Chapter 10
Cancer Stem Cells: Perspectives Beyond Immunophenotypes and Markers

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Abstract Decades of cancer research have failed to resolve therapeutic refractoriness and tumor dormancy that leads to disease recurrence. This presents formidable obstacles in achieving total remission for patients in several cancers. It is however realized that residual tumor regenerative potential resides in a rare population of cells with properties of self-renewal that permit them to remain quiescent yet contribute to recurrent disease. These cells are referred to as either Cancer stem cells (CSCs) or Tumor Initiating Cells (TICs), and their isolation, identification and extensive characterization followed through the establishment of several phenotypic and functional *in vitro* and *in vivo* assays. Notably, similarities with normal tissue stem cells have emphasized the need of developing new approaches for their specific targeting as opposed to current chemo- or radio-therapy. Thereby considerable interest and research has culminated in elucidating the behavior of CSCs vis-à-vis their deviations from normal stem cell performance, which might provide therapeutic novel cues. However, their identification, characterization and understanding of the cellular contexts in which they can be formidable yet have not been truly achieved beyond the development of convenient tools. This chapter outlines the present challenges in the field of CSC biology.

Keywords Cancer stem cells • Asymmetric cell division • Quiescence • Dedifferentiation • Transdifferentiation

10.1 Introduction

According to Greek mythology, when Prometheus defied the gods and stole fire for mankind, he was chained to Mount Caucasus where each day an eagle (emblem of Zeus, king of gods) would prey on his liver (believed to be the centre of emotions,

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G. Steinhoff (ed.), *Regenerative Medicine - from Protocol to Patient*,
DOI 10.1007/978-3-319-27610-6_10

not the heart!). Luckily for him, the high cell regeneration in this organ allowed him to tide over circumstances until he was freed by Hercules (Rosenthal 2003). Tumors sometimes appear to harbor such Promethean regenerative homeostasis that makes them virtually indestructible. This germ of an idea could well be the genesis of Cancer Stem Cells (CSCs), a concept supported by clinical observations of regeneration, unfortunately running awry. CSCs were termed so since they were believed to arise through transformation of normal tissue-derived stem cells. Hence similarities between these and normal stem cells were noted *vis-à-vis* expression of surface markers, derivation of a regenerative cell hierarchy comprising of CSCs purported to be a rare fraction within tumors that retains the capability of reversible quiescence; progenitors that constitute the proliferative tumor fraction and differentiated cells that represent the phenotypic and functional heterogeneity present in the organ in which the tumor establishes itself (Reya et al. 2001). Such extrapolation of cancer being a derivative of normal repair functions with CSCs driving tumor heterogeneity was first convincingly demonstrated in leukemia, wherein aberrant regeneration bestowed imbalanced growth advantages within the HSC hierarchy along with compromised tissue functioning from deficits in progenitor maturation (Bonnet and Dick 1997). This understanding also correlated with the clinical pattern of the disease and was hence accepted by researchers and clinicians alike, thus establishing a framework for further studies. CSCs in solid tumors were prospectively identified almost a decade thereafter (Al-Hajj et al. 2003; Singh et al. 2004; Bapat et al. 2005), with the leukemia model providing the prototype for initial studies drawing similarities of marker expression between normal tissue and tumor-derived stem cells and their regenerative capabilities.

10.1.1 Prospective Isolation and Identification of Cells in Tumors with Stem-Like Properties

Another decade thereafter has witnessed a deluge of research literature in the field with convictions though becoming stronger, also giving rise to severe criticisms. The most emphatic of these initially concerned isolation of CSCs through application of normal stem surface markers (Woodward and Sulman 2008; Duan et al. 2013). This strategy is widely applied and appreciated as a relatively easy and convenient tool; however excessive reliance on these techniques alone led to dilution of the basic concept. Since the regenerative CSC hierarchy is known to be associated with differentiation arrest and compromised tissue functions resulting from various combinations of genomic alterations, it also raises concerns of ambiguous correlations between normal and aberrant cellular subsets as identified by the same markers (Jaggupilli and Elkord 2012). Most importantly, in solid tumors the use of surface markers in discriminating between mature and immature cells is not established (Schulenburg et al. 2015). Thus the precision of prospective CSC isolation was thought to be compromised in certain tumors wherein marker correlation with

specific cell subsets is uncertain on the background of lack of similar functional correlations.

Yet another difficulty was that since normal stem cells and resolution of their functionalities is not achieved in several organs, isolation of CSCs in tumors arising in those was not possible. This problem was circumvented through development of 'universal' immunophenotyping approaches wherein screening was achieved using generic marker cocktails comprising of c-kit, CD44, CD24, CD133, Epcam, nestin, nanog, Oct-4, Aldh activity and their combinations (Medema et al. 2013). An understanding of such correlation however is unclear since none of these markers are exclusively associated with CSCs, and some of them are not even expressed in the normal tissue. While current understanding suggests acquisition of self-renewal and blocked differentiation to be most important in reverting to a stem cell state, these functions are not known to be associated with any known surface markers, further making such correlations rather incomprehensible. Thus 'marker-independent' approaches including sorting of side population (SP) stem cells within tumors that have a specific capability to efflux Hoechst dyes, growth of tumor derived cells as non-adherent spheroid cultures that could enrich CSCs within the population, label chase to identify differences in growth kinetics (quiescence vs. proliferation), etc. came to be explored for prospective CSC studies (Tirino et al. 2013).

