Chapter 1

Theoretical and Experimental Foundations of the "Cancer Stem Cell" Model

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Definition of Stem Cells

Many human tissues are in a state of constant flux, in which large numbers of mature differentiated cells are continuously eliminated and replaced. Despite the short-lived nature of many of their cells, tissues maintain their size and structure over time through a tightly regulated process of proliferation and differentiation. In each tissue, this process is orchestrated by a remarkable population of long-lived cells, called "stem cells" (Weissman, 2000).

Historically, the word "stem cell" originated to identify an ancestor/progenitor cell that stands at the "stem" of a genealogic tree, used to depict either evolutionary (i.e., phylogenetic) or developmental (i.e., ontogenetic) processes (Ramalho-Santos and Willenbring, 2007). Its meaning remains essentially intact today, where it is mainly used in developmental biology, to identify a cell capable to generate and sustain over time a specific set of diversified cell populations whose aggregate interaction leads to the formation of either an entire living organism (e.g., embryonic stem cells with pluripotent capacity) or a specific subset of its organs and tissues (e.g., adult stem cells with oligopotent or multipotent capacity) (Weissman, 2000).

Traditionally, stem cells are defined by three main properties:

1. Differentiation: the ability to give rise to a heterogeneous population of daughter cells, which then progressively diversify and specialize according to a hierarchical process, thereby replenishing tissues of fully mature, differentiated elements.
2. Self-renewal: the ability to form identical copies of themselves, other stem cells that retain intact potential for long-term proliferation, expansion, and differentiation, thereby maintaining a constant stem cell pool.
3. Homeostatic control: the ability to balance self-renewal and differentiation, and modulate the frequency of these two developmental outcomes based on environmental needs, and within the limits of a specific set of genetic constraints.

The "Cancer Stem Cell" (CSC) Model

Similar to normal tissues, tumor tissues are composed of a multiplicity of cell types, including various subpopulations of cancer cells. Traditionally, the cellular heterogeneity

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observed within an individual tumor is explained using a random or "stochastic" model. In this model, phenotypic differences observed between different subsets of cancer cells are explained as the result of the progressive and divergent accumulation of independent somatic mutations, which in turn lead to the formation of distinct genetic subclones. An alternative explanation is provided by the "cancer stem cell" (CSC) model. This model envisions tumors as "pathological organs," sustained in their aberrant growth by a mutated population of stem cells, in which normal homeostatic controls on tissue expansion have been lost. According to this model, phenotypic differences among cancer cells can also originate as the result of epigenetic diversity, secondary to multi-lineage differentiation processes akin to those sustained by normal stem cells (Dalerba et al., 2007a; Reya et al., 2001). The two models are not mutually exclusive, as they point to two independent sources of variability, both of which can act simultaneously and contribute to the observed diversity (Gerlinger et al., 2012; Lagasse, 2008).

Theoretical Considerations in Support of the CSC Model

The CSC model is supported by several considerations of theoretical nature. The concept that tumors can be viewed as "caricatures" of normal tissues, aberrant versions of normal developmental processes, is very old, and dates back to the beginnings of anatomic pathology (von Hansemann, 1897). A rapid look at a tumor's histology on a simple tissue-section usually reveals a complex three-dimensional structure, whose architecture and cellular composition usually mirror, though often aberrantly, those of the normal tissue from which the tumor originates. For instance, in colorectal carcinomas, cancer cells frequently organize themselves in crypt-like gland structures, and contain cellular subpopulations, such as mucus-producing goblet cells, that are reminiscent of normal colon tissues (Figure 1.1). In their daily practice, pathologists routinely rely on tissue-specific morphological features, such as tissue-architecture, cell composition, and expression of tissue-specific differentiation markers, to guide their diagnostic approach to tumor lesions, and for instance, to identify the anatomical origin of a metastatic tumor when the primary site is unknown (Pavlidis and Pentheroudakis, 2012). This approach presupposes that neoplastic tissues, even distant metastases, do usually retain, at least in part, the developmental patterns and differentiation programs of their tissue of origin. The degree by which a tumor retains the capacity to undergo differentiation, a feature usually referred to as "grading," is used as an important prognostic variable in many types of human cancer (Carriaga and Henson, 1995).

