

Cancer Stem Cell plasticity – a deadly deal

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Abstract

Intratumoral heterogeneity is a major ongoing challenge in the effective therapeutic targeting of cancer. Accumulating evidence suggests that a fraction of cells within a tumor termed Cancer Stem Cells (CSCs) are primarily responsible for this diversity resulting in therapeutic resistance and metastasis. Adding to this complexity, recent studies have shown that there can be different subpopulations of CSCs with varying biochemical and biophysical traits resulting in varied dissemination and drug-resistance potential. Moreover, cancer cells can exhibit a high level of plasticity or the ability to dynamically switch between CSC and non-CSC states or among different subsets of CSCs. The molecular mechanisms underlying such plasticity has been under extensive investigation and the trans-differentiation process of Epithelial to Mesenchymal transition (EMT) has been identified as a major contributing factor. Besides genetic and epigenetic factors, CSC plasticity is also shaped by non-cell-autonomous effects such as the tumor microenvironment. In this review, we discuss the recent developments in understanding CSC plasticity in tumor progression at biochemical and biophysical levels, and the latest *in silico* approaches being taken for characterizing cancer cell plasticity with implications in improving existing therapeutic approaches.

Key words: Cancer stem cells, Plasticity, Epithelial-Mesenchymal Transition, Metastasis, Microenvironment

Introduction

Heterogeneity in cancer biology has long been recognized and exploited in the clinical management of the disease (Rich 2016). Intertumoral heterogeneity within breast cancer patients for example, exhibiting different molecular subtypes based on immunohistochemical markers like Estrogen Receptor (ER) or Her2 has been the basis of successful targeted therapeutic approaches (Turashvili and Brogi 2017, Januskeviciene and Petrikaite 2019). The inbuilt cellular variation within a tumor has been shown to be an important driver for the emergence of therapy resistant clones which ultimately lead to recurrence and spread of the cancer cells resulting in patient mortality (Somasundaram, Villanueva et al. 2012, Dagogo-Jack and Shaw 2018, Akgul, Patch et al. 2019).

The development of intratumoral divergence in tumor cells has been widely attributed to two contrasting processes (Shackleton, Quintana et al. 2009, Prasetyanti and Medema 2017). The clonal evolution theory takes into account the intrinsic differences between all cells based on genetic and epigenetic programs as well as the influence of the tumor microenvironment (TME). The fitter clones are selected for and contribute to the diversity of the tumor cell population (McGranahan and Swanton 2017). The second model - Cancer Stem Cell (CSC) model - proposes that there are a sub set of cells (termed CSCs) which are predisposed to drive the tumor progression, metastatic and therapeutic resistance of the entire tumor. In this hierarchical model, CSCs can differentiate into less self-renewing populations of non-CSCs which form the bulk of the tumor, in an analogous fashion to stem cell development (Vermeulen, de Sousa e Melo et al. 2012). More recently, the ability of cells to switch states via different programs like Epithelial-Mesenchymal Transition (EMT) has given rise to the concept that plasticity can allow CSCs to interchangeably develop into non-CSCs and *vice-versa* (Chaffer, Brueckmann et al. 2011, Marjanovic, Weinberg et al. 2013, Gupta, Pastushenko et al. 2019). Moreover, different subsets of CSCs can lie on various points on the epithelial-mesenchymal axis (Liu, Cong et al. 2014, Bocci, Jolly et al. 2018, Bocci, Gearhart-Serna et al. 2019). The indirect implication of this plasticity model is that the clonal evolution and CSC models are not mutually exclusive and ushers in more complexity to the manner in which heterogeneous cell populations arise within a tumor (Cabrera, Hollingsworth et al. 2015).

The direct consequence of evolving populations in a tumor through inter conversion by plasticity programs is the rise of drug resistant and metastatic cells which are ultimately

responsible for the mortality associated with cancer (Biddle, Gammon et al. 2016, Doherty, Smigiel et al. 2016). The need of the hour is hence to understand the molecular underpinnings for CSC plasticity with a focus on discerning a detailed characterization and decoding the impact of bidirectional nature of CSC plasticity on the clinical management of the disease.

CSC heterogeneity and plasticity in tumor progression

The concept that CSCs are dynamic populations and can undergo spontaneous state transitions has been strengthened by various studies (Chaffer, Brueckmann et al. 2011, Gupta, Fillmore et al. 2011, Chaffer, Marjanovic et al. 2013). In the study done by Chaffer et al. (Chaffer, Brueckmann et al. 2011). using basal-like breast cancer cells, non-stem cells were shown to spontaneously switch to stem-like cells *in vitro* and *in vivo*; this plasticity was later found to be regulated by ZEB1 – a key regulator of EMT (Jia, Jolly et al. 2017). CSC heterogeneity and plasticity has been observed in different cancers. Just like their non-cancerous counterparts, identification of CSCs has been mainly based on the expression of cell surface markers (Chen, Dong et al. 2016). However, even within a single tumor type, different markers can identify distinct CSCs which are phenotypically distinct and could vary from patient to patient depending on the genetic make-up of the tumor (Tang 2012). For instance, in glioblastoma, multiple markers like CD133, CD44, A2B5, SSEA have been utilized for identifying the stem cell populations (Singh, Hawkins et al. 2004, Ogden, Waziri et al. 2008, Son, Woolard et al. 2009). However, the use of CD133 marker is controversial as CD133⁻ cells have also been shown to form tumors in glioma and CD133⁺ cells could be derived from CD133⁻ cells *in vivo*, implying the underlying plasticity (Wang, Sakariassen et al. 2008). A recent study by Dirkse et al found that in glioblastoma, the cell- membrane associated CSC markers such as CD133, A2B5, SSEA and CD15 does not represent a clonal entity but a plastic state which can be adapted by most of the cells in response to varying conditions in the microenvironment. They also proposed that the enhanced tumorigenic potential of CSC-like state is a result of faster adaptation of the cells to the microenvironment (Dirkse, Golebiewska et al. 2019).