Lack of information relating to normal homeostasis in certain organs also limits understanding the presence of CSCs in their tumor derivatives. In some tumors, normal stem cells may serve as the targets of oncogenic transformation to yield CSCs, while in others, transit-amplifying progenitors and/or differentiated cells may present the initial oncogenic genomic rearrangements. An essential feature accompanying the implied dedifferentiation in the latter is acquisition of self-renewal capabilities along with maturation defects (Quintana et al. 2010). Although such reprogramming is demonstrated at a functional level, convincing correlations with altered immunophenotypes are not established. During tumor formation and maintenance, CSCs like their normal counterparts continue to self-renew and generate differentiated derivatives that constitute the major bulk of a tumor. Disease progression further is believed to follow Darwinian principles of evolution in which continual genetic instability could establish several CSC clones of differential capabilities within the same tumor, to provide an increasing complexity of possibilities for survival. These complexities cannot be resolved through studies with surface markers.

10.1.2 Distinction Between CSCs, TICs and Cell-of-Origin of Tumors

A basic requisite for CSC isolation is that variability arising from execution of techniques such as differences in tumor digestion, source of tumors (cell lines vs. tumors, human vs. xenografts, extensively passed vs. early xenografts), stringency

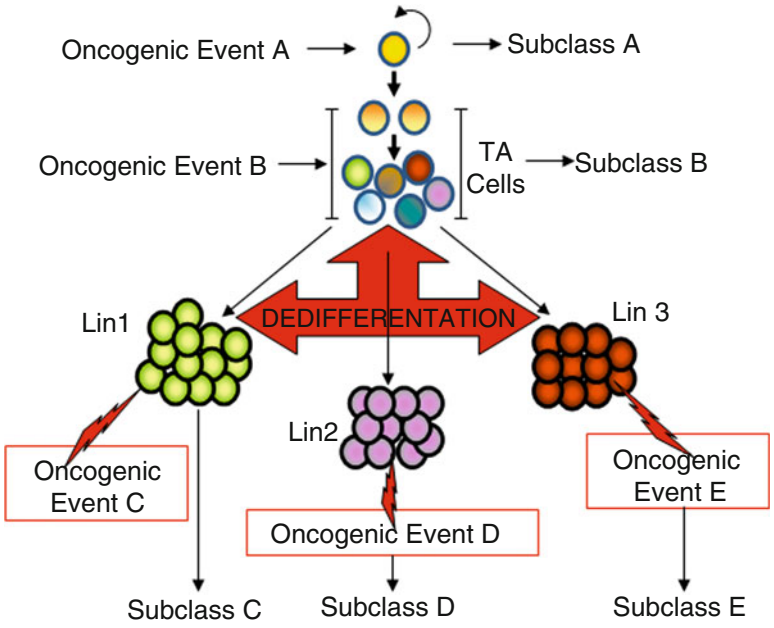

Fig. 10.1 Diverse cell(s)-of-origin and pathways to emergence of CSCs result in different sub-classes of tumors and deem implausible assigning of a single CSC immunophenotype (Lin1, Lin2, Lin3 – Lineage1, Lineage2, Lineage3 respectively)

of assays used in evaluation, etc. should be maintained at minimal. The diversity of CSC phenotypes (based on surface markers, SP sorting, label quenching, etc.) however leads to confounding interpretations when viewed across tumors from patients with the same histological and molecular subtype. This perspective of inter-tumor heterogeneity encompasses differences arising from ethnicity and genetic variability between individuals, environmental factors, cell-of-origin and type of oncogenic events (Marusyk et al. 2012). Such principles of tumor stratification rely on the type of oncogenic mutations or genomic rearrangements involved and/or their targeted lineage (cell-of-origin) within the organ (Visavader and Lindemann 2012; Fig. 10.1). Correlating across this variability to identify discrete sub-classes of tumors with similar molecular features forms the basis of tumor/patient stratification and is achieved in several cancers. In such a situation, it is difficult to comprehend how a single CSC phenotype could be associated with all known tumor subtypes arising from diverse cell(s)-of-origin and oncogenic events.

Tumor regeneration in immune compromised animal models from human tumor derived sorted cells thus increasingly gained recognition as a universal 'proof-of-function', besides providing a robust assay for functional validation of CSC self-renewal. Soon thereafter, Tumor Initiating Cells (TICs) became the new buzz word round the block that indicated definite emphasis on cell functions over phenotype.

While in most cases CSCs qualified as TICs, the latter were also reportedly isolated in instances wherein tumors do not conform to classical hierarchical regeneration (Rehe et al. 2013). This revealed that the frequency of TICs could vary from being relatively rare to comprising a significant fraction of tumors, thereby making their distinction from the rare CSCs (Bapat et al. 2009), implying that establishment of mitotic quiescence than expression of 'stemness' features was a more important characteristic of TICs (Wang et al. 2015).

However while functional readouts are indeed important and suggest non-exclusivity, CSCs nevertheless still retain an edge over TICs by providing a reasonable account of intra-tumor heterogeneity that assigns varying regenerative potential within a hierarchy, and cellular plasticity that is a key feature of stem cells besides self-renewal and regeneration. Under different conditions of stress or microenvironmental cross-talk, the coexistence of discrete CSC pools may be evident that reflects on emerging phenotypic heterogeneity within the same tumor. To address these basic questions in the field today, it becomes necessary to map out all possible correlates between the differentiation hierarchy, clonal selection capabilities and various molecular and cellular phenotypes within an organ/tumor that will better elucidate the subtle differences and identities between CSCs, TICs and cell-or-origin of tumors.