From a more mechanistic perspective, ample experimental evidence indicates that, in order to undergo malignant transformation, cells must progressively acquire a set of multiple and independent genetic mutations, frequently over a period of many years (Fearon and Vogelstein, 1990). Given their self-renewal capacity and long life span, stem cells are ideal targets for malignant transformation, as they have the opportunity to accumulate several mutations over long periods of time. On the contrary, their differentiated progeny is frequently short-lived, and thus less likely to survive long enough to complete the transformation process. Finally, one of the critical steps in tumorigenic transformation is the acquisition of the capacity to escape the limits on proliferation imposed by cellular senescence, a property often referred to as "immortality" (Hanahan and Weinberg, 2011). Given their capacity to self-renew, stem cells are naturally endowed with the potential for long-term growth and proliferation. Therefore, as compared to other cell types, stem cells appear better poised to undergo malignant transformation, as they do not need to artificially acquire immortal traits de novo. Taken together, these considerations suggest that, at least in the early stages of neoplastic transformation, a first set of oncogenic mutations might accumulate in the stem cell compartment of normal tissues, and that this population of mutated stem

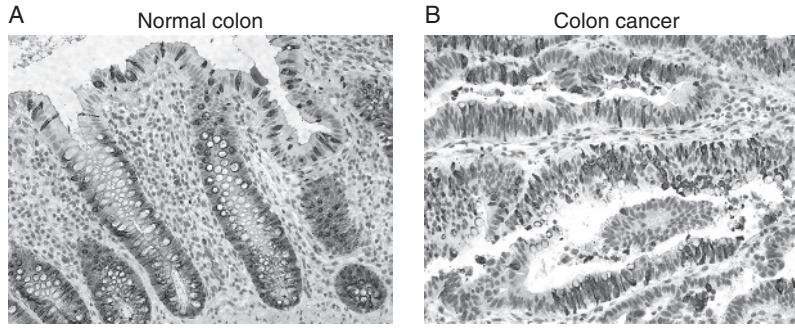

Figure 1.1. Tumors act as "*developmental caricatures*" of normal tissues. **A**. Normal colon epithelium analyzed by immunohistochemistry for expression of the MUC2 protein, a marker of mucus-producing, differentiated goblets cells (brown staining). **B**. Malignant colon cancer tissue analyzed by immunohistochemistry for expression of the MUC2 protein (brown staining). When compared to normal colon epithelia, human colon cancer tissues often reveal close similarities in terms of three-dimensional architecture (e.g., formation of crypt-like gland structures) and cell composition (e.g., presence of mucus-producing goblet-like cells). For color detail, please see color plate section.

cells might sustain the growth of a pathological tissue, which frequently retains the capacity for multi-lineage differentiation. This scenario, however, does not exclude that, over time, the progressive accumulation of additional mutations might ultimately disable the molecular constraints that prevent self-renewal outside of the stem cell compartment, and eventually "unleash" a fully malignant phenotype also in the more differentiated progeny of the neoplastic clone (Weissman, 2005).

Development of the CSC Model in Human Tumors

The experimental foundations of the CSC model, and especially of its more modern formulations, have been laid by studies that investigated the relationship between hematopoietic stem cells (HSC) and human myeloid types of leukemia. In the late 1970s, Philip Fialkow and colleagues (1977) initiated a set of seminal investigations on the monoclonal nature of hematological malignancies, using glucose-6-phosphate dehydrogenase (G6PD) as a marker. The gene encoding for G6PD is located on the X-chromosome. Secondary to random X-inactivation processes in female patients, one of the two X-chromosomes becomes inactivated in somatic cells. As a result, female patients who are heterozygous at the G6PD locus contain two different populations of somatic cells, each expressing either one allele or the other, and each found approximately at 50% frequency. The authors investigated the relative frequency of the two G6PD alleles in heterozygous female patients affected by different forms of hematological malignancies, including acute myeloid leukemia (AML), chronic myeloid leukemia (CML), essential thrombocythemia and polycythemia vera (Adamson et al., 1976; Fialkow et al., 1981a; Fialkow et al., 1977; Fialkow et al., 1981b). In all of these cases, while cells from healthy skin tissues were found to express either one or the other of the two G6PD alleles, and at similar frequency (50:50), circulating tumor cells were found to homogeneously express always the same allele, strongly pointing to a monoclonal origin of the disease. Most remarkably, the authors also showed that, in all of these disorders, the monoclonal expansions encompassed multiple independent lineages of mature circulating blood cells (e.g., granulocytes, erythrocytes, platelets) thus suggesting that the disease might have originated from a transformed hematopoietic stem cell (HSC) that

preserved the capacity for in vivo multilineage differentiation.

