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## **Cancer stem cells in tumor heterogeneity**

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## **Abstract**

Cancer cells within a given tumor were long regarded as a largely homogeneous group of cells originating from a common progenitor cell. However, it is increasingly appreciated that there is a considerable heterogeneity within tumors also on the tumor cell level. This heterogeneity extends to virtually all measurable properties of cancer cells, ranging from differentiation state, proliferation rate, migratory and invasive capacity to size and therapeutic response. Such heterogeneity likely represents a major therapeutic hurdle, but the mechanisms underlying its emergence remain poorly understood and a controversial topic. The cancer stem cell model of tumor progression has gained increasing support during the past several years. In this review, I will discuss some major implications of the cancer stem cell hypothesis on the origins of tumor heterogeneity, focusing both on heterogeneity within the tumor cells proper and on potential transdifferentiation of cancer stem cells into stromal and endothelial lineages, as well as on heterogeneity of the therapeutic response. Evidence for and against a direct and causal role of cancer stem cells in the emergence of tumor heterogeneity will be weighed and alternative explanations for apparently contradictory observations discussed. Finally, I will discuss the potential origins of cancer stem cells and the various implications of origin to the contribution to tumor heterogeneity, and outline some future directions.

## I. INTRODUCTION

Cancers are generally characterized by a remarkable phenotypical and functional inter- and intratumoral heterogeneity. Intratumoral heterogeneity results in part from the co-existence of tumor cells and a large number of varying stromal cells within tumors, but importantly, even within the tumor cell compartment there are plentiful diverse geno- and phenotypes. Such heterogeneity is likely a major therapeutic hurdle, yet the mechanisms underlying the development of tumor heterogeneity remain poorly understood. In this review, I will discuss the potential implications of the cancer stem cell hypothesis for the emergence of intratumoral tumor cell heterogeneity. Even though intratumoral heterogeneity is likely evident in virtually all measurable properties of tumor cells, like proliferation rate, invasiveness, migratory potential or therapeutic responses (Heppner, 1984), a lot of recent attention has been given heterogeneity with respect to tumor cell differentiation status (Shipitsin et al., 2007; Pietras et al., 2008; Pietras et al., 2010; Pistollato et al. 2010a).

Although there is little doubt that clonal evolution of tumor cells within any tumor inevitably occurs and drives development of distinct tumor cell subclones, the extent to which such clonal evolution within any cell type contributes to the massive heterogeneity within any individual cancer remains unclear. A major controversy in contemporary cancer research relates to how tumors are thought to be maintained and progress to metastatic disease. The classical Darwinistic view that cancers advance through a process of clonal evolution has recently been challenged by the increasingly supported

cancer stem cell hypothesis. The cancer stem cell hypothesis, if true, could have major implications for how we think of the origins of cellular heterogeneity in tumors and the role of clonal evolution.

## **II. A BRIEF INTRODUCTION TO MODELS OF TUMOR DEVELOPMENT**

### **A. The Clonal Evolution Model**

As first proposed by Nowell in 1976, the clonal evolution model for tumor progression suggests that within a population of tumor cells, a natural selection occurs that favors cells that have acquired (e.g. through additional mutations) the most aggressive phenotype (Nowell, 1976). The model suggests that cancers arise when a sufficient number of mutations have occurred in any given cell. Genetic instability within the tumor cell pool then inevitably leads to the accumulation of additional mutations within single cells; mutations that confer a growth advantage (e.g. by making cells less sensitive to cell death cues or more prone to proliferate) to the cell will survive as the cells give rise to new offspring. Thus, heterogeneity within tumors according to the clonal evolution hypothesis first and foremost arises from different genetic hits affecting different cells that originate from the same initial cell of origin. Importantly, it is also stipulated that such evolution within the tumor is what eventually gives rise to tumor cells with the capacities of invasion into neighboring tissues and metastasis. Furthermore, the same clonal evolution is believed to cause resistance to anti-cancer drugs and irradiation.

While direct evidence for any model of tumor progression is difficult to come by, the clonal evolution hypothesis is compatible with several characteristics of cancer including phenotypic heterogeneity (Marusyk and Polyak, 2009), genetic instability (Hanahan and Weinberg, 2000) and the frequent emergence of chemotherapy-resistant clones after treatment (Shah and Sawyers, 2003). The clonal evolution model further rests on the widespread notion that primary tumors and metastases frequently share the vast majority of genetic aberrations, but that metastases (that are thought to arise from cells generated by clonal evolution within the primary tumor) have acquired additional hits not present in the primary tumor. However, only few studies have addressed this matter by studying genetic aberrations in matched pairs of primary tumor and metastases. Intriguingly, although many reports at least partially support the prediction of the clonal evolution theory (Fujii et al., 1996; Kuukasjarvi et al., 1997; Jones et al., 2008), several of the studies performed to date report remarkably diverging patterns of genetic alterations in primary tumors as compared to metastases from the same patient (Albanese et al., 2004; Katona et al., 2007; Artale et al., 2008; Kalikaki et al., 2008). These data open for the possibility that in some tumors, metastases may arise from early disseminated tumor cells, or conceivably, a genetically more stable founder cell common to primary tumor and metastasis, rather than cells that have acquired a number of additional genetic aberrations by clonal evolution within the primary tumor.

Essentially, the clonal evolution hypothesis assumes that all cells within a tumor hold an equal potential to maintain and advance the tumor to metastasis, pending the acquirement of the necessary capacities.

