growth instead of counteracts it, comes from the evidence of different profiles of immune status between healthy subjects and cancer affected patients. The latter exhibit an enrichment in regulatory T-cells, at the expense of tumour-killing CD8+ CTL (Curiel et al., 2004). Neoplastic microenvironments indeed seem to promote a chronic pro-tumorigenic inflammatory states rather than acute anti-tumour responses (Zou, 2005).
Last but not least the tumour microenvironment is enriched in cytokines, chemokines, metalloproteinases and pro-angiogenic factors, such as Tumour Necrosis Factor alfa (TNFa), TGFb, VEGF, interleukin 1 (IL-1) and 6 (IL-6), which contribute to neoplastic cell proliferation and survival of damaged epithelial cells (Balkwill et al., 2005). In this context the matrix metalloproteinases (MMPs) play an important role, due to their capacity to remodel the ECM components and interfering with the cell–cell and cell-matrix adhesions. Despite some MMPs are produced by epithelial cells, the major source of their production are the stromal cell, such as fibroblasts, vascular cells but also immune cells (Egeblad and Werb, 2002). MMP-9 and MMP-7 are the two major proteins of this family having a role in regulation of inflammation and tumour angiogenesis (Bergers et al., 2000; Lynch et al., 2005) (Fig. 4).

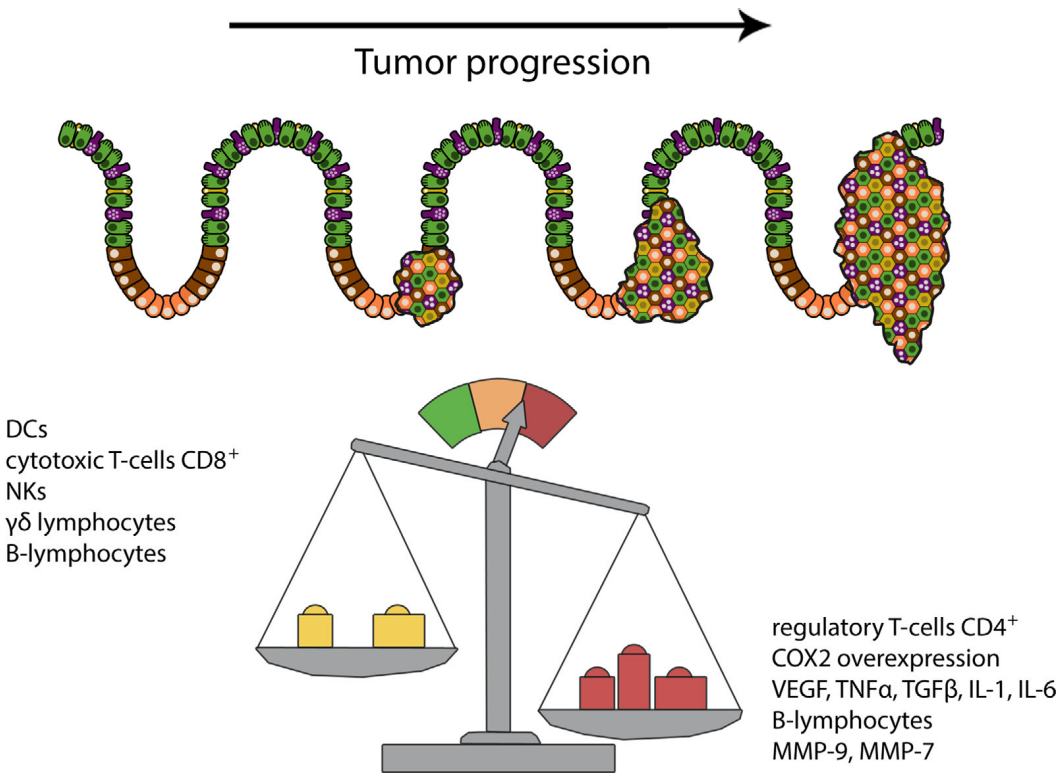

Fig. 4

Dual roles of innate and adaptive immune-cells and their released factors in colon cancer development and progression. The immune system possesses dual and controversial role regard cancer progression and the final result of its function seems to be caused by the correct balance of all its components. When cancer cells are recognized by the immune system the antigens that are present in early neoplastic cells are transported by dendritic cells (DCs) to lymphoid organs where they lead to the activation of adaptive immune cells, thus resulting in both promoting and counteracting tumour growth. The activation of B cells results in chronic activation of innate immune cells in neoplastic tissue thus promoting cell-cycle progression and survival due to the activation of mast cells, granulocytes and macrophages and the factors from them released. Moreover the tumour microenvironment is enriched in pro-inflammatory cytokines, chemokines, extracellular proteases and pro-angiogenic factors, such as TNFa, TGFb, VEGF, interleukin 1 (IL-1) and 6 (IL-6) that contribute to neoplastic cell proliferation and survival. By contrast the activation of adaptive immunity leads to an antitumor effect through T-cell-mediated toxicity in addition to antibody-dependent cell-mediated cytotoxicity and antibody-induced complement-mediated lysis.

Immunotherapy

All this advances carried out in recent years has fuelled many researcher group the purpose of developing new approaches of immunotherapy against tumours. The term ‘‘immunotherapy’’ indicates a treatment that includes the induction and enhancement (immunotherapies), or the suppression of immune response (suppression immunotherapies).

Adoptive cell transfer (ACT) approach, involving the transfer of ex vivo expanded autologous or allogeneic tumour reactive lymphocytes, has been reported to induce therapeutic efficacy and increased patients survival when combined with cytotoxic treatments (CTX, chemotherapy and radiotherapy). Ramakrishnan et al. demonstrated that CTX makes tumour cells more susceptible to the cytolytic effect of cytotoxic T

lymphocytes (CTLs), and that they are able to induce apoptosis also in neighboring tumour cells that do not express specific tumour antigens (Ramakrishnan et al., 2010). These data suggest that also a small numbers of CTLs could lead to a strong antitumor effect if combined with CTX.

Another important approach in tumour immunotherapy was sprouted from the idea to immunize patients against their own cancer using tumour killed cells, proteins, peptides, or DNA vaccines, even if the success was limited (Amato et al., 2010; Dougan and Dranoff, 2009; Giaccone et al., 2005; Testori et al., 2008).

As already mentioned the DCs are considered a bridge between innate and adaptive immunity. Early clinical trials showed that the vaccination with ex vivo generated DCs pulsed with tumour antigens can be useful even if the clinical benefit was observed only in a small percentage of stage IV patients (Palucka et al., 2008). In some cases of soft tissue sarcoma, the intratumoral treatment with DCs led to an increase in T-cell infiltration (Finkelstein et al., 2012), suggesting that the combined vaccines of DCs and CTX can counteract its immunosuppression effect on tumour microenvironment.

T_{regs} targeting in combination with CTX could be very important advance in cancer treatment, since it is known that enrichment of these cells into tumour microenvironment leads to block the anti-tumour immunity response, promoting also malignant proliferation and dissemination of cell through the expression of soluble mediators (Muzes et al., 2012). One of the most efficient strategies is based on CTL-4 inhibition. CTL-4 is a negative co-stimulatory molecule expressed on both T cells and T_{regs} and it acts inhibiting T cells and at the same time promoting Tregs function. A monoclonal antibody blocking CTL-4, ipilimumab, was recently recommended for the treatment of advanced malignant melanoma, displaying an extended overall survival, correlating with an increase in T-cell activation and Tregs inhibition (Hodi et al., 2010) in combination with CTX improved patient survival (Robert et al. 2011).

We have recently assessed that NKs can also play an important role in colon CSC recognition and killing (Tallerico et al., 2013). NKs are potent cytotoxic lymphocytes that can recognize tumour cells, in particular CCSCs. We showed that this different susceptibility is due to a different expression of ligands for NKp30 and NKp44 within the natural cytotoxicity receptor (NCR) group of activating NK receptors. The CCSCs express higher levels of these ligands and at the same time lower levels of MHC class I, known to inhibit NK recognition. This study strengthens the idea of a therapy based on the conventional CTX regimen (most effective on differentiated cells) coupled to NKs cells immunotherapy (specific for CSCs). Moreover it was recently demonstrated that is possible to generate mature and functional NKs from several different human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs). This innovative method could be extended to future studies improving clinical-scale expansion of anti-tumour lymphocytes (Knorr et al., 2013).

Most of the current strategies for immunotherapy aim to stimulation of the adaptive immune system dependent on MHC-restricted ab T cells, even if their loss is often observed in cancer cells (Gattinoni et al., 2006; Vesely et al., 2011).

We have recently highlighted the crucial role of cd T lymphocytes on anticancer therapy, since they exhibit potent MHC-unrestricted lytic activity against tumour cells (Todaro et al. 2009). In particular, this study emphasized the role of aminobisphosphonates (N-BP), as well as the treatment with zoledronate promoting the activation and proliferation of human Vc9Vd2. This subpopulation of T cells has been detected in the majority of CRC tumour infiltrating lymphocyte. Moreover Vc9Vd2 T cells can be obtained from patient blood and ex vivo expanded (Bennouna

et al., 2008; Kobayashi et al., 2007), thus retaining their migration efficiency (Viey et al., 2008). Finally the cd T transfer concomitant with the injection of bisphosphonates have been proved to be well tolerated and this fosters development of alternative, or better, of adjuvant therapies for treatment of tumour patients, including CRC.

Individualized therapies

Despite the improvements in cancer therapies and the application of new ‘‘target agents’’, the outlook for most of the patients is still poor, particularly in advanced solid tumours affected patients.
In the last years thanks to the increased diffusion of instruments for the proteomic and genomic characterization of tissues, it was possible to define different subgroups of patients based on the genetic and biological alterations present into colon tumour (De Sousa et al., 2013; Sadanandam et al., 2013). Each subtype shares similarities to distinct cell types of the normal colon crypt, showing different degrees of stemness and Wnt signaling activity.
These classifications could be useful in clinical practice to predict the best regimen to adopt for each patient. Another important approach for cancer treatment involves the culturing of freshly purified colon cancer cells to directly test different combination of drugs. The idea to introduce individualized therapy harbored from the possibility to individualize and select a small fraction of primary tumour cells with clonogenic capacity, the CSCs.
Three decades ago Salmon and Hamburger developed a culture method now well known as ‘‘clonogenic assay’’ or ‘‘human tumour stem cell assay’’ (HTCA). This methodological approach allowed to culture single cell suspensions from primary tumour in multilayer soft-agar and to treat the derived clone with specific drug (Salmon et al., 1978) introducing the concept of target therapy.
Based on these observations several clinical trials have been carried out, often with surprising results. Von Hoff et al. (1983) published a first study based on HTCS experimental model as guidance for treatment of 470 patients with 27 different advanced metastatic cancers (Von Hoff et al., 1983). They have found that the RR in the assay-guided therapy was 25%, compared to the 14% in the empiric treatment group. A second randomized clinical trial was performed on 211 ovarian cancer patients and it showed a higher RR in the assay-guided therapy (22%) compared to the empiric therapy group (3%) (Von Hoff et al., 1991). However the selection of chemotherapy treatment based on in vitro drug sensitivity testing is not currently recommended outside the clinical trial setting (Samson et al., 2004; Schrag et al., 2004).

The tumour heterogeneity puzzle

Despite the considerable progress made up on cancer research field, to date the majority of patients does not seem to take advantage of current therapies. These phenomena could be explained by tumour heterogeneity (Marusyk et al., 2012). The intra-tumoral heterogeneity was first proposed in the 70’s and justified by the continuous selection of dominant clones and the differentiation of malignant stem cells, underling tumour progression and resistance to treatments.
The new treatment strategies based on advance technologies, always faster and cheaper, aim to characterize individual tumour types reflecting a precise genomic/proteomic profile and to couple them with the best available treatment.

To stress the heterogeneity concept Gerlinger et al. recently demonstrated that about two thirds of the mutations found in single biopsies of renal cell carcinoma were not uniformly distributed throughout all the sample regions within the same patient's tumour (Gerlinger et al., 2012).
A key role in the maintenance of tumour heterogeneity it is certainly played by the surround microenvironment. According to the evolution principle, cells bringing different mutations should converge towards a common phenotype, able to guarantee the cell growth within the tumour. The variable architecture of a tumour (vascularization, infiltration degree, and connective tissue components) allows the selective growth of cell types carrying different mutations.
Since the majority of cancer-related mortality is due to the metastasis formation, and since most of the therapeutic decisions are based on the primary tumour analysis, should be important to extend the phenotypic and genotypic analysis to the metastatic foci (Stoecklein and Klein, 2010). Indeed, although metastatic lesions are related to primary tumours, sometimes they can also carried additional mutations in functionally important loci completely absent in the primary site (Shah et al., 2009; Yachida et al., 2010).

Conclusions

In recent years there have been great efforts and have been collected a lot of information about the causes of the onset and progression of CRC, but not always it has been found a direct application in the clinical setting, or sometimes the expected results in patients do not reflect those obtained in vitro or in various stages of clinical trials.
The different cell populations within the tumour mass not only restricted to the primary site, as well as the dual role of the immune system against the tumour during its different evolution stages, and finally the diverse response to the treatment of patients, highlight the urgency to obtain a higher resolution microscopic (molecular) analysis of the disease, without giving up on a macroscopic view of the problem.
The future of cancer care lies surely in the individualized treatment of the disease, this approach requires, however, higher costs and dedicated and specialized staff, nowadays still under progress. It will be also necessary to develop faster and more reliable study models than those currently considered valid for the study of this evil. For all these reasons. . .please be ''patient''.

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Chapter| 6

Tumor and its microenvironment: a synergistic interplay

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Abstract

The mutual and interdependent interaction between tumor and its microenvironment is a crucial topic in cancer research. Recently, it was reported that targeting stromal events could improve efficacies of current therapeutics and prevent metastatic spreading. Tumor microenvironment is a "complex network" of different cell types, soluble factors, signaling molecules and extracellular matrix components, which orchestrate the fate of tumor progression. As by definition, Cancer Stem Cells (CSCs) are proposed to be the unique cell type able to maintain tumor mass and survive outside the primary tumor at metastatic sites. Being exposed to environmental stressors, including reactive oxygen species (ROS), CSCs have developed a GSH-dependent antioxidant system to improve ROS defense capability and acquire a malignant phenotype. Nevertheless, tumor progression is dependent on extracellular matrix remodeling, fibroblasts and macrophages activation in response to oxidative stress, as well as Epithelial Mesenchymal Transition (EMT)-inducing signals and endothelial and perivascular cells recruitment. Besides providing a survival advantage by inducing *de novo* angiogenesis, tumor-associated vessels contribute to successful dissemination by facilitating tumor cells entry into the circulatory system and driving the formation of pre-metastatic niche. In this review, we focus on the synergistic effect of Hypoxia Inducible Factors (HIFs) and Vascular Endothelial Growth Factors (VEGFs) in the successful outgrowth of metastasis, integrating therefore many of the emerging models and theories in the field.

Keywords: CSCs, tumor microenvironment, ROS, hypoxia, angiogenesis.