In the 1980s, Irving Weissman and colleagues led a systematic effort to identify surface markers that could allow for the live isolation, and thus the prospective functional validation, of HSCs. Through a momentous sequence of studies, they progressively dissected the cellular hierarchy and the lineage relationships that underpin normal hematopoietic development in mammals (Weissman and Shizuru, 2008). Building on this experimental template, in the 1990s, John Dick and colleagues sought to understand whether human blood malignancies preserved not only the cell composition, but also the functional hierarchy observed in normal hematopoiesis (Bonnet and Dick, 1997; Lapidot et al., 1994). Working on human acute myeloid leukemia (AML), they used flow cytometry to sort different phenotypic subsets of the leukemic clone from individual human patients, and compared their functional properties side-by-side, in a prospective way, by transplantation into NOD/SCID immunodeficient mice (Bonnet and Dick, 1997; Lapidot et al., 1994). Remarkably, they observed that not all leukemic cells were equally tumorigenic (i.e., capable to initiate tumor growth upon transplantation), and that tumorigenic cells appeared enriched in the $CD34^{+}/CD38^{neg}$ subset of the neoplastic clone, whose phenotype corresponds to that of the immature stem/progenitor compartments of the normal human hematopoietic lineage. Importantly, leukemic expansions that originated in mice from $CD34^{+}/CD38^{neg}$ cells contained multiple phenotypic subsets and reconstituted the full phenotypic diversity that was characteristic of the original AML in corresponding patients. These studies provided the first evidence for the existence, within human AML, of a hierarchical organization reminiscent of a stem cell system.

In the last decade, the CSC model has been progressively applied to the study of many other tumor types, especially solid epithelial carcinomas. Progress has been slow, due to limited knowledge on the developmental hierarchy of many normal human tissues, and the frequent lack of known cell-surface markers for differential isolation of cellular subsets. In 2003, Michael Clarke and colleagues developed a flow cytometry method for the differential purification of distinct phenotypic subsets within human breast cancer epithelia and showed that $CD44^{+}/CD24^{neg/low}$ cells were substantially enriched for tumorigenic capacity in NOD/SCID mice (Al-Hajj et al., 2003). Importantly, tumors originated from $CD44^{+}/CD24^{neg/low}$ cells contained mixed populations of cancer cells, fully re-creating the phenotypic heterogeneity of the parent tumors. This study provided the first experimental evidence of a functional hierarchy reminiscent of a stem cell system in a human solid tumor (Clarke, 2005). Similar findings have since been obtained in many other human solid tumors, including glioblastoma multiforme (Galli et al., 2004; Singh et al., 2004), colon cancer (Chu et al., 2009; Dalerba et al., 2007b; O'Brien et al., 2007; Ricci-Vitiani et al., 2007), head and neck cancer (Prince et al., 2007), pancreatic cancer (Li et al., 2007), bladder cancer (Chan et al., 2009), prostate cancer (Collins et al., 2005; Patrawala et al., 2006; Rajasekhar et al., 2011), and cholangiocarcinoma (Wang et al., 2011).

Taken together, these studies contributed to define a common methodological framework for the experimental validation of the CSC model. As a general rule, a tumor is usually described as following a CSC model based on the fulfillment of three criteria:

1. Within tumor tissues, only a subset of cancer cells is endowed with tumorigenic capacity when transplanted into immunodeficient mice. These cells are characterized by a distinctive repertoire of surface markers and can be reproducibly purified from other cells that do not have this capacity.
2. Tumors grown from tumorigenic cells contain mixed populations of both tumorigenic and non-tumorigenic cancer cells, thus re-creating the phenotypic heterogeneity of the parent tumor.

3. Tumors grown from tumorigenic cells can be serially passaged multiple times.

According to this definition, the phenotypic subsets of cancer cells endowed with tumorigenic capacity are defined as "cancer stem cells" (CSC), as they display two of the hallmark properties of stem cells: self-renewal (i.e., the capacity to form other CSC with intact long-term proliferation and expansion potential), and differentiation (i.e., the capacity to form a variety of other cell types, thus re-creating the cellular diversity of the parent tissue) (Dalerba et al., 2007a).

Origin, Identity, and Evolution of CSCs During Disease Progression

It is important to note that, in its current use, the term "cancer stem cell" (CSC) entails a purely operational definition (i.e., it is based not on the display of a descriptive trait but on the experimental fulfillment of a specific set of functional properties). As a result, the term CSC indicates a tumorigenic cell endowed with self-renewal and differentiation capacity, but the term does not necessarily indicate a cell whose molecular or phenotypic identity can always be matched with that of a normal stem cell. From a theoretical perspective, stem cells represent ideal targets for malignant transformation, because they are naturally endowed with self-renewal properties, and because their long life span allows for the progressive accumulation of multiple rounds of oncogenic mutations. However, it is also possible that, over time, as more and more mutations accumulate in the neoplastic clone, some of the differentiated elements of the mutated stem cell's progeny might aberrantly acquire the capacity of self-renew (Weissman, 2005). As a result, during disease progression, the neoplastic clone could acquire additional CSC populations, some of whom are characterized by a more differentiated phenotype, and sometimes by higher proliferation rates.