10.2 Understanding the Cellular State

10.2.1 Asymmetric Cell Division

Asymmetric cell division is central to stem cell capabilities of long-term regeneration and maintenance of homeostasis, while remaining a fundamental means of generating cell diversity. Such functionality is manifested through generation of two daughter cells with discrete alternative fates of self-renewal (implying return to the stem cell state) vs. lineage commitment and differentiation (defining the state). While asymmetric division is the convention in normal homeostasis, adult stem cells may also undergo symmetric divisions under conditions wherein stem-cell pools are depleted by injury or disease (Kahn 2011). Two modes of regulation of asymmetric cell division are recognized, *intrinsic mechanisms* are driven by altered cell polarity and asymmetric segregation of cellular components during division, while *extrinsic mechanisms* are manifested by the reliance of a cell on its niche to receive cues that will drive it to self-renew or differentiate (Kelsom and Wange 2012). The exclusivity of these two modes remains to be determined. More important is an emerging concept that failure of asymmetric cell division could have widespread consequences in neoplastic growth. Perturbed stem cell activation and functioning is demonstrated to lead to imbalanced regeneration followed by tumorigenesis in several model systems including *Drosophila* neuroblasts (Siegrist and Doe 2006; Izumi and Kaneko 2012).

10.2.1.1 Intrinsic Regulation – Polarity, Asymmetric Segregation of Cellular Components, EMT

Establishment of cell fate suggests the subsistence of intrinsic features that drive asymmetric division, and extends beyond the simple principles of mitosis wherein equal segregation of sister chromatids ensures that each daughter cell receives a single, complete copy of the parent genome. Stem cells in their specific niche often exhibit an apical-basal axis of polarity. During mitosis, polarity establishes an asymmetrical localization of self-renewal regulators; concurrently asymmetric mitotic spindle positioning regulates the polarization of other determinants (Yamashita et al. 2007). Within the daughter cells thus formed, one inherits most of the polar and self-renewal determinants to re-establish polarity and revert back to the 'stem-cell' state, while the other loses these regulators and generates a critical mass of cells necessary for differentiation (Sugioka and Sawa 2012; Inaba and Yamashita 2012). Such differential distribution of determinants is reported at the RNA as well as protein expression levels (Gómez-López et al. 2014; Ganguly et al. 2012). Thereby, coordination of asymmetric protein localization and polarity with cell cycle progression through mitosis are important contributions of asymmetric cell division in maintenance of homeostasis. A large number of proteins including numb, par, pon, brat, Miranda, prospero, stuaufen, pins, gai, loco, inscuteable, aPKC, lgl, polo, aurora A, polo, pp2a, dpn, zif, etc. associated with asymmetric localization, involvement with spindle orientation and/or cell polarity are currently recognized as being determinants of asymmetric division (Kelsom and Wange 2012; Poulson and Lechler 2012). Significantly, specific mutations in the tumor suppressor Apc can influence spindle alignments and planar cell polarities that regulate daughter cell anisotropic movements away from niche-supporting cells (Quyn et al. 2010 ; Chang et al. 2012). Such processes are relatively easy to understand in simple epithelial layers like the ovarian surface epithelium; however, stratified epithelia further require cross-talk between layers to establish a delicate balance of proliferation, mitotic spindle orientation, differentiation and cell loss (Graham et al. 2010; Muthuswamy and Xue 2012). Failure to do so can trigger dysplasia through cooperation with other predisposing factors and progress to transformation.

In cancer, epithelial to mesenchymal transition (EMT) that was initially correlated with invasion and metastases, is now also being associated with disrupted cell polarity (Zheng and Kang 2014; Moreno-Bueno et al. 2008). This process wherein epithelial cells lose their characteristic features and undergo dissolution of cell–cell contacts to acquire a migratory, mesenchymal phenotype, is crucial to normal embryonic development wherein a tightly controlled program at the epigenetic and transcriptional levels achieves specific developmental milestones within specified parameters of time and space (Kerosuo and Bronner-Fraser 2012). This ensures formation of the three germ layers, differentiation and generation of organ structures within the developing embryo, thereby protecting against transformation. In the context of cancer, the process contributes to dissemination of tumor cells and metastases in a manner speculated to be an aberrant derivative of the normal program. The establishment of EMT has also been associated with re-acquisition of

'stemness' features in tumor cells and an indication of moving towards a recalcitrant, invasive stage of disease; aggressive tumors appear to be markedly associated with EMT (Beuran et al. 2015). Considerable diversity in the molecular profiles of this program in different tumors lends further support for this perception. For example, the conservation of definite EMT networks driven by specific transcriptional programs in a particular tumor type may define distinctive molecular classes (Scheela and Weinberg 2012). Expression of EMT- and CSC-associated genes in cells at the invasive edge of the tumor as well as within blood vessels further suggests EMT to be a first step in generation of migrating CSCs that ensure tumor regeneration at distant sites (Klymkowsky and Savagner 2009).