The evidence for plasticity of CSC states comes from melanoma as well. A slow cycling population of melanoma CSC-like cells were identified using H3K4 demethylase JARID1B as a biomarker (Roesch, Fukunaga-Kalabis et al. 2010). Intriguingly, the expression of this marker was dynamically regulated and JARID1B-negative cells could re-express the marker indicating the dynamic nature of the stemness trait. Another seminal study done on melanoma supports

the phenotypic plasticity model of stem-like cells. In this study, they found that in melanomas from patients, phenotypically distinct cells can recapitulate the original tumor and these cells can undergo reversible phenotypic changes *in vivo* (Quintana, Shackleton et al. 2010). In breast cancer, different subsets of CSCs were identified based on ALDH1, CD44, and CD24; and the two subpopulations (epithelial-like ALDH1+, mesenchymal like CD44+/CD24-) were shown to be capable of inter converting among themselves as well as give rise to non-CSCs (Liu, Cong et al. 2014). Moreover, in breast cancer, CSCs and non-CSCs were shown to exhibit dynamic equilibrium maintained by cytokine-mediated crosstalk among these distinct populations (Iliopoulos, Hirsch et al. 2011). These results suggest that at least in some cancers, phenotypic plasticity is reversible and does not necessarily depend on genetic alterations (Jolly, Kulkarni et al. 2018).

Another compelling evidence for CSC plasticity in tumor progression comes from studies on colorectal cancer. Targeted ablation of Lgr5+ CSC did not lead to tumor regression *in vivo* as the differentiated marker negative cells could give rise to marker positive population which sustained the tumor growth. But interestingly they could not form liver metastases (de Sousa e Melo, Kurtova et al. 2017), suggesting that the contribution of CSCs in primary tumor formation and that in metastatic settings may be different. More recently, some markers for metastatic CSCs have been identified across cancers (Celia-Terrassa and Jolly 2019).

CSC plasticity has also been observed alongside vasculogenic mimicry (VM) – a hallmark process of cancer cell plasticity in which cancer cells transdifferentiate and acquire endothelial cell like characteristics (Fernandez-Cortes, Delgado-Bellido et al. 2019). In triple negative breast cancer, a CD133+ cell population with CSC-like traits was found to show the ability to form tube-like structures (Liu, Sun et al. 2013). In renal cell carcinoma, using immunohistochemistry analysis of patient samples, the expression of stem cell like markers CD133 and CD44 was found to correlate with VM and high CSC marker expression and VM correlated with poor survival (Zhang, Sun et al. 2013).

Biochemical characterization of cancer stem cells and their subsets

The concept of cancer stem cell plasticity relies on the dynamic ability to shift from a CSC state to a non-CSC state and vice versa (Cabrera, Hollingsworth et al. 2015). CSC plasticity can be instigated by various components in the microenvironment such as the secretion of cytokines and chemokines, communication with different stromal cell types and extracellular matrix and hypoxia (Agliano, Calvo et al. 2017). Consequent activation of transcription factors

and/or epigenetic modifications have been shown to mediate this interconversion. To understand the biology of CSC plasticity and the mechanisms underlying their functional phenotype with the aim of developing efficient treatment strategies, an essential requirement is the characterization and methods to selectively isolate the plastic CSC population from heterogeneous tumors.

One of the serious and longstanding challenges in studying CSCs is the determination of appropriate methodology for the isolation and characterization of CSCs (Agliano, Calvo et al. 2017). One of the most widely applied method to identify CSCs is to sort the cells based on the expression of cell surface markers such as CD44, CD133, CD24, CD26, EPCAM, CD166 (Jaggupilli and Elkord 2012, Chen, Huang et al. 2013) or based on enzymatic activity of intracellular proteins like ALDH1 (Pattabiraman and Weinberg 2014). However, even these markers are not universally expressed on all CSCs, limiting their use in few cancers (Jaggupilli and Elkord 2012). To overcome this limitation, more than one markers are used together in several cancers (Agliano, Calvo et al. 2017). Although multiple markers have been described, the lack of reliable and accurate markers is still a stumbling block in the identification of CSCs. Moreover, recent single cell transcriptome analyses revealed that many CSC markers could be co-expressed by a single cell at the same time (Patel, Tirosh et al. 2014, Eun, Ham et al. 2017) and the expression of CSC markers could vary *in vivo* as a consequence of plasticity and adaptation to the microenvironment (Dirkse, Golebiewska et al. 2019). This clearly highlights the heterogeneity of CSCs and inefficiency of the markers currently in use in distinguishing CSCs and non-CSCs. These results pinpoint the need for better understanding of CSC heterogeneity and also the necessity to re-evaluate the idea of developing CSC marker-based therapeutic approaches. Therefore, combining marker-based isolation strategies with functional assays such as *in vitro* clonogenic and *in vivo* limiting dilution xenotransplantation assays are of paramount importance to validate the stemness trait of the cells (Dirkse, Golebiewska et al. 2019, Prager, Xie et al. 2019).