Once again, these concepts are best illustrated by investigations initially performed in hematological malignancies. In human CML, for instance, in the initial "chronic phase" of the disease, the malignant clone appears sustained by a mutated population of HSCs, which usually maintain the capacity for multilineage differentiation (Takahashi et al., 1998). However, as CML progresses toward its terminal "blast crisis," the leukemic clone acquires a second population with CSC properties, whose surface marker phenotype mirrors that of a more mature granulocyte-macrophage progenitor cell (GMP) (Jamieson et al., 2004). Conceptually similar findings hold true also for AML, where cells with CSC properties can be found in populations whose surface marker phenotype is not always strictly characteristic of hematopoietic stem-cells (HSC) but can encompass also multipotent progenitors (MPP) (Blair et al., 1997; Eppert et al., 2011; Miyamoto et al., 2000), and sometimes more differentiated oligolineage precursors, such as GMPs (Goardon et al., 2011).

Taken together, these studies suggest that the hierarchical organization of tumor tissues is likely to "evolve" during the natural history of each disease, and that the phenotypic and molecular identity of its CSC populations might change over time and across different patients (Jan et al., 2012). The molecular basis of these events is the subject of intense investigations. Experiments in mice have shown that aberrant acquisition of self-renewal capacity can result from the inactivation of classic tumor-suppressor genes, as observed in the case of mice bearing simultaneous inactivation of the Tp53, $p16^{INK4a}$ and $p19^{ARF}$ genes, a genetic lesion able to confer aberrant self-renewal capacity to normal blood MPPs, which are usually devoid of such property (Akala et al., 2008). In human CML, aberrant acquisition of self-renewal capacity by neoplastic GMP-like cells during progression to "blast crisis" is associated to abnormal mis-splicing of the GSK3β gene (Abrahamsson et al., 2009).

Validation of the CSC Model in Animal Tumors

Due to obvious ethical reasons, transplantation experiments using human cancer cells can be performed only in xenogeneic hosts (e.g., in immunodeficient animals). This experimental limitation raises the question of whether the lack of tumorigenicity observed for specific subsets of human cancer cells (i.e., their inability to form tumors when transplanted in immunodeficient mice) is caused by an incompatibility with the mouse tissue microenvironment rather than an intrinsic deficiency in long-term proliferation capacity (Quintana et al., 2008). Examples of cross-species barriers that could limit the in vivo engraftment of cancer cells in these experimental settings include the rejection of transplanted cells by residual elements of the host's innate immune system (e.g., macrophages, NK cells), or the inability of cancer cells to cross-talk with stromal populations of the host (e.g., fibroblasts, endothelial cells) due to a reduced capacity of specific growth factors to activate their receptor counterparts across different species (Quintana et al., 2008). To circumvent these problems, several authors set out to investigate whether the CSC model can be applied also to the study of animal tumors, where transplantation experiments can be performed in syngeneic systems (i.e., between genetically identical individuals), thus by-passing all cross-species barriers to engraftment. Studies on mouse epithelial tumors, such as the MMTV-Wnt1 transgenic mouse model of breast cancer and the DMBA/TPA-induced mouse model of skin squamous cell carcinoma, have shown that several forms of mouse cancer contain multiple phenotypic subsets of cancer cells, which differ substantially in their tumorigenic capacity (Cho et al., 2008; Malanchi et al., 2008). Importantly, in these animal models, the tumorigenic populations fulfilled the definition for CSC, as they were able to sustain the formation of tumor tissues that recapitulated the phenotypic diversity and hierarchical organization of the parent tissues, and included the presence of non-tumorigenic cancer cells. Similar to what observed in many human tumors, CSC from mouse tumors are frequently defined by a surface marker phenotype characteristic of more immature stem/progenitor cell compartments of the corresponding normal tissues (i.e., $CD24^{+}/Thy1^{+}$ in the mouse breast epithelium, $CD34^{+}/KRT10^{neg}$ in the mouse skin epidermis).