10.2.1.2 Extrinsic Regulation – Niche Effects

Paget's famous report outlining the "seed and soil" concept of metastases was amongst the first to recognize the contribution of tumor microenvironment in disease progression (Paget 1889). Even in the normal state, stem cells are localized at specialized positions within organs termed as niche, contact with which is crucial for its self-renewal and maintenance (Morrison and Spradling 2008). Initial equivalent daughter cells are impressed with different future fates following interactions with each other and with their environment (Jan and Jan 1998). Tumor niches comprise of cellular components including stromal, endothelial, inflammatory, mesenchymal stem cells and the extracellular matrix (ECM) that is a rich source of epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor (TGFβ), stem cell factor (SCF), chemokines etc. besides factors involved in Notch, Wnt and SHH signaling that promote stem/progenitor cell traits (Zhu et al. 2011). Niche cells may either be derived from the transformed stem cell lineage, another stem cell clone or normal host cells including the stroma, immune cells, etc. Significantly, heterogeneity within the tumor stroma generates a diversity of signals that influence the niche and CSCs (Costea et al. 2013). Some of the niche-associated ECM components are expressed by CSCs themselves (CD44, hyaluron, etc.), thereby ensuring self-sufficiency or independence of growth signals (Sebens and Schafer 2012). CSC niches are most frequently identified at hypoxic and/or perivascular locations or invasive fronts of tumors (Mimeault and Batra 2013; Filatova et al. 2012; Beck et al. 2011; Calabrese et al. 2007). In some subpopulations within gastrointestinal tumors, asymmetric cell division with non-random chromosomal cosegregation independent of cell-to-cell contact is regulated by Wnt pathway signals from the tumor niche in a heat-sensitive paracrine fashion (Xin et al. 2013). Another mechanism for ECM mediated influences on asymmetric division involves parallel alignment of the mitotic spindle axis mediated by hyaluronan-CD44 signaling from the apical surface cell membrane, while the same signaling from the basal surface-membrane aligns the mitotic spindle along an oblique-perpendicular axis. Fibronectin – integrinαβ6 signaling from the basal surface-membrane could also mediate similar alignments (Fujiwara et al. 2008). Such variations perturb the distribution of intrinsic factors. Stem cell (and CSC) niches

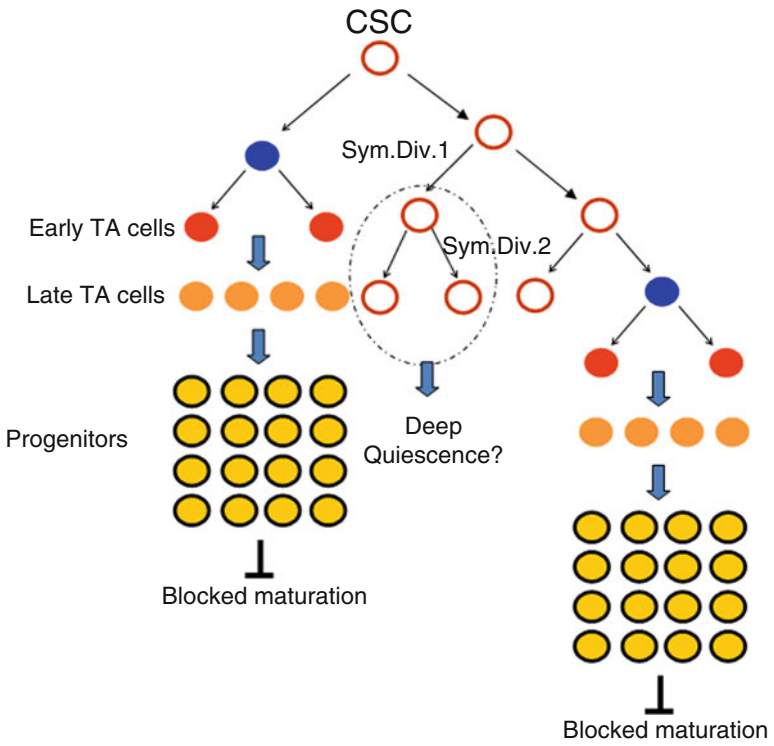

Fig. 10.2 States of asymmetric and symmetric divisions influencing quiescence and proliferation (Sym.Div. – symmetric division)

thus are gaining recognition as specialised ecological settings that generate the instructive cues for sustenance of specific metabolic states, signaling, feedback control and coordination between heterogeneous populations within the organ/tumor (Lander et al. 2012; Schepers et al. 2015).

10.2.1.3 Consequences of Asymmetric and Symmetric Divisions in Cancer

Studies in mouse models demonstrate perturbed asymmetric divisions of normal stem cells to lead to abnormal self-renewal and neoplastic transformation (Powell et al. 2010). The frequency of asymmetric division in tumors also negatively correlates with their proliferative capacity; more proliferative tumors are likely to harbor less asymmetric than symmetric divisions (Fig. 10.2). Thus the relevance of asymmetric cell divisions in maximizing tumor cell proliferation and generation of genetic diversity in a regenerative hierarchy is questionable since the basic caveat of such an arrangement is to balance cell generation with death that suggests effective apoptotic and homeostatic mechanisms. On the other hand, increased symmetric

divisions correlate with higher proliferative capacity and undifferentiated tumors, thereby appearing to be a more obvious path of regeneration. However, too rapid tumor growth itself can be self-limiting (due to stem cell exhaustion). Hence arriving at a balance between asymmetric and symmetric cell divisions may be a reasonable modality of retaining long-term regeneration in tumors. It has been shown that asymmetric division is more frequent in early- than late-stage tumors suggesting a major mechanistic shift in long-term regenerative capabilities during disease progression. Various factors including Akt, p53, EGFR and microenvironmental signaling can affect the balance of cell fate choice between symmetry and asymmetry (Dey-Guha et al. 2011). Recently, a tightly controlled miR-34a circuit has been shown to control the decision of a CSC to perform either symmetric or asymmetric division (Bu et al. 2013).