Biophysical characterization of CSCs and their subsets

The properties of stem cells such as self-renewal, multipotency and maintenance of an undifferentiated cell state have been shown to be governed by intracellular and extracellular components present in their microenvironment constituting the stem cell niche (Morrison and Spradling 2008). Similar to stem cells, properties of cancer stem cells (CSCs) are regulated by the tumor microenvironment (TME) which can enhance metastasis and tumor initiation capabilities of CSCs (Aponte and Caicedo 2017). CSCs, on the other hand, can also remodel ECM more strongly as compared to bulk cancer population (Srinivasan, Ashok et al. 2017); thereby setting a complex feedback loop among the CSC and its niche. Biochemical constituents of such loops have been well-characterized earlier (Korkaya, Liu et al. 2011). Recent evidence suggests how biophysical cues such as matrix stiffness, cell contractility and cell-matrix adhesion strengths can regulate the tumor-initiating properties of CSCs. For instance, blocking ROCK (Rho-associated protein kinase) can inhibit cell contractility and invasion potential of breast cancer stem cells (Srinivasan, Ashok et al. 2017). At a biophysical level, TME is often characterized by increased stiffness due to ECM remodeling, increased compressive stress due to confined growth, enhanced interstitial pressure and an increased interstitial fluid flow (Zanotelli, Goldblatt et al. 2018). Extrinsic mechanical forces exerted by ECM constituents can trigger biochemical changes inside cells such as cytoskeleton rearrangement, and changes in gene expression, protein-protein interactions and enzyme modifications, thus converging on various mechano-transduction and mechano-chemical axes (Ogden, Waziri et al. 2008, Broders-Bondon, Nguyen Ho-Bouldoires et al. 2018, Roy Choudhury, Gupta et al. 2019)

Cancer cells display variations in response to extrinsic biomechanical stimuli, leading to heterogeneity in their biophysical properties, which influences the overall nature of cells such as stemness and differentiation. Recent studies have shown that mechano-biological cues greatly influences the generation and maintenance of CSCs and eventually metastasis (Chen and Kumar 2017). In addition, mechanical properties such as deformability and adhesiveness are different for CSCs as compared to the bulk tumor population (Saliba, Saias et al. 2010, Zhang, Kai et al. 2012, Babahosseini, Ketene et al. 2014, Chen, Zhou et al. 2016). These advances have enabled attempts to isolate and identify CSCs based on biophysical marker using engineering techniques such as microfluidic devices (Saliba, Saias et al. 2010, Gossett, Tse et al. 2012, Zhang, Kai et al. 2012, Liu, Lee et al. 2015, Chen, Zhou et al. 2016) in a label-free manner.

For instance, CSCs were enriched based on their adhesive traits using a microfluidic chip having micro-channels coated with basement membrane extract. The cells entered into the chip driven by hydrodynamic forces. While highly adhesive cells were captured in micro-channels, less adhesive cells were collected from the outlet, which were shown to be enriched in cancer stem cells, had greater motility and were resistant to chemotherapeutic drugs (Zhang, Wu et al. 2015). This study emphasizes the interconnections between EMT, stemness, and drug resistance (Jolly, Somarelli et al. 2019), and the use of microfluidics in investigating these associations (Nath, Bidkar et al. 2019). Similarly, a microfluidic device fitted with microbarriers was used to isolate cancer cells based on their deformability *in vitro*. The more deformable flexible phenotype was associated with expression of many genes involved in motility, metastasis and greater mammosphere formation efficiency (Zhang, Kai et al. 2012). Consistently, *in vivo*, deformability has been shown to be crucial for efficient extravasation of tumor-repopulating cells during metastasis as seen in zebrafish models (Chen, Zhou et al. 2016).

Similar to biochemical heterogeneity observed within CSCs (Bocci, Gearhart-Serna et al. 2019, Moreira, Brayner et al. 2019), CSCs can be biophysically strikingly different too. In a recent study, Chen et al., showed the association of biophysical properties of CSCs and their ability to invade, migrate, and initiate tumors, using the SUM149 inflammatory breast cancer (IBC) stem cells (Chen, Allen et al. 2019). In this study, the authors sorted SUM149 CSCs based on the expression of aldehyde dehydrogenase enzyme (ALDH) and assayed biophysical properties of ALDH⁺ and ALDH⁻ cells in terms of deformability, adhesion strength and contractility. ALDH⁺ cells displayed greater deformability, lower adhesion strength and reduced contractility relative to ALDH⁻ cells, and resulted into enhanced functional phenotypes *in vitro* and greater tumor development *in vivo*. In addition, to validate the contribution of biophysical properties in metastasis, the authors isolated IBC cells based on their adhesive property using a microfluidic device and showed that the less adhesive cell population was ALDH⁺ enriched, displayed enhanced *in vitro* invasion and migration as well as increased *in vivo* tumor development. Further, exogenous alteration of cell stiffness also resulted in changes in metastatic potential of these cells with less stiff cells showing greater invasion and migration (Chen, Allen et al. 2019). The results observed in this study corroborated well with earlier studies showing that cancer cells with greater deformability, lower adhesion strength and lower contractile force show enhanced metastatic potential (Swaminathan, Myhreye et al. 2011,

Kraning-Rush, Califano et al. 2012, Byun, Son et al. 2013, Sun, Luo et al. 2016, Bongiorno, Gura et al. 2018).

Therefore, with increasingly detailed characterization of biomechanical properties of various subpopulations of cancer cells and ECM, a ‘mechanosome’ or ‘matrisome’ signature may be helpful in identifying and isolating the most aggressive cancer cell subpopulations (Roy Choudhury, Gupta et al. 2019).

Single cell methods to identify CSCs and their subsets

Much of our current understanding about CSCs comes from studies performed on bulk cancer cell populations. However, bulk analysis masks the underlying intra-tumor heterogeneity and does not inform much about rare cell subpopulations within the tumor (Navin 2015, Bhatia, Monkman et al. 2019). Therefore, it becomes extremely critical to study the cancer cells at a single-cell resolution to better comprehend the CSC heterogeneity and plasticity (Etzrodt, Endeale et al. 2014). Flow cytometry and Fluorescence Associated cell Sorting (FACS) is a widely used method for the isolation and characterization of single CSCs (Greve, Kelsch et al. 2012, Etzrodt, Endeale et al. 2014). In an interesting work done by Dirkse et al., by combining FACS analysis of CSC markers and functional assays such as proliferation, self-renewal and multipotency tests, they found that glioblastoma stem cell heterogeneity results from tumor plasticity which is determined by the microenvironment cues (Dirkse, Golebiewska et al. 2019). Lineage tracing methods have also been utilized by researchers to decipher the properties of CSCs and has huge potential in understanding the transition of cell states. Using intra-vital *in vivo* lineage tracing method in a genetic mouse model of breast cancer, Zomer et al, have shown the existence of CSCs in unperturbed mammary tumor for the first time. They also found that CSC state is plastic and can be activated, lost or deactivated (Zomer, Ellenbroek et al. 2013). In another study, lineage tracing and transcriptional analysis of Notch1 expressing cells of intestinal tumors has led to the identification of a previously uncharacterized and undifferentiated stem cell population that contribute to tumor progression and heterogeneity (Mourao, Jacquemin et al. 2019).