Experimental Proof of Multi-lineage Differentiation in Solid Tumors

In hematological malignancies, such as CML, leukemic populations can encompass multiple lineages of differentiated circulating blood elements (e.g., granulocytes, monocytes, B-cells), likely originated from multi-lineage differentiation of a mutated hematopoietic stem/progenitor cell. In solid tumors, however, the possibility that the phenotypic diversity observed among cancer cells could originate as the result of a multi-lineage differentiation process, similar to that normally observed in corresponding healthy tissues, remained historically controversial (Shackleton et al., 2009). Experimental efforts to address this question have traditionally been focused on human colon cancer, as malignant colon epithelia are frequently composed of multiple cell populations that mirror those of the normal tissue (Figure 1.1). Several pioneering studies provided evidence in support of multi-lineage differentiation (Kirkland, 1988; Odoux et al., 2008; Vermeulen et al., 2008), but could not take advantage of a comprehensive molecular characterization of the various malignant populations. Recently, analysis by single-cell PCR of the cell composition of human colon cancer tissues revealed that, indeed, malignant colon epithelia contain a diversity

of multiple cell subsets, whose transcriptional identity closely mirrors that of the various lineages and differentiation stages of the normal colon (e.g., goblet cells, enterocytes, $Lgr5^+$ columnar basal cells) (Dalerba et al., 2011). Formal demonstration of multi-lineage differentiation, however, requires a prospective experiment, in which the fate of a single (n = 1) cell is followed over time, together with careful molecular tracing of the monoclonal nature of its progeny (Figure 1.2). This experiment was performed by injection a single colon CSC ($EpCAM^{high}/CD44^+$), purified by flow cytometry from a xenograft line, and injected into a NOD/SCID/IL2R$\gamma^{-/-}$ mouse after infection with a lentivirus encoding for the enhanced green fluorescent protein (EGFP) (Dalerba et al., 2011). The resulting tumor's monoclonality was confirmed by the presence of a unique lentivirus DNA integration site in cancer cells, all of which expressed EGFP. Importantly, the monoclonal tumor generated in this experiment re-created the full phenotypic heterogeneity of the malignant parent tissue, in terms of both tissue histology and repertoire of cell populations. Most strikingly, a single-cell PCR gene-expression analysis of the monoclonal tumor demonstrated its heterogeneous lineage composition, again closely mirroring the cellular diversity of normal colon tissues.

Although transplantation experiments provided formal proof of the multi-lineage differentiation capacity of CSCs in human colon cancer, the artificial nature of transplantation procedures left open the question of whether this actually occurs in primary tumors, in the natural environment where cancer cells are normally found. To better clarify this point, a recent set of studies addressed the question of multi-lineage differentiation in mouse tumors, taking advantage of transgenic mouse strains genetically engineered to allow for in vivo lineage-tracing experiments. A classic example of such strains is represented by mice engineered to carry simultaneously: a) a copy of the gene encoding for the chimeric Cre/ER recombinase, placed under the transcriptional control of a lineage-specific promoter, such as the KRT14 promoter or the Lgr5 promoter, and b) a gene encoding for a fluorescent reporter protein (e.g., the yellow fluorescence protein or YFP), placed under the transcriptional control of a constitutive promoter (e.g., the Rosa26 promoter), but rendered inactive by the introduction of a poli-adenylation cassette flanked by LoxP sites between the promoter and the gene. In these mice, a pulsed injection of tamoxifen is able to cause transient activation of the Cre/ER recombinase but only in the specific subset of cells where the lineage-specific promoter is active (i.e., $KRT14^+$ or $Lgr5^+$ cells). Transient activation of the Cre/ER recombinase leads to excision of the poly-adenylation cassette and irreversible activation of the fluorescent reporter gene, resulting in the permanent in vivo labeling of individual $KRT14^+$ or $Lgr5^+$ cells and, most importantly, of all of their subsequent in vivo progeny. Experiments performed using this system were performed both in the DMBA/TPA-induced mouse model of skin papilloma, using a mouse strain engineered with a KRT14-Cre/ER construct active in the basal layer of the skin's epidermis (Driessens et al., 2012), and in the $APC^{fl/fl}$ mouse model of intestinal adenoma, using a strain engineered with the Lgr5-Cre/ER construct active in a subset of progenitor cells at the bottom of colonic crypts (Schepers et al., 2012). These studies allowed genetic labeling and prospective tracing of individual tumor cells in vivo, in a context of unperturbed tumor growth. In both models, this strategy revealed that a minority subset of neoplastic cells had both the capacity to persist long-term in tumor tissues (i.e., to self-renew) and to generate a diverse cellular progeny that encompassed all other mature cell types normally found in the original tissue (i.e., to undergo multi-lineage differentiation).