10.2.2 Quiescence

Stem cells exist in a transient state of cell cycle arrest from which they can be 'awakened' to re-enter into the cell cycle and perform regenerative functions. Such reversible quiescence distinguishes stem cells from differentiated cells that have permanently exited from the cell cycle. Asymmetric cell division is contextually linked to quiescence; conversely, symmetric cell division may be followed by a loss of quiescence. Surprisingly, despite the recognition that disrupted asymmetric cell divisions lead to tumorigenesis, evidences of quiescent cells in tumors are seriously lacking. This is largely due to a paucity of well-defined phenotypic quiescence-determining markers that could provide a convenient read-out, as well as experimental model systems to study the phenomenon. A large majority of hematopoietic stem cells (HSCs) are reported to be in the G0 stage of the cell cycle, whereas very few committed multipotent progenitor cells are actually quiescent although they initially appear to be slow-cycling (Wilson et al. 2008). Whether such distribution of quiescent fractions in stem vs. progenitor cells does actually exist within tumors and is modulated as in regenerative tissues, remains to be addressed. CSC quiescence *in situ* is often defined by their niche components and localization within the tumor. Perivascular and osteoblastic niches as well as several ECM components such as CD44, POSTN, tenascin C, etc. have been described as being essential for establishing and maintaining quiescence in CSCs.

Profiling of differential cell cycle kinetics within HSCs led to recognition of two discrete pools of slow-cycling/homeostatic stem cells vs. dormant/deeply quiescent stem cells (Wilson et al. 2009) both of which are likely to have distinct functions and underlying regulatory mechanisms. Slow-cycling HSCs exhibit relatively higher metabolic activities and active replication machinery than dormant HSCs in which metabolism is at basal maintenance levels and the replication machinery is almost shut off (van der Wath et al. 2009). The former contribute to homeostatic organ regeneration in a definite cyclic pattern, while the latter may be evoked only on drastic depletion of the stem cell pool under extreme circumstances of injury or

stress. Similar differential pools of CSCs with specific functionalities are yet to be completely resolved in tumors, although recent, label-chase studies for examining cycling over time through either thymidine analogues (bromodeoxyuridine – BrdU) incorporation, or chromatin-associated green fluorescent protein (GFP) expression under the control of a doxycyclin regulated transgenic allele, or lipophilic membrane labeling dyes such as PKH or CFSE have provided differential cycling-based, marker-free approaches for the identification of quiescent CSCs in tumors (Kusumbe and Bapat 2009; Foudi et al. 2009; Park et al. 2013; Terskikh et al. 2012; Mani et al. 2008). Such tumors kinetics suggest switching between slow-cycling and deep quiescence, especially on exposure to chemotherapy in mouse models. A hypothetical hierarchy of such CSC pools and their progeny is represented in Fig. 10.2. Such plasticity could be crucial in generating population asymmetry between different derivatives of a founder CSC, and becomes an effective mechanism to offset replicative aging with differential rates of regeneration. Quiescent cells are non-responsive to therapy; hence activation from a quiescent state could be a crucial first step of sensitization to chemotherapy. Deep quiescence however, presents an indeterminable, recalcitrant situation for a cancer patient in whom a state of remission is achieved but with an uncertain caveat. Tracing the subtle differences between the different modes of cell divisions in homeostasis *vs.* deep quiescence within tumors at the molecular level would unravel the differential mechanisms in quiescence and dormancy likely to be highly relevant at the clinical level, yet remain a challenge.

10.2.3 Dedifferentiation and Transdifferentiation

Dedifferentiation and transdifferentiation represent two extreme opposing options that a tumor cell can acquire during tumor progression. Dedifferentiation is suggested to arise from the plasticity acquired by tumor cells to mediate transition between invasive and proliferative states (phenotype switching), in which EMT plays an important role. In reconciling such events with the CSC hierarchy, it appears that the cross-talk of EMT with other pathways in response to microenvironmental stress leads to acquisition of stemness features and dedifferentiation of non-stem cells (Morel et al. 2008 ; Kurrey et al. 2009; Brabletz et al. 2005). Thus the EMT program not only enables physical dissemination of cells from primary tumors but through dedifferentiation, confers on them regenerative capabilities crucial for subsequent establishment of secondary metastases at sites of dissemination (Choi et al. 2012). Dedifferentiation is also derived from expression of embryonal markers like cancer testis antigens in tumors, which was one of the earliest suggestions of activation of developmental programs by tumor cells and considered as an indication of the acquisition of pluripotency in an aberrant setting.