Abbreviations: Cancer Stem Cells (CSCs), Colorectal cancer (CRC), Epithelial Mesenchymal Transition (EMT), Extracellular Matrix (ECM), Reactive oxygen species (ROS), Matrix Metalloproteinase (MMPs), Cancer-associated fibroblasts (CAFs), Cancer-associated macrophages (CAMs), Reduced Glutathione (GSH), Hypoxia-Inducible Factor (HIF), Vascular Endothelial Growth Factor (VEGF).

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the world and one of the major causes of death world-wide [1]. The prevention and the early diagnosis are surely the most important approaches for reducing the burden of CRC, given the symptoms of early disease occur just in 5% of cases. A significant portion of patients who receive surgery and adjuvant therapy still develop recurrences and metastasis and this phenomenon seems to be driven in some cell subsets by the acquisition of resistance to conventional therapy, such as chemo- and radio-therapy [2].

Growing evidence indicates that a cellular subpopulation with stem cell like features, commonly referred to as cancer stem cells (CSCs), is critical for tumor generation and maintenance.

A recent study showed that within the tumor population it is possible to identify a heterogeneous population of cells with different biological roles [3]. Recent advances in stem cell biology are revealing that this cellular fraction shares many properties with normal adult stem cells, including dormancy (quiescence), active DNA repair machinery, the expression of several ABC drugs transporters and an intrinsic resistance to apoptosis [4]. As their normalcounterpart, the colon CSCs reside in a specialized microarchitectonic structures or niches that respond to both local and systemic conditions providing also protection against conventional therapies [5]. Moreover, microenvironmental stimuli, such as those involved in the epithelial-mesenchymal transition (EMT) and hypoxia, indirectly contribute to chemoresistance by inducing in cancer cells a stem like-phenotype. Understanding the driving force of tumor progression and the relationship between cancer cells and microenvironment could be fundamental in developing innovative therapeutic strategies for a better and definitive response onpatient treatments.

CRC, stem cell niche and colon CSCs

It is widely accepted that CRC progression is driven by the acquisition of 4–5 progressive mutations in oncogenes or tumor suppressor genes [6]. Some driver mutations frequently occur in the same gene sequences and are shared by most of the people affected by this cancer, whereas some mutations are different and responsible of the final cancer phenotype in individual patients [7]. Most of the information about CRC derives from the study of familial adenomatous polyposis (FAP), an autosomal dominant colon cancer syndrome caused by APC gene mutation [8]. APC is involved in the regulation of Wnt pathway that, as we will discuss later inthis review, can regulate cell proliferation, differentiation, migration and apoptosis [9]. Tumor progression is also achieved by other mutations such as KRAS, SMAD2/4, TP53 and deletion of chromosome 18q [10].

It was recently demonstrated that despite the great heterogeneity and biological diversity of CRC it is possible to distinguish three different subtypes. De Sousa et al. indeed showed that two of these subtypes have already been identified for chromosomal-instableand microsatellite-instable cancer. A third one, prognostically unfavorable, is characterized by microsatellite stability and relatively more CpG island methylator phenotype-positive, thus rendering it impossible to be identified on the basis of characteristic mutations [11].

The presence of a distinct population with stem cell characteristics among disseminated and circulating cancer cells may be of clinical relevance, not only for

their putative role in metastasis formation and recurrence, but also for their role in resistance to conventional therapy. CSCs are likely to share many properties of normal stem cells as mentioned above, which may underlie their capacity to survive therapeutic protocols based on genotoxic agents targeting actively proliferating cells [12].

First invoked by Paget, the "seed and soil" hypothesis suggests that the successful growth of metastatic cells depends on the interactions and properties of cancer cells (seeds) and their potential target organs (soil). Additionally, new concepts include: (i) the role of cancer stem-like cells as putative cells of metastatic origin (the"seeds"); (ii) the mechanism of EMT in driving epithelial cell into the blood stream to avoid anoikis, or anchorage independent cell death; and (iii) the reverse process of EMT, or mesenchymal to epithelial transition (MET), which promotes conversion back to the parent cell morphology and growth of macrometastasis in the target organ, open a new broad of aspect on this issue [13].

The microenvironment plays a crucial role in maintaining the pluripotency of colon SCs at the base of colon crypts influenced by fibroblast, endothelium and inflammatory cells, cytokines and growth factors secreted by these cells (in particular HGF) thus finely regulating the balance between self-renewal and differentiation ofthe staminal population [14–16]. The most characterized pathway involved in the maintenance of colon stem cells is Wnt [17–19], and it is clearly highlighted by the different expression of Wnt members along the colon crypt [20], even if the maintaining of stemness and the differentiation pattern is actually the result of the fine collaboration with other important pathways, such as PTEN-PI3K-Akt[21,22], BMP [23], Notch [24] and Sonic hedgehog (Shh) [25].

EMT, pre-metastatic niche and metastasis formation

Metastasis formation is considered a complex multi-step process with sequential molecular and cellular events that permit transformed cells to gain access to the blood stream (intravasation), survive their journey through the blood stream, and ultimately traverse through the microvasculature of target organs (extravasation) to deposit, survive, and grow in a foreign tissue environment.The EMT represents the first step of this highly regulated cascade and it is an important biological process initially studied in normal tissues during the organogenesis and then extended in the pathogenesis of cancer diseases, particularly referred to the acquisition of of migratory phenotype in CRC cells [26]. After extravasation from the circulation into the target organ, aberrant cells must implant, proliferate, and induce angiogenesis in order to survive and grow in a foreign and presumably "hostile" environment. These phenomena are driven not only by genetic and/or epigenetic alteration of cancer cells, but also by the non-neoplastic stromal cells [27].

The EMT is characterized by the loss of epithelial properties,including the apico-basal polarity and cell adhesion, the E-cadherin, occluding and cytokeratins expression, and at the same time the acquisition of N-cadherin, vimentin, fibronectin, Twist1, zinc-finger proteins (SNAIL, SLUG, ZEB) and matrix metalloproteinases(MMPs) expression, all events that lead to an increased cell mobility[28]. Moreover, EMT-inducing factors released by the surrounding microenvironment [29] can affect the invasive phenotype in epithelial malignancies initiation. Key regulators of this process are TGF-β (by the activation of Twist, SLUG and ZEB2), PI3K/Akt (increasing the mTOR kinase expression), Shh and Wnt [30,31].

Currently, dissemination and spread of cancer cells during the tumor progression are elective events underling the invasion through the tissue extracellular matrix (ECM). It was recently shown that tumor cells have two different modes of motility : (1) the acquisition of a mesenchymal phenotype, as previously described that identifies a mesenchymal motility mode and (2) the amoeboid migration [32]. The mesenchymal mode is characterized by the acquisition of an elongated morphology and activation of the small GTPase Rac [33]; the amoeboid motility is defined by a rounded or ellipsoid cell morphology and weak interactions with the surrounding matrix, driven by Rho expression, which induce membrane blebbing through Rho-associated protein kinase (ROCK)-dependent myosin II phosphorylation and consequent actomyosin contractility [34]. These two migration modes are interconvertible and regulated by microenvironmental influences. The possibility to switch from one mode to the other one highlights the cell plasticity that accomplishes movement from the primary tumor, establishment in an ectopic site, and survival therein [35].
The balance between high levels of activated Rac and Rho proteins regulates finely the motility mode. Moreover, Rac signalling inhibits amoeboid movement through its effector WASP-family verprolin-homologous protein 2 (WAVE2), and in the same wayRho/ROCK suppresses Rac by the activation of ARHGAP22, aGTPase-activating protein (GAP) [36].
Although RHO gene mutations are extremely rare, their altered expression has been assessed in many human cancers, including CRC. In particular, RhoA is frequently overexpressed and its induction is rapidly mediated by TGF- β [37], while depletion of Rac1 strongly correlates with the inhibition of lamellipodia formation,cell migration and invasion in carcinoma cells [38].
Furthermore, recent study established the independent contribution of KRAS and BRAF mutations, which rarely co-exist inhuman tumors, to migration and invasion of CRC cells through RhoGTPases signaling. Although KRAS and BRAF are common members of the same pathway, Makrodouli et al. showed that BRAF mutation enhances cell migration through RhoA activation, and its effect is more pronounced compared to KRAS. These findings are expected to eventually result in tailor-made therapies against Rho pathway components, since it depends on the genetic background of the cancer patient [39].

Status redox and hypoxia: two sides of the same coin

In the absence of aberrant microenvironmental stimuli, genetic and epigenetic alterations in tumor cells are insufficient to induce primary tumor progression [27]. Either through structure and function-based mechanisms, including ECM remodeling, release of cytokines and growth factors, metabolic changes, or activation of stromal components, microenvironment enables tumor cells to achieve an aggressive phenotype [32].
As observed, reactive oxygen species (ROS) have emerged as an important factor affecting several cancer hallmarks. ROS are involved in the acquisition of self-sufficiency in proliferation signals by a ligand-independent receptor tyrosine kinase transactivation as well as loss of contact inhibition and anchorage-dependence cell growth. The development of a more aggressive phenotype is also promoted by ROS through MMPs secretion, EMT program activation, Met overexpression and regulation of cellular plasticity induced by the Rac1/RhoA antagonism [40, 41]. Moreover, ROS sustain de novo angiogenesis by inducing the recruitment of perivascular cells and the activation of endothelial progenitors through the vascular

endothelial growth factor (VEGF) and angiopoietin (Ang) release. Besides being involved in evading apoptosis by the activation of survival pathways, specifically PI3K/AKT, NF-kB, and anoikis resistance, ROS increase the sensibility to mutagenic agents and help escape from the immune surveillance system [42].
Oxidative stress can derive from either extrinsic or intrinsic source (Fig. 1).

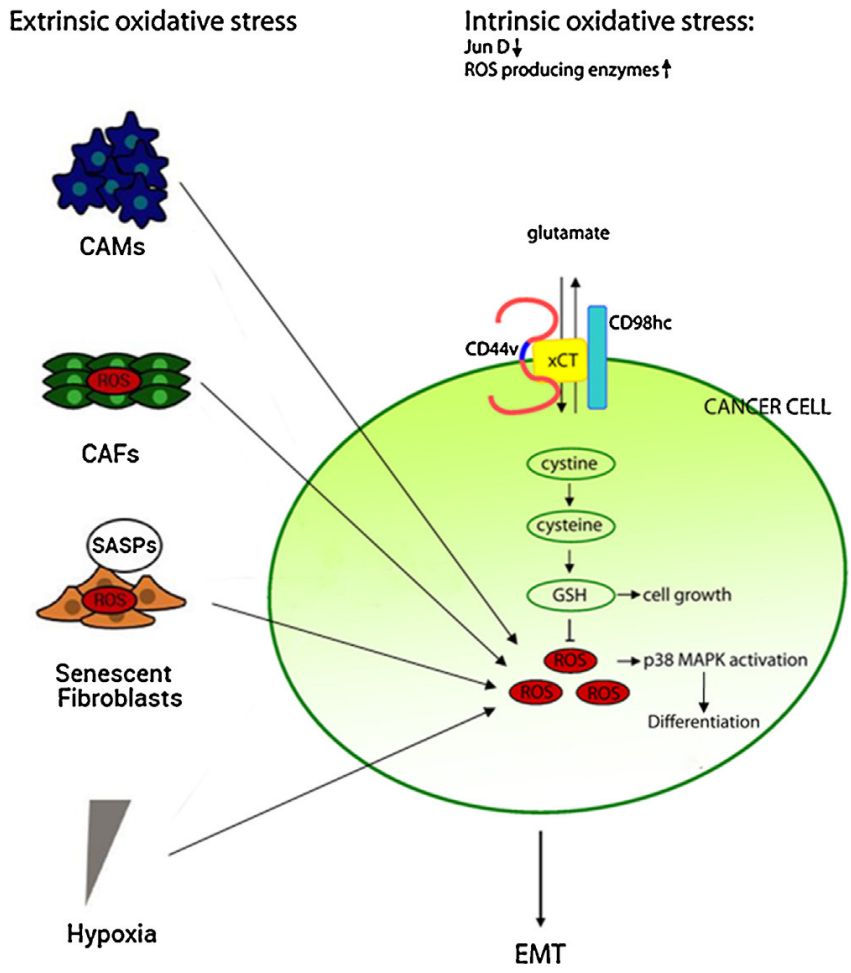

Fig. 1

Extrinsic and intrinsic production of oxidative stress. CAMs and hypoxiainduce a pro-oxidant environment, mandatory for CAF activation and senescent fibroblasts conversion into pro-inflammatory cells, affecting in turn EMT of cancer cells. Due to Jun D downregulation and increased activity of ROS-producing enzymes, cancer cells exacerbate the production of oxygen radicals. CD44v stabilizes the subunit xCT at the plasma membrane by promoting GSH synthesis and tumor growth. Cancer-associated macrophages (CAMs), cancer-associated fibro-blasts (CAFs), senescent activated secretory pathways (SASPs), reactive oxygen species (ROS), reduced glutathione (GSH), CD44 variant (CD44v), the light-chain subunit of cystine–glutamate antiporter system xc(-) (xCT), epithelial mesenchymal transition (EMT).

Cancer-associated-fibroblasts (CAFs) or -macrophages (CAMs) synergize in the induction of a pro-oxidantenvironment. Due to the activation of Nitric Oxide Synthase 2(NOX2), CAMs can directly produce ROS resulting in CAFs recruitment and MMPs activation [43]. Moreover, by secreting the master pro-inflammatory cytokine TNF α, CAMs prime the NF-kB activation in both stromal and cancer cells, which in turn up-

regulates SNAI1 expression [44]. In response to intrinsic and extrinsic oxidative stress, CAFs support tumor growth and promote EMT changesin cancer cells by secreting growth factors and ECM degrading proteases. Moreover, their production of extracellular matrix proteins promotes the recruitment of endothelial precursor cells from bone marrow [45]. Aging-induced oxidative stress concurs to transform fibroblasts into pro-inflammatory cells and induce an EMT program in the neighboring epithelial cells by secreting the so-called senescent activated secretory pathways (SASP) factors, which include pro-inflammatory cytokines and MMPs [46]. Klimova et al. demonstrated that hypoxia also improves ROS generation by deregulation of the mitochondrial complex III resulting in ROS release into thecytosol [47].

Interestingly, TGF-β has been correlated to redox control of EMT, either directly by the activation of MAPK or indirectly by ERK-mediated Smad 2 phosphorylation. As shown by Rhyu et al., in renal tubular epithelial cells, TGF-β 1 stimulation induces E-cadherin loss, α-SMA and fibronectin up-regulation. These EMT-related molecular events are prevented by the inhibition of both NADPH oxidase (NOXes) and mitochondrial electron transfer chain subunit I, suggesting that NOXes and mitochondrial metabolism are important sources of TGF-β -induced cellular ROS [48]. Similarly, Zhang et al. identified ferritin heavy chain (FHC) as a critical modulator of TGF-β -induced EMT. By repressing the synthesis of FHC, a cellular iron storage protein, TGF-β promotes iron release and subsequent increase in the intracellular labile iron pool (LIP), which is associated with redox-mediated activation of p38MAPK. Thus, FHC overexpression abrogates TGF-β -induced LIP increase resulting in ROS elimination and EMT suppression [49].