The advent of single-cell omics approaches has revolutionized our knowledge on CSC biology. Single cell genomic and transcriptomic analyses have provided invaluable insights regarding the intra-tumor heterogeneity and clonal evolution in different cancers (Patel, Tirosh et al. 2014, Eirew, Steif et al. 2015, Hou, Guo et al. 2016, Puram, Tirosh et al. 2017). In addition to

discerning the dynamics of clonal evolution, single cell OMIC methods have also enabled the identification of cancer stem cell state transitioning and its contribution to drug resistance (Puram, Parikh et al. 2018, Sharma, Cao et al. 2018). Considering the rarity of a cancer stem cell, combining single cell transcriptomics with enrichment strategies like flow cytometry or sphere assays will drastically improve the characterization of CSC (Akrap, Andersson et al. 2016, Jonasson, Ghannoum et al. 2019). Single cell multi-omics approaches which involves obtaining information from multiple components within a single cell is also gaining significant interest as it allows the assessment of genotype and phenotype relationship in regulating the cell states (Macaulay, Ponting et al. 2017, Liu, Liu et al. 2019).

Nonetheless, most of the single-cell omics analyses does not preserve spatial information as it requires the cells to be isolated from their microenvironment (Yuan, Cai et al. 2017). Another limitation is that a snap-shot analysis is inadequate to evaluate the dynamic nature of cellular processes and therefore lack the details regarding the temporal regulation of the cellular states (Skylaki, Hilsenbeck et al. 2016). Transcriptomic profiling of cells in-situ using fluorescence in-situ hybridization or sequencing will enable the decoding of the spatial regulation of cellular heterogeneity at single-cell resolution (Lee, Daugharthy et al. 2014, Chen, Boettiger et al. 2015, Suva and Tirosh 2019). Single cell RNA sequencing data can be mapped to spatial transcriptomic data using advanced computational methods (Satija, Farrell et al. 2015, Edsgard, Johnsson et al. 2018). Although acquisition techniques have greatly improved, better analysis tools are still lacking. Using a newly developed high-throughput automated single cell image analysis (HASCIA), the spatio-temporal factors regulating glioblastoma stem cell state transitions has been recently investigated (Chumakova, Hitomi et al. 2019). Integrating the transcriptomic and spatial data can significantly improve the interpretation of the CSC plasticity (Satija, Farrell et al. 2015, Yuan, Cai et al. 2017). A recent work coupling large scale single cell-resolution 3D imaging strategy, lineage tracing and RNA sequencing in pTEN/Trp53 deficient mice models, observed extensive molecular heterogeneity and clonal plasticity within tumors and found that EMT is not a rare event within the tumors (Rios, Capaldo et al. 2019). Live single-cell imaging techniques are also being developed which overcomes the limitation of static snap-shot analyses in studying the temporal regulation of cellular state changes (Fumagalli, Bruens et al. 2019). The number of genes analyzed by such studies are much less than snap-shot studies and the lack of specialized tools and computational methods for handling the large amount of data generated through such studies is a major challenge (Skylaki, Hilsenbeck et al. 2016).

Single-cell analysis has emerged as a powerful tool over the past few years and has the enormous potential to understand the biology of CSCs. Development of newer tools and analysis methods and integrative approach are required for better comprehending the cell state transitions and improved therapeutic strategies.

Computational methods to identify CSCs

With the deluge of preclinical and clinical data being generated at a high-dimensional level, computational approaches to extract meaningful information and generate testable hypotheses are becoming more common (Suhail, Cain et al. 2019). Various ‘top-down’ and ‘bottom-up’ computational methods provide a framework to unravel novel insights into various aspects of the dynamics of cancer progression such as role of intra-tumoral heterogeneity, dynamics of EMT, CSCs and its role in metastases, evolutionary dynamics of cancer initiation and progression, prediction of treatment response and therapy resistance. While ‘top-down’ methods use high-dimensional data and apply an inferential metric to identify patterns through machine learning and/or network reconstruction, the ‘bottom-up’ approaches aim to elucidate the emergent dynamics of a phenomenon based on its mechanism-based description through mathematical modeling. Both approaches can be synergistically used to predict and/or interpret cellular behavior (Altrock, Liu et al. 2015, Jolly, Tripathi et al. 2017).

Mechanism-based, i.e. ‘bottom-up’, mathematical models have been useful in understanding the dynamics of complex regulatory networks that modulate cancer stem cell behavior such as stem cell state transitions and dedifferentiation (Sehl and Wicha 2018). Recent studies using mathematical models have predicted that cells in one or more hybrid E/M phenotypes are associated more with stemness as compared to cells in purely mesenchymal or purely epithelial (Jolly, Huang et al. 2014). These predictions have since been validated *in vitro* and *in vivo* (Grosse-Wilde, Fouquier d'Herouel et al. 2015, Bieri, Pierce et al. 2017, Pastushenko, Brisebarre et al. 2018, Kroger, Afeyan et al. 2019) and has been supported by clinical data (Jolly, Somarelli et al. 2019). How are the pathways of EMT and stemness interconnected with each other? These questions can be addressed by investigating *in silico* the coupling between core regulatory circuits of EMT, CSCs and other connected signaling pathways such as Notch signaling; this model predicted that altering the coupling strength between EMT and CSC networks and/or modulating Notch signaling can change the position of ‘stemness window’ on the ‘EMT axis’, thus generating various subsets of CSCs in terms of EMT phenotypes (Bocci,