## **B. The Cancer Stem Cell Model**

Although Julius Cohnheim first put an early version of the cancer stem cell hypothesis forward as early as 1875 (Cohnheim, 1875), it is not until recently that it has gained wide support in the cancer research community. Cohnheim suggested that cancers arise from “embryonic rests”, remnants from development that were transformed into cancer, based on similarities between certain cancer cells and embryonic cells. Increased understanding of developmental and stem cell biology as well as technological advances have enabled researchers to investigate these ideas more thoroughly.

Unlike the clonal evolution hypothesis, the cancer stem cell model assumes that cancers are maintained and propagated by a presumably (but not necessarily) small subpopulation of cells within the tumor that have or have acquired properties of stem cells. Like most organs are believed to have a pool of organ-specific stem cells that have the capacity to self-renew as well as give rise to the various cell types within their respective tissue (Osawa et al., 1996; Reynolds and Weiss, 1996; Kim et al., 2005), so would cancer stem cells give rise to the multitude of cancer cell types within a tumor. Cancer stem cells themselves would have a near-unlimited proliferation capacity, whereas the more differentiated tumor cells it gives rise to would hold only a limited potential for proliferation. Thus, seeding of metastases and maintenance of tumors would primarily and in the long run depend on the stem cell population. While many believe that organ-specific stem cells may indeed be the cells of origin for most cancers, the cancer stem cell hypothesis itself does not stipulate how stem-like properties were acquired. Properties of cancer stem cells may equally occur through transformation of normal stem cells as dedifferentiation of more mature transformed cells (Jögi et al.,

2002; Mani et al., 2008; Heddlestone et al., 2009). Partly because of this potential confusion, cancer stem cells are frequently referred to as tumor-initiating cells (TICs), a term strictly based on the experimental evidence backing the hypothesis (see below). The cancer stem cell model suggests that metastatic spread occurs through dissemination of cancer stem cells, and that resistance to chemo- and radiotherapy is likely due to inherent resistance in the cancer stem cell pool rather than induced selection by clonal evolution.

Early experimental support for the cancer stem cell model comes from studies performed in the 1960s that would be (and should be) impossible to conduct today due to ethical considerations (Southam and Brunschwig, 1961). Transplanting human cancer cells back to patients subcutaneously post-surgery allowed Southam & Brunschwig to conclude that at least 1,000,000 injected tumor cells were required to seed a new tumor, suggesting that not all tumor cells had an equal capacity to induce tumor growth. The first successful isolation of a population of cells from a human cancer that appeared to have an exclusive ability to form new tumors was performed in John Dick's laboratory in 1994 (Lapidot et al., 1994). By transplanting cells from human acute myeloid leukemias into SCID mice, they found that the only cells capable of engrafting a new leukemia were cells that were isolated as positive for the cell surface marker CD34, but negative for CD38 (CD34+/CD38-). Xenotransplantation of CD34+/CD38+ or CD34- cells rendered no tumor growth in mice. Similar studies in a wide variety of leukemias and solid tumors (Al-Hajj et al., 2003; Singh et al., 2004; Taylor et al., 2005; Patrawala et al., 2006; Hermann et al., 2007; O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Eramo et al., 2008; Schatton et al., 2008), have followed to identify cell populations with an increased tumor-forming



ability as compared to other populations from the same tumor. The interpretation of these and similar results has recently been somewhat complicated by the finding that the tumor formation efficiency of different cell populations can be greatly affected by the host animal, most notably by the level of immunodeficiency in recipient mice (Quintana et al., 2008). Despite this insight, however, there are clearly qualitative differences in the tumor-forming ability of cancer stem cells versus non-stem-like cells in the most commonly used mouse models. Whether or not these differences represent an actual and important biological issue, however, remains an open question.

### **C. Cancer Stem Cells versus Cell of Origin: An Important Distinction**

Some controversial issues surrounding the cancer stem cell hypothesis relates to the origin of cancer stem cells, and the origin of cancer stem cells versus the cell of origin of the tumor as a whole. Many of these discussions boil down to more or less entirely semantic issues instigated by the use of the term “stem cells” in referring to “cancer stem cells”, and thus several other terms have been suggested to overcome these issues such TICs, tumor-propagating cells and cancer stem-like cells. Nevertheless, the issue of origin of the cancer stem cells is an interesting one and one of potential importance for the functional and practical implications of the cancer stem cell hypothesis for cancer biology and ultimately cancer therapeutics.

The first issue: does the cancer stem cell hypothesis imply that cancer stem cells are derived from normal tissue stem cells? Does the cancer stem cell hypothesis thereby imply that normal tissue stem cells act as cells of origin for most cancers? While normal tissue stem cells have been widely suggested as the most likely cells of origin for many

cancers (Holland, 2001; Gerdes and Yuspa, 2005; Shupe and Petersen, 2005; Hubbard and Gargett, 2010; Sell, 2010; Waters et al., 2010; Visvader, 2011), there is nothing in the cancer stem cell hypothesis that requires that cancer stem cells themselves are indeed the original tumor cell of the cancer. Instead, it is equally likely and equally compatible with the cancer stem cell model that cancers arise in a more differentiated cell and that cancer stem cells occur later in tumor progression. It is likely, however, that normal tissue stem cells require fewer alterations or hits in order to acquire cancer stem cell properties, due to their inherent stem cell abilities.

One likely origin of cancer stem cells in tumors arising from more differentiated cells is simply dedifferentiation of relatively differentiated tumor cells towards a stem cell-like state. Such dedifferentiation, although largely unheard of or at least still controversial during normal tissue development, has been demonstrated to occur in several tumor models and to be triggered by a number of extrinsic environmental stimuli such as tumor hypoxia (Jögi et al., 2002; Helczynska et al., 2003), or occur as a natural consequence of tumor progression (Delahunt, 1999), long before the cancer stem cell model regained mainstream interest in the research community.