Cancer cells exacerbate the oxidant microenvironment by enhanced basal metabolic activity through aberrant growth factors and cytokines signaling as well as increased activity of ROS-producing enzymes, such as NOXes, cyclooxygenase (COXes) or lipoxygenases (LOXes) [50]. Moreover, high levels of ROS may result from down-regulation of Jun D, a transcriptional activator of FHC that is known to minimize LIP-dependent ROS generation [51]. To protect themselves from oxidative stress, cancer cells develop adaptation strategies, including increased expression of scavenger anti-oxidative enzymes and pro-survival molecules. Particularly, reduced glutathione (GSH) is the major intracellular antioxidant factor by reducing the ROS levels and suppressing ROS-dependent activation of p38MAPK. Ishimoto et al. demonstrated that in gastrointestinal cancer cells a CD44 variant (CD44v) maintains high levels of GSH by stabilizing the xCT expression at the plasma membrane. xCT is the light-chain subunit of cystine–glutamate antiporter system xc(-), which exchanges extracellular cystine uptake for intracellular glutamate, thereby promoting GSH synthesis (Fig. 1). At first, glutamate–cysteine ligase couples glutamate and cysteine to form γ-glutamylcysteine. Glutatione synthetase then catalyzes the formation of GSH fromglycine and γ -glutamylcysteine. Since cysteine availability is a rate-limiting factor for GSH synthesis, CD44-mediated stabilization of xCT plays a key role in the GSH-dependent antioxidant system, promoting the proliferation of cancer cells and the formation of lethal gastrointestinal tumors. This is supported by the observation that CD44 depletion reduces the number of proliferating tumor progenitor cells and inhibits gastric tumor development in Gan (Gastric Neoplasia) mice through the ROS-dependent p38MAPK activation and p21CIP1/WAF1 upregulation. The antioxidant potential of gastric cancer cells confers resistance to ROS-inducing anticancer drugs, such as cisplatin and docetaxel. Consistently, in an HCT116 xenograft model, the specific xCT inhibitor sulfasalazine suppresses CD44-dependent

tumor growth in parallel with the activation of p38MAPK, suggesting that the suppression of xCT by sulfasalazine might impair the ROS defense ability of CD44v-expressing CSCs and improve the efficacy of currently available treatments [52] (Fig. 2).

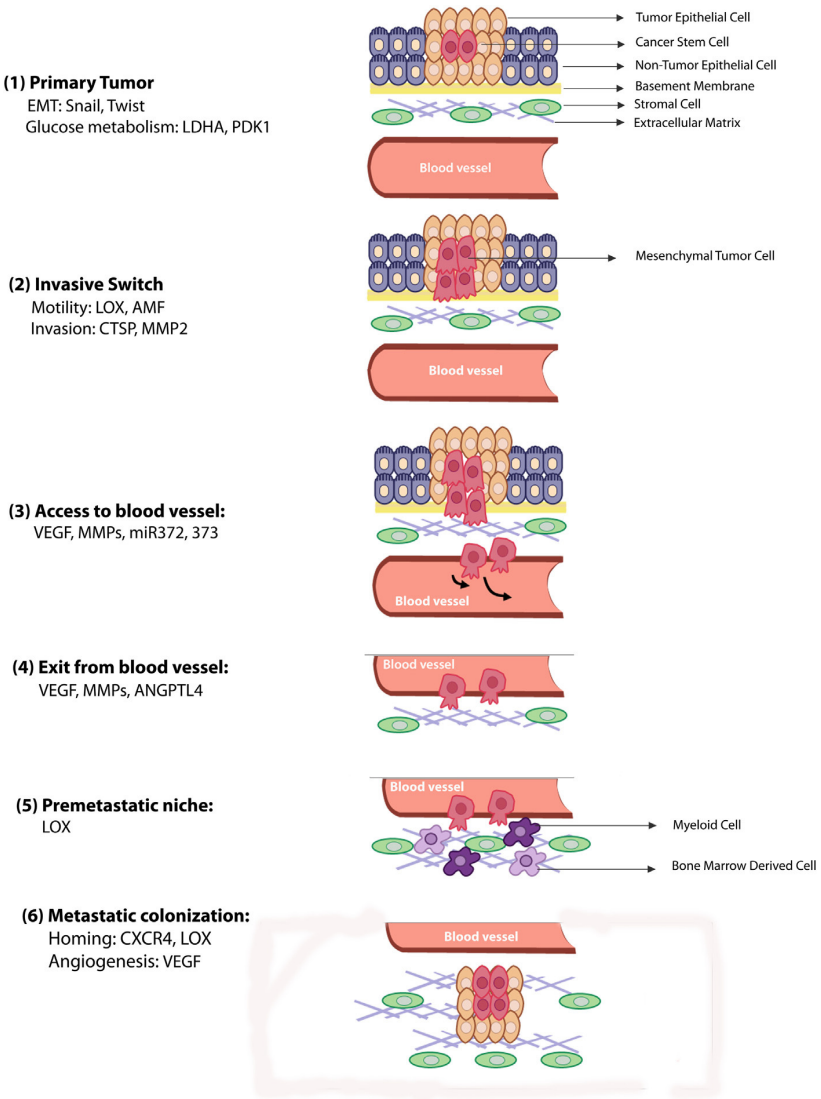

Fig. 2

Regulatory functions of hypoxia in different steps of metastasis. (1) During primary tumor growth, hypoxia acts as inductor of "glycolytic" phenotype and executor of EMT. (2) Under hypoxia, tumor cells gain an improvement in motility and invasion capacity, facilitating thereby detachment and dissemination from the primary site. (3 and 4) Increased expression of VEGF and MMPs induced by hypoxia is critical to penetrate the vasculature and promote the subsequent exit. (5) By the recruitment of bone marrow-derived cells and $CD11b^{+}$ myeloid cells to secondary organs, LOX secreted by hypoxic tumor cells forms the premetastatic niche. (6) Hypoxia-dependent induction of CXCR4 and angiogenesis contribute to the successful metastatic colonization. Epithelial mesenchymal transition (EMT), lactate dehydrogenase A (LDHA), pyruvate dehydrogenasekinase 1 (PDK1), Lysyl oxidase (LOX), autocrine motility factor (AMF), cathepsin D (CTSD), matrix metalloproteinase (MMPs), vascular endothelial growth factor (VEGF), angiopoietin-like 4 (ANGPTL4).

CD44 and its variant isoforms have already been identified as tumor metastasis-associated proteins. Ectopic expression of CD44v6 splice variant confers metastatic potential to non metastatic tumor cell lines, promoting Met activation by its ligand HGF that is mainly secreted by mesenchymal cells [53]. The importance of the CD44v6 and Met multimeric signaling in cancer progression has been strengthened by the observation that adenoma growth in the $Apc^{Min/+}$ mice model was reduced by inhibiting the CD44v6 expression through short hairpin RNA/nanoparticles technology [54]. Moreover, Jung et al. showed that CD44v6 supports tumor cell migration and apoptosis resistance since only the matrix assembled by CD44v6-competent but not-deficient cells induces metastasis formation [55]. Given that disseminating cells are exposed to high levels of ROS during tumor progression, metastatic growth requires also adequate ROS defense ability to successfully colonize secondary sites. Interestingly, knockdown of the redox protein thioredoxin-like 2 has been reported to inhibit tumorigenesis and metastasis of human breast cancer cell lines upon transplantation into immunodeficient mice by enhancing ROS levels and reducing NF- kB activity [56]. It has also been investigated the role of CD44v-xCT in lung metastasis. By pro-moting xCT-dependent GSH synthesis, CD44 expression allows mouse 4T1 breast cancer cells to evade high levels of ROS produced by neutrophils and colonize the lung. It is not surprising that knockdown of epithelial splicing regulatory protein 1 in $CD44^+$ subpopulation induces an isoform switch from CD44v toCD44s, resulting in reduced xCT expression and lung metastasis suppression [57].

Proliferating tumor cells distance themselves from the vasculature and colonize an environment deficient in oxygenand nutrients. Therefore, tumor cells need to reprogram their metabolism by increasing glycolytic activity and decreasing aerobic respiration rate. This shift is mediated by an increase in ROS levels generated by mitochondrial complex III, which accounts for hypoxia-inducible factor-1 (HIF-1) stabilization via oxidation/inactivation of prolyl hydroxylases and release from VonHipper-Lindau (VHL)-mediated degradation. When stabilized inhypoxia, HIF-1 α dimerizes with HIF-1 β and translocates into thenucleus. By interacting with the co-activators CBP/p300, the α/β heterodimer HIF-1, bound to hypoxia-response elements (HREs) in target genes, mediates the expression of proteins involved in the formation of new vasculature and metabolic adaptation to hypoxia [58]. HIF-1 α increases the transcription of glucose transporters and glycolytic enzymes as well as lactate dehydrogenase A (LDHA) and pyruvate dehydrogenase kinase 1 (PDK1), resulting in the diversion of pyruvate toward lactate away from mitochondrial oxidative phosphorylation [59]. Additionally, mutations of tumor suppressor genes (PTEN, VHL) and oncogenic pathways (Ras/MAPK, PI3K-Akt) converge on HIF-1 α activation through an oxygen-independent mechanism [58]. Specifically, in CRC hypoxia activation of wild-type K-Ras mediates Akt phosphorylation and resistance to apoptosis [60]. Similar to HIF-1 α, HIF-2 α is involved in the regulation of hypoxia tumor response. Interestingly, Heddleston et al. reported a role of HIF2 α in reprogramming non-stem cancer cells toward a stem-like phenotype by inducing the expression of key stem cell genes, like OCT4, NANOG and MYC. Concordantly, overexpression of HIF-2 α in glioma non-stem cells increased neurospheres formationand tumorigenic capacity [61]. Moreover, as shown by Xue et al., HIF2 α activation modulates colon tumorigenesis in $Apc^{Min/+}$ mice by overexpression of intestinal iron transport. The resulting iron intake contributes to dysregulation of local iron homeostasis, which in turn affects cancer progression through increasing cell survival and proliferation [62]. Hypoxia has been reported as an important driving

force for the multistep process of metastasis. The early EMT-related events induced by hypoxia support ROS-dependent GSK-3 β inactivation, followed by SNAIL nuclear translocation and E-cadherin loss [63, 64]. In response to hypoxic conditions, Notch signalling up-regulates Snail expression by two distinct but synergistic mechanisms, involving both direct transcriptional activation of SNAI1[65] and an indirect mechanism operating via the ECM protein lysyl oxidase (LOX) [66]. Moreover, Twist expression, directly induced by HIF-1 α through the HRE located in its promoter, contributes to cadherin profile changes with E-cadherin down-regulation followed by N-cadherin upregulation [64]. At a later stage, activation of Wnt/ β-catenin pathway and increased invasiveness are sustained by HIF-1 α- and VEGF-dependent events [63]. Particularly, hypoxia-induced invasion is associated with basement membrane degradation and ECM remodeling by upregulation of cathepsin D (CTSD) and MMP2 [58, 67]. Hongo et al. proposed that the up-regulation of β1 integrin expression by hypoxia in CRC cells increases the ability to adhere and migrate on collagen fibers [68].The role of HIF-1 α in cell migration is related to improved LOX expression. In hypoxic cancer cells, LOX mediates the covalent cross-linking of collagen fibers and elastin, thereby increasing cell focal adhesion kinase activity, known to induce cell motility by acting as a signal between integrins and actin cytoskeleton.These remodeled matrix events are essential for invasive cell movement and provide a metastasis freeway by which other tumor cells may walk and spread to adjacent tissues [69]. Hypoxia-induced “invasive switch” is also mimicked by Met and autocrine motility factor (AMF) overexpression. Pennacchietti et al. demonstrated that hypoxia synergizes with HGF to affect basal cell morphology and induce cell scattering by transcriptional activation of the MET proto-oncogene. Consistently, increased Met expression sensitizes tumor cells to HGF produced by fibroblasts, promoting thereby the invasive growth toward tissue parenchyma and blood circulation [70]. One of the most important tumor-secreted cytokines, AMF promotes resistance to apoptosis in tumor cells and angiogenesis induction via autocrine and paracrine mechanisms [71]. Hypoxia-selected tumor cells are able to evade the hostile milieu of primary site by promoting angiogenesis and affecting vascular integrity and permeability. Consistently, hypoxia-dependent expression of VEGF, MMP1 and MMP2 is essential to offend the vasculature and promote intravasation. MiR-372/373, upregulated in response to hypoxia through HIF-1 α, contributes to increased intravasation by targeting the MMP inhibitory protein RECK, resulting in excessive activation of MMPs [72]. Besides VEGF, MMP1 and MMP2, tumor cells extravasation is promoted by Angiopoietin-like4 (ANGPTL4), a member of vascular regulators angiopoietin family upregulated in the primary tumor by both TGF β and hypoxia [58]. As shown by Padua et al., the expression of ANGPTL4 in cancer cells primes these cells to disrupt vascular endothelial tight junctions and increase the capillary permeability, thereby affecting thetransendothelial passage [73].Recent reports suggested that the metastatic seeding at distant organs is influenced by hypoxia-induced factors released from primary tumor, critical for pre-metastatic niche formation. It has been reported that in breast cancer LOX, secreted by hypoxic tumor cells into the bloodstream, modifies the collagen cross-linking in the lungs and promotes the recruitment of $CD11b^+$ myeloid cells to pre-metastatic sites. By the consequent adhesion to cross-linked matrix, $CD11b^+$ myeloid cells produce MMP-2, which supports collagen remodeling by LOX and thereby increases recruitment and subsequent invasion of bone marrow-derived cells. This cell population is thought to create a favorable environment for the incoming primary tumor cells [69]. Hypoxia in primary tumor may also improve metastatic seeding of

tumor cells by heightening chemokine C-X-C motif receptor4 (CXCR4) expression. Specifically, CXCR4-mediated signal transduction can enable tumor cells to home to secondary organs where its ligand Stromal Derived Factor 1 (SDF1) is highly expressed (e.g., lymph nodes, lungs, liver, or bones). The responsiveness of $CXCR4^+$ cells to SDF-1 gradient is positively affected by several molecules produced during inflammation, specifically fibrinogen, fibronectin, C3a, and hyaluronic acid, suggesting that inflammation affects the spreading of $CXCR4^+$ tumor cells [74]. Similarly to primary tumor, hypoxia response molecules facilitate tumor–stromal interactions in secondary sites to support the metastasis colonies proliferation. However, the role of hypoxiain determining the organ-specific metastasis is still unknown. Microarray profiling revealed that hypoxia promotes the expression of lung-metastasis gene signature, including connective tissue growth factor, Osteopontin, IL-6 and -8, ANGPTL4, and primes ER−breast cancer cells in promoting lung colonization by activating an effective angiogenesis. Since bone marrow vasculature is already fenestrated facilitating the trans-endothelial passage of tumor cells, hypoxia-induced angiogenesis does not provide an advantage for bone metastasis seeding. Thus, it is not surprising that hypoxia activates a limited percentage of bone-metastasis genes, including CXCR4 and dual specificity phosphatase 1, which functions as a stress-inducible MAPK signaling activator [58,75]. Interestingly, experimental models and human cancers implicated TFG β in promoting distal metastasis formation. After seeding the lung parenchyma, ER−breast cancer cells take a proliferative advantage from local TGF β through induction of the cell differentiation inhibitor ID1 [76]. As shown by Kakonem et al., in mice inoculated by MDA-MB-231 breast cancer cells, osteolytic bone metastases require the recruitment and activation of osteoclasts. In particular, induction of IL-11 and parathyroid hormone-related protein production by TGF β promotes differentiation of osteoclast precursors and bone resorption, thereby increasing the osteoblastic expression of Receptor Activator for NF- kB (RANK) ligand [77]. Lastly, Batlleet al. speculated that IL-11, a TGF β-target gene in stromal cells,confers metastatic initiation capacity to CRC cells via GP130/STAT3 signaling, critical to induce a survival advantage and suppress apoptotic stimuli in metastatic sites [78].