Similarly, transdifferentiation is a pathologically established process in differentiated teratocarcinomas. From a wider perspective, CSCs may transdifferentiate into cell lineages other than that those from which the tumor arose including adipocyte, neuronal, vasculogenic, neuroendocrine and even along immune cell lineages

(Ramakrishnan et al. 2013). Another possibility is that transition of tumor cells into a mature, differentiated mesenchymal phenotype through EMT contributes to the generation of reactive stroma that is crucial for CSC maintenance. Transdifferentiation could be triggered by fusion events as recently demonstrated between tumor infiltrating hematopoietic cells and epithelial cancer cells (Shekhani et al. 2013). Further, the fact that tumor progenitors which are in a state of maturation arrest can be induced to progress into a post-mitotic terminally differentiated state on exposure to appropriate chemicals, presents an attractive therapeutic strategy which is not as yet fully exploited (Nör et al. 2013). All trans-retinoic acid (ATRA) has shown promise in such an approach in differentiation of acute promyelocytic leukemia blasts into mature granulocytes; along with demethylating agents or histone deacetylase inhibitors, it can also differentiate solid tumor cells along neuronal lineages (Politis et al. 2008; Zelivianski et al. 2001). Similarly, unsaturated fatty acids may drive CSC differentiation into adipocyte-like cells; while neuroendocrine differentiation is reported from prostate CSCs and in small cell lung carcinoma (Hanahan and Weinberg 2000). Transdifferentiation is evinced in the generation of reactive tumor stroma and vasculogenic mimicry, while dedifferentiation supports tumor survival and progression through multidrug resistance. Hence both necessitate the development of individualistic strategies. Understanding of such cellular plasticity through dedifferentiation and transdifferentiation may mediate reconciliation between the hierarchical and stochastic models of CSC emergence and tumor establishment (Hanahan and Weinberg 2011; Plaks et al. 2015).

10.3 CSCs and the Hallmarks of Cancer

By virtue of their nomenclature, besides an association with stem cell characteristics, it is expected that CSCs would also exhibit capabilities of tumor cells. Six primary hallmark features are proposed to be associated with transformation and progression of cancer (Gunes and Rudolph 2013); more recently, four additional enabling capabilities are identified (Gatti et al. 2011). It thus becomes pertinent to explore their association(s) with CSCs.

10.3.1 Primary Hallmark Features

10.3.1.1 Enabling Replicative Immortality

Cellular immortality is an intrinsic quality of stem cells achieved through maintenance of telomere length by telomerase. In tumor cells, telomere shortening often leads to genetic instability that reactivates telomerase to avoid genetic chaos thereby generating CSCs (Mo and Zhang 2013). While this suggests a reversal of direction towards reacquisition of immortalization and cell proliferation, it is yet distinct

from that of intrinsic telomerase expression in a stem cell which is targeted by oncogenic mutation(s) to undergone transformation. Thereby although the end result(s) are similar in the two states, a subtle and distinct difference exists in the pathways leading to self-renewal.

10.3.1.2 Resistance to Cell Death

Self-renewal in turn, defines capabilities of quiescence and regeneration of a cell. Most of the mechanisms mediating tumor cell death such as metabolic-, oxidative-, differentiation- or therapy-induced stress are ineffective in a quiescent stem cell which can ensure survival under adverse conditions. Stem cells and CSCs express several types of membrane ATP-binding cassette transporters including ABCG2, that provide an active mechanism for elimination of toxic intracellular components and drugs (Januchowski et al. 2013; Hanahan and Coussens 2012). The involvement of aldehyde dehydrogenase (Aldh) expressed by several CSCs is also demonstrated in tumor cell drug resistance (Faust et al. 2012). Thereby, acquisition of self-renewal possibly involves a multitude of mechanisms that complement and ensure establishment of stable intrinsic quiescence and impart to CSCs an option to select the most effective tool for combating specific adverse microenvironments during cancer progression.

10.3.1.3 Sustained Proliferation

The regenerative capability of a CSC provides the primary impetus for building up a primary mass of tumor cells. Continuing oncogenic mutations in several signaling pathways and transcriptional networks complements this aberrant growth, while disruption of feedback mechanisms further triggers expansion by blocking maturation of progenitors into functionally relevant, differentiated cells. This shifts the self-renewal kinetics of CSCs towards a state of frequent activation that culminates in accumulation of committed, yet proliferating progenitors with compromised functionality. Paracrine cross-talk between the transformed regenerative hierarchy and tumor stroma is also an important accessory in maintaining this state of sustained proliferation (Liu and Dean 2010).

10.3.1.4 Evasion of Growth Suppression

Maturation arrest consequently implies longer retention of progenitors and a slower turnover into terminally differentiated cells that disrupts homeostatic mechanisms that attempt to restore tissue functions. Such efforts at homeostasis may involve acquisition of resistance to apoptotic pathways that tissue cells normally commit to, or loss of contact inhibition that consequently leads to cell proliferation and has since long been associated with cancer cells grown in

culture. *In situ*, such homeostatic mechanisms regulate organ size and in cancer may be driven by Ras and FoxM1 mutations or activated EMT and Hippo-Yap pathways (Fuxe and Karlsson 2012; Kusumbe et al. 2009). One of the most significantly altered cellular responses involved in evasion of growth suppression is towards the growth factor TGFβ, which establishes extensive cross-talk with diverse downstream pathways involved in EMT, inflammation and immune evasion (Bao et al. 2006).

10.3.1.5 Inducing Angiogenesis

A rapidly proliferating tumor is slated to encounter the stresses imposed by limited availability of nutrients and gaseous exchange/diffusion. CSCs and progenitors are activated by such microenvironmental factors including hypoxia, hypoglycemia and iron deficiency into triggering an angiogenic switch in growing tumors. This effectively mediates the recruitment of primitive endothelial stem and progenitor cells by CSCs to primary and metastatic tumors in a paracrine manner through secretion of angiogenic growth factors such as VEGF (Doan and Chute 2012). CSCs also contribute to establishment of vasculature through their potential to transdifferentiate along endothelial and vascular smooth muscle-like lineages and give rise to non-endothelial channels through the process described as vasculogenic mimicry (Ping and Bian 2011). This active role of CSCs in establishing angiogenesis and/or vasculogenesis significantly contributes to niche development, and may be essential for their sustenance within tumors since both processes support survival of cells during tumor progression. Notably, the perivascular niche is known to play an important role in regulation and maintenance of CSC quiescence in leukemia, breast, skin and brain tumors (Ghajar et al. 2013). This further assigns an indispensable role to tumor vasculature in acquiring long-term dormancy.