In 2008, Weinberg and colleagues investigated the relationship between normal and neoplastic stem cells and cells that have undergone the process of epithelial-to-mesenchymal transition (for a general review on EMT in cancer, see Guarino et al., 2007) in a much-discussed paper (Mani et al., 2008) that initiated a possible bridge between the cancer stem cell and stochastic clonal evolution models of tumor progression. Importantly, this study clearly makes the point that cells with properties of

cancer stem cells need not be derived from actual tissue stem cells. The authors conclude that both normal and malignant human breast stem cells express high levels of molecular markers traditionally associated with epithelial-to-mesenchymal transition, such as N-cadherin and FOXC2. In line with that observation, induction of epithelial-to-mesenchymal transition in breast cancer cells lead to an upregulation of markers associated with the stem cell state (Mani et al., 2008). Importantly, forced epithelial-to-mesenchymal transition resulted in cells with the functional properties of breast cancer stem cells. These cells formed more mammospheres and colonies in soft agar as compared to wild-type controls, and interestingly formed tumors when xenotransplanted in mice with a significantly higher efficiency suggesting that epithelial-to-mesenchymal transition indeed generated cells that fulfill most criteria used to define cancer stem cells (Mani et al., 2008). Similar data have since been presented for several other epithelial cancers such as prostate (Giannoni et al., 2010) and lung (DiMeo et al., 2009) cancer and again for breast cancer (Santisteban et al., 2009) suggesting that epithelial-to-mesenchymal transition in these common cancers may be one general source of cells with cancer stem cell properties.

These concepts may be of more than strictly academic interest as they can significantly impact what strategies can be successfully used clinically to target the cancer stem cell pool. In the original and most strict version of the cancer stem cell model, it is inferred that the cancer stem cell pool is more or less constant and static. Successful targeting of these cells should, in theory, eliminate the tumor with time because of the supposedly limited survival/proliferation potential of the more differentiated tumor bulk cells (Fig. 1A). However, this strategy rests heavily on the assumption that tumor bulk cells cannot re-

acquire properties of stem cells and effectively become part of the cancer stem cell pool. Dedifferentiation and epithelial-to-mesenchymal transition as discussed above could clearly generate cancer stem cells from more differentiated bulk cells and thus, targeting the cancer stem cells in any given tumor does not permanently exclude the possible existence of cancer stem cells in that tumor (Fig. 1B). Does this apparent plasticity mean, then, that targeting the cancer stem cell pool is useless and that the cancer stem cell model adds no further insight as compared to the clonal evolution model? Not necessarily. The presence of tumor cells with stem cell properties may still be of crucial importance for long term tumor maintenance and progression. It is generally assumed that current anti-cancer therapeutics such as irradiation and chemotherapy successfully targets the bulk of tumor cells (see below), but not the cancer stem cell pool. This opens for the opportunity to target in parallel the cancer stem cell pool with novel and targeted therapeutics, while also targeting the bulk of the tumor with more traditional means (Fig. 1C). This strategy, if successful, would leave the tumor without viable cells that can repopulate the tumor and re-initiate tumor growth.

### **III. EVIDENCE FOR A ROLE OF CANCER STEM CELLS IN PHENOTYPIC TUMOR CELL HETEROGENEITY**

The mere existence of cancer stem cells, whatever their role in tumor biology in a broader sense may be, can be considered evidence of a role in promoting tumor heterogeneity. Cancer stem cells differ from their non-stem cancer cell counterparts in a wide variety of measurable properties, some of which include: proliferation rate (Moore

and Lyle, 2011), migratory and invasive behavior (Wei et al., 2010), metastatic potential (Liu et al., 2010), and DNA repair activation/mechanisms (Bao et al., 2006a).

A biologically more intriguing possibility, however, is that the whole range of phenotypically diverse tumor cells within any given tumor are all derived directly from the cancer stem cell pool. Compelling evidence in support of the cancer stem cell hypothesis demonstrate that putative cancer stem cells isolated from leukemias, breast cancers as well as gliomas, when xenotransplanted into immunocompromised mice give rise to cancers very similar to and containing all the different cell types of the original tumor (Lapidot et al., 1994; Al-Hajj et al., 2003; Lee et al., 2006). In contrast, non-stem cancer cells either give rise to no tumors at all or tumors that lack many key characteristics of the cancer observed in the patient (Lapidot et al., 1994; Al-Hajj et al., 2003; Al-Hajj et al., 2004; Al-Hajj and Clarke, 2004; Lee et al., 2006).

In their landmark contribution, Al-Hajj et al. were first to identify *bona fide* cancer stem cells in a solid tumor when they isolated CD44+/CD24- lineage cells from human breast cancer patients (Al-Hajj et al., 2003). These cells illustrate an important point in the biology of cancer stem and non-stem cells; in addition to having the exclusive ability to propagate tumors in recipient mice, they also give rise to tumors containing non-stem cancer cells while non-stem cancer cells never give rise to cancer stem cells. Importantly, non-stem cancer cells generated by cancer stem cells too are unable to form new tumors upon transplantation.