Jolly et al. 2018). Such CSC heterogeneity has been extensively seen across cancers (Liu, Cong et al. 2014, Giraddi, Chung et al. 2018, Zheng, Pomyen et al. 2018). A common unifying principle that has emerged upon investigating the regulatory networks underlying EMT, CSCs and related traits such as drug resistance has been the role of feedback loops in enabling multiple phenotypes (i.e. heterogeneity) and the ability to switch among them (i.e. plasticity) (Huang, Jolly et al. 2015, Mooney, Talebian et al. 2017, Tripathi, Fahrman et al. 2017, Jolly, Preca et al. 2018, Saha, Kumar et al. 2018).

‘Data-based’ models have also been valuable in decoding CSC signatures. A stemness index was derived using one-class logistic regression and observed to be higher in the metastatic breast cancer cells compared with primary tumors (Malta, Sokolov et al. 2018), suggesting the possibility of a signature specific to metastatic cancer stem cells. Similar logistic regression models have been used to quantify the extent of EMT (George, Jolly et al. 2017) that has revealed the heterogeneity of EMT phenotypes in various CSCs and their subsets (Bocci, Jolly et al. 2018), hence strengthening the insights from ‘mechanism-based’ or ‘bottom-up’ models.

Another set of questions where mathematical models have been useful is estimating the fraction of CSCs in a tumor. Many studies have used population-level models to understand the difference in growth kinetics of CSCs and non-CSCs, and used that to offer a potential mechanistic underpinning of ‘tumor growth paradox’ i.e. accelerated tumor growth with increased cell death (Hillen, Enderling et al. 2013). Typically, CSC represent a minor cell population within a tumor, major population being the non-CSCs which compete with the CSCs for space and resources. While induction of cell death results into death of bulk of non-CSCs which facilitates increase in CSC division, ultimately resulting into expansion of CSC population and increase in tumor progression (Hillen, Enderling et al. 2013). In contrary, another study showed that the CSC population within a tumor is homeostatically maintained such that reducing CSC population below a threshold triggers extensive phenotypic switching of non-CSC to CSC population (Sellerio, Ciusani et al. 2015). Thus, while the dynamics and mechanisms of CSC generation, plasticity and maintenance remain to be comprehensively understood (Enderling 2015), integrating these different modeling approaches with one another and with experimental and clinical data shall contribute to revealing this complex behavior at an intracellular and at a population level. Such an improved dynamic understanding can help identify optimal treatment strategies to reduce tumor burden, such as a combination of radiation and differentiation therapies (Bachman and Hillen 2013), or a sequential treatment of drugs to

tackle the *de novo* generation of CSCs (Gupta, Fillmore et al. 2011) and their functional attributes (Goldman, Majumder et al. 2015).

Spatial organization of CSCs

Spatial heterogeneity is a fundamental feature of tumor microenvironment (TME) that contains diverse cell types (Yuan 2016, Yuan, Cai et al. 2017). One canonical representation of spatial heterogeneity is the co-occurrence of vascular and hypoxic regions, as observed in solid tumors (Alfarouk, Ibrahim et al. 2013). This heterogeneity can alter cellular phenotypes, for instance, glioblastoma cells in hypoxic regions have been shown to over-express epidermal growth factor receptor (EGFR) while those over-expressing platelet-derived growth factor receptor (PDGFRA) were enriched in vascular regions (Little, Popov et al. 2012). Similarly, hypoxic TME induced by antiangiogenic agents can increase breast cancer stem cells (Conley, Gheordunescu et al. 2012). Thus, spatial heterogeneity of TME can give rise to differential spatial organization of cancer sub-populations within a tumor.

Indeed, varying levels of nuclear beta-catenin expression was observed in different sub-populations of well-differentiated colorectal cancer, suggested to be regulated by TME (Brabletz, Jung et al. 2001). Cells in the invasive front of the primary tumor as well as metastases expressed high levels of nuclear beta-catenin, and lacked the expression of membranous E-cadherin, indicative of an epithelial-mesenchymal transition (EMT). On the other hand, centrally located cells in the primary tumor and metastases showed cytoplasmic beta-catenin and membranous E-cadherin expression, perhaps due to a mesenchymal-epithelial transition (MET). These two transient phenotypic transitions could be driven by specific TME present in the invasive front and central regions of the tumor by modulating expression of intracellular beta-catenin (Brabletz, Jung et al. 2001). Consistently, the CD24–CD44+ mesenchymal breast CSCs (BCSCs) were found in the invasive edge of the tumor, while the more epithelial or hybrid E/M CSCs, identified by ALDH1+, were localized in the interior regions of the tumor (Liu, Cong et al. 2014). In vitro, these two subpopulations, when segregated, could give rise to the other and other non-CSCs; however multiple studies consistently report spatial heterogeneity with respect to EMT in a primary tumor (Jung, Schrauder et al. 2001, Schmalhofer, Brabletz et al. 2009, Bronsert, Enderle-Ammour et al. 2014, Liu, Cong et al. 2014, Grigore, Jolly et al. 2016). Therefore, put together, these observations beg the question of what mechanisms might underlie such patterning.

Recent efforts have attempted to elucidate the mechanisms underlying such spatial heterogeneity. A mathematical modeling analysis revealed that in the presence of a gradient of TGF- β (EMT inducing) signal, cell-cell communication among tumor cells mediated via Notch-Jagged signaling can recapitulate the experimentally observed spatial organization of CSCs sub-populations with varying EMT phenotypes (Bocci, Gearhart-Serna et al. 2019). Notch-Jagged signaling can be promoted by inflammatory cytokines like IL-6 which can stabilize hybrid E/M phenotype and increase the spatial proximity of hybrid E/M CSCs. In vitro knock down of JAG1 in SUM149 human breast cancer cells significantly reduced their tumor organoid formation, confirming the role of Notch-Jagged signaling in tumor progression (Bocci, Gearhart-Serna et al. 2019). Future studies can focus on gaining a understanding of other interconnected aspects of heterogeneity in TME.