CSCs and vasculature cells crosstalk: a mutualconvenience

Tumor cell growth and nurture require several strategies to supply the oxygen and metabolic demand, all involving new vessels formation and captivation from the surrounding stroma. Tumor neo vascularization can occur through (a) sprouting from existing vessels (sprouting angiogenesis), (b) lumen invagination and splitting of vessels (intussusceptive angiogenesis), (c) enfolding of vessels by cancer cells (vessel co-option), (d) simulation of endothelial features by tumor cells (vasculogenic mimicry), (e) formation of lymphatic vessels from pre-existing ones (lymphangigogenesis) and finally (f) endothelial progenitor cells recruitment [79].
Angiogenesis has been defined as a key process for tumor and metastasis formation and CSCs are predicted to be strong promoters of this phenomenon. For instance, Bao et al. demonstrated a profound interplay between CSCs and tumor vasculature. Injection of glioblastoma stem cells (GSCs) $CD133^+$ in the right frontal lobes of athymic nude mice displays strongly angiogenic and hemorrhagic tumors compared to the $CD133^-$ counterpart. The angiogenic advantage of the $CD133^+$ fraction may be supported by a 10–20 fold increase of VEGF secretion. Significantly, conditioned medium from these fractions fosters human endothelial cells migration and tube

formation [80]. According to these data, the concomitant presence of CSCs correlates with more angiogenic tumors in terms of enhanced resident endothelial cells function and recruitment of bone marrow-derived endothelial progenitors to the tumor site. VEGF and SDF1 are the main powering determinant of these CSCsproperties [81].
On the other hand, it is likely conceivable a possible impact of endothelial cells on CSCs state. A paracrine signaling by endothelial cells may induce CRC cells to gain CSC properties with Notch pathway as the main player of this conversion. Indeed, Jagged-1, a Notch-activating ligand, is released from endothelial cells as a soluble form by ADAM17 proteolitic cleavage and its binding to Notch receptor of adjacent CRC cell triggers the onset of stem-like features. Co-culturing CRC cells either with endothelial cancer cells or with endothelial cell-conditioned medium leads to an increase of the $CD133^+/ALDH^+$ subpopulation compartment and sphere forming capability as well as in vivo tumor growth and spreading [82]. Similarly, as showed by Calabrese et al., it was demonstrated that endothelial-derived factors support self-renewing of brain tumor cells and keep them in an undifferentiated state. The sestem-like cells closely interact with $CD34^+$ capillaries and are strictly dependent on microvasculature density. Co-injection of primary human endothelial cells and $CD133^+$ medulloblastoma cells accelerates initiation and promotion of brain tumor xenografts by expanding the CSCs pool. Thus, tumor microenvironment orchestrates a vascular niche formation determining the CSCs fate [83]. Furthermore, the presence of 'mosaic' blood vessels in which both endothelial and tumor cells are located into the lumen surface of tumor vessels has long been described [84]. Consistent with these findings, glioblastoma stem cells can be induced to differentiate into endothelial cells and directly contribute to tumor vasculature architecture when injected in immunocompromised mice, as proven by the presence of $CD34^+/CD144^+/VEGFR2^+$ human-derived derived endothelial cells [85]. Likewise, vasculogenic mimicry can occur viaa multipotent intermediate ($CD133^+/CD144^+$) that can differentiate either into a tumoral or endothelial phenotype [86]. Another related possibility is that, rather than differentiation into endothelial lineage, CSCs generate vascular pericytes that mainly support endothelial cells to maintain vessels function and integrity. It was recently shown that, after GSC differentiation induction, a fraction of 4–11% cells expressed several pericyte markers such as α-SMA, NG2, CD146 and CD248. Significantly,in vivo cell lineage tracing with specific fluorescent reporter confirmed that the majority of pericytes had GSC origin. Of note, selective deletion of GSC-derived pericytes hampered microvessel development and tumor growth. CXCR4 expressing GSCs were recruited toward epithelial cells by an SDF-1 chemoattractant gradient and then induced to pericytes differentiation upon TGF-β release by endothelial cells [87].

Angiogenic pathways orchestrate CSCs survival andmotility

Although CSCs represent a minority of tumor cells population, deregulation of pathways involved in cell self-renewal and motility contributes to cancer conversion and promotion. In addition to well established CSCs radioresistance and chemoresistance mechanisms, an increasing adaptability to antiangiogenic treatment was shown [88]. These cells can elicit resistance and increase their tumorigenic and invasive potential by exploiting an hypoxic microenvironment [89] as well as the activation of an anti-apoptotic program [88] (Fig. 3).

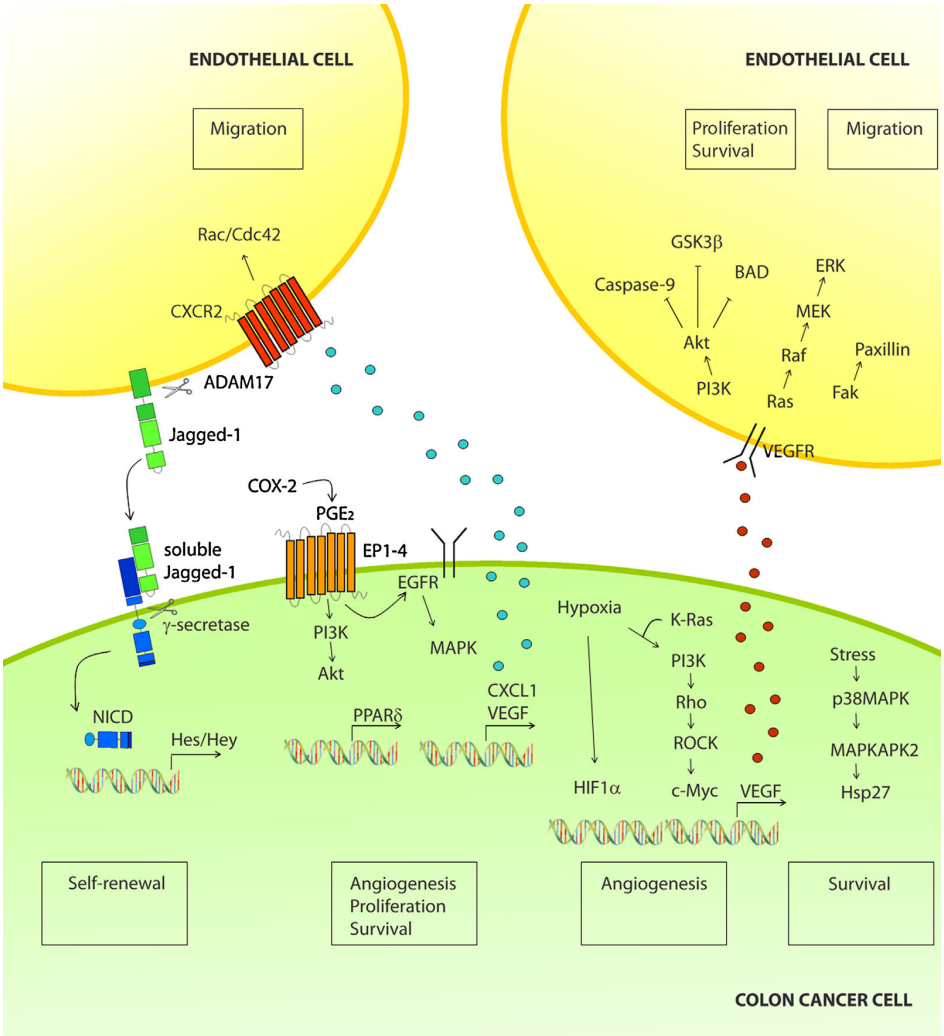

Fig. 3

Tumor microenvironment is conducive to angiogenesis promotion. A truncated soluble form of Jagged-1 is released by endothelial cells and its binding to Notch receptor on nearby colon cancer cells promotes a stem-like phenotype. PGE2 mediates the release of the angiogenic factors CXCL1 and VEGF in colon cancer cells, via an EP1-4/EGFR/MAPK cascade. CXCL1 secretion stimulates endothelial cell migration by CXCR2 binding and Rac/Cdc42 pathway activation. Furthermore, PGE2 induces colon cancer cell proliferation and survival trough PI3K/Akt signaling and transcriptional activation of PPAR δ. Under hypoxic conditions, induction of HIF1 α and alternative K-Ras pathways results in further VEGF release from cancer cells. In endothelial cells, VEGF/VEGFR interaction promotes cell proliferation, survival and migration via PI3K, Ras and FAK pathways. Finally, activation of pro-survival signals in tumoral cells is triggered by microenvironmental stress and p38MAPK, MAPKAPK2 and Hsp27 cascade. Notch intracellular domain (NICD), prostaglandin E2 (PGE2), chemochine C-X-C motif ligand 1 (CXCL1), prostaglandin E receptor 1–4 (EP1-4), epidermal growth factor receptor (EGFR), mitogen-activated protein kinase (MAPK), chemochine C-X-C motif receptor 2 (CXCR2), cell division control protein 42 (Cdc42), perixisome proliferator-activated receptor δ (PPAR δ), Rho-associated protein kinase (ROCK), MAP kinase-activated protein kinase 2 (MAPKAPK2), Heat shock protein 27 (Hsp27).

Among molecules that regulate tumor angiogenesis, such as plateled-derived growth factor (PDGF), FGF, HGF and TGF- α/β,VEGFs and their cognate receptors

(VEGFRs) are the driving force of angiogenic response due to their specific expression on endothelial and tumoral cells, resulting in multiple signal pathways activation.VEGF family is represented by five members (VEGFA, VEGFB,VEGFC, VEGFD and placental growth factor [PGF]) coupled with three tyrosine kinase receptors (VEGFR1 [Flt1], VEGFR2 [KDR/Flk1]and VEGFR3 [Flt4]). As a soluble factor, VEGF serum concentration, in preoperative CRC, reflects the stage and correlates with disease progression. Both VEGFs and VEGFR2 are associated with a worse prognosis, tumor spreading and enhanced microvessel density. Particularly, VEGF expression increases during the colonic adenoma–adenocarcinoma pathogenesis conversion and prior to the invasive phenotype switch [90].VEGFR1 is mostly expressed on endothelial cells, monocytes,macrophages, hematopoietic stem cells and some tumoral cells,including CRC cells [91]. VEGFB and PGF have been identified as its exclusive ligands. VEGFR2 is not restricted to endothelial cells but it is also shared by, for example, colitis-associated colon cancer epithelial cells [92] and GSCs [93]. Furthermore, VEGFR3, the first normal lymphatic endothelium marker [94], together with VEGFC is involved in cancer lymphangiogenesis [95].
VEGFA/VEGFR2 interaction is recognized as a potent pro angiogenic stimulus increasing survival, proliferation, migration, and vascular permeability of endothelial cells [96]. Although VEGFA has a higher binding affinity for VEGFR1, VEGFR2 possesses a greater tyrosine kinases activity that governs the activation of MAP-kinase, PI3K, Fak and Rac pathways. Interestingly, phosphorilation of p38MAPK, in colon CSCs, protects them from antiangiogenic treatment through the activation of Heat shock protein 27 (Hsp27)[88]. Hypoxic induction of VEGF is not merely dependent on HIF-1 α. It was already reported that CRC cells are forced to express VEGF through a K-Ras/PI3K/Rho/ROCK/c-Myc axis. Indeed, a putative Myc-Max binding site was found on VEGF gene promoter [97].
It was extensively observed that Prostaglandin E2 (PGE2) is abundantly secreted by both colon cancer cells and stromal cells and promotes the release of the angiogenic factors C-X-C motif ligand 1 (CXCL1) and VEGF through the Prostaglandin E receptor 1–4 (EP1-4)/Epidermal growth factor receptor (EGFR)/MAPK cascade. Tumor-derived CXCL1 stimulates endothelial cell migration and in vivo tumor growth and microvessels density by CXCR2 binding and Rac/Cdc42 pathway activation. Furthermore, PGE2, via PI3K/Akt signaling, enhances transcriptional activation of Perixisome proliferator-activated receptor δ (PPAR δ) required for colorectal adenoma growth [98, 99].The angiogenic properties of VEGF may be amplified when tumoral endothelium is previously destabilized by other growth factors, such as Ang-2. Ang-1, 2 and 4 bind the same endothelial receptor Tie2. While Ang-1 is expressed by pericytes, smooth muscle cells and tumor cells, Ang-2 is exclusive to endothelial cells. Ang1 preserves vascular integrity by reducing cell-to-cell gapswhereas Ang2 increases pericytes dissociation and vessels destabilization, rendering endothelial cells more receptive to foreign stimuli, for instance, VEGF [100]. A broad spectrum of clinical data reports that activating KRAS mutations could occur up to 50% of early stages CRC patients [101]. Interaction of Ras with the catalytic subunit p110 of PI3K appears to be extremely relevant to the induction of VEGF gene expression. PI3K phosphorylates Akt, which subsequently inhibits GSK-3 β leading to β-catenin nuclear translocation. Mutated KRAS enhancesthe stability of β-catenin and promotes the formation of nuclear β-catenin/TCF4 complexes [102]. In addition, further evidence ofa cooperative interaction between K-Ras and Wnt pathway in CRC lies in the presence of a consensus TCF4 element in the VEGF

promoter [103]. At the early onset of colon neoplastic lesion, a crosstalk between Ras and the microenvironment has been described.
Particularly, RAS oncogene can orchestrate endothelial and inflammatory cells recruitment to the tumor site in an IL-8-dependent manner [104]. On the other hand, as previously mentioned, in wildtype KRAS CRC and in presence of a hypoxic microenvironment, VEGF expression is strictly regulated by Akt and c-Src pathways [60]. Entirely conflicting with other Ras oncoprotein features, R-Ras is described as a supporter of tumor vessels normalization by counteracting VEGF angiogenic potential. Tumor vasculature differs from the normal counterpart for the presence of saccular, tortuous and high permeable vessels with fibrin-gel matrix deposition. Pericytes are poorly associated with endothelial cells supported by an irregular basement membrane. Vessel leakiness allows cancer cells to easily penetrate into the bloodstream and thus colonize distant organs. In addition, plasma leakage from vessels, due to an higher interstitial hydrostatic pressure at the tumor site, reduces the delivery of chemotherapeutic agent [105]. However, R-Ras does not affect the oxygen-sensing mechanism of vessel normalization exerted by PHD2 or HIF-2 α under hypoxic condition. Conversely, it facilitates the accumulation of VE-cadherin on cell-to-cell junction, favoring the stabilization of the endothelial barrier. Indeed, it reduces phosphorylation of Ser665 in the cytoplasmic domain of VE-cadherin, suppressing its internalization on endothelial cells. Interestingly, this phenomenon antagonizes VEGF-mediated VE-cadherin phosphorylation. Furthermore, R-Ras activity in pericytes increases their interaction with endothelial cells, leading to normal vessels morphogenesis [106]. Based on this observation, antiangiogenic therapies may contribute to the normalization of tumor vasculature architecture and consequently improve their distribution and efficacy [107]. Finally, the BMPs pathway was observed aberrantly regulated in the majority of sporadic CRC and germline mutation on BMP receptors and downstream substrates were detected in juvenile polyposis [108]. Furthermore, BMP signaling has been shown to be essential in human intestinal development and regeneration regulating also the number and the self-renewal state of colonic stem cells [109]. To date, little is known about BMPs role in angiogenesis. Recently, BMP9 was identified as a ligand of the orphan Activin receptor-like Kinase 1 (Alk1) in endothelial cells and the resulting interaction affects several angiogenic steps. BMP9/Alk1 signaling counteracts bFGF-stimulated endothelial cells proliferation and migration as well as VEGF-induced angiogenesis. Indeed, BMP9/Alk1/BMP receptor II (BMPRII) complex abolished VEGF expression through suppression of TGFβAlk5/BMPRII signalling [110]. Certainly, further investigations are needed to identify the underlying mechanism of BMP engagement during angiogenesis promotion.