In the paper by Howard Fine and colleagues, the authors describe the parallel isolation of cancer cells from human gliomas in two different ways; one culture was established traditionally in medium containing serum (similar to how most cancer cell lines are established and cultured), while another culture from the same specimen was established in cancer stem cell media (notably without added serum). Within weeks, the traditional culture had altered its phenotype and developed into a homogenous cell line with an epithelial-like morphology. Importantly, while cells cultured under stem cell conditions formed xenograft tumors in mice that recapitulated most features of the clinical gliomas, conventionally cultured cells formed tumors that were remarkably well circumscribed and did not show any tumor cell infiltration into surrounding tissues. SNP analysis and karyotyping revealed that serum-cultured cells had acquired several novel genetic aberrations not seen in the original tumor, while the cancer stem cell culture had remained true to the original genotype. Overall, data presented by Lee et al. emphasize that cancer stem cell cultures under certain circumstances may serve as more reliable models of human cancer biology than conventional cancer cell lines do, whether or not tumor progression follows the cancer stem cell model.

These data indicate that the heterogeneity seen in tumors may arise from differentiation of a cancer stem cell into different lineages rather than from clonal evolution of equal tumor cells. This is particularly compelling, perhaps, because the cancer stem cells isolated from human tumors typically represent only a minor fraction of all tumor cells. The inherent heterogeneity in the non-stem cell population at isolation should allow for fully heterogeneous tumors to form, if this was not dependent on the cancer stem cell compartment as a driver of tumor heterogeneity. That is, most of the heterogeneity seen

in the clinical specimen should be represented instantly in the non-stem cell injection, while only a minor population would be represented in the cancer stem cell injection. Thus, formation of tumors with all cell lineages of the clinical specimen from the cancer stem cell but not from the non-stem cancer cell compartment strongly suggests that the cancer stem cells give rise to the various lineages also in the tumor.

A recent study of heterogeneity in human malignant melanoma cultures gives further support for the role of cancer stem cells in tumor heterogeneity. Melanomas are derived from the neural crest, and melanoma biology in some ways mimics normal neural crest development. Thus, in a malignant melanoma lesion, cells expressing neural crest stem cell markers such as SOX10 and CD271 are frequently present together with more differentiated tumor cells reminiscent of neural crest derivatives. Civenni et al. found that sorting of melanoma cells expressing the neural crest stem cell marker CD271 resulted not only in cells with an exclusive ability to form melanomas when transplanted into recipient mice, but further found that the resulting tumors contained all the various lineages expected from the original tumor (Civenni et al., 2011). This study, however, also shed some further light on one of the major caveats of the predominant method to identify and define cancer stem cells by xenotransplantation (and as also discussed elsewhere in this review). When transplanting sorted cells into mice that were more severely immunocompromised, both CD271 positive and negative fractions gave rise to xenograft tumors. Intriguingly, in these mice none of the fractions gave rise to tumors with the full heterogeneity of the original melanoma represented, suggesting that cancer stem cells themselves are not sufficient for multi-lineage differentiation.

#### **IV. CLUES FROM METASTATIC AND RECURRING LESIONS**

Clues to how tumors are maintained and progress to metastatic disease may be found in comparisons of the genetic identity of the diagnostic clone of the primary tumor with that of recurring or metastatic lesions. In some interpretations of the cancer stem cell model, it is assumed that the bulk of tumor cells contains clones with additional genetic alterations as compared to the cancer stem cell compartment. As the recurring and/or metastatic tumors theoretically stem from the cancer stem cell compartment, these would likely be clonally related to, but significantly different from the dominant clone in the primary tumor. In the more classical view of the clonal evolution model, on the other hand, metastases and recurring tumors, given similar selective pressures, are likely to be highly similar to the most advanced clone in the primary tumor. In the relatively few studies dealing with this issue directly to date, interestingly, most of the time the dominant clone at diagnosis differed significantly from the recurring or metastatic lesion's identity (Li et al., 2003; Zuna et al., 2004; Mullighan et al., 2008). These data suggest that the recurring tumor did not evolve directly from the dominant clone at diagnosis, instead, the recurring tumor and the primary tumor likely shared a common ancestor further back in evolution.

Intriguingly, despite these data there is frequently a striking histological similarity between primary tumor and corresponding metastases (Ma et al., 2003; Weigelt et al., 2003). While this fact could conceivably be explained by any number of reasons, it is also consistent with the idea of a common progenitor cell giving rise to both tumors, i.e.



the cancer stem cell. It is conceivable that a cancer stem cell, that according to the hypothesis remains only a minor population of the primary and metastatic lesion, has acquired a number of genetic hits, then gives rise to the bulk of tumor cells that will in turn acquire additional aberrations. The metastatic or recurring tumor, then, would also be seeded directly by the cancer stem cell. The bulk of tumor cells within the metastatic or recurring lesion would then acquire its own set of additional aberrations, each likely different from the additional hits present in the bulk of the primary tumor. A comparison of the primary tumor and the metastatic or recurring tumor would then, as was frequently the case in the studies referenced above, suggest that indeed these lesions were clonally related, but from a common ancestor that lies further back evolutionary than the dominant clone in the bulk of each tumor type. Furthermore, it is likely that two individual tumors initiated and maintained by the same pool of cancer stem cells would obtain strikingly similar histological features, despite the fact that they each acquire a unique set of additional genetic aberrations.

## **V. CANCER STEM CELLS AND ENDOTHELIAL/STROMAL DIFFERENTIATION**

Recent publications have hinted at further complexity in the contribution of cancer stem cells to intratumor heterogeneity beyond the diversity of tumor cells proper (Fig. 2). An emerging concept in cancer biology is the transdifferentiation of actual tumor cells into various lineages that were not typically perceived as tumor-derived. In a landmark contribution, Maniotis et al. first described the process of vasculogenic mimicry of malignant melanoma cells over 10 years ago (Bissell, 1999; Maniotis et al., 1999). While

blood vessels in tumors are generally thought to form from the recruitment and differentiation of normal host endothelial cells (Phng and Gerhardt, 2009), the authors described the existence of apparently functional vascular channels in human malignant melanomas were lined not by classical endothelial cells, but instead, were lined by tumor cells proper (Maniotis et al., 1999). Similar results have since been obtained in other tumor forms including the childhood tumor neuroblastoma, adding to the notion that vascular support in tumors can sometimes come from the tumor itself (Pezzolo et al., 2007). However, until recently, the mechanisms and the precise cellular players involved have been largely unknown.