Spatial heterogeneity of tumors can be used as a predictor of cancer prognosis and treatment response across different cancer types (Yuan 2016). For example, colorectal cancer patients with high density of tumor infiltrating lymphocytes responded to anti-cancer therapy (Gong, Anders et al. 2018). Spatial heterogeneity can also significantly impact the time to occurrence in cancer cells exposed to continuous as well as adaptive therapies (Gallaher, Enriquez-Navas et al. 2018). Thus, the spatiotemporal dynamics of phenotypic changes induced by TME can be pivotal in aggravating aspects of tumor progression.

Mechanisms controlling CSC plasticity

CSC plasticity is controlled by both cell-intrinsic and cell-extrinsic factors (Poli, Fagnocchi et al. 2018). Several studies have implied the importance of key transcription factors in modulating the generation of CSCs and regulation of cellular plasticity (Eun, Ham et al. 2017). The transcription factors like OCT3/4, SOX2, NANOG, KLF4 has been found to induce reprogramming of somatic cells into pluripotent stem cell [induced pluripotent stem cells (iPSC)] (Liu, Yu et al. 2013). A plethora of research has shown the expression of iPSC transcription factors in CSCs of many cancers that contribute to tumor progression (Gu, Yuan et al. 2007, Ben-Porath, Thomson et al. 2008, Liu, Yu et al. 2013). For example, the introduction of OCT3/4, NANOG and KLF4 retrovirally into human colon cancer cells resulted in enhanced CSC properties and the xenografts of these cells actually resembled the original human tumor tissue (Oshima, Yamada et al. 2014). Similarly, in glioblastoma, Suva et al., identified a core set of neurodevelopmental transcription factors (POU3F2, SOX2, SALL2,

and OLIG2) that were sufficient to reprogram differentiated glioblastoma cells to CSCs (Suva, Rheinbay et al. 2014). Tumor suppressor transcription factors like p53, pTEN has also been associated with CSC plasticity (Cabrera, Hollingsworth et al. 2015, da Silva-Diz, Lorenzo-Sanz et al. 2018, Santoro, Vlachou et al. 2019). Loss of p53 lead to increased expression of Nestin and enable the dedifferentiation of hepatocytes and thereby contributes to cellular plasticity in liver carcinogenesis (Tschaharganeh, Xue et al. 2014). Combined loss of p53/pTEN in clonal prostate epithelial cells caused transformation of multipotent progenitors and lead to epithelial to mesenchymal transition (Martin, Liu et al. 2011). Genetic mutations of oncogene like KRAS and tumor suppressor like APC is also linked to the generation of stem-like cells (Easwaran, Tsai et al. 2014).

Many studies have pointed out various mechanisms of epigenetic regulation such as bivalent chromatin state, DNA methylation, histone modifications in mediating CSC plasticity (Poli, Fagnocchi et al. 2018). For example, in basal like breast cancer cells, Chaffer and colleagues observed that Zeb1 promoter of non-CSCs are maintained in a bivalent configuration and in response to TGF β , the chromatin switches to an active state leading to the transcription of Zeb1, consequently converting the non-CSCs to CSCs (Chaffer, Marjanovic et al. 2013). Homeobox genes are master regulators of development and maintains the balance between proliferation and differentiation. A study by Shah et al., has shown that a loss of function of HoxC8, a homeobox gene, in non-tumorigenic mammary epithelial cells due to promoter DNA hypermethylation is associated with the expansion of CSC pool, increased self-renewal and a transformed phenotype (Shah, Cardenas et al. 2017). A histone modifier, enhancer of zeste homolog 2 (EZH2) is a core member of polycomb repressor complex 2 (PRC2) and mediates transcriptional repression of target genes via trimethylation of lysine 27 of histone 3 (H3K27me3) (Gan, Yang et al. 2018). EZH2 is upregulated in many cancers and its enhanced expression of EZH2 is associated with invasion, migration and stemness (Yamaguchi and Hung 2014). In breast cancer, overexpression of EZH2 has increased mammosphere formation and self-renewal ability in breast CSCs (Chang, Yang et al. 2011, Wen, Cai et al. 2017). In glioblastoma, loss of H3K27me3 leads to aberrant activation of a transcription factor, ASCL1 and results in Wnt activation which is required for tumorigenicity and CSC maintenance (Rheinbay, Suva et al. 2013). On the other hand, in pediatric glioblastomas, the mutations in histone variants H3.1 and H3.3 results in reduced activity of EZH2 and consequently reprograms towards a stem cell-like state (Lewis, Muller et al. 2013). Another histone modifier, JARID1B, a histone demethylase is associated with CSC plasticity in melanoma (Roesch,

Fukunaga-Kalabis et al. 2010). These observations suggest that CSCs hijack and exploit the reversible nature of epigenetic modifications to achieve their plastic nature (Wainwright and Scaffidi 2017). However, this reversibility also offers an attractive opportunity that needs to be harnessed for therapeutic targeting.