Therapeutic advances

Quiescent cells within the stemness niche have been associated with tumor recurrence and relapse after chemotherapy. Targeting the molecular mediators and signaling pathways affecting EMT and tumor progression may provide novel therapeutic strategies to prevent CSCs-dependent distant metastasis formation. Fighting neovascularization to counteract cancer promotion is a crucial step of the long-standing theory of Folkman [111]. Based on this hypothesis, the first antiangiogenic compound approved by the FDA, in 2004, was Bevacizumab. It is a monoclonal antibody againstVEGF recommended in first and second line settings, either with FOLFOX (5-Fluorouracil, Leucovorin and Oxaliplatin) or FOLFIRI (5-Fluorouracil,

Leucovorin and Irinotecan). As shown by preclinical data, Aflibercept is a VEGFA, VEGFB and Placental growth factor (PIGF) decoy receptor, composed of VEGFR1 and VEGFR2 extra-cellular domains fused to the constant portion of immunoglobulin gamma chain. In 2012, FDA approved the administration of this this compound plus FOLFIRI in patients with metastatic CRC with disease progression after oxaliplatin treatment. Recently, advanced clinical trials validate the efficacy of Regorafenib as a VEGFR1/2/3 and Tie2 tyrosine kinase inhibitor [112]. Despite initial therapeutic benefits in patients with metastatic CRC, classic antiangiogenic strategies failed to improve long-term clinical outcomes [113]. Since new development of tumor vasculature implies several complex signaling, alternative angiogenic or anti-apoptotic mechanism could be devised by cancerous cells [88]. Indeed, it has been recently pointed out, by Lu et al., that glioblastoma multiforme treatment with Bevacizumab developed more invasive tumors, as the blockade of VEGF enhances HGF-induced MET phosphorylation [114]. Another attractive approach takes into account that anti-angiogenic treatments favor a hypoxic microenvironment that gives to CSCs population a metabolic advantage and preserves their self-renewal state [89]. Given that anti-angiogenic drugs may enhance tumor invasiveness by blocking de novo angiogenesis and inducing hypoxia, the development of HIF-1 α targeted therapies may reduce or prevent metastasis [58]. There are several agents that affect directlyor indirectly the HIF-1 α expression or activity. The binding of HIF-1 α to the co-activator p300/CBP has been attenuated by the chetomin, a small molecule that interferes with hypoxia-inducible transcription [115]. In addition, the proteasome inhibitor bortezomib, approved for treatment of patients with multiple myeloma and mantle cell lymphoma, impairs the interaction with the co-activator p300/CBP by inducing the hydroxylation of Asn803 in the C-terminal transactivation domain [116]. By blocking HIF-1 α binding to HRE sequence, a step required for transcription induction, anthracyclines have been reported to significantly reduce the prostate tumor growth and vascularization in a mouse model [117]. The topoisomerase I inhibitor topotecan, cardiac glycoside digoxinand PX-478 have also been implicated in HIF-1 α expression, consistent with their remarkable antitumor activity in a variety of human tumor xenograft models [118]. HIF-1 α protein translation is also inhibited by the chaperone Hsp90, which induces its proteasomal degradation in a VHL-independent manner [119]. Nontoxic prodrugs that generate active species in hypoxic tissue by selective bioreduction have now reached advanced clinical trials. Nitroaromatics, quinones, tertiary amine N-oxides, and transition metals are selectively reduced and activated in the absent of O2 to release or activate toxic effectors to eradicate surrounding hypoxic tumor cells. Similarly, the gene-directed enzyme prodrug therapy uses HRE sequence to improve the expression of reductase enzymes, including P450 reductase, HSV thymidine kinase and cytosine deaminase, which kill hypoxic tumor cells by converting a prodrug into a cytotoxin [58]. Nevertheless, a robust validation of hypoxia inhibitors in clinical trials is needed to support the hypoxia-targeted therapies. Overall, these findings suggest that advanced compounds need to be developed to selectively target cancer microenvironment.

Conclusions

The reviewed data emphasize the supporting role of the microenvironment in primary tumor establishment and dissemination to distant sites. The critical event of EMT depends on the complex signals produced by stromal components ensuring the generation of CSCs phenotype with increased proliferative capacity and metastatic

potential in hostile milieu. In addition, perivascular, hypoxic and premetastatic niches have been proposed to enhance the resistance of CSCs to therapy. Based on this observation, combination therapies targeting hypoxia and de novo angiogenesis may have enormous therapeutic implications by blocking the successful homing of cancer cells to metastatic sites. Thus, a better understanding of cancer microenvironment framework could be a crucial key to improving patient cure.

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Chapter| 7

Lipid droplets: a marker for colorectal cancer stem cells

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Summary

The Cancer Stem Cell (CSC) model is describing tumors as a hierarchical organized system and CSCs are suggested to be responsible for cancer recurrence after therapy. The identification of specific markers of CSCs is therefore of paramount importance. Here we show that high levels of Lipid Droplets (LDs) are a distinctive mark of CSCs in colorectal cancer. This increased lipid content was clearly revealed by Raman spectroscopy. The elevated LD content was confirmed by fluorescence and electron microscopy techniques and directly correlated with the other CSC markers, such as CD133 and WNT/□□catenin transcriptional activity. From a functional point of view, LD^{High} cells were found to contain more clonogenic potential as compared to LD^{Low} cells.

All together our results indicate that LDs are a defining feature of the CSC compartment and can be identified with Raman spectroscopy, which is a powerful and label-free method. Additionally, we show that simple flow cytometric analysis of LDs represents a fast identification procedure of CSCs.

Keywords

Colon Cancer Stem Cells, Lipid Droplets, Raman Spectroscopy

Introduction

Colorectal cancer is one of the most frequent neoplasms and the second leading cause of cancer-related death in the Western world (1). Colorectal cancer seems to originate from clonal expansion of a single cell located at the bottom of the colorectal crypt (2, 3) that underwent genetic or epigenetic alterations (4, 5). Moreover, different studies have indicated that CSCs (6), a cell subset belonging to the tumor initiating cell compartment (7) , in colorectal cancer are more resistant to therapy than differentiated tumor cells (8, 9). For these reasons colorectal-CSCs (CR-CSCs) have been recognized as key components in colorectal carcinogenesis and recurrence (10-12).

At present, identification and isolation of CR-CSCs by means of biological tools is a critical task (13). *In vitro* analysis commonly used to this task include serial colony forming unit assays, which confirm the self-renewal capacity of CR-CSCs, propagation as tumor spheres in stem cell culturing conditions (14), and their identification by means of CR-CSCs markers (15). To prove the tumorigenic potential of the isolated CR-CSCs it is then necessary to perform serial injections of the spheres into immune compromised mice (16). Moreover, nearly all of the potential markers of CR-CSCs so far proposed, such as CD133 (16, 17), CD44 (18), ESA (EpCAM) (17), CD166 (18), ALDH-1(8), Musashi 1 (Msi1) (19), and LGR5 (20) require staining and are not full proof.

It would be then highly desirable to develop an alternative, rapid and reliable technique for CR-CSCs identification and sorting. The identification of such a method could also reveal new relevant cellular/functional aspects of the CSCs subpopulation (13).

In this regards Raman techniques have been recently used for biological and medical studies, thanks to the sensitivity of the chemical structure of biomolecules, non-invasive sampling capabilities, label-free imaging and high spatial resolution. As examples, Raman micro-spectroscopy has been proposed to study DNA and protein distribution inside cells (21, 22), the cellular uptake and distribution of liposomal drug carriers (23), label-free mitochondrial distribution (24), lipidomics in leukocytes (25), lipids imaging in human lung-cancer cells and in brain tissues (26). Furthermore, latest developments in Raman techniques (27, 28) could lead in the near future to a very high spatial resolution spectroscopic tool (in the nm-scale, well beyond the diffraction limit) with the capability of protein detection in highly diluted samples (29).

In this study Raman spectroscopy, fluorescence microscopy, flow-cytometry and electron microscopy are used to investigate the presence of distinctive features of CR-CSCs compared to differentiated tumor cells and healthy colon cells.

Here we show that Raman micro-spectroscopy highlights a higher content of lipids in CR-CSCs compared to all the other cells of the tumor bulk. Fluorescence microscopy with hydrophobic dyes, BODIPY (30) and LD540 (31), clearly identifies the origin of the larger lipid content as an increased expression of lipid droplets (LDs). The high content of LDs is also confirmed and quantified by flow cytometry and electron microscopy. As a remarkable point we find that LDs content in CSCs subpopulation is directly correlated with the overexpression of CD133 and Wnt/β-catenin pathway activity, two well-accepted markers for CR-CSCs.

From a detection point of view, the large amount of LDs produces remarkable increased intensities of the Raman peaks corresponding to specific vibrations of fatty acids, and the intensity differences are so unambiguously evident that these Raman modes are ideal candidates as Raman markers for a fast, robust and label-free method

for CR-CSCs identification. From a biological/functional point of view, LDs can be an ideal target for future colon cancer therapies.

Results

Colorectal Cancer Stem Cells show a specific lipid Raman signature

In this work, primary CR-CSC lines and sphere derived adherent cells (SDACs), derived from seven different human colorectal cancer specimens from 7 patients (stage II-IV) undergoing colorectal resection (see SI), were analyzed by Raman spectroscopy. To study the possible role of LDs in healthy tissue and colon cancer progression, to have a representative panel of colon cell lines, we, also used for our measurements the healthy epithelial colon cells (HCCs) and 2 immortalized colon cancer cell lines (CCCs). Fig. 1A shows a typical Raman imaging result recorded on a single CR-CSC. By comparison of the spectra measured across the cell area, two spatial regions (named region * and **) with different Raman features were identified. The characteristic Raman spectra (Fig. 1A* and 1A**) from these regions exhibit clear differences for peak intensity at 1300, 1440, 1740 cm^{-1} and for the Raman band at 2800-3000 cm^{-1}. The assignment of all these Raman bands has been thoroughly discussed in literature, with the peaks at 1300 and 1740 cm^{-1} unambiguously assigned to lipids molecular vibrations (32, 33), while the 1440-1450 cm^{-1} and 2800-3000 cm^{-1} bands are indicators for the lipid to protein ratio (see SI).
Comparison of the two spectra of Fig. 1A revealed that region 1A* has a larger content of lipids. Besides the intensity increase observed for the peaks at 1300 and 1740 cm^{-1}, which are characteristic only of lipids, both the aforementioned lipid (1440-1450 and 2800-3000 cm^{-1} bands) to protein indicators denote larger lipids content. In fact a detailed analysis of the 1440-1450 cm^{-1} band shows a shift towards the 1440 cm^{-1} vibration typical of lipids, while in the 2800-3000 cm^{-1} region the CH_2 symmetric stretching at 2850 cm^{-1} is noticeably more pronounced (CH_2 groups are more frequent in fatty acids than in proteins). Overlapping the brightfield image of the cell with the Raman map at 2850 cm^{-1} clearly shows that these lipid-rich areas correspond to the presence of granulated (or droplet-like) morphological structures. Also, imaging at 1300, 1440 and 1740 cm^{-1} (data not shown) exhibit the same spatial correlation with the brightfield image as for 2850 cm^{-1}. These peaks are then space-correlated which confirmed the lipid nature of the observed droplets.
Fig. 1B shows the comparison of typical Raman imaging for all of the measured cell lines, with intensity maps at 1300 and 2850 cm^{-1}, along with whole-cell-averaged Raman spectra on the last column. CR-CSCs clearly exhibit a distinctive Raman signature with remarkable intensities for both CH_2 twist and CH_2 symmetric stretching vibrations (second row in Fig. 1B). Again, these features are localized in spatial regions corresponding to granules observed in the brightfield image of the cell. The SDACs (third row in Fig. 1B), have partially inherited this characteristic, but at a smaller extent. Even if some spots are still noticeable in the brightfield picture of the cell (mostly in the left-bottom part of the SDAC in Fig.1B), the peaks intensities at 1300 and 2850 cm^{-1}, on the averaged Raman spectra, are much smaller compared to the case of CR-CSCs. CCCs (fourth row in Fig. 1B) exhibit, instead, few spots. Accordingly, Raman intensities at 1300 and 2850 cm^{-1} drop to smaller values, and Raman spectra from CCCs generally resemble to Raman profile of region ** of Fig. 1A. Finally, the HCCs (first row in Fig. 1B) shows the most uniform appearance, with

a nearly absence of spots in the brightfield image, and also their Raman spectra have small intensities at the characteristic frequencies of lipids vibrations.