Two recent reports have indicated that the ability of human glioblastoma multifome tumors to form tumor-derived endothelial cells rely on the cancer stem cell compartment (Ricci-Vitiani et al., 2010; Wang et al., 2010). The authors of both papers identify apparently functional vessels within human glioblastomas that carry genetic alterations typical of the bulk of tumor cells within their respective tumors. Isolating and culturing cancer stem cells by use of cell surface markers, both studies go on to show that under in vitro conditions, at least a proportion of cancer stem cells but not their non-stem cell counterparts can form capillary-like networks in matrigel, much like normal cultured endothelial cells (Ricci-Vitiani et al., 2010; Wang et al., 2010). Furthermore, in vivo studies confirmed that cancer stem cell-derived xenograft tumors in mice contained vascular endothelial cells derived directly from the injected tumor cells (Wang et al., 2010). Importantly, Ricci-Vitiani et al. demonstrated that specifically targeting cancer stem cell-derived endothelial cells in xenograft tumors significantly impaired tumor

growth, suggesting a functional importance of endothelial differentiation of cancer stem cells (Ricci-Vitiani et al., 2010).

The concept of tumor-derived endothelial cells carries major implications for anti-tumor therapeutics. A key advantage in therapeutic targeting of angiogenesis and tumor vasculature is, or is thought to be, the relative genetic stability and “normal” behavior of the tumor-associated vasculature as compared to the tumor cells proper. If tumor cells themselves, on the other hand, are capable of forming functional blood vessels within the tumor, classical strategies to target endothelial cells may be inefficient in targeting these plastic and genetically unstable tumor-derived cells with endothelial differentiation. If this heterogeneity within the endothelial cell pool in a tumor is a direct consequence of the multi-lineage differentiation potential of the cancer stem cells, therapeutic targeting of this subpopulation of cells may aid cancer therapeutics regardless of whether these cells are the only cells capable of propagating and maintaining a particular tumor.

Similarly, cancer stem cells of glioblastoma multiforme tumors have been shown to have the ability to differentiate into mesenchymal lineages (Ricci-Vitiani et al., 2008). Ricci-Vitiani et al. demonstrated that both in vivo and in vitro, a subset of glioblastoma stem cells was able to give rise to both neuronal and osteo-chondrogenic lineage cells. These studies again highlight that targeting “normal” cells of the stromal compartment in tumors may be complicated by stromal differentiation of a small subset of bona fide tumor cells. Furthermore, they are suggestive of an even broader role for cancer stem cells in regulating intratumoral cellular heterogeneity (Fig. 2).

## VI. CANCER STEM CELLS IN ANGIOGENESIS

Accumulating evidence additionally support a direct and crucial role for cancer stem cells in the recruitment, expansion and differentiation of normal host-derived tumor vasculature, thereby contributing to another well-documented intratumoral heterogeneity (Fig. 2). Different regions of the same tumor may have dramatically different vascular density and tumor cells themselves play a key role in the recruitment of vessels. Early on, VEGF was identified as a key secreted factor produced by CD133+ human glioblastoma cancer stem cells (Bao et al., 2006b), and Bao et al. demonstrated that VEGF secreted from cancer stem cells contributed to and promoted glioblastoma angiogenesis. Subsequent studies have revealed that cancer stem cells or putative cancer stem cells in a number of tumor forms exhibit higher than normal levels of the hypoxia-inducible transcription factors and specifically, HIF-2 $\alpha$ , regardless of oxygen tension (Pietras et al., 2008; Kim et al., 2009; Li et al., 2009; Bar et al., 2010; Mendez et al., 2010; Pietras et al., 2010; Pistollato et al., 2010a; Seidel et al., 2010). The mechanisms underlying this pseudo-hypoxic phenotype of cancer stem cells remain poorly understood, but it is increasingly clear that both metabolic and angiogenic heterogeneity within tumors are affected by aberrant expression of key players of the hypoxic response.

It is increasingly evident that the same pathways that regulate maintenance of stem cell properties in both normal and malignant stem cells play important roles also in regulation of tumor angiogenesis. This is particularly true perhaps for the Notch signaling pathway,

that has emerged as a popular target in tumor biology both for targeting stem cell phenotypes and aberrant tumor vascularization (Gustafsson et al., 2005; Zeng et al., 2005; Noguera-Troise et al., 2006; Ridgway et al., 2006; Siekmann and Lawson, 2007; Thurston et al., 2007; Bar et al., 2010; Charles et al., 2010; Pistollato et al., 2010b; Yustein et al., 2010). Indeed, there is accumulating evidence that the pseudo-hypoxic phenotype of cancer stem cells itself will lead to increased Notch signaling or specific expression of elements of the Notch signaling pathway, subsequently leading to an increase in both tumor “stemness” and tumor vascularization (Jögi et al., 2002; Gustafsson et al., 2005; Sahlgren et al., 2008; Chen et al., 2010; Elias et al., 2010; Pietras et al., 2011).