CSC plasticity and EMT

EMT is a reversible dynamic process which is critical during embryonic development and also aberrantly activated during various pathological processes like wound healing, fibrosis and cancer progression (Kalluri and Weinberg 2009, Jolly, Ward et al. 2018). EMT is characterized by the loss of apico-basal polarity, rearrangements in the cytoskeleton and the acquisition of mesenchymal gene expression signature (Kalluri and Weinberg 2009). The activation of EMT program is associated with the acquisition of stem like characteristics and has been implicated in different cancers (Mani, Guo et al. 2008, Shibue and Weinberg 2017, Singla, Kumar et al. 2018, Dongre and Weinberg 2019). Initial reports suggested that activation of an EMT program endowed cells with traits similar to CSCs, such as enhanced colony formation *in vitro* and enhanced tumorigenesis *in vivo* (Shibue and Weinberg 2017). Recent studies have, however, presented a more nuanced understanding of the interconnection between EMT and CSCs. Cells that undergo a more extreme version of EMT can lose the stemness gained during the initiation of EMT; thus, cells in a hybrid epithelial/ mesenchymal phenotype are much more likely to be stem-like as compared to those on either end of the spectrum – pure epithelial or pure mesenchymal (Bierie, Pierce et al. 2017, Jolly, Mani et al. 2018). A recent study by Kroger et al., has found that the acquisition of a hybrid phenotype a critical for the maintenance of tumorigenicity of basal breast cancer cells. Based on CD104/CD44 cell surface antigen expression and by regulating the expression of transcription factors like Zeb1 and Snail, they isolated highly tumorigenic cell population residing stably in a hybrid EMT state. This hybrid E/M cell population showed enhanced stemness which was mediated by increased expression of Snail and Wnt signaling pathway (Kroger, Afeyan et al. 2019). Another interesting study by Pastushenko et al., looked at the spectrum of EMT states that exist in a tumor rather than the binary fixed state that was accepted for long. The hybrid E/M tumor cells were associated with differences in their transcriptional and epigenetic programs, metastatic potential and also the location within a tumor (Pastushenko, Brisebarre et al. 2018). It would be interesting to further understand whether these different hybrid states also respond differently to cues like chemotherapeutic treatment leading to resistance and ultimately relapse in cancer patients.

The effect of the tumor microenvironment on CSC plasticity

The tumor microenvironment is a complex and dynamic ecosystem consisting of diverse stromal cell types like fibroblasts, endothelial cells, adipocytes and immune cells which are influenced by the cancer cells and vice versa. In addition to the juxtacrine crosstalk with each other and cancer cells through direct cell to cell contacts, there are also factors secreted by the different cell types that form complex interacting networks which ultimately contribute to tumor growth and metastasis (Swartz, Iida et al. 2012, Quail and Joyce 2013, Peltanova, Raudenska et al. 2019). Accumulating evidence suggests that crosstalk with the stromal cells have significant implications on the stem-like behaviour and phenotypic plasticity of cancer cells (Pattabiraman and Weinberg 2014, Cabrera, Hollingsworth et al. 2015, Prasetyanti and Medema 2017). Cancer associated fibroblasts (CAFs) are a major component of the tumor microenvironment and play a pivotal role in various aspects of tumor progression (Kwa, Herum et al. 2019). CAFs were found to modulate the stem cell plasticity in hepatocellular carcinoma through c-Met/FRA1/HEY1 signaling (Lau, Lo et al. 2016), in pancreatic adenocarcinoma through FAK signaling through direct interactions mediated by collagen (Begum, McMillan et al. 2019) and regulate lung cancer stem cell plasticity by IGF-II/IGF1R signaling pathway (Chen, Ho et al. 2014). In a recent study, the extent of intracellular Notch1 signalling in mesenchymal stem cell-derived dermal fibroblasts was found to determine the ability of these cells to regulate melanoma aggressiveness, stemness and phenotypic plasticity (Du, Shao et al. 2019). Another key component is the immune system which plays a crucial role in regulating CSC plasticity. In response to chemotherapy, macrophages can secrete factor like Oncostatin-M (OSM), an IL-6 family cytokine which in turn can activate the dedifferentiation of triple negative breast cancer cells into aggressive stem cells (Doherty, Parvani et al. 2019) and this could be mediated through co-operative STAT3/SMAD3 signaling (Junk, Bryson et al. 2017). OSM can also be secreted by cancer associated adipocytes which can also promote stemness (Wolfson, Eades et al. 2015).

The physical and chemical composition of the microenvironment like acidic pH, low oxygen and nutrient availability, rigidity and porosity of the ECM also play an important role in regulating the cancer stem cell behaviour (Hjelmeland, Wu et al. 2011, Nallanthighal, Heiserman et al. 2019, Prager, Xie et al. 2019). A classic example would be hypoxia which is a hallmark of tumor progression in solid tumors and is associated with metastasis, therapeutic

resistance and poor survival (Lequeux, Noman et al. 2019). A hypoxic microenvironment is known to regulate various aspects of malignant progression including cellular plasticity. In Glioblastoma, hypoxia was found to promote self-renewal in non-stem cells by upregulating important factors like OCT4, NANOG and cMYC (Heddleston, Li et al. 2009). Also, the hypoxic microenvironment can select the fate of breast cancer stem cells *in vivo* as shown by Kim et al (Kim, Lin et al. 2018). Using flow cytometry, hypoxic and non-hypoxic breast cancer cells were isolated from hypoxia sensing xenografts of MDA-MB-231 and MCF7 breast cancer cell lines. Hypoxic tumor cells showed enhanced cancer stem cell characteristics compared to non-hypoxic cells which is attributed to the PI3K/AKT signaling. Interestingly, this differential cell fate was observed only in tumor cells isolated from hypoxic tumor microenvironment *in vivo* and not in tumor cells treated by hypoxia *in vitro* alone (Kim, Lin et al. 2018).

These studies underscore the importance of the microenvironment in sculpting intra-tumoral heterogeneity and CSC plasticity and highlight the need to better understand the tumor-microenvironment crosstalk for the development of effective therapeutic strategies. However, it is still controversial whether the CSC heterogeneity arises as a consequence of the microenvironment exerted selection pressure or whether plasticity is an intrinsic, default feature of the cancer cells that enable to adapt to varying cues from the microenvironment (Poli, Fagnocchi et al. 2018, Dirkse, Golebiewska et al. 2019). Recent evidence from the study on glioblastoma, suggests that intrinsic plasticity of tumor cells enables them to stochastically transition between different states defined by distinct expression of cancer stem cell markers and adapt to the microenvironment. Although all cell subpopulations are capable of phenotypic adjustment, they vary in their speed of adaptation (Dirkse, Golebiewska et al. 2019).