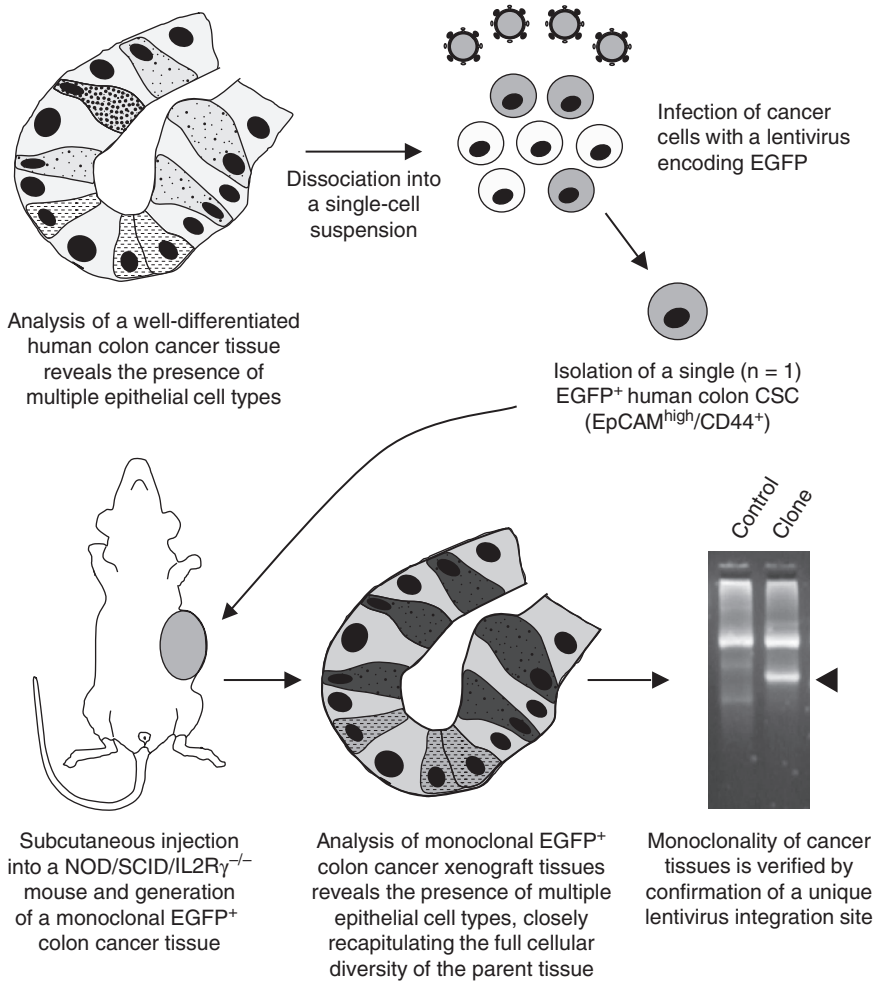

Figure 1.2. Prospective evaluation of multi-lineage differentiation capacity using transplantation experiments. Tumor tissues are frequently composed of a heterogeneous population of cancer cells, containing multiple cellular subtypes. According to the CSC model, the cellular diversity of tumor tissues can originate as the result of multi-lineage differentiation processes, akin to those sustained by normal stem cells. Formal demonstration of multi-lineage differentiation requires a prospective experiment, in which the fate of a single (n = 1) cancer cell is followed over time, together with careful molecular tracing of the monoclonal nature of its progeny. A possible way to perform this experiment is to inject into an immunodeficient animal host a single (n = 1) CSC purified from a tumor tissue known to contain a heterogeneous population of cancer cells (e.g., a single $EpCAM^{high}/CD44^{+}$ human cancer cell from a well-differentiated human colon cancer xenograft line) after infection with a lentivirus encoding for the enhanced green fluorescence protein (EGFP). The monoclonal nature of resulting tumors can be demonstrated by showing the presence of a unique lentivirus DNA integration site in the genome of cancer cells, all of which must also express EGFP (*green cells*). The observation that monoclonal tumor tissues generated by transplantation of a single cancer cell are able to recapitulate the phenotypic diversity and lineage composition of parent tumors (*various shades of green cells*) provides formal evidence of the multi-lineage differentiation capacity of the transplanted "*cancer stem cell.*" For color detail, please see color plate section.