## **VII. CANCER STEM CELLS IN HETEROGENEITY OF THE THERAPEUTIC RESPONSE**

Data obtained from studies of putative cancer stem cells provide an alternative explanation for how resistance to chemo- and radiotherapy occurs in tumors. Several experiments have demonstrated in human and animal systems that when treated with these modalities, the cancer stem cell pool survives whereas the non-stem cell pool dies (Al-Hajj et al., 2004; Bao et al., 2006a; Hambardzumyan et al., 2008a; Hambardzumyan et al., 2008b; Bleau et al., 2009; Creighton et al., 2009; Chappell and Dalton, 2010; Singh and Settleman, 2010). The point that stem-like cells in tumors may be more resistant to radiation therapy has been best demonstrated in brain tumors. Bao et al. sorted stem-like cells from gliomas based on their expression of CD133, and subjected them and their corresponding non stem-like CD133 negative cells to irradiation *in vitro*

and *in vivo* (Bao et al., 2006a). Specifically, the authors showed that the stem-like cells more efficiently activated the DNA damage checkpoint and thus were better at repairing radiation-induced DNA damage. While these data were mainly derived from isolates of stem-like and non stem-like cells from human tumors, Holland and colleagues elegantly demonstrated similar findings in several physiologically relevant mouse models of the childhood brain tumor medulloblastoma (Hambardzumyan et al., 2008b). The mouse models used, induced by Sonic Hedgehog in combination with N-Myc or AKT, show remarkable similarity to human brain tumors in general and medulloblastomas in particular, with a well-defined and relatively small stem cell compartment located in a perivascular niche, clearly separated from the bulk of tumor cells with more differentiated phenotypes. These mouse models can thus be used to study the biology of cancer stem cells without isolation of cell populations and disturbing the tumor microenvironment. Irradiating these medulloblastomas, Hambardzumyan et al. demonstrated that indeed, while the bulk of tumor cells rapidly underwent apoptotic cell death upon irradiation, the stem-like cells of the perivascular niche simply went into cell cycle arrest for up to 72 h, then begun to proliferate again (Hambardzumyan et al., 2008b). These data clearly demonstrated that in these models, tumor resistance to irradiation and most likely recurrence after treatment despite successful “debulking” of the tumor by radiotherapy was due to resistance of the cancer stem cell pool. Interestingly, the cancer stem cell compartment appeared to preferentially activate PI3K/AKT signaling in these tumors, and inhibiting these pathways during irradiation greatly increased the level of cell death in the perivascular compartment. These data promise a therapeutic benefit of specifically targeting pathways hyperactivated in cancer stem cells specifically in combination with more conventional anti-tumor therapeutics.

Similar mechanisms appear to be in place for resistance to chemotherapy in gliomas. In PDGF-induced murine gliomas, Bleau et al. first demonstrated that the side population assay (based on Hoechst 33342 exclusion via the drug efflux pump ABCG2, reviewed in Goodell et al., 2005) can be used to isolate highly tumorigenic cells with properties of cancer stem cells (Bleau et al., 2009). Strikingly, treating neurospheres *in vitro* with the standard chemotherapy for human gliomas – temozolomide – resulted in a greatly increased proportion of side population cells. Remarkably, this effect was seen despite that temozolomide is not a known substrate for the ABCG2 pump and indeed, the increase in side population cells did not appear to be the result of selection for ABCG2 expressing cells as temozolomide was as efficient in killing ABCG2 positive and negative cells (Bleau et al., 2009). Like in the medulloblastoma models, it was found that the PI3K/AKT pathways regulate the side population phenotype in stem-like cancer cells.

Taken together, these results suggest that resistance may occur not due to a selection of tumor cells that happen to have acquired properties of resistance (as generally assumed by the clonal evolution hypothesis), but rather due to a cancer stem cell pool that is intrinsically resistant to chemo- and radiotherapy.

## **VIII. UNRESOLVED ISSUES REGARDING THE CANCER STEM CELL MODEL IN TUMOR HETEROGENEITY**

While the cancer stem cell model attractively answers several key questions about the origins of intratumoral phenotypic heterogeneity, a number of issues remain unresolved. Surprisingly, only a limited number of studies have convincingly addressed the clonal relationship between the cancer stem cell compartment and the bulk of tumor cells within any given tumor. Early studies from the Polyak laboratory on breast tumor heterogeneity were indeed consistent with the cancer stem cell model: isolating cancer stem cells in breast tumors based on the expression of CD44, the authors were able to show that CD44+ and CD24+ are clonally related, with the more differentiated non-stem cancer cell CD24+ pool having acquired additional genetic hits not present in the CD44+ cancer stem cell pool (Campbell and Polyak, 2007; Shipitsin et al., 2007). However, more recently, another study from the same laboratory (Park et al., 2010) paints a more complex picture. Within the same tumor, Park et al. found remarkable genetic heterogeneity within phenotypically similar tumor cell subgroups, i.e. even within the cancer stem cell pool there were a multitude of genetic clones. The authors conclude that their data is inconsistent with the cancer stem cell model altogether and specifically with the concept that CD24+ more epithelial-like cells would be derived from the CD44+ cancer stem cell pool. However, these data are inconsistent only with the strictest definition of the cancer stem cell model. The cancer stem cell model per se arguably does not necessarily exclude the possibility that there is significant diversity within the cancer stem cell pool. In fact, genetic diversity within the cancer stem cell pool would be a pre-requisite for long-term expansion of diverse subclones within a tumor. Clonal evolution will occur in any population of cancer cells, however, only those genetic aberrations acquired in the cancer stem cell pool would survive in the long run (Gupta et al., 2009). Similar results were recently reported in a study of leukemia-initiating cells in