Discussion/Conclusion

The issue of phenotypic plasticity presents a clear and present danger in the treatment of cancer patients. Accumulating evidence suggests that CSCs consist of different sub-populations that can interconvert among different states through intracellular and intercellular regulatory networks. Over expression of one or more transcription factors or activating trans-differentiation processes such as EMT can drive the switch among CSCs and non-CSCs as well as between different subsets of CSCs. These adaptive strategies adopted by cells must be taken into account while devising new therapeutic strategies in the clinic in order to target all populations effectively.

The challenges to addressing this issue are multifold. The ability to identify the plastic CSC population using markers has its inherent problems which are further confounded by each individual patient's unique biochemical and biomechanical signatures. The general consensus is that drug resistance is achieved through a transition to a slow cycling state which is reversible once the stress is removed. Understanding how the cells switch from a slow cycling state and reenter the proliferative phase of the cell cycle will be key to targeting this population which contributes to minimal residual disease.

Using latest developments in computational and experimental methods will allow us to map the different states of tumor cells within multiple locations in a tumor, thus enabling a more comprehensive view of the genetic and non-genetic heterogeneity that exists within a cancer. Correlating such intratumoral heterogeneity with cellular phenotypes will be key to devising better therapeutic options in patients to ablate the tumor cells stably within a patient.

Author contributions

RN and MKJ designed the review. AT, KS, RM, MJ and RN wrote, read and approved of the manuscript.

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Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures

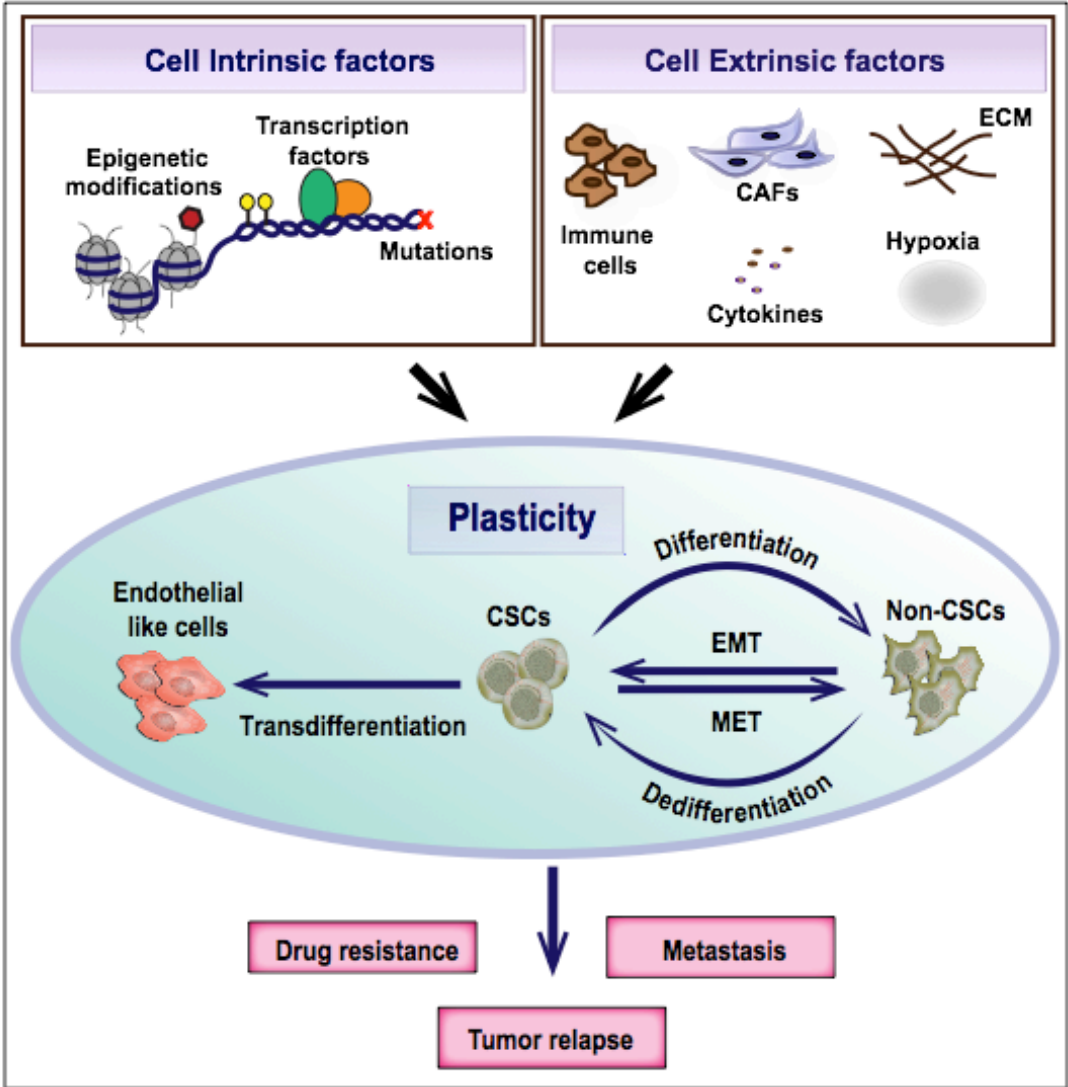


Fig. 1. Cancer stem cell plasticity is the ability to dynamically switch between CSC and non-CSC states. It is a complex process regulated by both cell intrinsic and extrinsic factors. Plasticity plays an important role in the evolution of therapeutic resistance, tumor relapse and metastasis.