10.3.1.6 Activating Invasion and Metastasis

EMT has been considered as the primary mediator of the program of invasion and metastasis in several tumor types. Epigenetic and microenvironmental influences on hypoxia, autocrine – paracrine signals from Wnt, tyrosine kinases, NF-κB and growth factor (TGFβ, EGF) pathways etc. trigger a transcriptional program that initiates intercellular junction dissolution and modulate phenotypic changes. Effectively this leads to enhanced plasticity within the regenerative hierarchy that strikingly generates variations in the development-associated invasive program. Acquisition of a mesenchymal phenotype is stated to facilitate invasion through the basement membrane and/or tumor stroma into blood vessels and lymphatics. Such dissemination poses a challenge to a migrating cell that relies on several intrinsic and acquired survival mechanisms of CSCs. On homing to secondary sites, these stem-like cells mediate a regenerative program that is inclusive of mesenchymal to epithelial transition (MET) to establish metastatic colonization.

Surprisingly, in some cancers EMT is *not* indicated to be a modality of invasion and metastases of tumor cells and CSCs. Cohesive or cooperative cell migration (CCM) of cell groups that exhibit intact cell junctions (a characteristic epithelial morphology) and dissemination through passive implanting in lymphatic spaces and blood vessels have been described. Such features may provide a survival advantage to the migrating cell clusters with respect to protection from immune attack and sheer forces of circulation, to increase the probabilities of generating micrometastases. The relevance of CCM in a clinicopathological setting is presently considered as being more robust than that of EMT (Chui 2013). These differing metastases modalities *viz.* EMT vs CCM have been recently applied as a classifier in high-grade serous ovarian carcinoma to achieve patient stratification into discrete subtypes (Gardi et al. 2014). Notably, in each subtype, different pathways of stem cell activation and regulation are noted.

As in the case of CSCs/TICs, the terminology of EMT is often used rather ambiguously to imply any process associated with loss of cell polarity, invasion, metastasis, acquisition of stemness, resistance to apoptosis and generation of cancer associated fibroblasts. A specific cellular shape/morphology may not sufficient to achieve all these effects without a defined molecular context in terms of the associated genes and activation of specific networks. This is important since the apparently diverse outcomes reported in association with EMT are reached under specific cellular contexts, and hence it becomes pertinent to define specific tissue and systems network associations wherein some functions either complement each other or may be exclusive. In doing so, one could achieve improved understanding of context-specific EMT pathways and their cross-talk in driving de-differentiation and/or trans-differentiation.

10.3.2 Enabling Characteristics and Emerging Hallmarks

10.3.2.1 Genome Instability and Mutation

Maintenance of genome integrity is a critical mission of the stem cell hierarchy; compromise of the same results in transformation and posits the generation of CSCs. Random oncogenic insults in progenitors/differentiated cells also trigger cellular reprogramming towards dedifferentiation that is accompanied by acquisition of self-renewal properties and a ‘stem-like’ phenotype. Several tumors are established as being monoclonal, which ensures faithful inheritance and propagation of the oncogenic mutation(s) within the CSC progeny, besides imparting a specific molecular identity to the tumor. In the last few years, considerable evidence has been additionally presented that genetic instability is crucial in the generation of intratumor heterogeneity and clonal evolution (Naik et al. 2015). Differential clone dominance is established through the principles of Darwinian selection in which clones exhibiting genomic rearrangements in addition to the founder mutations are selected for by the microenvironment and may interact in either a competitive and mutualistic manner (Ng et al. 2012). Consequently, genome protection from further

oxidative damage by the niche may be achieved through synthesis of hyaluron that scavenges ROS, maintenance of the tumor cell at low hypoxic levels, etc. that play a major role in protecting the transformed clone against continual DNA damage and (mis)repair cycles that could lead to genetic chaos (Darzynkiewicz and Balazs 2012).

10.3.2.2 Tumor-Promoting Inflammation

Several chronic inflammatory states are considered high risk factors for the onset of cell transformations likely to progress towards malignancy. Persistent tissue injury and repair leads to increased localized levels of several inflammatory cytokines and growth factors like tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8, TGFβ, nuclear factor kB (NFkB), etc (Shigdar et al. 2014). Such cyclic/recurrent activation of developmental signaling and regenerative pathways also perturbs stem cell self-renewal and tissue homeostasis resulting in aberrant cell expansion and hyperplasia. Production of ROS and hypoxia in the reactive stroma further sets the stage for transformation of those CSCs that are continuously activated (Tanno and Matsui 2011). Tumor-promoting inflammatory niches also recruit components of the innate immune system that precipitate and support progression of incipient wounds associated with inflammation into full-blown tumors.