*BCR-ABL1* positive lymphoblastic leukemia from the Dick laboratory (Notta et al., 2011). Notta et al. found that, in samples from *BCR-ABL1*-related acute lymphoblastic leukemia patients, functionally defined tumor-initiating cells of diverse genetic clones were present, suggesting a multi-clonal evolution of acute lymphoblastic leukemias. Intriguing data showed that in mouse xenografts derived from patient samples, the dominant clone at diagnosis in the clinic was frequently not the clone that grew as a xenograft. Rather, a genetically related clone from an earlier evolutionary stage, as a few genetic aberrations besides the *BCR-ABL1* fusion were indeed often shared. Of note, the same patient sample could give rise to xenografts in mice arising from distinct clones that were all different from the dominant clone at diagnosis. These data are in line with the recent report indicating that ALL relapse tumors frequently arise from a clone that's related to but not identical to the predominant clone at diagnosis (Mullighan et al., 2008). Together, these reports indicate that there may be a considerable heterogeneity and clonal evolution going on even within the cancer stem cell pool. The cancer stem cell hypothesis, thus, would take a giant leap towards finally merging with the stochastic clonal evolution model of tumor progression. It is noteworthy, however, when interpreting and extrapolating the results of Notta et al., that although they have studied functionally defined tumor-initiating cells, *bona fide* cancer stem cells have to my knowledge not yet been isolated from acute lymphoblastic leukemias.

Further evidence pointing towards a high plasticity in the tumor-initiating cell pool comes from studies on melanoma-initiating cells in severely immunocompromised animals (Quintana et al., 2010). In addition to concluding that, as previously demonstrated by the same group (Quintana et al., 2008), melanomas contain a high proportion of cells

capable of forming xenograft tumors if the recipient mouse is the right one, there were no consistent markers or no consistent phenotype that represented the tumor-initiating cells (Quintana et al., 2010). Furthermore, the authors demonstrate that a large variety of tumor cell subpopulations were able to recapitulate the full heterogeneity of the parent tumor, again arguing strongly against a simple cancer stem cell model in malignant melanoma (Quintana et al., 2010).

There is little direct evidence to prove that in fact cancer stem cells do give rise to the bulk of tumor cells. One intriguing possibility arises from the fact that many normal cell types with properties of stem cells are recruited into solid tumors. It is well-established that mesenchymal stem cells are recruited from the bone marrow into tumors (Birnbaum et al., 2007) and in brain tumors, neural stem cells exhibit great tropism towards the tumors (Aboody et al., 2000). Indeed, such tropism has been widely suggested to potentially function as a way to specifically deliver anti-cancer therapeutics into tumor tissue (Bexell et al., 2010). Although the topic remains controversial, mesenchymal stem cells have been suggested to have a more direct role in tumorigenesis than previously anticipated. In a mouse model of gastric cancer, using a GFP-tagged mesenchymal lineage Wang and colleagues demonstrated that in fact, resulting gastric cancers after inflammation consisted largely of epithelial bona fide tumor cells derived directly from the mesenchymal (i.e. GFP positive) cells (Houghton et al., 2004). These data demonstrate clearly that at sites of injury, mesenchymal stem cells are prone to acquire alterations in behavior ultimately leading to tumor formation. The milieu within tumors typically contains many elements that may conceivably alter normal cell behavior towards malignancy. The abundance of growth factors produced by tumor cells themselves

(Hanahan and Weinberg, 2000) will undoubtedly exert effects also on adjacent non-tumor cells that are present within the tumor. Specifically in gliomas, Holland and co-workers demonstrated that platelet-derived growth factor exposure induces dedifferentiation of neural cells (Dai et al., 2001). In line with these results, paracrine PDGF-PDGFR signaling from tumor cells to adjacent neural progenitor cells was demonstrated to result in seemingly unrestricted expansion of the neural progenitor cell compartment in a murine model of brain tumors (Assanah et al., 2006). Similarly and as discussed above and at length elsewhere (Axelson et al., 2005; Edsjo et al., 2007; Pietras et al., 2010), hypoxic environments have been shown dedifferentiate human tumor cells of various lineages towards stem cell-like phenotypes (Jögi et al., 2002; Jögi et al., 2004; Heddleston et al., 2009). Furthermore, microenvironmental cues can lead to the induction of epithelial-to-mesenchymal transition (Lopez-Novoa and Nieto, 2009; Sullivan et al., 2009), a process that in itself may render cells with stem cell-like properties (Mani et al., 2008). Taken together, it is not unlikely that neural and mesenchymal stem- and progenitor cells that are recruited into sites of tumor growth are affected by the tumor microenvironment in ways that ultimately increase the risk of these cells turning tumorous themselves (Fomchenko and Holland, 2005). Given that these cells already possess many of the features associated with stem cells in general and cancer stem cells in particular, it is certainly possible that cancer stem cells in fact represent recruited stem- and progenitor cells unrelated to the bulk of tumor cells, or at least, unrelated to the tumor-initiating cells, that have turned cancerous within the tumor microenvironment. This speculation calls for a rigid separation of the cancer stem cell hypothesis from issues of cell of origin, as it implies that cancer stem cells in most cases by no means have a direct relationship to the tumor-founding cell that acquired the first

tumorigenic genetic hits. However, this would not exclude the possibility that stem- and progenitor cells recruited to sites of injury in fact do represent both cell of origin and cancer stem cell in certain specific cases (Houghton et al., 2004).