Clinical Implications of the CSC Model

Beyond its intellectual appeal for the theoretical modeling of cancer biology, the CSC theory has profound clinical implications in medical oncology, especially for the discovery of novel prognostic biomarkers and for the design and validation of novel anti-tumor treatments. For instance, the CSC model suggests that variations in the cellular composition of tumor tissues, including both variations in the relative content of distinct subsets of mature cell types (e.g., produced by perturbations in multi-lineage differentiation processes), and variations in the frequency and phenotype of CSC populations (e.g., produced by aberrant acquisition of self-renewal capacity by mature cell types), might have substantial impact in disease biology. Furthermore, these variations might underpin changes in growth rate and differential sensitivity to individual drugs.

Given the ominous capacity of CSCs to self-renew and form tumors in mice, the CSC model suggests that tumors characterized by a higher content of CSCs could be characterized by a more aggressive natural history, and worse clinical outcomes. This concept was explored in a study that investigated whether the gene-expression profile of purified human breast CSCs ($CD44^{+}/CD24^{neg/low}$) could be used to generate a gene signature for prognostic applications (Liu et al., 2007). Indeed, the study showed that breast cancer patients whose whole tumor gene-expression profile displayed higher similarity to the CSC-derived signature were characterized by higher incidence of tumor recurrence and lower overall survival rates. Very similar results have recently been obtained also in the case of AML (Eppert et al., 2011; Gentles et al., 2010) and colon cancer (Merlos-Suarez et al., 2011). Microarray-derived gene-expression signatures are often difficult to implement for routine clinical use, but they can be frequently reduced to more simplified marker sets, focusing on a few robust genes (Gentles and Alizadeh, 2011). In the case of CSC signatures, a detailed understanding of multi-lineage differentiation processes can lead to the identification of reference markers for individual cell types, which in turn can be exploited as a measure of tissue cell composition. For example, a careful dissection of the differential gene-expression profile of the various epithelial cell lineages of the colon epithelium led to the development a two-gene classifier system (i.e., KRT20 versus CA1, SLC26A3, MS4A12, or CD177) that allowed the stratification of large cohorts of human colorectal carcinomas into discrete biological subgroups, characterized by very different clinical outcomes (Dalerba et al., 2011). Once again, tumors characterized by a more immature, undifferentiated phenotype (e.g., $KRT20^{neg}/CA1^{neg}$) were associated with worse survival outcomes. Remarkably, the prognostic power of this two-gene classifier system appeared superior to that of pathological grade, which is currently one of the few parameters used in the design of treatment algorithms for colon cancer. A similar strategy was successfully applied also to bladder cancer, using a limited set of gene-expression markers known to be differentially expressed during the various differentiation stages of the normal bladder epithelium (e.g., KRT14, KRT5, KRT20) (Volkmer et al., 2012).

Another important implication of the CSC model is related to the design and evaluation of anti-tumor treatments. According to the CSC model, eradication of tumor tissues is strictly dependent upon elimination of their CSC component, as CSCs are endowed with self-renewal capacity and hold the potential to sustain tumor recurrence (Figure 1.3). Historically, many conventional cancer treatments, such as many forms of chemotherapy and radiation, have been selected based on their capacity to induce tumor response (i.e., to rapidly reduce tumor size). As a result, these treatments are usually capable of killing a majority of cancer cells, and frequently target rapidly proliferating cells, but are not specifically designed to target CSC populations. Importantly, several studies suggest that CSCs might be preferentially