10.3.2.3 Reprogramming Energy Metabolism

The metabolic switch of cancer cells to glycolysis even in the presence of oxygen first reported by Warburg is paradoxical given its poor efficiency of energy generation (Warburg 1956). More recently, it has been reported that CSCs overexpress metabolism-related genes like glycine decarboxylase and pyruvate kinase M2 along with metabolites such as 2-hydroxyglutarate, lactate and kynurenine that are suggested to act as continual oncogenic metabolic triggers that contribute to disease progression by mediating epigenetic and genetic reprogramming (Zhang et al. 2013).

10.3.2.4 Evading Immune Destruction

Overcoming the host immune surveillance mechanisms is now recognized to be essential for tumor establishment and progression. CSCs have been demonstrated to lose MHC class I molecules and selectively silence expression of tumor-associated antigens towards such an evasion (Guerry et al. 1984). Yet other mechanisms of immune evasion of CSCs include altered immunogenicity, production of regulatory molecules by tumor cells, interactions with tumor-infiltrating immune cells (Qi et al. 2012), functional inactivation of antigen-reactive T lymphocytes, activation of Treg cells by secretion of immunosuppressive cytokines, including IL-4, IL-10 and TGF-β (Dancescu et al. 1992). Tolerance may also be achieved through clonal

anergy of macrophages and DCs, or expression of tolerogenic molecules including B7-H1, B7.1, CD47, SIRPa, CD200 by CSCs that facilitate immune evasion (Quesnel 2008).

10.4 Future Perspectives

The diversity of specific mechanisms involved in the generation of tumors through reprogramming of the intracellular circuitry to perturb regenerative homeostasis as well as acquire hallmark capabilities suggest that these may be achieved in a tumor – and tissue- specific manner. Feed-forward mechanisms of CSC emergence potentiate normal stem cell transformation and generation of maturation arrested derivatives; while feedback mechanisms suggest dedifferentiation or reprogramming towards a loss of differentiated identity. Both mechanisms emphasize that fidelity of either the quiescent state or a stable differentiated identity cannot be taken for granted and is susceptible to loss of intuitive, instructive cellular networks that could present either as transient ablation of homeostasis, or more seriously as stable transformation (Holmberg and Perlmann 2012). While there is increasing knowledge in the field regarding cell fate determination, lineage commitment and differentiation, mechanisms ensuring long-term stable stem cell state maintenance or phenotypic and functional differentiation remains poorly understood, yet can be highly relevant to cancer biology.

The existence of CSCs is convincing enough in certain cancers, yet mere expression of a certain phenotype and aberrant regeneration provides a narrow comprehension for inter-tumor heterogeneity between different tumor subtypes, and intra-tumor heterogeneity between specific cellular subsets within the same tumor. These phenomena in turn represent a wide range of variation of cellular and molecular cross-talks. While the present review emphasizes cellular aspects of CSCs, an equal if not more extensive research efforts currently focus on understanding how such cellular states and phenomena are regulated in tumors that are notoriously heterogeneous at the cellular and molecular levels. Hard wiring of gene expression is known to control normal tissue homeostasis; in contrast in tumors these are suggested to be flexible and completely erratic. Further integrative analyses across the various levels of gene regulation including epigenetic, genetic, proteomic, protein translation and signaling may thus be necessary to define specific tissue contexts.

While understanding a cellular state *in situ* is the objective of most research programs, development and use of appropriate *in vitro* model systems is necessitated on logistic and ethical grounds. In doing so, it should be ensured that such models are as close to the *in situ* state as is possible through addressing various issues. Can immortalized cell lines be termed as CSC lines? Do these or prospectively isolated CSCs retain a memory of the complex developmental process by which the tissue was first constructed, and can it be reproduced in experimental models? Are patient-derived xenografts more relevant than cell line derived xenografts? In the latter are humanized animal models harbouring human stroma and/or human

immune cells more relevant? Given the complexity of inter-tumor and intra-tumor heterogeneity how could one develop the most relevant models of specific cancers? Although related processes such as asymmetric cell division are currently addressed using models, their systemic homology in human disease opens a whole new door of complexities (Matsumura and Toyoshima 2012). This list of newly arising queries obviously is relentless, but it is quite likely that integration of inter-disciplinary studies including simulation, computation and mathematical modelling to channelize information from reductionism approaches towards construction of a systems view will address them in the long run.

Most if not all the above discussions regarding the future of CSCs seems to be centered on elucidation of their very existence and underpinning various characters crucial for their identity and functions. However, these have serious clinical implications that can contribute to development of cancer therapy in eliminating the regenerative tumor hierarchy to improve patient prognosis. Translation of these basic concepts thus is imperative and involve the development of specific drugs, inhibitors and antibodies to stabilize for example, disrupted polarity mechanisms, restore homeostasis mechanisms, identify 'druggable molecular targets' within the cellular systems networks, restrict migrating CSCs, stabilize innate immune responses to CSCs, neutralize the reactive tumor niche (Lathia et al. 2011; Bliss et al. 2014; Fan et al. 2015; Pan et al. 2015; Ajani et al. 2015), etc. The wider expectation of these efforts is to achieve optimal combinatorial therapies that would improve drug tolerance and patient prognosis in a personalized manner. Thus the process of pursuing the elusive quiescent CSC promises to be an exciting endeavor, and leads to an appreciation of the intrinsic drive of these populations in surviving against all odds in a manner akin to the Darwinian principles of survival-of-the-fittest. Being fraught with frustrations it might at times appear to be an academic exercise; however such information will be of immense important in view of developing appropriate personalized, specific and more effective therapies in cancer.

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