## **IX. FUTURE DIRECTIONS**

Complete understanding of tumor biology and investigations into the true contribution of cancer stem cells to tumor heterogeneity have thus far largely been hampered by lack of reliable and advanced experimental systems. As discussed elsewhere, the most commonly used in vivo assays to study the biology of cancer stem cells come with several serious caveats (Quintana et al., 2008), and importantly, frequently require a total disruption of the heterogeneous tumor microenvironment in which putative cancer stem cells normally act. For instance, cancer stem cells like their non-malignant counterparts are increasingly recognized to depend on a cancerous equivalent to the stem cell niche. Such dependence on microenvironmental elements such as tumor vasculature or specific oxygen tensions have been demonstrated both for leukemic and solid cancer stem cells. It is likely that most other cell types present in tumors, too, contribute to maintenance of cancer stem cells and/or differentiation of cancer stem cells into more differentiated progeny, as was recently demonstrated in transplantation models of melanoma into immunodeficient mice with varying levels of immunodeficiency (Civenni et al., 2011). It is thus likely that tumor models in immunodeficient mice, where tumors essentially lack key components of the normal tumor microenvironment, may never fully mimic human tumor biology.

A major improvement would be studying mouse models of cancers that naturally contain stem-like cancer cells within a complete tumor microenvironment. Many genetically engineered mouse models, however, appear to not necessarily follow the cancer stem cell model, an issue that is likely derived from the way these cancers are initiated and may call for caution when interpreting and extrapolating from data on tumor progression generated by such models. Considering this, it would be of great interest to characterize the presence and possible function of cancer stem cells in the most commonly used mouse tumor models. While the vast majority of studies on cancer stem cells are presently performed on human tumor material with xenotransplantation into immunocompromised mice, some of the key studies mentioned in this review indeed investigated these phenomena in mouse models of cancer. For both of the brain tumors glioma and medulloblastoma, the RCAS/tva system (reviewed in Huse and Holland, 2009; Momota and Holland, 2009) can give rise to tumors that appear remarkably faithful to human brain tumor biology given that the right vectors and initiating cells are employed (Hambardzumyan et al., 2008b; Bleau et al., 2009; Charles et al., 2010). First, as was described for a large variety of human brain (and other) tumors (Calabrese et al., 2007; Pietras et al., 2008), a cell compartment expressing high levels of stem cell-associated markers as compared to the bulk of tumor cells appear located preferentially in a perivascular niche (Hambardzumyan et al., 2008b; Bleau et al., 2009). More importantly, upon irradiation, these cells persist while the bulk of the tumor undergoes apoptosis (Hambardzumyan et al., 2008b), again mimicking what has been widely proposed for human cancer stem cells. Clearly, the identification of and subsequent

study of such models will have great advantages as compared to using transplantation models of FACS-sorted human tumor cells.

To resolve the remaining issues regarding the origin of and the contribution to tumor heterogeneity and therapy resistance of cancer stem cells, true lineage tracing models of cancer *in vivo* must be used to study these phenomena without disrupting tumors and their microenvironment by cell sorting and transplantation. Mapping the fate of individual tumor cells and their progeny during tumor progression and therapy should aid in defining both origins and functions of cancer stem cells and tumor bulk. Given the recent advances in lineage tracing models and the ability to functionally define the role of normal tissue stem cells *in vivo* (Snippert et al., 2010; Weber et al., 2011), there is hope that similar studies can inform us further on the functional properties of cancer stem cells.

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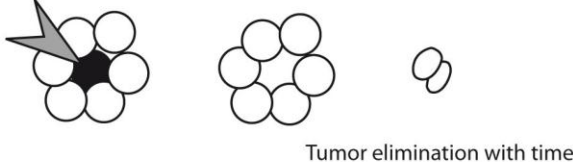
## Figure legends

**Figure 1.** Strategic implications for therapeutics of the cancer stem cell model. (A) In the most strict interpretation of the cancer stem cell model, therapeutic targeting of this subset of tumor cells will eliminate tumor growth in the long run, as the more differentiated tumor cells of the tumor bulk will have a limited proliferative capacity. (B) Dedifferentiation and epithelial-to-mesenchymal transition may render differentiated tumor bulk cells capable of acquiring cancer stem cell properties and thus, therapeutic targeting of the cancer stem cell pool only may not exclude the existence of tumor cells with cancer stem cell properties with time. (C) Targeting of the cancer stem cell compartment in parallel with conventional therapeutics that target the bulk of tumor cells should eliminate tumor growth.

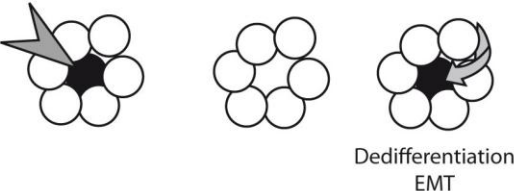
**Figure 2.** Cancer stem cells in tumor heterogeneity. Cancer stem cells contribute to tumor heterogeneity by differentiating and transdifferentiating into various tumor lineages as well as mesenchymal/stromal and endothelial cells of tumor origin. In addition, cancer stem cells aid in the recruitment of host-derived endothelial and mesenchymal/stromal cells through secretion of growth factors.

**Figure 1**

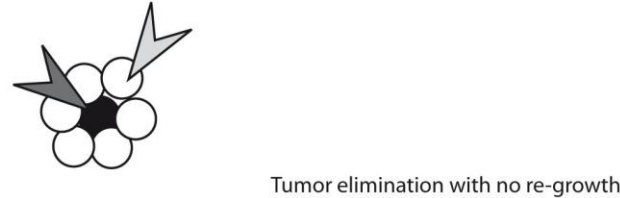
A



B



C



	Cancer Stem Cell
	Tumor Bulk Cell
	Cancer stem cell-targeted therapy
	Conventional therapeutics targeting tumor bulk



**Figure 2**