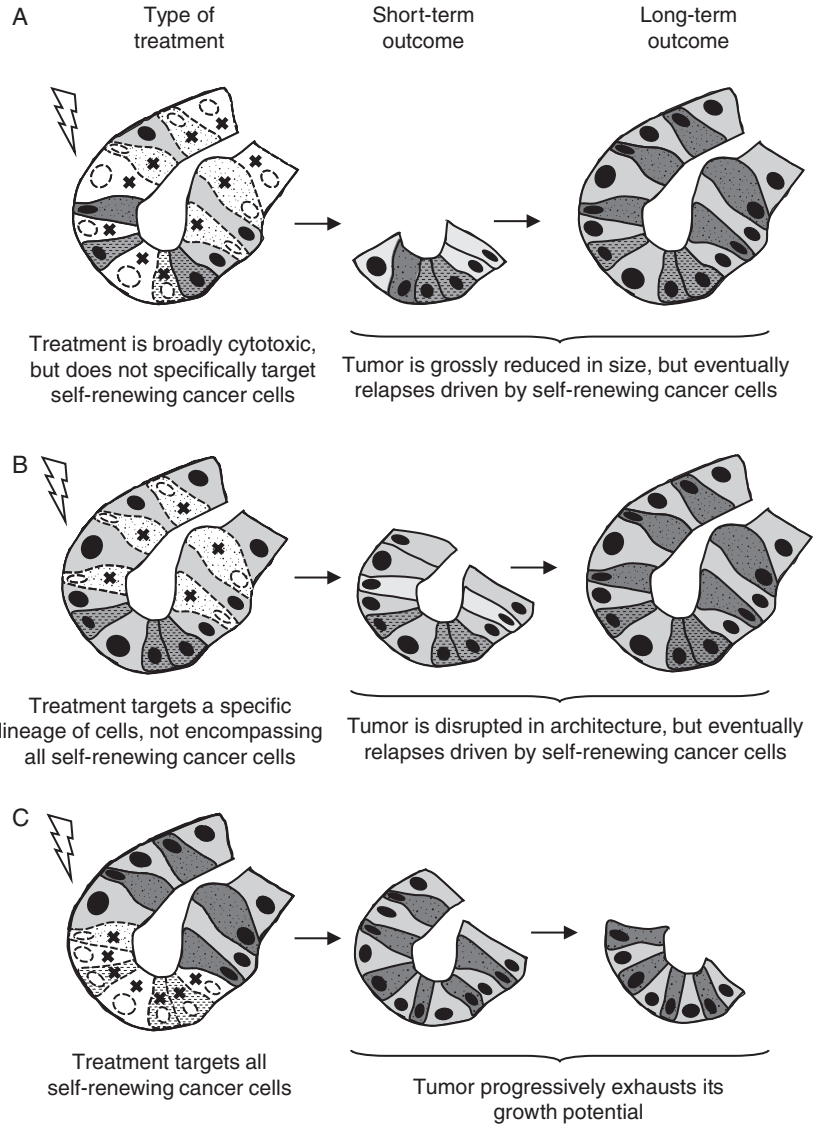

Figure 1.3. Implications of the CSC model for the design and evaluation of anti-tumor treatments. The heterogeneous cell composition of cancer tissues implies the possibility of a differential sensitivity of different cell types to various treatments. **A**. Anti-tumor treatments endowed with broad cytotoxic activity (e.g., treatments targeting rapidly proliferating cancer cells) might be able to hit multiple cell types (*yellow cells with red crosses*) and induce substantial reductions in tumor size. However, if cancer cells endowed with self-renewal capacity (*dark grey cells*) are spared, they hold the potential to cause tumor relapse. **B**. Anti-tumor treatments targeted to a specific lineage of cancer cells (*black cells*) might induce tissue disruption and temporary reduction of tumor volume. However, if the targeted lineage does not encompass all cells with self-renewal capacity, tumor tissues are likely to relapse, sustained by self-renewing cancer cells. At relapse, cells of the targeted lineage might be re-created as a result of multi-lineage differentiation processes. **C**. Anti-tumor treatments able to target and eliminate all cancer cells with self-renewal capacity, although not necessarily designed to induce rapid reductions in tumor volume, could cause long-term exhaustion of tissue growth, and hold the potential for permanent tumor eradication. For color detail, please see color plate section.

resistant to several standard anti-tumor treatments, including both chemotherapy (Dylla et al., 2008; Guzman et al., 2001) and radiotherapy (Bao et al., 2006; Diehn et al., 2009), and that they might be responsible for fueling tumor recurrence after initial response (Chen et al., 2012). Based on these findings, a new wave of studies is currently being initiated, aimed at a detailed molecular characterization of CSCs across the full spectrum of human tumors. Hopefully, a deeper understanding of the biochemical pathways that define CSC properties will identify novel therapeutic targets, and pave the way to new, more effective therapies.

Future Perspectives

During the last 10 years, the CSC model progressively established itself as a powerful research tool in cancer biology, endowed with substantial heuristic value. It provided us with a better understanding of intra-tumor heterogeneity and of the hierarchical organization that exists within cancer tissues. According to this model, the cellular heterogeneity observed within tumors originates, at least in part, as the result of multi-lineage differentiation, an epigenetic diversification process characteristic of normal stem cell systems. Perturbations in multi-lineage differentiation can translate into variations in the cellular composition of malignant tissues, and likely underpin important differences in tumor behavior across patients. As a general rule, for instance, tumors charaterized by a gene-expression profile characteristic of more immature, undifferentiated cells display a more aggressive clinical course and associate with worse survival. Identification of genes differentially expressed across cell populations holds the potential for the discovery of both novel prognostic biomarkers and highly specific cellular targets for drug development. Ultimately, a deep molecular understanding of the similarities and differences between CSCs and normal stem cells will allow the development of a new generation of therapies, able to target cancer tissues specifically, while leaving normal tissues unharmed.

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