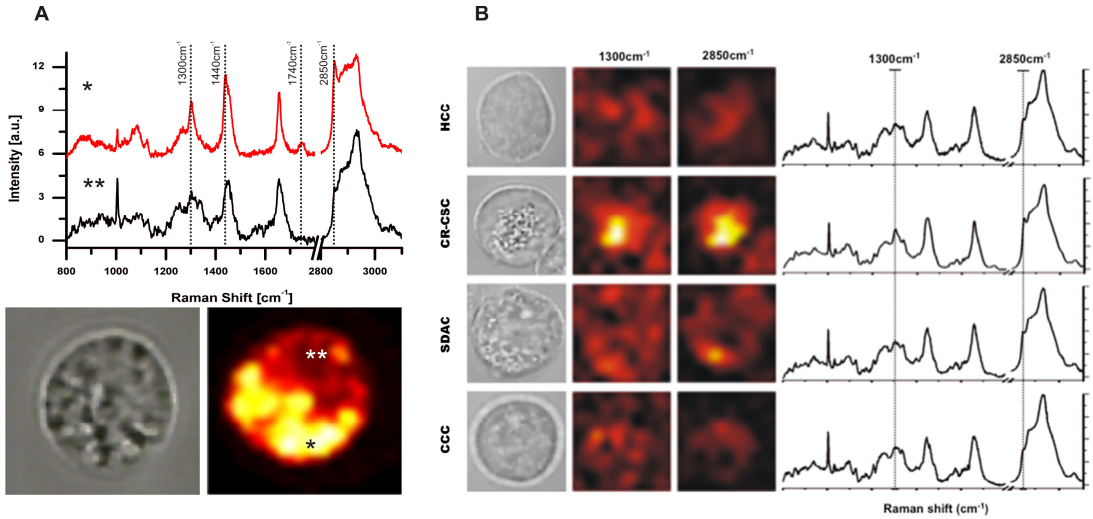

Fig. 1. Raman characterization and mapping of colon cell samples.

(A) Two different cell regions, indicated as * and ** respectively, can be clearly identified in the cell according to their Raman spectra. The Raman differences are due to 4 main peaks located at 1300, 1440, 1740 and 2850 cm^{-1}. (*) Typical spectra from region 1 (top curve) and region ** (bottom curve) show that region * has higher expression of all these mentioned peaks compared to region **; bottom are reported the brightfield image of the CR-CSC and the Raman imaging at 2850 cm^{-1} of the same cell, highlighting the two different regions. (B) From the top row to the bottom one: Healthy Colon Cells (HCCs), Colorectal Cancer Stem Cells (CR-CSCs), Sphere Derived Adherent Cells (SDACs) and Colon Cancer Cells (CCCs). Brightfield images are reported on the first column, while Raman images calculated at 1300 and 2850cm^{-1} are reported in the second and third column respectively. The fourth column shows spectra averaged over the whole cell area, from each cell line. Raman images of the second column are similar to the corresponding ones of the third column, thus revealing that the two Raman modes at 1300 and 2850cm^{-1} are space correlated. Mostly important, both from the Raman images as well as from the spectra, it is clear that peaks related to lipidic vibrations are more pronounced in the CR-CSCs (second row) and their intensities decrease as moving through the cancer differentiation, i.e. passing from CR-CSCs to SDAC (third row) and finally to CCCs (fourth row). Healthy colon cells (HCCs) reported in the first row express the lowest Raman intensities of lipidic vibrations.

In order to prove that Raman spectroscopy can provide a fast tool for CR-CSCs detection (and for future sorting applications), besides point-by-point Raman mappings we have measured a single Raman spectrum in the 800-1800 cm^{-1} range for each cell by using a line-focused laser excitation extending for the whole cell diameter. In these measurements (see also Fig. S1 and S2) the 1300 cm^{-1} Raman peak of CR-CSCs cells has an intensity level undoubtedly more pronounced than in healthy cells and in other non-stem colorectal cancer cell lines, and can be used as reliable Raman marker for detecting CR-CSCs. We notice that, due to the clear spectra change between CR-CSCs and the other cell types, no data treatments were necessary (for instance PCA, Principal Component Analysis (34)) to distinguish the different cell populations (See SI).

Lipid Droplets quantification

To confirm the presence, and assess the amount of the lipid-rich regions revealed by Raman spectroscopy, fluorescence imaging and flow cytometry measurements have been performed on the above cell lines by using BODIPY 493/503 or LD540 staining, which are a consolidated dyes for cellular LDs visualization (31, 35, 36).
Confocal images have been collected for all the colon cell lines and z-projections have been created by using the ImageJ software (37). The acquired images clearly show the "Lipid Droplet" nature of the same granular structures visible in the brightfield image, which are responsible for the high lipid-related Raman peaks. A comparison of typical LDs content among the considered samples is shown in Fig. 2A. It is clear that the number of LDs increases from the healthy cells to the CR-CSCs.
Flow-cytometric analysis allows, moreover, for a statistical assessment of the LD expression difference among the investigated cell lines. Comparison of histograms for CR-CSCs cell lines from 3 different patients and their related SDACs established the higher LDs expression in multiple CR-CSCs cells.

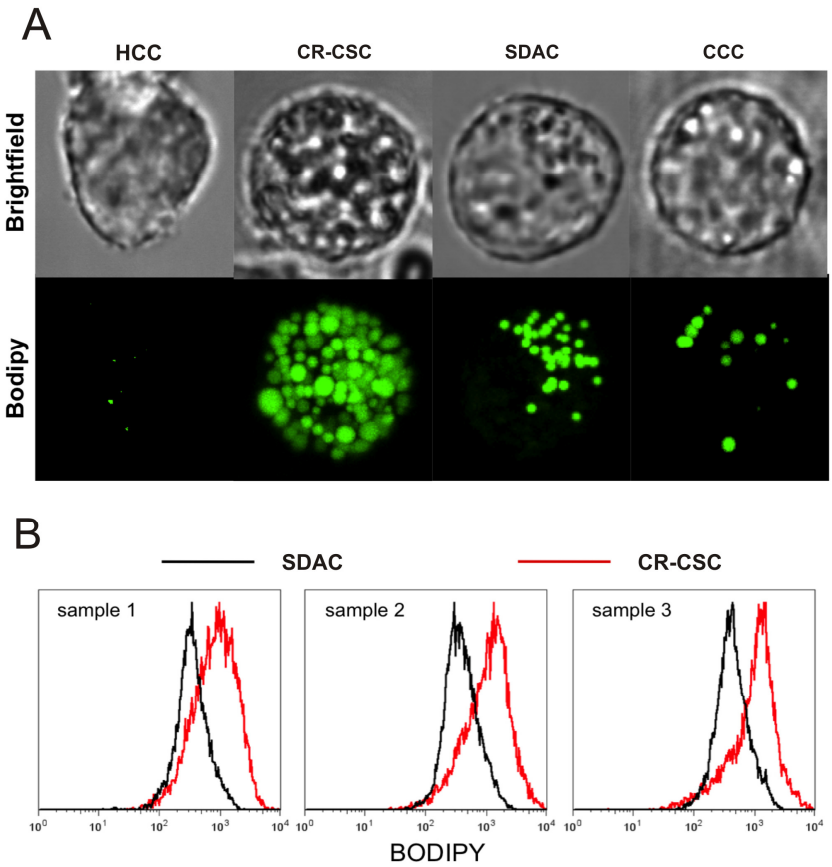

Fig. 2. Lipid Droplets quantification

(**A**) Comparison of typical z-projected confocal fluorescence images of the investigated cell lines stained with BODIPY 493/503. It is clear the higher LDs content on CR-CSCs compared to all of the other cell lines; (**B**) Histograms overlay for flow-cytometry BODIPY fluorescence measurements regarding three of the CR-CSCs (red) cell lines and their SDACs (black).

The ultrastructural analysis performed with Transmission Electron Microscopy (TEM) on HCCs, two different CR-CSCs samples, their relative SDACs, and CCCs, corroborated both Raman and fluorescent microscopy results (Fig. 3).

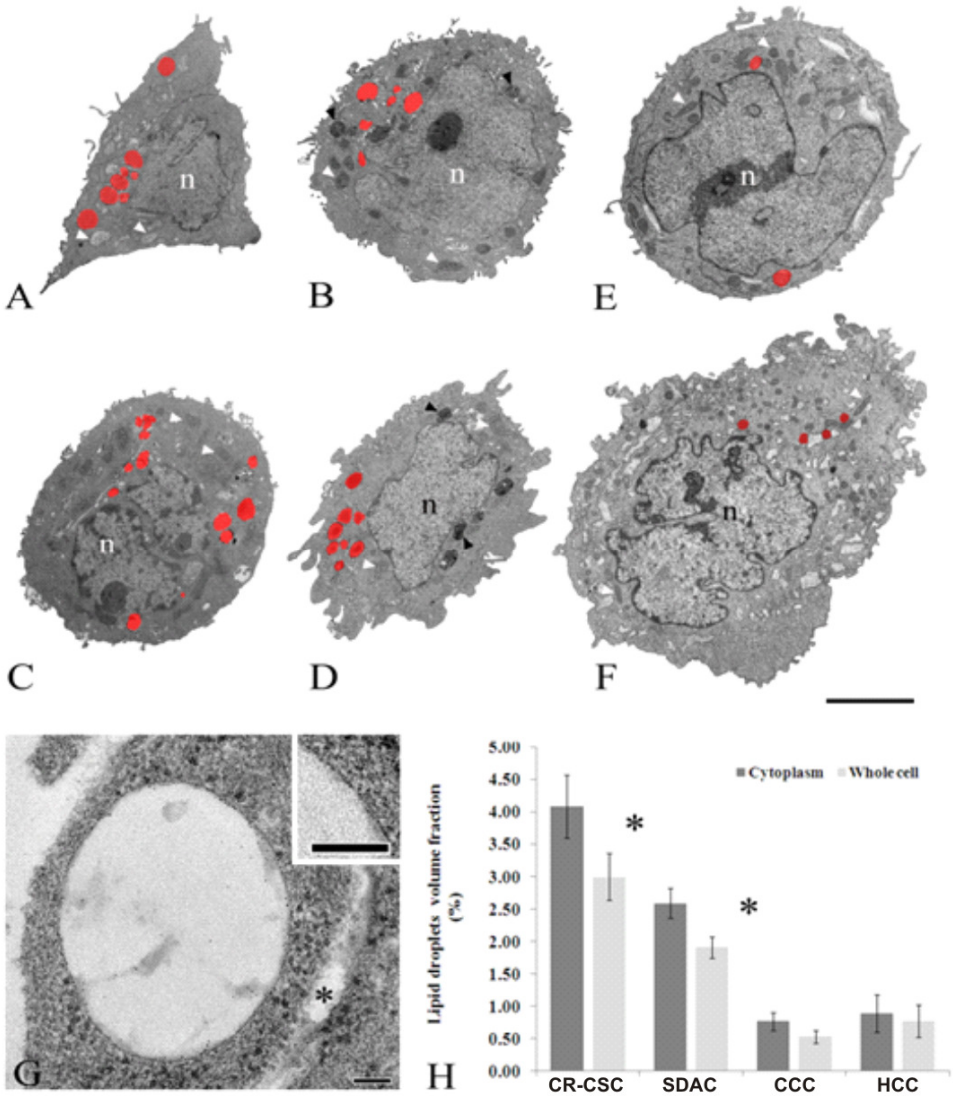

Fig. 3. Transmission Electron Microscopy to reveal Lipid Droplets on examined samples.

(**A**-**F**) TEM images of the Colorectal Cancer Stem Cells (CR-CSCs), their differentiated forms (SDACs), Colon Cancer Cells (CCCs), and Healthy Colon Cells (HCCs). (**A** and **B),** parasagittal sections of (**A**) CR-CSCs and (**B**) SDACs belonging to human patient 1; (**C** and **D),** parasagittal sections of (**C**) CR-CSCs and (**D**) SDACs belonging to human patient 2; (**E**), parasagittal section of a CCC. (**F**), parasagittal section of a HCCs. White arrowheads point to mitochondria; black arrowheads point to multivesicular bodies (MVBs) and late-endosome/lysosome hybrids. The lipid droplets are colored in red. n, nucleus. (**G**), TEM image of a lipid droplet in cross section. The asterisk points to the endoplasmic reticulum. Inset: detail of the lipid droplet single membrane leaflet. (**H**), volume fraction of lipid droplets in the cytoplasm and in the whole cell. Error bars, SEM. Statistical significance is denoted by * ($p \leq 0.01$, Student's t test). Scale bars are 4 µm for **A-F** and 100 nm for **G**.

The LDs were unambiguously identified in the cell cytoplasm, often close to the endoplasmic reticulum, as sub-spherical structures delimited by a single membrane

leaflet (Fig. 3G) (35). The stereological analysis, performed to quantify the volume fraction of the LDs in the various cell lines analyzed, further confirmed the insights from Raman and fluorescent microscopy, suggesting a significant decrease of LDs paralleling the tumorigenic progression (Fig.3H). We measured a LDs volume fraction expressed as % of cytoplasmic volume ranging from 4.09 ± 0.48 %, for the CR-CSCs, to 0.89 ± 0.29 %, for the HCCs, value that falls into the range reported for other cell types (Fig 3H) (38). In the CR-CSCs the LDs volume fraction in the whole cell and in the cytoplasm resulted to be significantly higher ($p \leq 0.01$), compared to that measured for SDACs (Fig 3H). Furthermore the LDs volume fraction in the SDACs was largely higher ($p \leq 0.01$) compared to that measured inside the HCCs and the CCCs (Fig 3H).

Correlation between CD133, WNT and Lipid Droplets

In order to investigate the existence of a correlation between LDs content and the expression of CR-CSCs markers, we have performed flow cytometry measurements involving CD133 and the Wnt/β-catenin pathway activity. In a first experiment, different CR-CSCs samples were double-stained for LDs and CD133 with BODIPY 493/503 and anti-CD133 antibody APC-conjugated, respectively. Flow cytometric analysis (Fig. 4A and B) showed a clear correlation between the two markers. In a second experiment, LDs and Wnt correlation was studied by using two CR-CSC cultures with a TOP-GFP reporter gene (39).

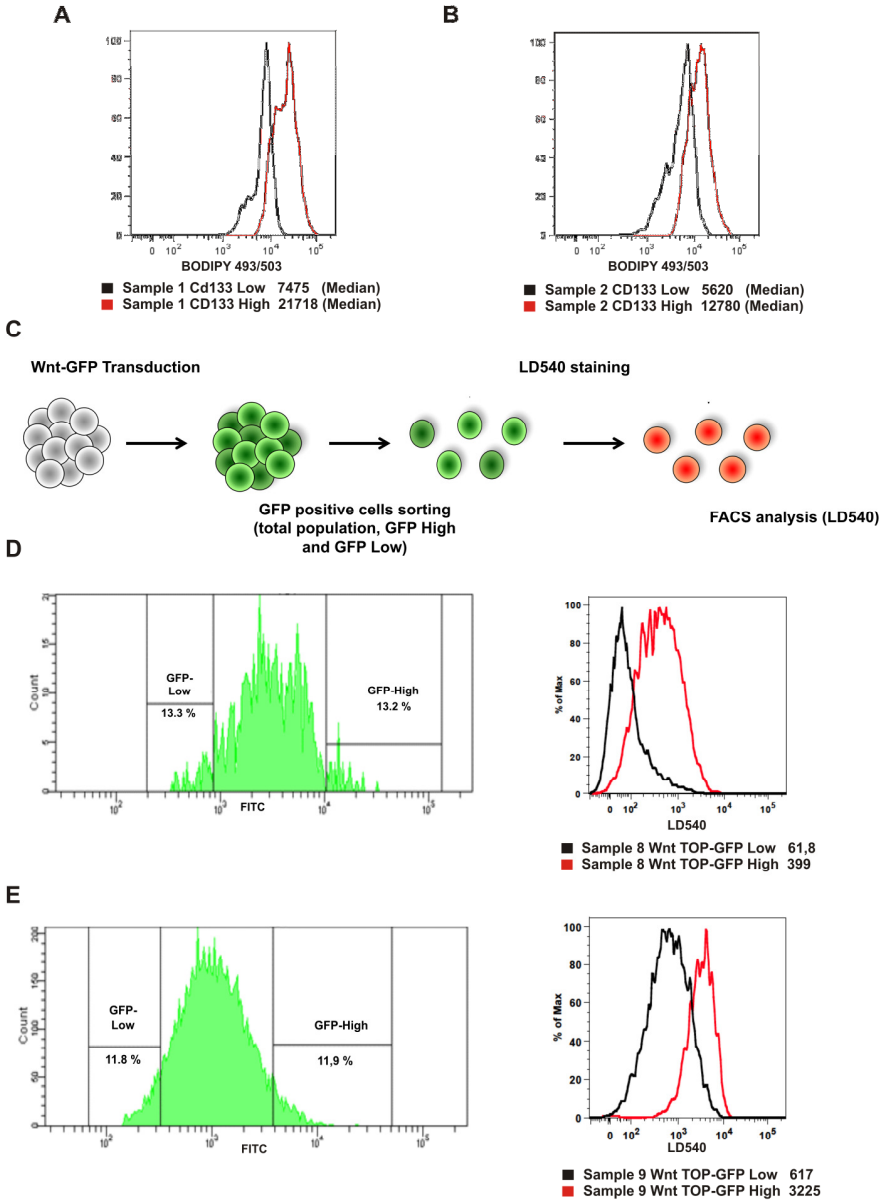

Fig. 4. Correlation of the expression levels of the Lipid Droplets with CD133 and Wnt/β catenin

(**A** and **B**) The expression of the LDs in CD133^{High} and CD133^{Low} cells were analyzed by flow cytometry. Cells were stained with an anti CD133 APC-conjugated and then with BODIPY 493/503. Both CD133^{High} samples (A and B red lines) have a higher expression of LDs compared to the CD133^{Low} (black lines); (**C**) Schematic representation of the TOP-GFP Wnt construct. Cells were sorted for GFP expression (GFP^{High} and GFP^{Low}) and then stained for LDs with LD540; (**D** and **E**) Both TOP-GFP samples have the same behavior showing as Wnt/β-catenin pathway expression clearly correlates with LDs quantity.

Importantly, cells derived from these single cell cloned TOP-GFP cultures still showed a big heterogeneity in Wnt signaling level (39). The two cell lines were firstly sorted based on the GFP fluorescence, as an indicator for Wnt expression, into two subsets, Wnt^{High} and Wnt^{Low}. Sorted cells were then stained for LDs content by using the LD540 dye, taking advantage of the fact that it can be used in combination with

GFP (Green) since its emission spectrum extends to the red (Fig. 4C-E). It is evident the correlation between the LDs expression and the Wnt signaling level. It is important to note that the different expression of LDs is not due to the use of different cell media, since Wnt^{High} and Wnt^{Low} cells were sorted from the same population, such as for the case of CD133, as reported above.
These results, showing a clear correlation between CD133, Wnt and LDs content, indicate that LDs could be eligible as CR-CSCs markers, and suggest a possible functional or metabolic link between these markers.
We point out that our discovery is a compelling argument that supports the emerging interest on LDs as organelles with additional and very important functions compared to how they were considered in the past (40, 41).

A high LDs content reveals an increased clonogenic potential of CR-CSCs

Different CR-CSC cell lines were stained with the LD540 dye and sorted for LDs^{High} and LDs^{Low} content. The sorted cells were used to perform a limiting dilution assay (LDA) to test their clonogenic potential. The results reported in Fig. 5 show that LDs^{High} cells possess a higher clonogenic potential compared to the LDs^{Low} in all the CR-CSC lines analyzed, suggesting a possible key role of these lipids in giving an advantage in promoting and sustaining cell growth, and tumorigenesis.

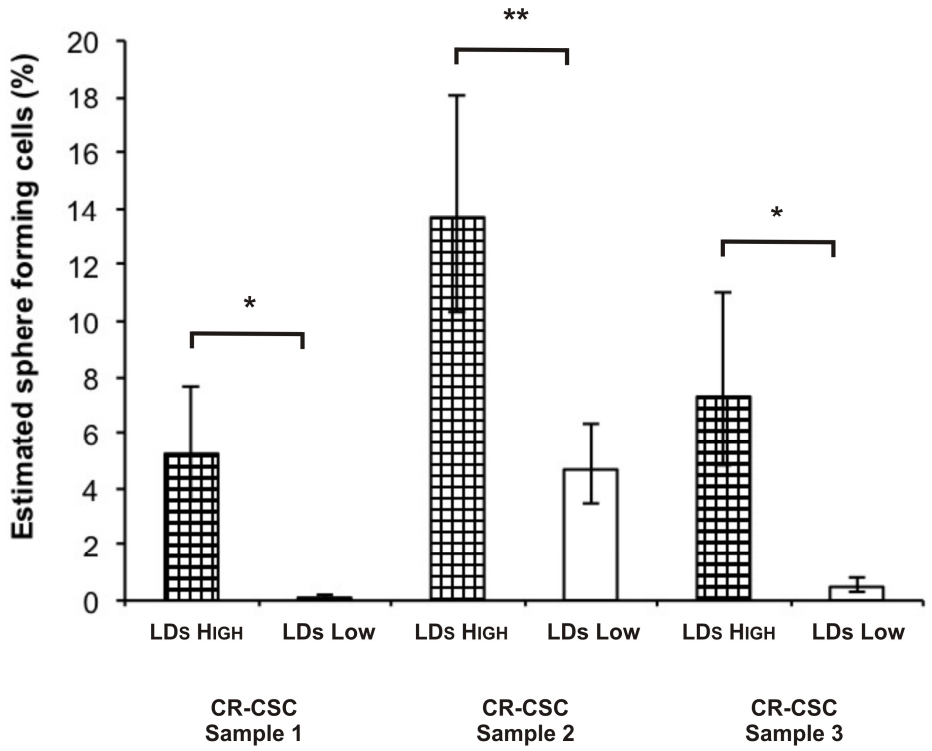

Fig. 5: Clonogenic assay

Three different CR-CSCs samples were tested for their clonogenic potential. CR-CSCs were sorted for LD^{High} and LD^{Low} by Fluorescence Actived Cell Sorting for LDs using LD540 dye and then deposited 1, 2, 4, 8, 16, 32 and 64 cells per well. The estimated sphere forming cells were analyzed using the Extreme Limiting Diluition Analysis (ELDA) as reported in the graph. All the three LD^{High} cell samples have a significantly increased clonogenic potential than the LD^{Low} cell samples.

Only a small fraction (5-14 %) of cells composing the LDs^{High} cells exhibit stem cells properties (according to the CSCs model), much more higher compared to the stem cells fraction that was found in the LDs^{Low} subset (0-5%). These data show that CRC contains a sub-population of cells able to self-renew, and that the lipid droplets content can be used alone as an identification marker for the CSC subset present within the tumor bulk.

Discussion

Although it was already known that colon cancer cells have a large number of LDs (40, 42), here we have shown that CR-CSCs can be identified for having the largest amount of LDs when compared to differentiated tumor or healthy cells. This finding has been confirmed by measurements carried out on CR-CSCs from 7 different patients. This result opens to a new identification approach to measure the patient's cancer stemness, based on Raman spectroscopy, which is suitable also for *in vivo* detection. Moreover, the present method finds a clear confirmation with flow cytometry, reinforcing its efficacy in clinical early cancer studies and consequently could help to improve the individual tailored therapy protocols aiming at targeting the cancer stem cell compartment.

The novel identification approach could solve previous limitation in the quantification of the CR-CSCs as the lacking of specific surface markers and the need to use labeled monoclonal probes.

We can speculate that the higher expression of LDs in CR-CSCs could be part of the disease pathogenesis confirming the increasing interest towards these organelles, shown by the recent literature (40, 41). In fact, LDs in neoplastic cells act as distinct intracellular domain for regulated eicosanoids production (Prostaglandin E2, PGE2) starting from arachidonic acid (AA) (42). The metabolism of arachidonic acid is directly implicated in the generation of chronic inflammatory tissue environment that could promote carcinogenesis. Different products of AA metabolism are indeed implicated in carcinogenesis: 80% to 90% of colon carcinomas show an enhanced cyclooxygenase-2 (COX-2; prostaglandin H synthase) expression compared with normal intestinal mucosa (43-45). COX-2 is the enzyme that catalyzes the rate-limiting step in eicosanoids synthesis, converting AA into prostaglandins. Our results could then lead to the conclusion that CR-CSCs, overexpressing LDs, may function as main tissue compartment engaged in inflammatory process in cancer.

Moreover, besides of its functions as organelles involved in the generation of eicosanoids, LDs constitute sites of compartmentalization of several signaling-relevant proteins, which may have functions beyond AA metabolism. Indeed, proteins with well-established roles in oncogenic cell transformation, tumorigenesis and metastasis, including PI3K, ERK2, p38, PKC and Caveolin, were shown to localize in LDs in a variety of cell types (46-48).

Furthermore, the LDs may play a role even in the multidrug resistance of CR-CSCs with at least two mechanisms: i) evidences reported in literature demonstrate that LDs contain also glucosylceramide that directly control the multidrug resistance of tumor cells, thus it is conceivable that the CR-CSCs with high LD content may be related their drug resistance (49); ii) the role of LDs in the maintenance of inflammation can contribute to the CSCs multidrug resistance mechanism recently demonstrated by Todaro et al. (9). They observed that a high level of inflammatory cytokine

Interleukin 4 (IL-4), produced by CR-CSCs, is implicated in their protection against the apoptosis induced by chemotherapy. This ectopic IL-4 over-expression could be related to the continuous inflammatory state and then
to LDs.
The positive effects reported for the treatment of colorectal cancer with different classes of drugs which interfere with LDs formation, like Statins (50) and Aspirin (51-53) witness the relevance of LD in the cancer pathogenesis.
All these data put in evidence that LDs, could be considered as a new CSC markers over-expressed in CR-CSCs, and a rather interesting cellular target for future innovative anti-cancer therapy.

Acknowledgements

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Experimental procedures

Cell cultures

CR-CSC cultures were generated as previously described by Ricci Vitiani et al., (17) and cultured in ultra-low adhesion flasks (Corning, Lowell, MA) in DMEM/F12 serum-free medium (Life Technologies) supplemented with fresh epidermal growth factor (20 ng/ml) and basic fibroblast growth factor (10 ng/ml) (Sigma-Aldrich, St. Louis, MO) to promote their growth. A GFP^+ subculture was obtained by lentiviral transduction as previously described (39). Differentiated cells (SDACs) were obtained by dissociating CR-CSCs and culturing them in Dulbecco's modified medium supplemented with 10% FCS in adherent conditions for at least 25 days. Healthy epithelial colon cell lines (CCD841-CoN, CRL-1790)) and colon adenocarcinoma (HCT116 and RKO, ATCC CCL-247 and CRL-2577 respectively) were purchased from ATCC and cultured with RPMI and alpha-MEM completed with 10% of FBS and 1% of P/S respectively.

Raman Measurements

Raman microspectroscopy is carried out by means of a Renishaw InVia Raman microscope, equipped with a motorized stage for the laser-scanning of the sample. The excitation wavelength is 633 nm and the incident light is focused on the sample through an Olympus 60×/1NA water immersion objective. The laser power at the sample level is about 3mW. A notch-filter is used to block the Rayleigh back-scattered light. Before recording Raman measurements, the cells are passaged by trypsinization, washed three times with Phosphate Buffered Saline (PBS) and then resuspended in the same buffer. During the measurements, CaF_2 slides are used as substrates because of their negligible Raman signal background.
For the imaging experiments, cells are scanned through the laser focus in a raster pattern with a typical step-size of 1 μm. Raman spectra are recorded in the 800-3200 cm^{-1} range and the accumulation time is 5 sec per each pixel. Subsequently, Raman images are created by plotting the integrated intensity of a specific Raman band as a function of position. Since different biomolecules exhibit different characteristic Raman bands, this technique allows for a label-free imaging of the spatial distribution of biomolecules inside the cell.

Confocal Microscopy

Fluorescence images have been collected using a Nikon A1 confocal-laser-scanning microscope with a PlanApo 60x oil immersion objective with a 1.40 numerical aperture. In suspension live cells have been stained for LDs using BODIPY 493/503, (Molecular Probes, Invitrogen). BODIPY 493/503 was dissolved in PBS at 1 mg/mL and used at 1 μg/mL. Cells were washed with PBS 1x and incubated with BODIPY 493/503 for 15 minutes at room temperature.

Flow Cytometry

All cells were collected from the flasks, washed with PBS 1x and incubated with BODIPY 493/503 (Molecular Probes, Invitrogen) or LD540 for 15 and 10 minutes

respectively at room temperature in the dark. CD133 was stained using an anti CD133 antibody (MiltenyiBiotec) APC-conjugated (Invitrogen). Stained cells were washed twice with PBS 1x and resuspended in the same solution. Samples were analyzed by FACSAria II flow cytometer (BD Biosciences). To allow for comparison of the different cell lines, gains for forward-scattering, side-scattering and fluorescence PMTs are kept the same on all of the measurements.

TEM measurements

HCCs, CR-CSCs, SDACs and CCCs were processed for transmission electron microscopy. The volume fraction of the cell occupied by LDs was estimated using point counting stereology techniques.

Cell Sorting

Two different CR-CSC lines bringing the TOP-GFP construct were collected from the flasks and sorted for GFP^{High} and GFP^{Low} (both the sorted fractions consist of about 12% of the total GFP^{+} population), using a FACS Aria II, and then stained for LDs content using the LD540 dye.

Limiting Dilution Assay

The self-renewal capacity of the CR-CSCs was assayed by dissociation of primary tumor cells and plating them at serial dilution (1, 2, 4, 8, 16, 32, 64 and 128 cells per well). Results were statistically evaluated after 4 weeks by using the Extreme Limiting Dilution Analysis (ELDA) software (54).

Supplemental Data

Figure S1, related to Figures 1 and 2.

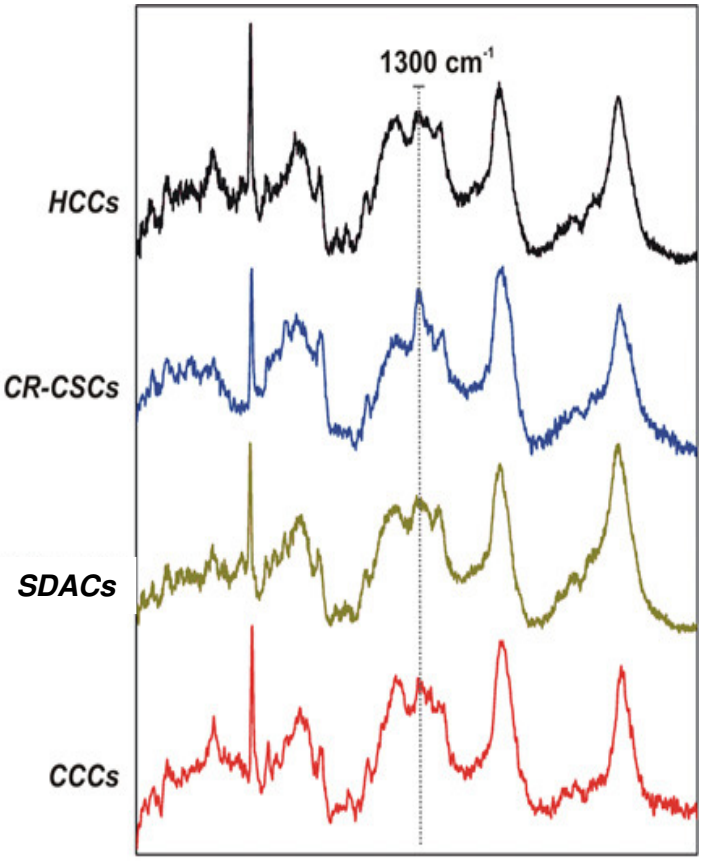

Fig. S1. CR-CSCs Raman detection through 830 nm laser power.

Raman fingerprint of all colon cell lines performed with 830 nm laser type. CR-CSCs show a unique behavior at 1300 cm^{-1}; the signal intensity for this Raman shift is clearly more pronounced than in other non-stem colon cancer cell lines.

Figure S2, related to Figures 1 and 2.

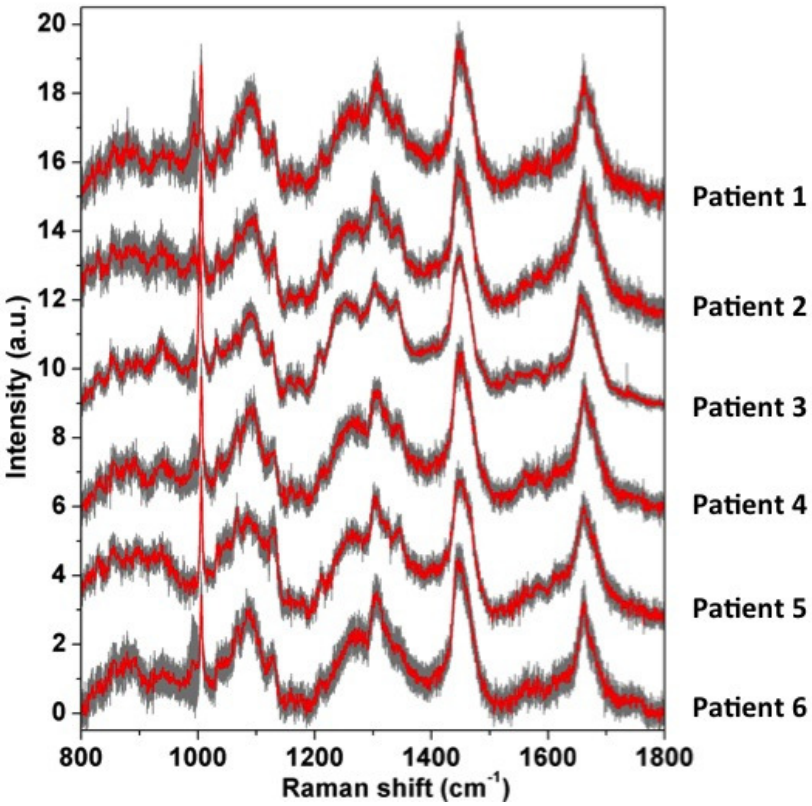

Fig. S2. Raman measurements on CR-CSCs from 6 different patients.

The average measurements (red curves) of CR-CSCs from six different patients are reported along with the standard deviations (gray shadows). The spectral acquisition is performed in the 800-1800 cm^{-1} range. All the curves exhibit a similar behavior with a pronounced intensity of the 1300 cm^{-1} Raman peak, thus confirming the reproducibility of this spectral feature.

Figure S3

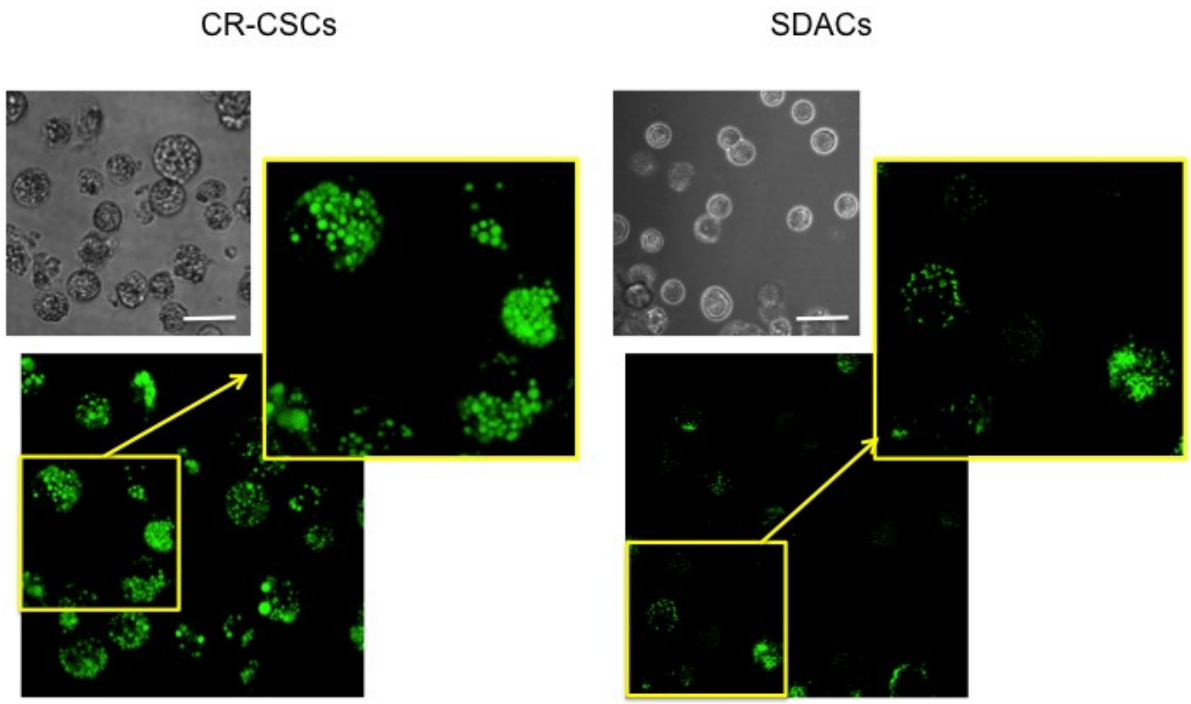

Fig. S3. LDs detection through Confocal-Fluorescence Microscopy

Comparison in the high field of typical z-projected confocal fluorescence images of the investigated cell lines stained with BODIPY 493/503. CR-CSCs (left panels) over-express LDs both in terms of number and size, compared to the differentiated counterpart (right panels). This difference is more appreciable by looking at the magnification (one representative experiment of 3 is shown) Scale bar is 20□m.

Supplemental Experimental Procedures

Cell Cultures

Human colon carcinomas specimens were obtained from 7 patients (stage II-IV) undergoing colorectal resection, in accordance with the ethical standards of the institutional committee. Tumor diagnosis was based on anatomical and histopathological analysis. Tumor tissues were mechanically and enzymatically digested using collagenase (1.5 mg/mL; Gibco) and hyaluronidase (20 ìg/mL; Sigma-Aldrich). The tumor digest was divided into different culture conditions to obtain different cell populations enriched in particular cell subsets. To obtain the 7 different CR-CSC samples, part of the tumor digest was cultured on ultralow adhesion flasks (Corning, Lowell, MA) in the presence of serum-free medium supplemented with epidermal growth factor (20 ng/mL) and basic fibroblast growth factor (10 ng/mL, both from Sigma-Aldrich, St. Louis, MO 63103, USA) to promote the growth of CSCs as spheres in 1–2 months, as previously described (reference). Tumor digest was also cultured in the presence of Dulbecco's modified Eagle medium (EuroClone Ltd.) and supplemented with 10% heat-inactivated fetal bovine serum, Antibiotic/Antimycotic (Euroclone), Penicillin/Streptomycin (Euroclone), L-glutamine (Euroclone), to obtain primary tumor cells. Just the sphere cultures that were validated for a colorectal-CSC phenotype and for the ability to form a xenograft in immune-compromised mice, resembling the parental tumor, were considered as CR-CSCs and were used for subsequent studies. To achieve the *in vitro* differentiation of CR-CSCs, dissociated sphere cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% FBS in adherent conditions for at least 25 days, obtaining four Sphere Derived Adherent Cells (SDACs) (from patients 1-4). Human CCD841-CoN healthy colon cell line (ATCC CRL-1790) was cultured in □-Mem (Invitrogen) with 10% FBS (Invitrogen) and antibiotics (100 U/ml penicillin, 100 □g/ml streptomycin) (Invitrogen). Human HCT116 and RKO colorectal carcinoma cell lines (ATCC CCL-247) were cultured in RPMI (Invitrogen) with 10% FBS (Invitrogen) and antibiotics (100 U/ml penicillin, 100 □g/ml streptomycin) (Invitrogen).

Raman peaks assignment

The assignment of all the Raman shifts reported in the main text has been thoroughly discussed in the literature. The peaks at 1300 and 1740 cm^{-1} are unambiguously assigned to lipids molecular vibrations and correspond respectively to the CH_2 twist and to the C=O in $–CH_2$-COOR, this last due to triacylglycerol (1, 2). The Raman band at 1440-1450 cm^{-1} is assigned to the CH_2 bending, occurring at 1440 cm^{-1} for lipids and 1450 cm^{-1} for proteins (3). Depending on the lipid to protein ratio, this peak shifts from 1440 to 1450 cm^{-1}. Concerning the spectral region between 2800 and 3000 cm^{-1}, CH_2 symmetric and asymmetric stretching are respectively found at 2850 and 2885 cm^{-1}, while the analogous vibrations for CH_3 are located at 2933 and 2950 cm^{-1} (3). Similarly to 1440-1450 cm-1 band, the CH_2 stretching intensities are suitable indicators for the lipid to protein ratio, due to the fact that CH_2 groups are more

frequent in fatty acids chains than in proteins. Finally, the band at 1050-1100 cm^{-1} correspond to C-C stretching vibrations in hydrocarbon chain (2).

Raman spectroscopy as sorting tool for CR-CSCs

The unambiguously difference of intensity of the 1300 cm^{-1} Raman peak offers the possibility to discriminate between CR-CSCs and the other cell types involved in the cancer progression. An 830 nm diode laser is used to perform Raman measurements over all the cell samples. In the present case the laser focus has not a point-like shape but an elliptical shape with one of the two axes much larger than the other. This kind of line-focus allows for probing the cell almost in its entirety with a single Raman measurement, thus saving a lot of time compared to Raman mappings. This is a crucial point for future applications in cancer stem cell sorting.

First, we measured Healthy Colon Cells (HCCs), Colorectal Cancer Stem Cells (CR-CSCs), Sphere Derived Adherent Cells (SDACs), and Colon Cancer Cells (CCCs) in the spectral region between 800 and 1800 cm^{-1}. The differences highlighted in the main text for this spectral region are still evident in these measurements (Fig. S1), with the 1300 cm^{-1} peak reporting the most evident difference between the CR-CSCs on one side and the other cells types on the other side. Subsequently we have performed similar measurements on other six CR-CSCs lines from six different patients. For each patient, at least 30 cells are probed and Fig. S2 reports the average spectra with the standard deviations. For all the patients the peak at 1300 cm^{-1} is clearly more pronounced than the two side peaks at 1260 and 1340 cm^{-1} respectively, while in the other cell types of the cancer progression the intensity level of all the three peaks (1260, 1300 and 1340 cm^{-1}) is comparable.

Sample preparation for Electron Microscopy measurements

HCCs, CR-CSCs, SDACs. and CCCs were processed as follows. Briefly the cell monolayers were fixed for 45 min at room temperature in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH=7.4). The cells were then harvested from the Petri substrate with a cell scraper, transferred in Eppendorf tubes and centrifuged at 10000 g for 10 min. Once fixed in fresh fixative for 1 h at room temperature, the cell pellets were washed three times for 10 min in 0.1 M cacodylate buffer. Secondary fixation was carried out in 1% aqueous osmium tetroxide for 2 h at room temperature, followed by three washes, 10 min each, in 0.1 M cacodylate buffer. After two short washes in bi-distilled water, the specimens were stained overnight in 1% uranyl acetate in 70% ethanol at 4 °C, and then dehydrated through a graded series of ethanol. The cell pellets were then placed in propylene oxide for 15min twice. Infiltration was accomplished by placing the pellets in a 1:1 mixture of propylene oxide and Epon resin for 4 h at room temperature. The pellets were then left in Epon resin for 2 h at room temperature, and finally embedded in fresh resin for 48 h at 65 °C.

Transmission Electron microscopy and stereological analysis

Sections of about 70 nm were cut with a LeicaEMUC7 ultramicrotome, stained with 1% uranyl acetate in bi-distilled water and lead citrate and observed with a JEOL JEM 1011 electron microscope operating at an accelerating voltage of 100 KV. Images

were recorded with an 11 Mp GatanOrius SC100 Charge-Coupled Device (CCD) camera.

The volume fraction of the cell occupied by lipid droplets was estimated using point counting stereology techniques (4). For each cell sample, 5 arbitrarily selected sections were systematically random sampled and quantified at a magnification of 4,000 to unambiguously recognize lipid droplets. Sections and areas were chosen such that no cell was sampled more than once. The number of cells in any given area varied, but the total cellular area sampled averaged 2,700 mm^2 for each sample. The mean and standard error were calculated for each grid (n = 5). Comparison of the volume fraction of cell occupied by LDs for each sample was done with Student's t-test.

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Curriculum vitae

The author of this thesis was born in Palermo (Italy) on the 4th of May 1986. Since his childhood he was convinced to become a doctor, so after the primary school he attended the scientific high school Galielo Galilei, where he obtained his scientific diploma in July 2004 with the score of 100/100. After few months, in September, he decides to give a different slant to his career starting the Biotechnology course at the University of Palermo, to work in research instead of doing medicine. In 2007 he obtained his bachelor degree with 110/110 *cum magna laude*. He continued his study in the Biotechnologies for Industry and Scientific Research course. During his studies he gained interest for cancer research and for this reason he decided to spend the internship period in the Prof. Stassi laboratory, thus studying in particular the role of cancer stem cell in tumor progression. He obtained the master degree on 2009 with a score of 110/110 *cum magna laude*.
In 2010 he was selected by the PhD programme in Immunopharmacology at the University of Palermo, bringing a project that focused on the mechanisms that underlie the tumor progression, in particular in breast and colon tissues. After his doctorate he wants to continue his research in oncological research applying for a post-doc position.

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